Further characterization of retinoblastoma gene-mediated cell growth and tumor suppression in human cancer cells

(gene transfer/tumorigenicity/cell growth regulation/tumor suppressor resistance)

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ABSTRACT We have transfected the osteosarcoma cell line Saos2 and the bladder carcinoma cell line 5637 with additional retinoblastoma (RB) expression plasmids. The RB-reconstituted Saos2 and 5637 cells showed only slightly lower ratios of cells undergoing DNA synthesis compared to their parental RB⁻ tumor cells, and there were no noticeable changes in cell morphology. Furthermore, we have isolated long-term RB⁺ clones from Saos2, 5637, and the retinoblastoma cell line WERI-Rb27 after transfection/transduction with a RB expression plasmid or retrovirus. These clones were similar to their parental cell lines in terms of morphology and growth rates, and they all expressed functional RB protein (p110^{RB}) as evidenced by its potential of phosphorylation, simian virus 40 large tumor antigen binding, and nuclear tethering. No mutation or deletion of the exogenous RB gene was detectable by PCR and single-strand conformation polymorphism analysis. In addition, either the individual or pooled RB⁺ clones did form malignant tumors in nude mice but usually with a longer latency period and lower frequency. Such tumors also retained normal RB expression, suggesting that at least a portion of the RB-reconstituted tumor cells were still tumorigenic. This phenomenon is referred to by us as tumor suppressor resistance (TSR).

The retinoblastoma gene (RB) is one of the best-studied "tumor suppressor" genes (1) or "anti-oncogenes" (2) and has been implicated in a diverse group of human malignancies (3). This gene encodes a nuclear phosphoprotein of ≈110 kDa (4). The RB protein pattern seen in normal cultured cells demonstrates a major unphosphorylated RB protein band with an apparent relative molecular mass of 110 kDa (p110^{RB}) and a more variable region above this band with a mass ranging from 110 to 116 kDa (pp110^{RB}), representing the phosphorylated forms of the RB protein (5). In addition, differences in the ratio unphosphorylated/phosphorylated RB protein forms were observed between normal fibroblasts growing in logarithmic and G₁ phases with more unphosphorylated p110^{RB} being seen in G₁-arrested cells, suggesting that changes in the ratio phosphorylated/unphosphorylated RB proteins are related to the fluctuation of the cell cycle (5). Four subsequent papers have described the cell cycle-dependent phosphorylation of RB protein in detail (6–9). Another recognized property of the functional RB protein is that p110^{RB} is able to form stable complexes with the oncoprotein encoded by the transforming genes of human adenovirus, simian virus 40 (SV40), and human papilloma virus. Nonfunctional mutants of the RB protein are characterized by defects in phosphorylation, viral oncoprotein association, and nuclear tethering (10).

The suggestion that RB inactivation could play a broad role in the development of human malignancies has been rein-

forced by a number of studies indicating that replacement of the normal RB gene in RB-defective cells from widely disparate types of human cancers could suppress their tumorigenic activity in immunodeficient mice (11–18). In several other studies, attention was paid to cell growth suppression and morphological changes of the RB-defective osteosarcoma cell line Saos2 after transfection with RB expression plasmids (10).

Although, in general, the results of these previous studies, except for one recent report (19), indicated that replacement of the RB gene has a significant effect on cell growth and/or tumorigenicity of RB-defective tumor cells, there were inconsistent statements on the tumorigenic phenotype of RB-reconstituted tumor cells (11–18). This has raised a question as to whether correction of the RB gene defect alone, for example, in retinoblastoma or in osteosarcoma and bladder carcinoma, the latter two of which carry multiple genetic alterations, is sufficient to completely revert their malignant phenotypes (20). A solution to this question may not only provide real insight into the molecular mechanisms of cell growth regulation acting beyond the level of RB protein expression but may also need to be considered carefully when evaluating the feasibility of RB gene therapy for human cancers as postulated by some authors (13).

MATERIALS AND METHODS

Construction of RB Expression Plasmids and Transfection. A plasmid, F7 (15) or p4.95BT (21), containing the full-length RB gene cDNA was digested with appropriate restriction enzymes at nucleotides –4 and +3548 (the A of the AUG start codon was designated nucleotide +1). The resultant RB cDNA fragment was inserted into the unique *Bam*HI site of plasmid vectors pCMV-Neo-Bam (22) and pHAPr-1-neo (23). The RB expression plasmids were designated pCMV-f-RB35 and pBA-f-RB33, respectively. The RB⁺ long-term mass culture derived from the RB retrovirus-transduced retinoblastoma cell line WERI-Rb27 has been described (12). Tumor cells were transfected with the RB expression plasmid or mock expression plasmid via Lipofectin reagent (GIBCO/BRL) according to the manufacturer's specifications.

