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NEWLY ESTABLISHED HUMAN RETINOBLASTOMA CELL LINES EXHIBIT AN "IMMORTALIZED" BUT NOT AN INVASIVE PHENOTYPE *IN VITRO*

Sabine GRIEGEL[1], Chen HONG[1], Roland FRÖTSCHL[3,4], Dieter F. HÜLSER[3], Valerie GREGER[2], Bernhard HORSTHEMKE[2] and Manfred F. RAJEWSKY[1,5]

[1]*Institut für Zellbiologie (Tumorforschung) and* [2]*Institut für Humangenetik, Universitätsklinikum Essen, Hufeland-Strasse 55, D-4300 Essen 1; and* [3]*Abteilung Biophysik, Biologisches Institut, Universität Stuttgart, Pfaffenwaldring 57, D-7000 Stuttgart 80, FRG.*

Retinoblastoma (RB), an intraocular childhood tumor occurring in a hereditary (mostly bilateral) or non-hereditary (unilateral) form, is associated with the inactivation of both alleles of a putative tumor suppressor gene (RB-1) located on chromosome 13q14. Both the process of RB development and the biological characteristics of RB cells are as yet poorly understood. We have established 7 new RBL lines (RBL13, RBL14, RBL18 and RBL30, derived from unilateral RB; and RBL7, RBL15 and RBL20, derived from bilateral RB). Southern blot analyses of restriction fragment length polymorphisms in DNA samples from 6 cell lines revealed loss of constitutional heterozygosity at one or several polymorphic loci on chromosome 13 in 4 cases. Gross deletions involving the RB-1 locus and amplification of the N-*myc* gene were not detected in any of the RBL lines. The phenotypic properties of the RBL lines were analyzed in comparison with cells from the original RB tumors, with 4 RB lines established by others (RB383, RB355, RB247C3 and Y79) and with the adenovirus-E1A-transformed human retinoblast line HER-*Xho*1-CC2. It was found that RB tumors consist of phenotypically heterogeneous cell subpopulations with varying nutrient requirements and differentiation potential *in vitro*. All cell lines showed the typical characteristics of established ("immortalized") cells. In some cases, cells from original RB tumors or cell lines were able to form colonies when cell aggregates of 2–10 cells were suspended in semi-solid agar medium; however, anchorage-independent colonies never developed from single cells. Cell lines RBL13, RBL18, RB247C3, RB355, RB383 and Y79 were tested for invasion into embryonic chick heart fragments *in vitro* and found to be non-invasive. None of the RBL or RB lines were tumorigenic in nu/nu (T^-) mice. Y79 cells (propagated in culture for many years) exhibited properties distinctly different from those of the other cell lines, and thus cannot be considered phenotypically representative of RB cells.

Retinoblastoma (RB) is the most common intra-ocular neoplasm of childhood, appearing in most cases during the first year of age with a frequency of 1 in about 15,000 births. About 40% of RBs are caused by germ-cell mutations, and predisposition to the development of RB is transmitted as an autosomal dominant trait (Schappert-Kimmijser *et al.*, 1966). These patients usually develop multiple tumor foci in both eyes (bilateral RB) and have a strongly increased risk of development of second primary cancers, particularly osteogenic sarcoma (Matsunaga, 1980). In contrast, the non-hereditary form of RB typically affects only one eye (unilateral RB). Tumor formation is assumed to be initiated by the inactivation of both alleles of a putative tumor suppressor gene (RB-1) located on chromosome band 13q14 (Benedict *et al.*, 1983; Cavenee *et al.*, 1983). The RB-1 gene has been molecularly cloned (Friend *et al.*, 1986; Lee *et al.*, 1987) and its product (p105-RB) is currently being characterized with respect to its role in the normal cell (Whyte *et al.*, 1988).

Contrary to the genetic background of RB, little is known about the process of RB development and about the phenotypic and functional properties of RB cells. While it has been notoriously difficult to grow RB cells *in vitro* for prolonged periods, 2 groups have described methods of establishing cell lines from RB tumors (Gallie *et al.*, 1982; Bogenmann and Mark, 1983). Contrasting with most other malignant cell lines, RB cells exhibit some very characteristic properties *in vitro*, *e.g.* the formation of 3-dimensional aggregates in suspension culture. Although the cell type(s) of origin of RB has not been unequivocally identified, recent evidence suggests that RB may be derived from a primitive neuroectodermal precursor cell capable of differentiating into various neural cell types present in the embryonic and mature retina (Chader, 1987; Detrick *et al.*, 1988).

Under cell culture conditions, RB cells sometimes tend toward spontaneous differentiation to Flexner-Wintersteiner rosettes. Light- and electron-microscopic examinations revealed that the cells in these organized structures have many characteristics of mature photoreceptor cells, such as polarized shape, a cilium, and sometimes stacks of lamellated membranes (Bogenmann, 1986; Reid *et al.*, 1974; T'so *et al.*, 1970). Cell cloning has so far only been possible with the RB line Y79 (Reid *et al.*, 1974). This line was established 15 years ago, and Y79 cells are often used as prototype cells to describe phenotypic properties of RB cells (Gentleman *et al.*, 1985; Ueda *et al.*, 1980; Amy and Bartholomew, 1987). Y79 cells form colonies in semi-solid agar medium (Reid *et al.*, 1974) and produce rapidly growing tumors in nu/nu (T^-) mice after subcutaneous (s.c.) injection of high cell numbers (Gallie *et al.*, 1977). Cells isolated from primary RB tumors proliferate *in vivo* after intra-ocular implantation to the anterior eye chamber of nu/nu (T^-) mice, but only Y79 cells can also invade the orbit, the optic nerve and the brain (Gallie *et al.*, 1977).

