Hybrids were prepared between Y79 retinoblastoma (RB) and nonmalignant NIH3T3 cells and studied to confirm the presumed recessiveness of RB. Twenty hybrids containing both of the dominant gene markers, pSV2neo and pSV2GPT, initially transfected into the parent cells were isolated. All of the hybrids showed a fibroblastic morphology and anchorage-dependent growth. None of the tested hybrids or the parent NIH3T3 cells showed growth in soft agar; the parent RB cells showed a 15% growth in soft agar. The results indicate suppression of the malignant phenotype in the hybrids, confirming the recessiveness of the malignant phenotype of RB at the cellular functional level. Karyotyping of selected hybrids and the parent cells indicated a cumulative representation of all of the Y79 chromosomes in the hybrids, excluding loss of a specific Y79 chromosome causing the suppression of malignancy. Northern analysis of RNA from the hybrids demonstrated the mRNA of the reported putative mouse RB gene, consistent with the complementation of the Y79 RB gene defect by the normal mouse RB gene in the hybrids. Such a complementation may be a factor in the suppression of the malignant phenotype. Interestingly, the abnormal Y79 RB mRNA was absent in the hybrids, suggesting a possible negative feedback control by the normal mouse RB gene product.
of RB as recessive cancer, was confirmed in the hybrids. Expression of the normal murine RB gene and an interesting modulation of the expression of the abnormal Y79 RB gene were observed.

Materials and Methods

Materials

The Y79 RB and NIH3T3 were the original cell lines used. The neomycin (NEO)-resistant plasmid, pSV2neo, and the bacterial xanthine-guanine phosphoribosyltransferase (GPT) plasmid, pSV2GPT, were used as dominant gene markers. Cell culture reagents were obtained from Gibco (Gaithersburg, MD), chemicals from Sigma (St. Louis, MO), and restriction enzymes from Bethesda Research Laboratory (Gaithersburg, MD).

Cell Culture

The RB-NEO and RB cells were cultured in RPMI-1640 containing 15% fetal calf serum, 200 units/ml penicillin, 200 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (RB media) with or without NEO selection, respectively, in dishes treated with poly-D-lysine and fibronectin to obtain attachment cultures. The NIH3T3-GPT and NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium containing 15% fetal calf serum, 200 units/ml penicillin, 200 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (NIH3T3 media) with or without GPT selection.

Preparation of Cell Lines Containing Dominant Gene Markers

The RB-NEO cells were prepared by transfection of Y79 RB cells with the NEO-resistance marker pSV2neo and subsequent isolation in RB media containing 0.5 mg/ml NEO (G418). The NIH3T3-GPT cells were prepared by transfection of the NIH3T3 cells with the bacterial GPT gene marker pSV2GPT and subsequent isolation in NIH3T3 media containing 1 mM L-glutamine, 0.025 mg/ml mycophenolic acid, 0.001 mg/ml thymidine, 0.002 mg/ml aminopterin, 0.25 mg/ml xanthine, and 0.015 mg/ml hypoxanthine.

Preparation and Isolation of Hybrids

Hybrids were prepared by growing the NIH3T3-GPT cells, 6 x 10^4 to a 100-mm dish for 3 days, adding the same number of RB-NEO cells, continuing the culture in a 1:1 mixture of the NIH3T3 and RB media for 3 more days, and then fusing the cells using polyethylene glycol. The treated cells were cultured in a double-selection media containing NEO, aminopterin, and mycophenolic acid as described, and the doubly resistant hybrids were isolated using a cylinder and subcloned. Cells found suspended in the liquid media after fusion were also collected over time and observed under double selection to check for the presence of anchorage-independent hybrids.

Southern Blot Hybridization Analysis

Genomic DNAs were isolated from the cell lines and digested with EcoRI. They underwent electrophoresis in 0.8% agarose gel, were transferred onto nitrocellulose or nylon filters by the Southern blot method, and were hybridized to 32P-labeled DNA probes. The probes were excised pSV2neo, pSV2GPT, or the human RB probe (pGEM1-4.5Rb, a gift from Dr. W. H. Lee). The hybridized filters were washed and autoradiographed as described.

Analysis of the Cell Lines for Growth in Soft Agar

For determination of growth in soft agar, 10^2-10^3 cells in 0.3% agar were grown on a bed of 0.5% agar in 100-mm dishes as described by others, with a weekly feeding of 0.3% agar containing the appropriate media. Visible colonies were counted after 3 weeks.

Northern Analysis

Total RNA was isolated from cells using guanidine thiocyanate. The samples underwent electrophoresis in denaturing agarose gel, were transferred onto nylon membranes by the Southern blot method, and were used for northern hybridization with 32P-labeled DNA probe. The hybridized blots were washed and autoradiographed as described.

