Finite Life Span of Hybrids Formed by Fusion of Different Simian Virus 40-Immortalized Human Cell Lines

NOEL J. WHITAKER, ELSA L. KIDSTON, AND ROGER R. REDDEL*

*Children's Medical Research Foundation, P.O. Box 61, Camperdown,
New South Wales 2050, Australia*

Received 17 June 1991/Accepted 25 October 1991

Simian virus 40 (SV40) genes are able to induce immortalization of normal human cells after a culture crisis during which unknown cellular genetic changes presumably occur. To determine whether these genetic changes are always identical, we performed somatic cell hybridization analysis of an SV40-immortalized human bronchial epithelial cell line, BET-1A. Fusion of BET-1A with an SV40-immortalized fibroblast cell line resulted in hybrids that senesced, indicating that these cell lines are in different complementation groups for immortalization.

Immortalization is an important aspect of cancer cell biology. Whereas normal diploid mammalian cells have a finite life span in culture (3), tumors often contain cells that exhibit an apparently unlimited proliferative potential. In many experimental systems, immortalization appears to be an obligatory prerequisite for the induction of tumors (e.g., see references 6-8, 12, and 13). Human cells rarely, if ever, spontaneously immortalize in vitro, but transfer of genes from DNA tumor viruses (e.g., simian virus 40 [SV40] early region genes) into normal human cells sometimes induces immortalization (reviewed in reference 2). SV40 early region genes usually extend the in vitro life span of normal human cells, but immortalization occurs only in rare variant cells that grow out after a period of crisis. These genes are therefore not sufficient for immortalization, and presumably cellular genetic events are required for escape from crisis and thus immortalization. The nature of these putative genetic changes is currently unknown.

Somatic cell hybridization studies indicate that the immortal phenotype is recessive, since hybrids between normal and immortal cells undergo senescence (1, 5, 9). This technique has also been used to assign immortalized human cells to four complementation groups for immortality (referred to as groups A through D) (10). By definition, when cell lines from different complementation groups are fused, some or all of the hybrids undergo senescence, i.e., complementation occurs to yield the senescent phenotype. With the exception of one SV40-immortalized cell line derived from an individual with a DNA repair deficiency, all SV40-immortalized cell lines previously analyzed have been assigned to group A, which may imply that SV40 always induces immortalization via the same cellular genetic event(s) (10).

To use SV40-immortalized cells to study the processes of immortalization, it is necessary to determine whether SV40-immortalized cell lines may be found in other complementation groups. Here we report studies of a human bronchial epithelial cell line (BET-1A) that was established following transfection of normal human bronchial epithelial cells with a plasmid containing the SV40 early region genes (11). Fusion with representatives of each of the four immortalization complementation groups showed that BET-1A is not in complementation group A but is in group D. All the hybrids

continued to express the SV40 T-antigen (T-Ag) genes regardless of whether they eventually senesced or remained immortal, confirming that expression of these genes is not sufficient for immortalization. Further, some hybrid clones which remained immortal were suppressed for tumorigenicity, demonstrating that induction of senescence is not necessary for tumor suppression.

Cell lines. Universal hybridizer cell lines representative of each of the four complementation groups for immortalization (10) were kindly provided by O. M. Pereira-Smith, and were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum. The cell lines are GM847 (SV40-immortalized fibroblasts, group A), HeLaCOT (cervical carcinoma, group B), 143BTk⁻ (osteogenic carcinoma, group C), and A1698 (bladder carcinoma, group D). Each of these four lines is sensitive to hypoxanthine-aminopterin-thymidine (HAT) medium. 143BTk⁻ is G418 resistant, and the other three are ouabain resistant. The growth of BET-1A cells (11) is inhibited by serum, and therefore they were cultured in serum-free LHC-8 medium (4). In preliminary studies, it was found that LHC-8 medium supplemented with 3% chemically denatured serum (i.e., fetal bovine serum that had been denatured with reducing and acetylating reagents [14]) supported the long-term growth of BET-1A cells and the universal hybridizer cells. Therefore, all hybrids were

TABLE 1. Proliferative potential of cell hybrids formed by fusion of BET-1A cells with cell lines representative of immortalization complementation groups

Cell line (group) ^a	No. of senescing clones ^b	PD ^c before senescence (mean ± SD)	Days before appearance of segregants ^d (n)	PD/day for immortal clones or segregants (n)
GM847 (A)	21/21	18 ± 1	50, 150	0.19, 0.30
HeLaCOT (B)	24/36	20 ± 9	128 ± 82 (4)	0.22 ± 0.06 (16)
143BTk ⁻ (C)	18/18	18 ± 1	89, 98	0.26, 0.26
A1698 (D)	0/15	NA ^e	NA	0.60 ± 0.05 (15)

^a Cell lines representative of immortalization complementation groups A through D, to which BET-1A cells were hybridized.