In order to analyze in more detail some of the phenotypic and functional characteristics of RB cells, we have established 7 new cell lines (RBL lines) from 4 unilateral and 3 bilateral RB tumors. The properties of these RBL lines were compared with those of the cells of their tumors of origin, with 4 RB lines previously established by others (Gallie *et al.*, 1982; Reid *et al.*, 1974), and with the adenovirus-E1A-transformed human embryonal retinoblast line HER-*Xho*1-CC2 (Vaessen *et al.*, 1986). In particular, we investigated whether RB cells exhibit invasive properties in an *in vitro* assay for invasion into chick heart fragments (Mareel *et al.*, 1979). Our results show that RB tumors consist of heterogeneous cell subpopulations in

[4]Present address: Institut für Viruskrankheiten der Pflanzen, Biologische Bundesanstalt, Messe-Weg 11-12, D-3300 Braunschweig, FRG.

[5]To whom reprint requests should be addressed, at Institut für Zellbiologie (Tumorforschung), Universität Essen, Hufeland-Strasse 55, D-4300 Essen 1, FRG.

Abbreviations: FCS, fetal calf serum; RB, retinoblastoma; s.c., subcutaneous(ly); PDGF, platelet-derived growth factor; EGF, epidermal-derived growth factor.

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terms of varying nutrient requirements, cellular differentiation potential, and stage in the process of malignant transformation. The newly established RBL lines proved to be non-invasive in the *in vitro* assay and exhibited typical properties of established ("immortalized") cells rather than those characteristic of malignant (invasive) cells.

MATERIAL AND METHODS

DNA analysis

Total genomic DNA from peripheral blood cells, tumor specimens and cells of the RBL lines was isolated as described by Kunkel *et al.* (1977) and digested with the appropriate restriction enzymes. DNA fragments were separated by gel electrophoresis, transferred to DURALON-UV nylon membranes (Stratagene, La Jolla, CA) and hybridized with ^{32}P-labelled DNA probes (Horsthemke *et al.*, 1987). Filters were exposed at −70°C to Fuji RX film with a Dupont Lightning Plus intensifying screen for 1 day. To detect mutations at the RB-1 locus, the DNA was digested with *Hind*III and probed with the 0.9 kb and 3.8 kb *Eco*RI fragments of the RB4.7 cDNA clone (Friend *et al.*, 1986). In some experiments, a 0.6-kb *Hpa*I-*Eco*RI fragment of the 0.9-kb probe was also used. Loss of constitutional heterozygosity at the RB-1 locus and other chromosome 13 loci was investigated with the help of the following DNA polymorphisms [locus/probe/enzyme(s)]: (RB-1/p123/*Bam*HI); (RB-1/p88/*Xba*I); (RB-1/p68/*Rsa*I); (RB-1/p35/*Asp*1; Wiggs *et al.*, 1988); (ESD/ESD14.1.1/*Ban*II; Squire *et al.*, 1986*a*); (D13S11/pG2E3.1/*Pst*I, *Msp*I; Scheffer *et al.*, 1986); (D13S1/p7F12/*Msp*I, *Taq*I); (D13S3/p9A7/*Msp*I, *Hind*III); (D13S2/p9D11/*Msp*I, *Taq*I); (D13S4/p1E8/*Msp*I; Cavenee *et al.*, 1984); (D13S5/pHUB8/*Eco*RI, *Hind*III); (D13S6/pHU10/*Xmn*I, *Eco*RI); and (D13S7/pHU26/*Bgl*II; Dryja *et al.*, 1984).

To test for amplification of the N-*myc* gene, DNA samples were digested with *Eco*RI and probed with the 1-kb *Eco*RII-*Bam*H_{II} fragment of the pNB-1 (N-*myc*) plasmid. DNA samples from cell lines Y79 and RB355, and from cell lines RB247 and RB383, respectively, served as positive and negative controls (Squire *et al.*, 1986*b*).

Tumors

Primary tumor material was obtained from hemisected eyes and immediately transferred into ice-cold "standard medium" (Gallie *et al.*, 1982), *i.e.*, Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% heat-inactivated fetal calf serum (FCS; Sebak, Aidenbach, FRG), penicillin (100 units/ml), streptomycin (100 μg/ml), D-glucose (4.5 mg/ml), insulin (Sigma, Deisenhofen, FRG; 10 μg/ml), and 2-mercaptoethanol (5×10^{-5} M). The tissue was minced with surgical blades and further dissociated by pipetting. Necrotic material and cell debris were removed by 3 successive sedimentation steps at 1 *g*. The last cell pellet was split into aliquots, and the samples were either used immediately for cell culture (see below) or frozen (after addition of 10% dimethylsulfoxide) by lowering the temperature at a rate of 1°C per min with a Scientific Biological Freezer (Sy Lab, Burkersdorf, Austria) and thereafter stored in liquid nitrogen.

RBL cell lines

Seven new cell lines (RBL lines) were established from RB tumors (Table I). At the time of the present analyses, these RBL lines had been maintained as established (proliferative) cell lines for 4–18 months.

