Expression of the \textit{RB} Gene under the Control of MuLV-LTR Suppresses Tumorigenicity of WERI-Rb-27 Retinoblastoma Cells in Immunodefective Mice\textsuperscript{1}

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Abstract
Retinoblastomas arise by the loss of the retinoblastoma (\textit{RB}) gene (1). The isolation of the \textit{RB} gene and its expression in \textit{RB} protein defective tumor cells permits direct tests of the ability of the protein to act as a tumor suppressor. We demonstrate that a functional \textit{RB} gene introduced into WERI-Rb-27 retinoblastoma cells by retroviralmediated gene transfer can suppress their tumorigenicity in immunodefective mice.

Introduction
Structural changes leading to the inactivation of both alleles of the \textit{RB} gene represent an essential step in the genesis of the tumor (1, 2). In the familial form of retinoblastoma, a defective \textit{RB-1} allele is inherited (1). The second allele is lost during somatic development by a variety of cytogenetic mechanisms, such as the loss of normal chromosome 13 with or without the duplication of its defective homologue by somatic crossing over or interstitial deletion (2). Cytogenetically invisible deletions or mutations may be responsible as well. Retinoblastoma patients cured of their primary tumors often develop osteosarcoma at a later age (3). The osteosarcoma cells also lack functional \textit{RB} genes. It is therefore generally believed that the loss of the second \textit{RB} gene represents an essential rate limiting event for the development of retinoblastoma and osteosarcoma in persons who inherited a mutated \textit{RB} gene (4).

The isolation of the retinoblastoma gene has permitted the detailed analysis of the genetic defect and its functional consequences on the protein level (5–7). It has become possible to test the hypothesis that the tumorigenicity of retinoblastoma and osteosarcoma cells is dependent on the lack of \textit{RB} protein with the use of direct reconstitution experiments. In the first reported experiment of this type, W.-H. Lee’s group (8) found that a retrovirally expressed \textit{RB} cDNA\textsuperscript{2} suppressed the tumorigenicity of \textit{RB} defective WERI-Rb-27 retinoblastoma cells in nude mice. Although this was consistent with the hypothesis that the malignant behavior of these tumors is dependent on the lack of a functional \textit{RB} gene, it was not uncontroversial. The delay of confirmatory reports from other laboratories on successful expression of exogenous \textit{RB} gene in transfected tumor cells raised skepticism about the feasibility of suppressing the tumorigenic phenotype, in a sufficiently large proportion of the transfected cells, to make an impact at the population level.

The purpose of our study was to examine the claim of W.-H. Lee and his coworkers by essentially repeating their experiments.

Results
The human retinoblastoma line WERI-Rb-27 was infected with the \textit{RB} and LUX viruses, respectively. The infection of 10\textsuperscript{7} cells by the PA12 supernatants (40 ml) was performed in the presence of Polybrene (4 \mu{g/ml}) for 32 h. The cells were collected at 4-h intervals by centrifugation and subsequently resuspended in 40 ml virus containing PA12 supernatant. Selection on 1 mg/ml (active concentration) of G418 was started immediately after infection. The cell density was 0.25 \times 10\textsuperscript{5}/ml at the beginning of the selection. In parallel with the retrovirus infected cells, unmanipulated WERI-Rb-27 cells were also grown in the presence of 1 mg/ml G418, starting at the same initial cell density. They were killed by G418 invariably after 16 days.

The LUX virus infected cells grew into dense cultures in the presence of G418 after 24 days. They were morphologically unchanged compared to the original parental cells. In the \textit{RB} virus infected cultures, larger and more flattened cells appeared in the course of the G418 selection and became the dominating cell type after 4 weeks. In order to test whether the \textit{RB} virus infected WERI-Rb-27 cells expressed a normal sized \textit{RB} protein, 10\textsuperscript{5} cells were lysed and exposed to anti-\textit{RB} IgG antibody in order to precipitate the \textit{RB} protein, and the immunoprecipitate was analyzed by Western blotting. A normal sized \textit{RB} protein was readily detectable in the \textit{RB} virus infected but not the LUX virus infected cells (Fig. 1). Its level was comparable to the human lymphoma lines BJAB and Ramos that had an intact \textit{RB} gene (Fig. 1). The tumorigenicity of the unmanipulated, LUX virus and \textit{RB} virus infected retinoblastoma cells was compared in immunodefective and nude mice, respectively.