^b Hybrid clones that ceased proliferation for at least 30 days.

^c PD, population doublings.

^d Days from cessation of proliferation (senescence) to appearance of segregant proliferating cells. Mean ± SD.

^e NA, not applicable.

* Corresponding author.

FIG. 1. Growth curves for hybrid clones. Fifteen to 36 hybrid clones were selected from fusions of BET-1A cells to cell lines representative of the four complementation groups for immortalization and were individually subcultured. Cumulative population doublings were calculated at each passage.

cultured in LHC-8-3% chemically denatured serum in culture flasks or dishes coated with fibronectin, collagen, and bovine serum albumin (4).

Cell hybridization and selection. Each of the universal hybridizer cell lines was plated at 2×10^6 cells per T75 coated flask, followed 4 h later by 2×10^6 BET-1A cells. After a further 4 h, the growth medium was removed and the cells were fused by adding 2 ml of 48% polyethylene glycol in serum-free growth medium for 1 min. After overnight incubation in growth medium, the cells were plated out at 4×10^4 cells per T75 coated flask for selection. For each fusion, control flasks containing either the parental cells that had been subjected to the polyethylene glycol fusion reagent or mixed unfused cells were also plated out for selection. In each case, there were no viable cells in any of the control flasks at completion of selection. Hybrids were selected with HAT (10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, 1.6×10^{-5} M thymidine) (Sigma) and either ouabain (10^{-6} M) or G418 (250 μ g/ml). The HAT-plus-ouabain selection was maintained for 2 weeks, then HT (HAT minus aminopterin) was used for 1 week, and then HAT plus 3.2×10^{-6} M ouabain was used until colonies of hybrid cells were isolated (approximately 1 week). For the HAT-plus-G418 selection,

G418 was maintained in the medium until no viable cells remained in the BET-1A control flask (7 to 10 days), and the HAT selection was performed as for HAT-ouabain.

Assignment of complementation group. A minimum of 15 colonies per fusion were individually isolated by using cloning cylinders and then subcultured continuously until senescence or 100 population doublings, at which stage they were considered to be immortal (9). Senescence was defined as cessation of population growth for at least 30 days, and this was usually also manifested by the appearance of many large cells, often with bizarre morphology. The senescent hybrids were maintained in culture for at least 60 days from cessation of proliferation to determine whether a segregant proliferating subpopulation would appear.

For each fusion, there were at least some hybrids that were able to grow long term, indicating that the cell culture conditions supported the growth of hybrid cells. The majority of hybrids arising from fusion of BET-1A with each of the cell lines representative of groups A, B, and C senesced (Table 1), indicating that complementation occurred in these fusions and that BET-1A is therefore not in group A, B, or C. Of 21 BET-1A-GM847 hybrid clones, all senesced, but 2 of these (clones F and J) recommenced proliferation after 50

FIG. 2. DNA content (left panel) and morphology (right panel) of hybrid cells. (a, c, e) DNA content of parental and hybrid cells was determined by flow cytometry of propidium iodide-stained cells. Examples shown are BET-1A (a), HeLaCOT (c), and BET-1A-HeLaCOT hybrid clone A (e). (b, d, f) Phase-contrast morphology of the same cells. Magnification, ca. $\times 128$.

and 150 days, respectively (Fig. 1A). Of 36 BET-1A-HeLaCOT clones, 24 senesced; 4 of the senescing clones gave rise to segregant colonies of immortal cells. The remaining 13 hybrid clones showed no evidence of senescence (Fig. 1B). Each of 18 BET-1A-143BTK⁻ hybrid clones senesced, and from 2 of these clones immortal segregants arose (Fig. 1C). The observation of some hybrids that never underwent senescence and also immortal segregants in the BET-1A fusions with the group A, B, and C representatives is consistent with the occurrence of chromosomal segregation. In contrast, none of the 15 BET-1A-A1698 hybrid clones senesced (Fig. 1D). The BET-1A cell line may therefore be assigned to the same complementation group as A1698, i.e., group D.

DNA content. To confirm that fusion had taken place, we compared the DNA content of the hybrids as measured by DNA flow cytometry of propidium iodide-stained cells with the DNA content of the parental cell lines (Fig. 2). This indicated that all hybrid clones had a DNA content greater than that of either of the two parent lines, but usually less than the sum of the parents (Fig. 2; Table 2). The DNA content corresponded to the cell size as shown by phase-contrast microscopy (Fig. 2).