Three RB lines (RB383 and RB355, both derived from unilateral tumors; and RB247C3, originating from a bilateral tumor) were kindly provided by Dr. B. Gallie (Hospital for Sick Children, Toronto, Canada; Gallie *et al.*, 1982). The RB cell line Y79 (Reid *et al.*, 1974) was obtained from the American Type Culture Collection (ATCC HTB 18; Rockville, MD). Cell line HER-*Xho*1-CC2 (Vaessen *et al.*, 1986) was a gift from Dr. A.J. van der Eb (Department of Medical Biochemistry, The Sylvius Laboratories, State University of Leiden, Leiden, The Netherlands).

Cell culture

In order to establish the RBL lines, 2×10^7 cells were seeded into T25 Falcon culture flasks in 2 independent experiments for each RB tumor. In cultures of one type, RB cells were grown on feeder layers of normal human fibroblasts (see below) in "primary medium", *i.e.*, standard medium containing, instead of FCS, 15% heat-inactivated human serum from known donors (isolated as described by Bogenmann and Mark, 1983), and supplemented with L-glutamine (4 mM), 1% non-essential amino acids (Flow, Meckenheim, FRG), transferrin (Sigma; 1 μg/ml), and oxalacetic acid (10 mM). In some experiments, pyruvate (1 mM), platelet-derived growth factor (PDGF; Seromed/Biochrome, Berlin, FRG; 0.25 units/ml), and epidermal growth factor (EGF; Boehringer, Mannheim, FRG; 10 ng/ml) were added to the medium. In cultures of the second type, cells were seeded at the same density into standard medium without feeder layers. During the first week of culture, the spent medium was replaced by fresh medium at 2-day intervals every second day, and thereafter twice weekly. After the RBL lines had become established in primary medium, cells were adapted to standard medium in a stepwise manner.

Feeder layers

Monolayers of human fibroblasts (passages 4–6) were grown in T25 Falcon flasks and mitotically arrested by mitomycin-C (Sigma; 100 μg/ml; 15 min; 37°C), followed by extensive washings with standard medium. The cells were used as feeder layers at the earliest 4 weeks after mitomycin-C treatment. In a number of experiments, fibroblast feeder layers were omitted, but the surface of the culture flasks was coated with type-1 gelatine from pig skin (Sigma; 2% w/v), poly-L-lysine (Sigma; 0.2 mg/ml), poly-L-ornithine (Sigma; 0.1 mg/ml), human fi-

TABLE I – CHARACTERISTICS OF 7 NEWLY ESTABLISHED HUMAN RETINOBLASTOMA CELL LINES (RBL LINES)

Designation of cell line	Type of original RB tumor	Age of patient at surgery (months)	Population doubling time *in vitro* (days)	Flexner-Wintersteiner rosettes	Cell line requires "primary medium" and feeder layer	Duration of cultivation phase 2 (months)[1]
RBL7	Bilateral	16	6	+	+	4.5
RBL13	Unilateral	18	6	−	−	−
RBL14	Unilateral	23	10	−	−	−
RBL15	Bilateral	11	8	+	+	6
RBL18	Unilateral	15	9	+	−	−
RBL20	Bilateral	16	14	−	+	1
RBL30	Unilateral	30	6	+	+	3.5

[1]See text.

bronectin (Sigma; 5 μg/ml) or ECM membrane, a natural membrane-like substratum (Product Review, 1984).

Cell population doubling times in vitro

To determine cell-population doubling times in culture, 5×10^5 cells of each line were seeded into T25 Falcon flasks. Cells from 2 flasks per time point were harvested and counted by hemocytometer twice weekly over a period of 2 months.

Colony formation in semi-solid agar medium

Cloning experiments were performed using suspensions either of single cells or of small aggregates of 2–10 cells by gently passing large aggregates 1–3 times through a 21-gauge needle. Cells excluding Trypan blue were counted in a hemocytometer. Colony formation frequencies (either from 10^2, 10^3 and 10^4 single cells per 40 ml-flask, or from the same numbers of 2–10 cell aggregates per flask) were determined as described by Laerum and Rajewsky (1975), using primary medium, and standard medium, containing 0.15% agar (Noble agar; Difco, Detroit, MI). Colonies with diameters >0.5 mm were counted after 4–8 weeks of incubation (Table II). Alternative attempts to improve plating efficiencies and clonogenicity included the use of (i) serum-impoverished DMEM for mature neural cells (ffrench-Constant and Raff, 1986) with and without addition of growth factors (see above); (ii) serum-depleted, hormone-containing B27 medium (Lang and Brunner, 1983); (iii) 2 layers of agar medium with different agar concentrations (lower layer, 0.5%; upper layer, 0.3%) and a combination of FCS and horse serum (Inomata *et al.*, 1986); (iv) methylcellulose (Sigma; 0.96%), 20% FCS, and 20% medium conditioned by phytohemagglutinin-stimulated leukocytes (Ito *et al.*, 1987); (v) FGF (Boehringer; 75 ng/ml; Rizzino and Ruff, 1986); (vi) 1,α,25-dihydroxyvitamin-D3 (10–100 nM; kindly provided by Dr. N. Huh, Institute of Medical Science, University of Tokyo, Tokyo, Japan; Huh *et al.*, 1987); (vii) medium conditioned by log-phase Y79 cells (50%); or (viii) X-irradiated (10 Gy) Y79 feeder cells interspersed between the RB cells in the semi-solid agar medium.