Results

The Y79 RB cell line was established from a tumor removed from a 2½-year-old patient with a family history of RB. The Y79 cells are tumorigenic in nude mice. No visible deletion is present in the q14 region of chromosome 13 in the Y79 RB, but a small deletion that eliminates exons 2-6 of the RB gene has been demonstrated in one of the alleles at the molecular level. The Y79 cells are small, round, undifferentiated cells that grow in suspension naturally but can be made to attach to the surface of a culture.
dish when grown in a dish treated with fibronectin and poly-D-lysine. Attachment cultures were used routinely to facilitate close observation and manipulation of these cells. In such cultures, the Y79 cells grow in a monolayer, in clusters, or in chains with occasional rosettes (Fig. 1A).

The choice of cells to fuse with the Y79 cells to test for RB gene complementation was problematic. Human embryonic retinal cells might have been the most appropriate cells for this experiment, but they are extremely difficult to obtain. Even if obtainable, their viability in culture (especially after fusion) is questionable. Rodent cells have been used to fuse with human tumor cells to study malignancy, and the advantage of interspecies hybrids in the study of malignancy, especially with respect to tracing chromosomes, has been described. Therefore, we chose NIH3T3 mouse cells for fusion with the Y79 RB cells. The NIH3T3 cells are embryonic mouse fibroblasts that show an anchorage-dependent, strongly contact-inhibited pattern of growth in culture (Fig. 1B) and are nontumorigenic in animals.

To facilitate isolation of the hybrids between the Y79 RB and NIH3T3 cells, dominant gene markers were put into these cells to allow selection of cells containing both markers. Accordingly, the NEO-resistance marker pSV2neo and the bacterial xanthine GPT marker pSV2GPT were transfected into the Y79 and NIH3T3 cells, respectively. A clone of Y79 cells that had chromosomally integrated the pSV2neo marker was isolated by selection with NEO and named RB-NEO. Similarly, a clone of NIH3T3 cells that had chromosomally integrated the pSV2GPT marker was isolated by selection with media containing aminopterin and mycophenolic acid and named 3T3-GPT. The presence of the pSV2neo and pSV2GPT sequences in the hybrids was confirmed by a genomic Southern blot analysis using the excised pSV2neo and pSV2GPT as probes. The result indicates the presence of NEO-containing (phosphotransferase; 6.3- and 5.3-kilobase pair fragments) and GPT-containing (7.5-kilobase pair fragment) sequences in the genomes of the hybrids (Fig. 2, lanes 1-7) in agreement with their phenotypes.

Having confirmed our cellular clones as hybrids at the phenotypic and genomic DNA levels, we next analyzed the growth characteristics of the hybrids and the parents. Analysis of these cells for growth in soft agar was most useful to identify the transformed phenotype. The RB-NEO cells, which grow in suspension naturally and are malignant, formed rapidly growing, visible colonies in soft agar at a rate of 15% (Table 1). None of the tested hybrids or 3T3-GPT cells were able to grow in soft agar to form colonies even after 3 weeks of culturing at high concentration (10^6 cells in a 100-mm dish), confirming their nonmalignant phenotype.

The parent RB-NEO and 3T3-GPT cells, along with five hybrid clones, also underwent karyotypic analysis. The karyotype of the 3T3-GPT cells was identical to the original NIH3T3 cells. The RB-NEO cells contained specific chromosomal changes identifying them as the Y79 NH-77 subline of the original Y79 cell line. Chromosomal analysis of the hybrids indicated that a loss of some Y79 human chromosomes had taken place in each of the hybrids analyzed as expected for human–rodent somatic hybrids (Ta-
To determine the state of expression of the reported putative RB gene\textsuperscript{10-11,20} in the hybrids, RNA was isolated from the hybrids and the parental cell lines and
TABLE 1. Cell growth in soft agar

<table>
<thead>
<tr>
<th>Cells</th>
<th>Percentage of colony formation in soft agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>RB</td>
<td>15</td>
</tr>
<tr>
<td>3T3</td>
<td>=0.001</td>
</tr>
<tr>
<td>Hybrid 1</td>
<td>=0.001</td>
</tr>
<tr>
<td>Hybrid 2</td>
<td>=0.001</td>
</tr>
<tr>
<td>Hybrid 3</td>
<td>=0.001</td>
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<tr>
<td>Hybrid 4</td>
<td>=0.001</td>
</tr>
<tr>
<td>Hybrid 5</td>
<td>=0.001</td>
</tr>
<tr>
<td>Hybrid 6</td>
<td>=0.001</td>
</tr>
<tr>
<td>Hybrid 7</td>
<td>=0.001</td>
</tr>
</tbody>
</table>

NIH3T3 and all of the hybrids (Fig. 3A). In the Y79 lane, a faint band around 5 kilobases was present, corresponding to nonspecific hybridization of the 28s ribosomal RNA, since there is no RB mRNA of this size in Y79. Analysis with a human RB probe demonstrated the expression of the abnormal human 4-kilobase RB mRNA (normal, 4.7) in the Y79 cell line and apparent absence of this message in all of the hybrids (Fig. 3B). The mouse RB mRNA did not show much cross hybridization with the human probe. To determine if the absence of the abnormal human RB mRNA in the hybrids was a result of loss of the human RB gene on chromosome 13 due to elimination of the chromosome from the rodent–human hybrid, genomic DNA from the hybrids and parental cells underwent Southern blot analysis. Analysis using the human RB probe at low stringency demonstrated the pattern of the RB gene bands in the 3T3 and Y79 parental cells and evidence of both patterns being intact in all of the hybrids (Fig. 4).