SV40 T-Ag immunostaining. The presence of SV40 large and small T Ag in cell lines and hybrids was detected by indirect immunofluorescence staining, using a mouse monoclonal antibody against SV40 T Ag (Ab-1; Oncogene Science Inc.) and a fluorescein-conjugated goat anti-mouse immuno-

TABLE 2. DNA index^a of hybrids between BET-1A and representatives of four immortalization complementation groups

Hybrid cells ^b	Predicted DNA index ^c	Observed DNA index
BET-1A-GM847	3.04	2.61, 2.35
BET-1A-HeLaCOT	2.90	2.69 $\pm$ 0.25 (19) ^d
BET-1A-143BTk ⁻	2.79	2.18, 3.38
BET-1A-A1698	3.29	3.00 $\pm$ 0.50 (15) ^d

^a A Becton-Dickinson FACscan was used to quantitate the DNA content by comparison with the chicken erythrocyte nuclei internal control; the DNA index of a normal human diploid cell was defined as 1.00.

^b Sufficient cells for DNA flow cytometry were available only for immortal hybrids and immortal segregants.

^c Predicted DNA index for hybrid cells is the sum of the indices of the parental cell lines. These were BET-1A, 1.70; GM847, 1.34; HeLaCOT, 1.20; 143BTk⁻, 1.09; A1698, 1.59.

^d Mean $\pm$ SD (*n*). To assess reproducibility, independent preparation and analysis of three samples of one hybrid clone was done on every occasion that DNA analysis was performed, and this showed that the DNA content never deviated more than 0.83% from the mean.

globulin G antibody (Jackson ImmunoResearch). Of the parent cell lines, only BET-1A and GM847 were SV40 T Ag positive (Fig. 3a to e), as expected. Forty-six hybrid clones were tested, including senescing hybrids, proliferating segregants, and immortal hybrids, and all were positive for SV40 T Ag (Fig. 3f to i). This is consistent with many previous studies (reviewed in reference 2) showing that expression of SV40 early region genes is not sufficient for immortalization of human cells.

Dissociation between tumor suppression and induction of senescence. A1698 is a tumorigenic bladder carcinoma cell line, but previous studies have shown that BET-1A is nontumorigenic in athymic nude mice for at least 12 months (11, 12a). To determine whether the BET-1A genome is able to suppress the tumorigenicity of A1698 cells, BET-1A and A1698 cells and five BET-1A-A1698 hybrid clones were tested for tumorigenicity by injection of 5×10^6 cells subcutaneously in athymic nude mice. A1698 was highly tumorigenic, but BET-1A and four of the five hybrids failed to form tumors within 30 days (Table 3). This indicates that BET-1A is able to suppress the tumorigenicity of A1698 cells and is further evidence that cell hybridization has occurred. Further, since BET-1A-A1698 hybrids did not senesce, tumor suppression in these hybrids is clearly independent from induction of senescence.

Genetic events in immortalization of human cells. As has been shown previously (10), we found that fusion of different immortal cell lines (BET-1A with group A, B, and C cell lines) resulted in hybrid cells which senesced. This is consistent with the notion that the mortal phenotype is dominant over the immortal phenotype and that defects responsible for the loss of mortality can be complemented to reimpose the normal mortal phenotype. These results also support the model of complementation groups for immortality, suggesting that more than one genetic lesion results in immortalization (10).

Not all SV40-transformed cell lines are in the same complementation group for immortalization. Fusion of the SV40-immortalized BET-1A and GM847 cell lines resulted in mortal hybrids, demonstrating that these cells are able to complement each other for the senescent phenotype. These results indicate that SV40-immortalized human cells may be useful for analyzing the molecular genetics of immortalization of more than one complementation group. Many, but not all, of the SV40-immortalized cell lines previously re-

FIG. 3. Immunostaining for SV40 T Ag. Indirect immunofluorescence staining with a primary antibody recognizing SV40 large and small T Ag and a fluorescein-conjugated second antibody, visualized with a Wild-Leitz confocal microscope. (a) BET-1A; (b) GM847; (c) HeLaCOT; (d) 143BTk⁻; (e) A1698; (f) BET-1A-GM847; (g) BET-1A-HeLaCOT; (h) BET-1A-143BTk⁻; (i) BET-1A-A1698. Cells stained only with fluorescein-conjugated second antibody gave similar levels of cytoplasmic fluorescence.