Tumorigenicity tests

Different numbers ($1–8 \times 10^6$) of log-phase cells (RBL lines, RB lines, Y79 cells, and HER-*Xho*1-CC2 cells), washed twice and resuspended in 0.1 ml of Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline, were injected s.c. into the flancs of NIH Swiss nu/nu (T^-) mice. The animals were checked for tumors during a period of ≥8 weeks.

Invasiveness of RB cells into embryonic chick heart fragments in vitro

Six lines (RBL13, RBL18, RB247C3, RB355, RB383 and Y79 cells) were tested for the capacity of cells to invade embryonic chick heart muscle fragments *in vitro* (Mareel *et al.*, 1979). Pre-cultured heart fragments were confronted with RB cell aggregates (Bräuner and Hülser, 1990). The rat mammary carcinoma-derived cell line BICR/M1R_k (Rajewsky and Grüneisen, 1972) was used as a positive control. In cases where stable attachment of cells to the heart fragments could not be achieved, the fragments were covered with RB cell aggregates in stationary culture. Histological sections of the heart fragments were analyzed for the presence of invasive RB cells after 93, 120, 140, and 166 h of culture.

RESULTS

DNA analyses of RB tumors and RBL lines

Seven primary RB tumors were used in the present study. Four of these, from which cell lines RBL13, RBL14, RBL18, and RBL30 were established, were unilateral tumors. Three tumors, giving rise to cell lines RBL7, RBL15, and RBL20, were bilateral (Table I). DNA samples from peripheral blood cells, fresh tumor material (except from RB tumor 7), and the RBL lines were used to investigate molecular mechanisms responsible for inactivation of the RB-1 gene. Loss of constitutional heterozygosity at one or several polymorphic loci on chromosome 13 was detected in 4 RB tumors and the corresponding RBL lines (RBL14, RBL18, RBL20 and RBL30). RB tumor 13 and the corresponding cell line RBL13 main-

TABLE II – CAPACITY OF RB CELLS TO FORM COLONIES IN SEMI-SOLID AGAR MEDIUM: FREQUENCY VALUES FOR DIFFERENT RBL LINES AND THE CORRESPONDING PRIMARY RB TUMOR CELLS, AND FOR THE ESTABLISHED HUMAN RETINOBLAST CELL LINE HER-*Xho*1-CC2 (VAESSEN *ET AL.*, 1986)

Designation of cell lines	Frequency of colony formation (%)	Average diameter of colonies (mm)	Time[3] (weeks)
Y79	30	2.0	4
Y79a[1]	100	1.5	4
Y79b[1]	12	1.5	4
Y79c[1]	18	1.5	4
RB247C3	5	1.0	6
RB383	0.1	0.5	6
RB355	2.5	0.5	6
RBL7	$<1 \times 10^{-4}$	NCD	8
RBL13[2]	1×10^{-3}	1.5	8
RBL13	0.25	1.0	8
RB13a[1]	5×10^{-2}	0.5	8
RB13b[1]	0.1	0.5	8
RB14[2]	$<1 \times 10^{-4}$	NCD	8
RBL14	$<1 \times 10^{-4}$	NCD	8
RB15[2]	1×10^{-3}	0.5	8
RBL15	0.2	0.5	8
RB18[2]	$<1 \times 10^{-4}$	NCD	8
RBL18	$<1 \times 10^{-4}$	NCD	8
RB20[2]	$<1 \times 10^{-4}$	NCD	8
RBL20	$<1 \times 10^{-4}$	NCD	8
RB30[2]	$<1 \times 10^{-4}$	NCD	8
RBL30	$<1 \times 10^{-4}$	NCD	8
HER-*Xho*1-CC2	$<1 \times 10^{-4}$	NCD	8

[1]Sublines established from colonies formed in semi-solid agar medium.–[2]Primary cells from RB tumors.–[3]Time of cultivation after which colonies were counted. NCD: No colonies detected. For experimental details, see "Material and Methods".

tained heterozygosity. RB tumor 15 showed partial loss of heterozygosity (the intensity of one allelic band was markedly reduced, Fig. 1*a*). The corresponding cell line was heterozygous.

In order to detect mutations at the RB-1 locus, DNA from both the RB tumors and the corresponding RBL lines were analysed by Southern blot hybridization with the RB-1 cDNA probe. No gross structural alterations of the RB-1 gene were detected (not shown). Tentative evidence for a point mutation was obtained in tumor RB30. Using the 0.6-kb *Hpa*I-*Eco*RI subfragment of the cDNA (Friend *et al.*, 1986), which detects exons 3–8 (McGee *et al.*, 1989), an abnormal 12-kb *Taq*I band was found in the tumor DNA that was not present in blood cell DNA. On the other hand, the normal 10-kb band was missing (Fig. 1). Digestion with *Hind*III, *Eco*RI, *Xba*I or *Sac*I did not reveal any abnormal bands, suggesting that the first mutation was a point mutation within a TaqI site, and that the second gene copy was lost by mitotic non-disjunction or recombination. The recognition site for *Taq*I contains the dinucleotide CpG which appears to be a "hot spot" for point mutations (Cooper and Youssoufian, 1988).