Western Blotting and Immunochemical Staining. Direct Western immunoblotting and RB protein staining were performed as described (24, 25). *In situ* extraction was done directly on the coverslips with hypotonic buffer (26). The extracted coverslips were fixed in 45% acetone/10% formaldehyde in 0.1 M phosphate buffer and were processed for immunocytochemical staining.

Tumorigenicity Assay in Nude Mice. Equal numbers of parental and RB-reconstituted tumor cells were injected subcutaneously into the flanks of nude mice (WERI-Rb27, 5637, and Saos2) or intraocularly into the anterior chamber of

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Abbreviations: SV40, simian virus 40; T antigen, large tumor antigen; SSCP, single-strand conformation polymorphism.

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nude mouse eyes (WERI-Rb27) as described (12, 14, 15). Xenograft tumors were scored 1 or 2 months after injection and were excised for further analysis.

RESULTS

Effects of Transient and Stable Expression of Exogenous RB on Tumor Cell Growth and Morphology. To determine the effects of p110^{RB} on cell growth and morphology, two established human tumor cell lines, an osteosarcoma cell line, Saos2, and a bladder carcinoma cell line, 5637, were transiently transfected with the RB expression plasmid pCMV-f-RB35 or p β A-f-RB33. The rationale for choosing Saos2 and 5637 as recipient cells was that, according to the literature, dramatic cell growth suppression and morphological changes were observed in Saos2 cells after RB replacement (10, 11), while introducing the *RB* gene into 5637 cells had no effect on their growth and cellular morphology using standard culture medium containing 10% fetal bovine serum (14, 15). In contrast to the former observations, as shown in Fig. 1

Upper, we found the RB-expressing Saos2 cells as determined by immunocytochemical staining in the primary RB-transfected mass cultures had no significant morphological changes when compared to those lacking RB protein. Although numerous large and flat senescent-like cells did appear in the RB-transfected Saos2 mass culture after geneticin selection, the majority of these cells were RB⁻ rather than RB⁺ by RB protein staining (data not shown). The same finding was true for the 5637 cells after transfection by RB expression plasmids (data not shown), which was consistent with previous reports (15).

To further study the cell growth suppression of p110^{RB} on Saos2 and 5637 cells, we used an assay described earlier by Baker *et al.* (22) to measure DNA synthesis in RB-transfected cells. The results are summarized in Table 1. The percentage of cells undergoing DNA replication was only slightly lower in RB-expressing Saos2 and 5637 cells compared to cells that were RB⁻. Moreover, such differences were diminished 96 hr after RB plasmid transfection, when the total number of RB⁺ cells in the mass cultures dropped to 2–5% (data not shown).

FIG. 1. Immunocytochemical staining of RB nuclear protein. (*Upper*) Saos2 cell mass cultures 48 hr after transfection with the mock expression plasmid (*a*), RB expression plasmid pCMV-f-RB35 (*b*), and RB expression plasmid p β A-f-RB33 (*c*). Cells were also counterstained with Mayer's hematoxylin to compare the cellular morphology. ($\times 400$.) (*Lower*) Long-term clones and xenograft tumors of RB-reconstituted WERI-Rb27 retinoblastoma cells. (*a*) Parental WERI-Rb27. (*b*) RB⁺ clone WERI-27R20 cells prior to subcutaneous injection. (*c*) RB⁺ clone WERI-27R20 cells attached on polylysine-coated coverslips and extracted *in situ* by hypotonic buffer containing 0.1% Triton X-100 before fixation, showing the exogenous RB protein was able to form a tight nuclear association (26). (*d* and *e*) Paraffin sections of xenograft tumors R20T1 and R20T3, respectively. Tumors were excised 1 month after injection of WERI-27R20 cells. Note that only $\approx 5\%$ of R20T3 tumor cells were stained RB positively. (*f*) Cell culture reestablished from the mouse tumor R20T3 shown in *e* in regular growth medium containing no geneticin. (*a-c* and *f*, $\times 1000$; *d* and *e*, $\times 400$.)

Table 1. RB protein expression and DNA synthesis of RB-defective Saos2 and 5637 cells after transfection with RB expression plasmids

Recipient cells	Plasmids	Cells expressing RB protein, %	Cells incorporating [³ H]thymidine, %	
			RB ⁺	RB ⁻
Saos2	pCMV-f-RB35	11	19	32
	pβA-f-RB33	8	28	40
5637	pCMV-f-RB35	14	21	38
	pβA-f-RB33	12	34	45

Forty-eight hours after transfection, the cells were labeled with [³H]thymidine and then stained for nuclear RB protein with monoclonal anti-RB antibody, mAb-1 and autoradiographed as described (22, 24). About 1000 RB⁺ or RB⁻ cells were counted for each determination. Each entry represents at least two independent experiments.