The effect of the \textit{RB} gene reconstitution on tumorigenicity was tested in five independent experiments. Each experiment involved the preparation of new virus stocks, new infection and selection, and assessment of \textit{RB} protein expression. The results are summarized in Table 1. The parental retinoblastoma cells formed large, progressively growing tumors in immunodefective mice within 20 days after s.c. inoculation of 10\textsuperscript{3} cells. In nude mice, the tumors grew out only after 4–5 weeks. The

\textsuperscript{1} This work was supported by Inga-Britt och Arne Lundbergs Forskningsstiftelse and by National Cancer Institute Grant 5K01CA14054. J. S. and E. U. are recipients of fellowships from the Cancer Research Institute and Concern Foundation.

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\textsuperscript{4} The abbreviations used are: cDNA, complementary DNA; SDS, sodium dodecyl sulfate; FCS, fetal calf serum.
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(Experiment 5), and 1 (Experiment 5), presence of pp110 RB protein in the WERI-Rb-27 retinoblastoma cells infected with RB virus and used in the tumorigenicity test. reconstituted retinoblastoma cells that expressed the RB protein failed to form tumors over an observation period of 2–3 months in a total of 31 mice inoculated with 10\(^7\) cells (Table 1).

**Discussion**

These data confirm the earlier report of Huang et al. (8) in showing that the reconstitution of RB deficient WERI-Rb-27 retinoblastoma tumor cells with a functional RB gene suppresses their tumorigenicity. Our data corroborate their conclusion that the tumorigenicity of RB defective retinoblastoma and osteosarcoma cells can be suppressed by the expression of an artificially induced, but fully expressed, RB protein. This fact is more puzzling than it appears at first sight, however. Selection for a drug resistance marker, linked to a gene of interest, does not usually lead to the expression of the second gene in 100% of the cells. Since we have only selected for G418 resistance, this must either imply that the RB gene was regularly expressed in all drug resistant cells or, alternatively, that expression in less than 100% of the cells is sufficient to suppress tumorigenicity.

The latter possibility, that the gene would be expressed in 100% of the reconstituted cells, is also suggested by the consideration that variants that have lost the gene would have an immediate selective advantage. Such variants would be expected to arise equally readily as their counterpart, nontumorigenic revertants from cultures transformed by a single retroviraly carried oncogene (9). We have actually found that the loss of expression of normal sized RB protein during 90 days of in vitro cultivation of RB reconstituted WERI-Rb-27 cells was accompanied by the reappearance of tumorigenicity (data not shown). One might expect that this would also happen after the inoculation of the reconstituted cells in the immunodefective mice. The fact that it did not happen in vivo suggests either an unusually high stability of the retrovirally introduced construct, and perhaps inte-
igration of more than one exogenous RB gene, or, alternatively, the possibility that the exogenous RB gene may not have to be expressed in 100% of the cells in order to suppress tumorigenicity at the population level.

Inhibition of a small number of tumor cells by a majority of nontumor cells may occur, in principle, in several different ways. Involvement of the host immune mechanism is unlikely, since the tests were done in immuno-defective mice. Still, it cannot be completely excluded, since the mice were mainly defective with regard to T-cells. Conceivable immunological models can be derived from the experimental demonstration of neighboring normal cells acting through diffusible substances and/or intercellular contacts (10).

The mechanism by which the RB protein suppresses the tumorigenic phenotype at the level of the individual cell remains another, open question. Since the reconstituted cells appear to remain immortalized in vitro but fail to grow in vivo, terminal differentiation in response to the appropriate host signals is one possibility. A precedent can be found in the terminal differentiation of in vitro immortal HeLa cell/keratinocyte hybrids into squamous cell pearls upon in vivo inoculation (11). This possibility will be investigated by histological examination of the inoculation sites in the present system.