Amplification of the N-*myc* gene was not detected by the pNB-1 (N-*myc*) probe in any of the RBL lines (Southern blots not shown).

Establishment of RBL lines

The 7 RBL lines were established by seeding cells from the corresponding primary RB tumors onto fibroblast feeder layers in "primary medium". RB cell aggregates and single cells attached firmly within 24 hr. The period of establishment of the RBL lines could be subdivided into 3 successive stages:

During the first 2 weeks of cultivation, all single cells and many of the aggregated cells died. Addition of pyruvate, PDGF or EGF, to the culture medium had no supportive effect on cell viability or proliferation. Similarly, the use of ECM membranes, gelatine from pig skin, human fibronectin, poly-L-lysine, or poly-L-ornithine, instead of fibroblast feeder layers, failed to give better results.

During the second stage of cultivation, cell numbers remained constant without evidence of further cell death or cell proliferation. The duration of this stage was 4 weeks to nearly 6 months, depending on the individual RB tumor (Table I).

During the third stage of cultivation, cell aggregates began to round up. They could easily be shaken off and transferred to cell culture flasks without feeder layers. The cells then generally began to proliferate continuously as established RBL lines in suspension culture. Growth curves revealed population doubling times of 6 to 14 days (Table I).

In parallel, we also attempted to establish RBL lines in "standard medium" without feeder layer. In most cases RB cells were rapidly overgrown by fibroblasts and glial cells, and died within 6 weeks of cultivation. Exceptionally, cells from RB tumors 13, 14 and 18 started proliferation in suspension culture under these conditions after 2 weeks.

Phenotypic characteristics of cell lines

With the exception of line RB355 (Gallie *et al.*, 1982), most of whose cells attach to the cell culture flask, all other lines, including the newly established RBL lines, grow in suspension. The cultured cells have retained the same morphology and exhibit the same 3-dimensional aggregate formation as the primary cells from RB tumors when these are first examined *in vitro*. Cell aggregates are composed of 10 to >100 cells with an anaplastic appearance and a high nuclear/cytoplasmic ratio (Fig. 2). Disaggregation of these structures to single-cell suspensions reduced cell viability: either cells died within 1 week or cell aggregates developed again over a period of 2 to 3 months before proliferative activity resumed.

Since malignant cells often multiply even at reduced serum concentrations and neural cells prefer a low serum content in the culture medium, we tried to cultivate the lines in a serum-impoverished medium specially developed for mature neural cells and their progenitors (ffrench-Constant and Raff, 1986), or in serum-depleted, hormone-containing B27 medium (Lang and Brunner, 1983). With the exception of Y79 cells, the cells of all other lines died within 6 weeks of culture. Addition of PDGF or EGF had no supportive effect on viability or proliferation of the cell lines. Y79 cells showed a prolonged popu-

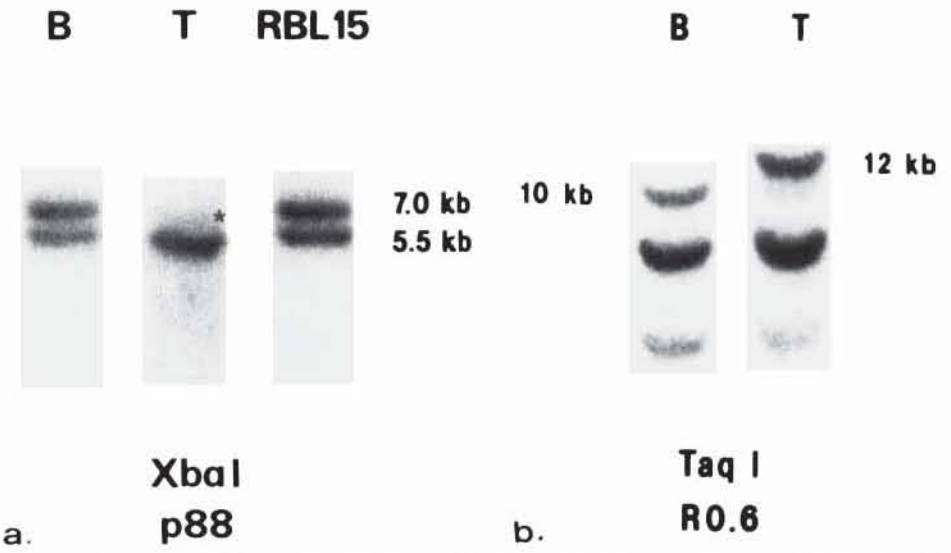

FIGURE 1 – *(a)* DNA polymorphism analysis of cell line RBL15. DNA isolated from peripheral blood cells (B), primary RB15 tumor material (T), and from RBL15 cells, was digested with *Xba*I and probed with the intragenic RB-1 fragment p88 (Wiggs *et al.*, 1988). In the tumor the intensity of the upper allelic band is markedly reduced. The RBL15 line is heterozygous. *(b)* Southern blot analysis of the RB-1 gene in the tumor of origin of cell line RBL30. DNA isolated from peripheral blood cells of the patient (B) and primary tumor material (T) were digested with *Taq*I and probed with the 0.6-kb *Hpa*I-*Eco*RI fragment of RB-1 cDNA. An abnormal 12 kb is present in the tumor DNA, while the normal 10-kb band is missing.