Furthermore, 7 of 48 geneticin-resistant Saos2 clones derived from transfection with the RB expression plasmid pCMV-f-RB35 were found to express the exogenous RB protein. We have also isolated four stable RB⁺ clones from the bladder carcinoma cell line 5637 transfected with the same RB plasmid. In fact, expression of RB protein in these various types of RB-reconstituted clones was assessed over a 4-month to 2-year period of continuous culture by direct Western blotting and immunocytochemical staining. The results will be discussed later in more detail. These clones, however, were indistinguishable from their parental cells in terms of morphology (data not shown) and growth rate (Fig. 2; data not shown).

Functional RB Protein Expression in Stable Clones of the RB-Reconstituted Tumor Cells. We have previously described a RB⁺ long-term mass culture, WERI-27R, which was derived from the RB-transduced retinoblastoma cell line WERI-Rb27 (12). It has been reported that intraocular inoculation of WERI-27R cells produced xenograft tumors in nude mice after a longer latency period (12). In our present study, ≈30% of the single cell clones isolated from this mass culture were RB⁺ as determined by the presence of normal RB protein nuclear staining. Ten random RB⁺ clones were grown for further analysis. Eight of the 10 WERI-27R clones have stably expressed the normal-sized RB proteins over a 2-year period of continuous culture. On Western immunoblots, the normal RB proteins comprised unphosphorylated and phosphorylated forms as illustrated in Fig. 3A. The majority of these clonal WERI-27R cells, ranging from 80%

FIG. 2. Effect of RB gene replacement on cell growth of the osteosarcoma cell line Saos2. Parental Saos2 (◇), the RB-reconstituted RB⁺ clone Saos2EC35.1 (□), and Saos2EC35.3 (Δ) as well as the RB⁻ revertant Saos2EC35.2F3 (○) were seeded at a concentration of 1×10^5 cells per well in six-well tissue culture plates and three wells were assayed each day for 6 days. Average log cell number is plotted vs. time in days after seeding.

to 95%, were stained RB positively in the exponentially growing cultures (for example, see Fig. 1 Lower b). Moreover, the exogenous RB protein expressed in the RB-reconstituted WERI-27R clones tended to form a tight association with the nuclear structure, as evidenced by the fact that the RB staining was retained in most of the nuclei after *in situ* Triton X-100/low-salt buffer extraction before fixation (Fig. 1 Lower c). It is notable that 1 of the 10 clones, WERI-27R3, expressed the intact RB protein at an extremely low level as detected by immunocytochemical staining (data not shown). Another clone, WERI-27R7, lost RB expression ≈9 months after its isolation.

We next tested the viral oncoprotein-binding function of the RB protein. As shown in Fig. 3B, the p110^{RB} extracted from a representative RB-reconstituted clone WERI-27R20 was able to assemble a specific complex with SV40 large tumor antigen (T antigen), just as the natural RB protein did (10).

Finally, we have used the PCR single-strand conformation polymorphism (SSCP) technique (27) to screen for small mutations and point mutations in the integrated, exogenous RB cDNA sequences from the genomic DNA of these RB-reconstituted WERI-27R clones. Fourteen pairs of oligonucleotide primers were synthesized to cover the full length of the RB cDNA insert. Each PCR-amplified fragment was ≈250 bp (ranging from 176 to 337 bp). A typical result of the PCR-SSCP analysis is demonstrated in Fig. 4, in which the PCR-amplified fragment was 337 bp. No mutation of the exogenous RB cDNA was found in any of the RB⁺ WERI-27R clones screened, including the clone WERI-27R3, which

FIG. 3. Expression of the functional RB protein in long-term clones of RB-reconstituted retinoblastoma cell line WERI-Rb27. (A) Direct Western immunoblotting. Lanes: 1, normal human fibroblasts (WI-38); 2, parental WERI-Rb27 cells; 3–5, RB⁺ clones WERI-27R10, -R43, and -R20, respectively; 6, RB⁻ revertant clone WERI-27R7; 7, pooled RB⁺ WERI-27R clones; 8–10, cell cultures reestablished from xenograft tumors R20T1, R20T2, and R20T3, respectively. These tumors were obtained from subcutaneous injection of WERI-27R20. (B) Complex formation of the clone WERI-27R20-expressed RB protein with SV40 T antigen (T-Ag). Lanes: 1, cell lysate of clone WERI-27R20; 2, purified T antigen; 3, coprecipitation of T antigen with p110^{RB} from the mixture of WERI-27R20 cell lysate and T antigen by anti-T antibody pAb419. The blot was subsequently incubated with both pAb419 (Oncogene Science) and anti-RB antibody mAb-1 (Ciba-Corning Diagnostics, Triton Products).