Materials and Methods

Cells. WERI-Rb-27 retinoblastoma and two amphotropic retrovirus producing PA12 cell lines were obtained from Dr. W.-H. Lee (8). The retrovirus vectors produced by the PA12 cell lines carried the cDNA of the human RB gene or the cDNA of the firefly luciferase gene, respectively, and were designated as PA12-RB and PA12-LUX (8). The PA12-RB produced human RB protein detected by immunoprecipitation and Western blot analysis (data not shown). The virus titer of the PA12-RB supernatant was 1–2 x 10^8/ml; the PA12-LUX produced about 5 times more virus.

The cells were grown in RPMI 1640 supplemented with 10% FCS.

Preparation of Immunosuppressed Mice. The immuno-defective mice were prepared by thymectomy at the age of 3–4 weeks followed by i.p. injection of 200 mg/ml cytisine arabinoside 3–5 weeks later. Forty-eight to 72 h after the cytisine arabinoside treatment, the mice were irradiated with 735 Gy X-rays. Twenty-four h after irradiation, the mice were inoculated s.c. in the lower flank with a single inoculum of cells from growing cultures in a total volume of 0.2 ml. Such immunosuppressed mice are more receptive to heterografted tumors than marrow grafted or nude mice (12).

Immunoprecipitation and Western Blot. Cellular extracts were prepared from 10^7 cells. They were washed twice with phosphate-buffered saline and collected by centrifugation. After the last wash and centrifugation, the cells were resuspended in 1 ml of lysis buffer (25 mM Tris-HCl, pH 7.4-5 mM NaCl-0.5% sodium deoxycholate-0.1% SDS-0.2% Nonidet P-40-0.05 mg/ml aprotinin-0.05 mg/ml leupeptin). The cell lysates were kept on ice for 15 min, vigorously vortexed every 5 min, and then centrifuged in a microcentrifuge for 15 min at 14,000 rpm at 4°C. To the supernatants, 35 μl of anti-RB, a polyclonal rabbit anti-human RB antibody (received from W.-H. Lee), were added, and the samples were incubated for 1 h at 4°C. Then 100 μl of Protein A-Sepharose (Pharmacia) were added, and the incubation was continued for an additional 1 h at 4°C. The extract was continuously mixed using a rotator. After 1 h, the samples were centrifuged in a microcentrifuge for 2 min at 14,000 rpm, and the supernatants were carefully removed. The Protein A-Sepharose antibody-antigen complex was mixed with an equal volume of twice-concentrated electrophoresis sample solution (20 mM Tris-HCl, pH 7.0–5 mM EDTA-4% SDS-20% glycerol-10% mercaptoethanol-0.5 mg/ml bromphenol blue). Each sample was electrophoresed through a 7.5% polyacrylamide-SDS gel. After electrophoresis, the proteins were transferred to a nitrocellulose filter using a Bio-Rad trans-blot apparatus. After transfer, the membrane was washed overnight in blocking B solution (25 mM Tris-HCl, pH 8.0–125 mM NaCl-0.1% Tween 20–4% nonfat milk powder). The next morning, the nitrocellulose filter was incubated with mouse monoclonal anti-human RB antibody (received from W.-H. Lee) in blocking B for 3 h at room temperature. This was followed by two 30-min washes in blocking B. A 1:3000 dilution (in blocking B) of the second antibody, a goat anti-mouse IgG alkaline phosphatase conjugate (Bio-Rad), was then incubated with the membrane for 45 min at room temperature. The nitrocellulose membrane was washed extensively with blocking B three times for 15 min at room temperature and once with blocking B without nonfat milk powder. Finally, the membrane was incubated in a buffer (100 mM Tris-HCl, pH 9.5–100 mM NaCl-5 mM MgCl2) containing 165 μg/ml of 5-bromo-4-chloro-3-indolyl phosphate and 330 μg/ml nitro blue tetrazolium (Promega Biotec). The specific pp110RB bands become visible after 15 min of incubation at room temperature. The membrane was washed, dried, and photographed.

Acknowledgments

We thank Dr. Wen-Hwa Lee for the LUX and RB retroviruses and for the RB antibodies.

References