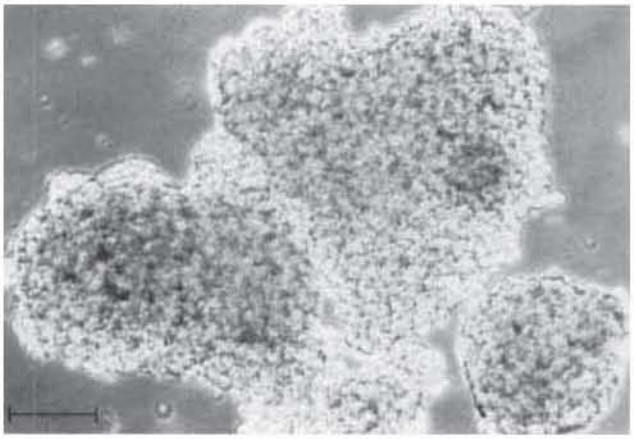

FIGURE 2 – Characteristic cell aggregates in a suspension culture of retinoblastoma cell line RBL13 (phase-contrast; bar = 100 μm).

lation doubling time of 68 hr, but still proliferated continuously.

In 4 of the 7 RBL lines (RBL7, RBL15 and RBL18) the formation of Flexner-Wintersteiner rosettes was observed during the second stage of cultivation (Table I), *i.e.*, when the aggregates were still attached to the feeder layers (Fig. 3). With continued cultivation, up to 90% of cells participated in the formation of these structures. After prolonged cultivation, line RBL7 lost its ability to form rosettes; however, lines RBL15 and RBL18 have retained this ability also as suspension cultures for more than 2 years.

Colony formation in semi-solid agar medium

Colony formation was obtained with cell lines Y79, RB247C3, RB355, RB383, RBL13 and RBL15 when small aggregates of 2–10 cells were used as "starters" (Table II). Under these conditions the line Y79 (established 15 years ago; Reid *et al.*, 1974) exhibited a high frequency of colony formation (approx. 30%) after 4 weeks of incubation. The lines RB247C3, RB355, RB383 (established more than 7 years ago; Gallie *et al.*, 1982) exhibited low frequencies (approx. 0.1–5%) within a period of 6 weeks, as often found with malignant human cell lines (Grofova *et al.*, 1987; Hamburger and Salmon, 1977). Like the human embryonal retinoblast line HER-*Xho*1-CC2 (Vaessen *et al.*, 1986), the RBL lines, after cultivation for 4–18 months, exhibited very low frequencies of colony formation, or developed no agar colonies at all even after prolonged incubation periods of ≥8 weeks (Table II).

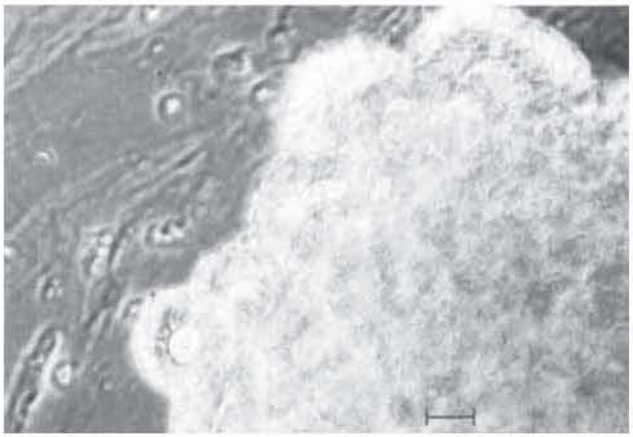

FIGURE 3 – Flexner-Wintersteiner rosettes in cell aggregates of retinoblastoma cell line RBL7 after 3 months of cultivation on a feeder layer of human fibroblasts (phase-contrast; bar = 200 μm).

When small aggregates of primary cells from RB tumors 13 and 15 were suspended in semi-solid agar medium, they formed colonies at very low frequencies ($2–10 \times 10^{-4}$). Passage of these colonies in semi-solid agar medium was possible for up to 4 months (longer intervals not tested). Colonies of Y79 cells and cells from RB tumor 13 were isolated (Y79a, Y79b, Y79c, RB13a, RB13b), grown in liquid culture for 4 weeks, and replated in semi-solid agar medium. However, the cell aggregates of these sublines exhibited the same low colony formation frequencies as their cells of origin. Only aggregates of Y79a cells exhibited a colony formation frequency of 100% in semi-solid agar medium.

Colony formation was never observed after single cells had been seeded from any of the cell lines into semi-solid agar medium. To improve culture conditions in semi-solid agar medium in order to achieve higher frequencies of clonability and colony formation of single RB cells and small aggregrates, several published methods were tested (Inomata *et al.*, 1986; Ito *et al.*, 1987; Rizzino and Ruff, 1986; Huh *et al.*, 1987; ffrench-Constant and Raff, 1986; Lang and Brunner, 1983). Neither the application of 2 differentially concentrated agar layers with a combination of FCS and horse serum, nor the use of methylcellulose instead of agar and supplementation with phytohemagglutinin-stimulated leukocyte-conditioned medium improved the efficiency of colony formation. FGF and 1,α,25-dihydroxyvitamin D3, both reported to increase colony formation in semi-solid agar medium, also had no effect. The use of a serum-impoverished special medium for neurons (with and without the addition of PDGF or EGF), a serum-depleted, hormone-containing B27 medium, addition of Y79-conditioned medium (which probably contains an activity promoting the proliferation of these cells), or of X-irradiated Y79c cells interspersed as feeder cells in the semi-solid agar medium were equally unsuccessful.