FIG. 4. PCR-SSCP analysis of *RB* cDNA coding region in the long-term RB-reconstituted WERI-Rb27 clones. Genomic DNAs from the pooled RB⁺ WERI-Rb27 clones (lane 3); the individual RB⁺ clones WERI-27R10 (lane 4); R43 (lane 5); R3, which expressed the extremely low level of RB protein (lane 6); R20 (lane 7); and the xenograft tumors R20T1 (lane 8) and R20T3 (lane 9) were amplified by using primers RB12S (5'-GAACATATCATCTGGACCC-3') and RB12AS (5'-TGAGGTATTGGTGACAAGG-3') in the presence of [α -³²P]dATP. cDNAs synthesized by reverse transcriptase from total cellular RNAs of normal human fibroblasts, WI-38 (lane 1), and small cell lung carcinoma cell line H209 (lane 2) were also amplified by using the same pair of primers as controls. The H209 harbored a point mutation at codon 706. The PCR-amplified fragment was 337 bp. The PCR products were analyzed on 6% nondenaturing polyacrylamide gel containing 10% (vol/vol) glycerol.

expressed the extremely low level of the intact RB protein (lane 6).

The comprehensive studies described in this section have also been used to characterize the RB⁺ clones derived from the bladder carcinoma cell line 5637 and the osteosarcoma cell line Saos2 after transfection with the RB expression plasmids, and identical results were obtained (data not shown). We therefore conclude that the great majority of these long-term RB⁺ clones obtained by RB replacement in our present study expressed the intact, functional RB protein.

Tumorigenicity of the Stable Clones of the RB-Reconstituted Tumor Cells. As described above, all the RB⁺ clones obtained from the RB-reconstituted WERI-Rb27, Saos2, and

5637 tumor cells had quite similar morphology and growth rates in culture when compared to the corresponding parental cell lines. We have also assessed their neoplastic phenotypes *in vivo* by using the nude mice tumorigenicity assay. The results are summarized in Table 2. In general, xenograft tumor formation in nude mice was considerably suppressed from these single cell clones or pooled clones stably expressing the functional RB protein. The only exception was that the RB⁺ clone WERI-27R3, which expressed a very low level of the intact RB protein, was as tumorigenic as the parental WERI-Rb27 and the real RB⁻ revertant clone WERI-27R7 (Table 2).

On the other hand, the majority of the RB⁺ clones isolated still formed tumors in nude mice after a longer latency period (Table 2). When these xenograft tumors were excised from the nude mice, it was found that they all expressed normal human RB protein as analyzed by Western blotting and immunohistochemical staining in paraffin-embedded tumor sections. Examples of the results are shown in Figs. 1 *Lower d* and 3, lanes 8–10. By histopathological analysis, these RB⁺ tumors were malignant and frequently invasive (Fig. 1 *Lower d* and *e*).

All the xenograft tumors showed a heterogeneous RB staining pattern (24) in which a significant number of tumor cells had RB nuclear staining ranging from 70% to 95% (Fig. 1 *Lower d*). Of particular interest was that, occasionally, there were only 5–10% of tumor cells having RB⁺ staining in the pathological sections of xenograft tumors formed after injection of the RB⁺ single cell clones (Fig. 1 *Lower e*), whereas >90% of the cells in the exponentially growing cultures before injection had prominent RB nuclear staining (Fig. 1 *Lower b*). Surprisingly, when such tumors were excised and reestablished in growth medium containing no geneticin, the great majority of the tumor cells regained the typical RB nuclear staining after 2 weeks of continuous culture, indicating that tumor cells even in such xenografts were actually RB⁺ (Fig. 1 *Lower f*). There were no detectable mutations of the RB protein coding region in these tumor cells by PCR-SSCP analysis (Fig. 4, lanes 8 and 9).