TABLE 3. Tumorigenicity of BET-1A-A1698 hybrid clones

Cell line ^a	No. of tumors ^b	Days ^c
BET-1A	0/10	NA ^d
A1698	30/30	11.7 ± 5.5
BET-1A-A1698 clone A	0/10	NA
BET-1A-A1698 clone B	0/10	NA
BET-1A-A1698 clone C	1/10	7
BET-1A-A1698 clone D	5/10 ^e	10.8 ± 4.3
BET-1A-A1698 clone F	1/10 ^f	9

^a Cells were injected subcutaneously in the right and left subscapular regions of athymic nude mice, 5×10^6 cells per site.

^b Number of tumors greater than 5 mm in diameter within 30 days of injection/number of sites injected.

^c Days taken to reach an average tumor diameter of 5 mm, from time of injection.

^d NA, not applicable.

^e Two of these tumors subsequently regressed totally; the remaining three reached a maximum diameter of 5 to 7 mm before ceasing growth.

^f This tumor subsequently regressed totally.

ported to be in group A are of fibroblastic origin (10). We have commenced similar analyses of three other SV40-immortalized cell lines of epithelial origin and have shown that each cell line has a limited life span when fused with GM847 group A cells. It will be of considerable interest to determine whether there is any correlation between complementation group and histological origin of SV40-immortalized cells.

This study was supported by the New South Wales State Cancer Council and the National Health and Medical Research Council of Australia.

REFERENCES

- Bunn, C. L., and G. M. Tarrant. 1980. Limited lifespan in somatic cell hybrids and cybrids. *Exp. Cell Res.* **127**:385-396.
- Chang, S. E. 1986. *In vitro* transformation of human epithelial cells. *Biochim. Biophys. Acta* **823**:161-194.
- Hayflick, L., and P. S. Moorhead. 1961. The serial cultivation of human diploid cell strains. *Exp. Cell Res.* **25**:585-621.
- Lechner, J. F., and M. A. LaVeck. 1985. A serum-free method for culturing normal human bronchial epithelial cells at clonal density. *J. Tissue Culture Methods* **9**:43-48.
- Muggleton-Harris, A. L., and D. W. DeSimone. 1980. Replicative potentials of various fusion products between WI-38 and SV40 transformed WI-38 cells and their components. *Somatic Cell Genet.* **6**:689-698.
- Newbold, R. F., and R. W. Overell. 1983. Fibroblast immortality is a prerequisite for transformation by EJ c-Ha-ras oncogene. *Nature (London)* **304**:648-651.
- Newbold, R. F., R. W. Overell, and J. R. Connell. 1982. Induction of immortality is an early event in malignant transformation of mammalian cells by carcinogens. *Nature (London)* **299**:633-635.
- O'Brien, W., G. Stenman, and R. Sager. 1986. Suppression of tumor growth by senescence in virally transformed human fibroblasts. *Proc. Natl. Acad. Sci. USA* **83**:8659-8663.
- Pereira-Smith, O. M., and J. R. Smith. 1981. Expression of SV40 T antigen in finite lifespan hybrids of normal and SV40-transformed fibroblasts. *Somatic Cell Genet.* **7**:411-421.
- Pereira-Smith, O. M., and J. R. Smith. 1988. Genetic analysis of indefinite division in human cells: identification of four complementation groups. *Proc. Natl. Acad. Sci. USA* **85**:6042-6046.
- Reddel, R. R., Y. Ke, B. I. Gerwin, M. G. McMenamin, J. F. Lechner, R. T. Su, D. E. Brash, J. B. Park, J. S. Rhim, and C. C. Harris. 1988. Transformation of human bronchial epithelial cells by infection with SV40 or adenovirus-12 SV40 hybrid virus, or transfection via strontium phosphate coprecipitation with a plasmid containing SV40 early region genes. *Cancer Res.* **48**:1904-1909.
- Reddel, R. R., Y. Ke, M. E. Kaighn, L. Malan-Shibley, J. F. Lechner, J. S. Rhim, and C. C. Harris. 1988. Human bronchial epithelial cells neoplastically transformed by v-Ki-ras: altered response to inducers of terminal squamous differentiation. *Oncogene Res.* **3**:401-408.
- Reddel, R., et al. Unpublished data.
- Rhim, J. S., G. Jay, P. Arnstein, F. M. Price, K. K. Sanford, and S. A. Aaronson. 1985. Neoplastic transformation of human epidermal keratinocytes by AD12-SV40 and Kirsten sarcoma viruses. *Science* **227**:1250-1252.
- van Zoelen, E. J. J., T. M. J. Oostwaard, P. T. van der Saag, and S. W. de Laat. 1985. Phenotypic transformation of normal rat kidney cells in a growth factor-defined medium: induction by a neuroblastoma-derived transforming growth factor independently of the EGF receptor. *J. Cell. Physiol.* **123**:1511-1516.