Tumorigenicity in nu/nu (T^-) mice

When varying cell numbers of the present cell lines, the semi-solid agar sublines (cultured in liquid medium for 4 weeks), and the HER-*Xho*1-CC2 line (Vaessen *et al.*, 1986) were injected s.c. into the flanks of nu/nu (T^-) mice, no tumors were detected during an observation period of ≥8 weeks.

Invasiveness into chick heart fragments

This assay is based on the confrontation of stable "spheroids" of potentially invasive tumor cells with precultured chick heart fragments *in vitro* (Mareel *et al.*, 1979). In the present experiments, the lines RBL13 and RBL18 could be grown as "spheroid-like" structures. While RBL13 and RBL18 cells attached firmly to the heart fragments, the aggregates of all other RB lines only formed loose contacts. In the case of the RB lines RB247C3, RB355, RB383 and cell line Y79, aggregates from the suspension cultures were cultured in close contact with the chick heart fragments. It is well established that the size of precultured aggregates of malignant cells does not significantly influence their ability to invade the adjacent heart tissue (de Ridder and Laerum, 1981). None of the lines tested exhibited any invasive potential in this assay (Fig. 4) even after prolonged cultivation for 166 hr. Invasion of BICR/M1R_k cells (which were used as positive controls) into the chick heart fragments could be detected at many sites after 65 hr. Pronounced destruction of the heart tissue by the invading tumor cells was apparent after 120 hr, and after 160 hr only the central part of the heart fragment remained (Bräuner and Hülser, 1990).

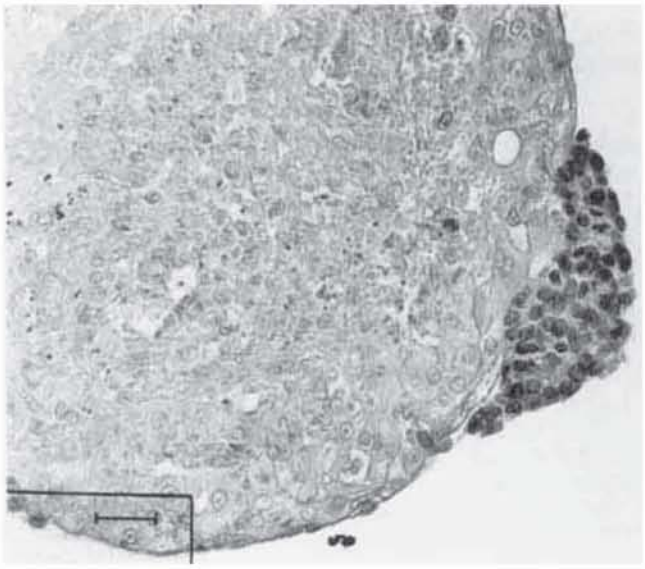

FIGURE 4 – Non-invasiveness of human retinoblastoma cells in the embryonic chick heart fragment *in vitro* assay (Mareel *et al.*, 1979). Confrontation culture of an RBL18 cell "spheroid" with an embryonic chick heart fragment after 120 h of incubation (phase-contrast; bar = 25 μm).

DISCUSSION

Loss of RB-1 gene function is considered to be causative in the development of RB. In 4 of 6 RB tumors and in the corresponding RBL lines, loss of heterozygosity was detected at one or several polymorphic loci on chromosome 13. In these cases one of the 2 genetic events was the loss of one RB allele by mitotic non-disjunction or recombination (Cavenee *et al.*, 1983). Tumor RB 13 is probably the result of 2 mutations at the homologous RB-1 loci. The case of RB tumor 15 is more complex. While this tumor showed partial loss of heterozygosity, the corresponding cell line RBL15 is heterozygous. This indicates that the tumor was heterogeneous and that the cell line is derived from cells that maintained heterozygosity.

The N-*myc* gene is sometimes amplified and overexpressed in neuroblastoma (Schwab *et al.*, 1984) and in RB cell lines (Amy and Bartholomew, 1987; Squire *et al.*, 1986*b*). It has, therefore, been suggested that the N-*myc* gene might play a role in RB tumorigenesis (Lee *et al.*, 1984). However, we did not detect N-*myc* amplification in any of our newly established RBL lines. As already observed by Squire *et al.* (1986*b*), amplification of N-*myc* in RB appears to be a rare event not obviously associated with initiation or progression of this tumor.

Our analyses indicate that RB tumors are composed of heterogeneous subpopulations of cells differing in their requirements for nutritive factors, their differentiation capacity, and their stage in the process of oncogenic transformation. Establishment of continuously proliferating RBL lines on feeder layers takes up to 6.5 month (in the longest case). It seems probable that, during this long period of *in vitro* cultivation, the cells either acquired additional properties supporting cell proliferation *in vitro*, *e.g.*, the production of (a) growth factor(s), or that selection for an initially small number of cells already possessing these characteristics occurred. The fact that the cells of 3 RB tumors (RB tumors 13, 14, 18) started proliferation in suspension culture after 2 weeks in "standard medium" is remarkable, suggesting that feeder layers and the addition of human serum and other supplements may not be absolute requirements for the establishment of all RBL lines. The need for aggregate formation for survival and proliferation of the RBL lines may reflect the situation *in vivo*. This can also be the reason why cloning of cells derived from RB tumors *in vitro*—with the exception of Y79 cells—has so far never been possible.