Table 2. Incomplete suppression of tumor formation in nude mice by stable expression of the functional RB protein in retinoblastoma, osteosarcoma, and bladder carcinoma cell lines

Cell line	RB protein expression	Mice with tumor/ mice injected*	Average latency, days	Average tumor size, mm ³
10 ⁵ cells injected intraocularly				
WERI-Rb27	–	9/9	16.2	63.3
WERI-27R12	–	5/5	19.2	42.3
WERI-27R10	+	3/9	26.3	17.6
WERI-27R43	+	4/9	29.3	25.6
2 × 10 ⁷ cells injected subcutaneously				
WERI-Rb27	–	5/5	13.6	770
WERI-27R7	–	10/10	11.2	810
WERI-27R3	+ [†]	10/10	15.0	695
WERI-27R20	+	3/12	19.7	220
WERI-27R, pooled [‡]	+	3/10	17.1	215
WERI-27R20T [§]	+	18/30	13.1	784
5 × 10 ⁶ cells injected subcutaneously				
Saos2	–	10/10	10.3	90.8
Saos2R, pooled [‡]	+	4/10	20.4	34.5
5637	–	8/10	8.4	84.7
5637R, pooled [‡]	+	2/10	34.2	18.0

*Tumors were scored at 1 month, except for 5637 and 5637R, in which tumor formation was scored 2 months after injection.

[†]Clone WERI-27R3 expressed an extremely low level of the intact RB protein, and no point mutation of the RB cDNA insert was detected by PCR-SSCP from this RB-reconstituted cell line.

[‡]Mixed with equal numbers of cells from four to eight RB⁺ single cell clones of the indicated RB-reconstituted tumor cell line.

[§]Clone WERI-27R20T is a subline reestablished from a RB⁺ nude mouse tumor after subcutaneous injection of the clone WERI-27R20.

Finally, RB⁺ sublines reestablished from RB⁺ nude mouse tumors were more tumorigenic than their RB⁺ parental counterparts (for example, see Table 2, WERI-27R20T vs. WERI-27R20).

DISCUSSION

In our present study, the effect of functional RB protein expression on cell growth, morphology, and tumorigenicity was assessed in RB-defective tumor cell lines from a diverse group of human cancers. In general, the study suggests that the wild-type RB-mediated tumor suppression in RB-defective tumor cells is substantial, but often incomplete, and the RB replacement appeared to have no major effect on cell growth and morphology of the RB-reconstituted tumor cells studied. The RB cDNA used in this study came from three independent sources (12, 15, 21) and there were no mutations of the RB cDNA coding region detectable from genomic DNAs of the cloned RB-reconstituted tumor cells in which expression of the intact functional RB protein was also verified by protein size, phosphorylation, SV40 T-antigen binding, and nuclear tethering (10).

The RB-mediated partial (incomplete) tumor suppression may reflect the biological heterogeneity of the tumor cell population—i.e., some tumor cells in a given RB-defective tumor cell population are resistant to the tumor suppression function rendered by RB gene replacement. We believe that a fundamental aspect of tumor cell biology providing the basis for this phenomenon is the fact that the RB gene may play a role in tumor development of only a portion of the tumor cells—e.g., in bladder, lung, prostate, and breast carcinomas. Furthermore, even though the RB gene may play a critical role in the initiation of all or the majority of the tumor cells—e.g., in retinoblastomas and osteosarcomas—these tumor cells are likely to acquire additional random and/or specific genetic defects late in tumor progression. In general, some tumor cells in a given tumor cell population may have inherited or acquired the ability to surmount the complex cellular growth regulatory pathway(s) in which the RB gene normally functions or the ability simply to inactivate the RB gene product by hyperphosphorylation.

Consequently, as demonstrated in our present studies, a significant percentage of the RB-reconstituted tumor cells still formed tumors in the nude mouse assay. Such tumors, although retaining normal RB expression, were histologically malignant and invasive. This phenomenon is referred to by us as tumor suppressor resistance (TSR). Since inactivation of the RB gene is restricted to a subset of human tumors, in a broad sense, besides a portion of the RB-defective tumor cells, other tumors with the normal endogenous RB gene probably have also inherited or acquired TSR for RB. In fact, TSR could be a general concept extending to cover other tumor suppressor genes. For instance, p53 gene-mediated tumor suppression may be abrogated by changing the protein conformation in a certain cellular environment. The TSR phenotype of the tumor cells may be encoded by amplified/activated oncogenes, tumor suppressor-associated protein genes, or other cellular growth regulatory genes in the signal transduction pathway. Identification of the respective TSR genes for each tumor suppressor gene should provide real insight into the varied mechanisms of tumor suppression.

Finally, from a practical point of view, the present study suggests that RB gene resistance may reduce the efficacy of the emerging RB gene therapy for human cancers. New

strategies, therefore, would be required to improve the potential treatment. One approach to overcome the tumor suppressor resistance is to create a modified RB gene with enhanced tumor suppression function, and this has provided encouraging results (H.-J.X., K.X., Y.Z., J.L., W.F.B., and S.-X.H., unpublished data).

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