Discussion

Since the first demonstration in 1961 of somatic cell hybrids between mouse fibroblasts possessing varying malignant phenotype, the cell fusion technique has often been used to study the nature of malignancy at the cellular level. The general scheme in these studies has been to fuse a malignant cell with a nonmalignant or less-malignant cell and examine the result of the interaction of the two genotypes at the phenotypic level in the hybrids. The results of the early normal–malignant hybrid studies indicated that the hybrids are malignant in phenotype suggesting dominance of the malignant genotype. Later studi...
ies demonstrated, however, that suppression of the malignant phenotype can be seen frequently in such hybrids, giving rise to the concept of recessive cancer genes.\textsuperscript{24-27} The importance of the chromosomal content of hybrids in interpreting the hybrid phenotype also became well known since fusions between certain cell types result in a high degree of chromosome segregation and loss.\textsuperscript{24,31}

Despite the abundance of data consistent with the presumed recessiveness of RB, functional confirmation of this point using the classic somatic cell hybrid technique has not been reported. The use of cell fusion to confirm the presumed recessiveness of RB is appropriate in that the concept of dominance and recessiveness of cancer was derived from malignant-normal hybrid studies.\textsuperscript{4,24,52,53} Our results support the recessiveness of RB at the cellular level. All 20 of the original hybrids showed anchorage-dependent growth, and all 7 hybrids tested along with the parent NIH3T3 cells showed no growth in soft agar. The malignant parent Y79 RB cells showed good growth in soft agar. No hybrids were isolated that showed anchorage-independent growth or growth in soft agar after the initial fusion. Although in vitro phenotypic criteria of malignancy can be ambiguous at times, the ability to grow in soft agar is considered the most reliable criterion of a transformed phenotype.\textsuperscript{47,54,55} It is noteworthy that the fibroblastic morphology of the hybrids is essentially that of the nonmalignant parent NIH3T3 cells, a pattern usually observed when a malignant phenotype is suppressed in a normal-malignant hybrid.\textsuperscript{56}
The use of NIH3T3 cells in our fusion experiment was necessitated partly by the unavailability of the most logical model tissue, normal human embryonic retina, but the results obtained with NIH3T3 indicate that interspecies complementation of the RB gene defect in Y79 RB can occur, assuming that the malignant phenotype is suppressed by this mechanism. Studies showing modulation or suppression of malignancy in human–rodent hybrids have been reported, and the gene(s) that is complemented in these cases, including RB, is most likely an important regulatory gene of growth and/or differentiation that is highly conserved among species. This is consistent with the high degree of conservation observed in the RB gene among species, specifically up to 95% between mouse and human. The fact that none of our original hybrids had a malignant phenotype indicates that a dominant oncogene, such as ras, which can transform NIH3T3 cells most likely does not play a primary role in the malignant transformation of Y79 RB. The use of interspecies hybrids in our experiment also allowed us to monitor the status of chromosomal segregation in the hybrids; this is critical for a correct interpretation of the results. Although variable loss of Y79 human chromosomes was observed in each of the hybrids analyzed, all of the human chromosomes including chromosome 13 are represented in the hybrids, excluding the possibility that the suppression of malignancy in our hybrids was due to a loss of a specific human chromosomal gene.

Our results with the hybrids are similar to those obtained by others who showed suppression of tumorigenicity of RB cells (WERI-Rb27) after transfection and expression of the normal RB gene in them. They did not examine their RB-transfected WERI cells for growth in soft agar. The original WERI RB cells do not grow in soft agar. Presumably, the RB gene defect in the WERI cells is being complemented by the transfected RB gene, similar to the putative interspecies complementation in our hybrids. These authors noted, however, a relatively high frequency of malignant revertants among their RB-transfected cells. The original WERI RB cells were viable because suppression was not complete without additional gene(s).

Our demonstration of expression of the mouse RB mRNA in the hybrids was consistent with the putulated complementation of the RB gene defect of Y79 by the normal mouse RB gene of NIH3T3. The apparent disappearance of the abnormal human RB mRNA in the hybrids was interesting. Since the abnormal RB message is stable in the original Y79 cell, the data suggested possible destruction of the message or cessation of expression of the message in the hybrids. The expression of the abnormal RB gene may have become suppressed at the transcriptional level by the normal mouse RB gene product in the hybrids by a negative feedback control. Such a negative feedback control, also at the transcriptional level, was found for the oncogenes, c-fos and c-myc. Presumably, the suppression of the malignant phenotype in the hybrids was not related to the disappearance of the abnormal RB message since the latter does not produce an abnormal RB protein which might conceivably interfere with normal functions.

Key words: Y79 retinoblastoma, NIH3T3, hybrids, suppression, malignancy, RB gene

Acknowledgments

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