In 4 out of 7 RBL lines up to 90% of the cells temporarily participated in the formation of Flexner-Wintersteiner-rosettes, indicating that RB tumors originally consist of heterogeneous cell subpopulations with a different capacity for differentiation. The fact that this ability is retained in 2 RBL lines for more than 2 years points to the stability of the differentiation potential. In the case of Y79a the selection pressure probably led to the isolation of a subclone in which additional genetic events have resulted in a stable genetic alteration.

Most of the cells from RB tumors and from the corresponding RBL lines were found to be incapable of forming colonies in semi-solid agar medium. Only small subpopulations of primary RB cells exhibited a transient capacity for anchorage-independent proliferation. Thus, colony formation in semi-solid agar medium is not a characteristic property of RB cells but may rather be acquired, or selected for, during prolonged cultivation (*e.g.*, Y79 cells).

Contradictory results have been reported concerning the ability of RB cells to form tumors upon implantation into nu/nu (T^-) mice. In some studies, the formation of s.c. tumors has been described (Benedict *et al.*, 1980), as well as the possibility of multiplying RB cells in the anterior chamber of the eye (Benedict *et al.*, 1980). Other authors reported the survival of RB cell implants in the eyes of nu/nu mice, but after s.c. injection the cells were not tumorigenic or formed tumors only when the immunological defense of the recipients was further reduced by treatment with cyclophosphamide (Gallie *et al.*, 1977). To our knowledge, Y79 is the only RB cell line destroying the eye of the recipient mice and invading the orbit, optic nerve and brain (Gallie *et al.*, 1977). We could not detect any tumors after s.c. injection of varying cell numbers of the present cell lines in NIH Swiss nu/nu (T^-) mice. Tumorigenicity of RB cells in immune-deficient recipient animals is, therefore, at least equivocal.

The expression of more advanced neoplastic phenotypes by RB cells appears to be acquired, or selected for, during prolonged cultivation. The RB cell line Y79 exhibited exceptional properties in all of our experiments. While we found these cells to be incapable of forming tumors in nu/nu (T^-) mice, tumorigenicity has been reported by other authors who used higher cell numbers for implantation (Gallie *et al.*, 1977). It appears, therefore, that RB cell lines such as Y79 (or WERI; McFall *et al.*, 1977), which have been propagated for prolonged periods, have proceeded further toward the development of fully malignant phenotypes and thereby have acquired properties no longer characteristic of primary RB tumor cells or early RB-derived cell lines. Huang *et al.* (1988) have reported suppression of neoplastic properties following introduction of an intact RB-1 gene into human osteosarcoma cells and WERI cells. The indicator chosen to demonstrate suppression was the loss of tumorigenicity upon s.c. implantation into nu/nu (T^-) mice, a property which is not characteristic of RB cells.

All of the present RBL lines thus exhibited properties similar to the established retinoblast cell line HER-*Xho*1-CC2 (Vaessen *et al.*, 1986); *i.e.*, very long population doubling times; no proliferation under reduced serum conditions; very low frequencies or absence of colony formation in semi-solid agar medium (with instability of the cellular capacity to form colonies); lack of invasiveness into embryonic chick heart fragments *in vitro;* and inability to form tumors upon implantation into nu/nu (T^-) mice. How can this lack of properties frequently associated with malignant phenotypes be explained? Malignant tumors of the central nervous system are characterized by local destruction and invasion into adjacent tissue, but only rarely by metastasis. At the time of clinical detection, RB

tumors are usually restricted to the retina and vitreous cavity (in rare instances also to the anterior chamber of the eye), and metastases are rarely observed. Signs of tumor progression are invasion into the optic nerve or, via blood vessels, into the orbit, choroid, and sclera. It is not unlikely that the primary RB cells analyzed in the present study were still at a stage largely characterized by an increased proliferation potential in the absence of overt invasive properties. In other experimental systems, the acquisition of invasiveness (as assayed *in vitro*) was found to be a late event in the process of tumorigenesis, corresponding in time with the first observation of tumorigenicity upon re-implantation of the cells *in vivo* (de Ridder and Laerum, 1981; Messmer *et al.*, in press).

Whyte *et al.* (1988) reported that the E1A protein of adenovirus forms a complex with the Rb-1 gene product p105-RB, thereby probably inhibiting its function. P105-RB may thus be a critical effector element in a signalling pathway ensuring the response of cells (retinoblasts) to microenvironmental inhibitors of proliferation. Proliferation-competent retinal precursor cells unable to respond to these signals could thus be forced into unrestrained proliferation. A case in point is the human retinoblast cell line HER-*Xho*1-CC2 (Vaessen *et al.*, 1986). These embryonal retinoblasts were transformed to permanent proliferation ("immortalized") by introduction of an Ad5-E1A-containing plasmid. Based on these considerations, a model may be considered in which inactivation of both alleles of the "tumor suppressor gene" RB-1 primarily results in the unrestrained clonal proliferation of retinoblasts rather than in the expression of fully malignant phenotypes. Additional events (*e.g.*, gene or chromosome mutations, or gene amplification) may then be responsible for the emergence of RB cells with more advanced malignant properties (invasiveness, and sometimes metastasis).

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