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SPONTANEOUS TRANSFORMATION OF A CLONED CELL LINE OF NORMAL DIPLOID BOVINE VASCULAR ENDOTHELIAL CELLS

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SUMMARY

We report here the spontaneous transformation of a normal diploid bovine fetal aortic endothelial cell line. This cell line showed a period of rapid proliferation, followed by a period of declining proliferative activity, as judged by both a decline in the number of population doublings achieved from seeding to subcultivation and a decrease in the fraction of cells incorporating [^{3}H]thymidine. During the decline in proliferation, foci of small cells appeared amid a background of larger senescent-appearing cells. The cultures then regained proliferative activity and have been maintained in our laboratory for more than 22 months. The transformants are characterized by (a) an indefinite life span, (b) a morphology that is more spindle shaped as compared to pretransformants, and (c) heteroploidy with chromosome translocations.

Key words: aging; bovine; endothelial cells; heteroploidy; transformation; translocations.

INTRODUCTION

A number of investigators have reported that cultures of bovine-derived fibroblasts (1-4), smooth muscle cells (4,5), and vascular endothelial cells (6-10) have a finite replicative life span and can be useful for studies of cellular aging. Vascular endothelial cells are a particularly useful model because one can study both proliferative capacity and the expression of at least two differentiated functional capacities, Factor VIII antigen and angiotensin-converting enzyme. In addition, studies of chemical (11) and viral (Gorman, manuscript in preparation) transformation and their effects on the above should provide important information on the mechanisms of transformation.

Spontaneous transformation of bovine cells into cultures with an indefinite life span is considered a low frequency event (3,12) and until now only five have been reported: two from kidney-derived cultures (13,14), one from turbinate-derived cultures (15), one from lens epithelial cells (16), and one from cloned vascular endothelial cells (17,18). These reports, however, do not document adequately the characteristics of the cultures surrounding the event of the growth decline and subsequent growth increase that accompany the transformation. During the course of our studies on the proliferative characteristics of normal clones of bovine vascular endothelial cells we observed, on two separate occasions, one clone to transform spontaneously. The growth characteristics during active normal growth, senescence, and transformation as well as the changes in morphology, life span, and karyotype during this period comprise the basis of this report.

MATERIALS AND METHODS

Cell culture. Bovine fetal aortic endothelial cloned cell lines designated BFA-39e and BFA-39b were obtained at cumulative population doubling levels 38 and 42, respectively, from the laboratory of Dr. Elliot M. Levine of the Wistar Institute. They had been isolated as described and their endothelial origin was verified by expression of the Factor VIII antigen and angiotensin-

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converting enzyme (4,10). Cells were cultured by our routine procedures (19,20) using either autoclavable Eagle's minimal essential medium (MEM) (21) containing Earle's balanced salt solution (Auto-Pow, Flow Laboratories, Rockville, MD) supplemented with an additional mixture of vitamins as formulated for Eagle's BME (22) or F12 medium (23), each supplemented with 10% (vol/vol) fetal bovine serum (Flow). No antibiotics were used. Cultures were maintained at 37° C in 5% CO_2:95% air and checked for extent of growth each week. Subconfluent cultures were refed weekly with fresh medium, whereas confluent cultures were harvested by treatment for 2 to 5 min with 2 ml/T-75 flask of a trypsin-EDTA solution consisting of a mixture of 0.25% (wt/vol) trypsin, and 0.09% (wt/vol) EDTA in calcium- and magnesium-free phosphate buffered saline (PBS), seeded at a density of 1×10^4 cells/cm² in a T-75 plastic tissue culture flask with 40 ml medium. Cellular proliferation was measured by determining the number of population doublings (PDs) by cell counts and correcting for the 24 h seeding efficiency (usually 0.70 to 0.95) as described (4). The increase in PDs at each subcultivation was added to the cumulative PDs of the culture to give the new cumulative population doubling level (CPDL). BFA-39e and BFA-39b were received as single confluent T-25 flasks in F12 medium and comparison of their proliferative life histories began when subcultured into either MEM for a first life history or F12 medium for a second life history. Different media were used for reasons unrelated to this report. Cultures were considered to be at the end of their life span when the cell density did not double beyond the seeding inoculum after 4 consecutive weeks in culture with weekly refeedings. All cultures were free of mycoplasma contamination as determined by biweekly anaerobic agar-plate cultivation (24) and by 33258 Hoechst dye staining for cytoplasmic DNA fluorescence (25). Cultures were frozen on numerous occasions throughout their life histories for possible future studies.

The trypsin-resistant cultures described in the text were removed from the flasks by sequentially incubating with trypsin-EDTA solution (2 ml/T-75 flask) for 10 to 15 min, decanting the released cells, rinsing the cultures with trypsin-EDTA solution, and incubating the cultures with additional fresh trypsin-EDTA solution, until a majority of the cells had detached. The released cells were pooled and collected by centrifugation at 250 ×*g* for 10 min at 4° C before seeding.

Tritiated thymidine labeling index. The percentage of proliferating or rapidly dividing cells was estimated from the fraction of cells incorporating tritiated thymidine ([³H]TdR) into their nuclei as described previously (19). Harvested cells were seeded at a density of 1×10^4 cells/cm² in 60 × 15 mm petri plate with a sterile 22 mm² cover glass in 5 ml medium. After 24 h, [³H]TdR (sp act 2.0 Ci/mmol) was added to the medium for a final concentration of 0.1 μCi/ml and the cells were incubated for an additional 30 h. The cells were rinsed in PBS, fixed in absolute methanol for 5 min, and air dried overnight. The cover glasses were mounted on slides (cell side up) with Harleco (Gibbstown, NJ) synthetic resin and processed for autoradiography using Kodak NTB-2 emulsion (19). Random fields of cells were scored until at least 400 cells were counted. Any cell with five or more grains over the nucleus was considered labeled.

SV40 T-antigen determination. Simian virus 40 (SV40) T-antigen was assayed by indirect immunofluorescence (26) with either the hybridoma-derived antibody PAb5 (27,28) or the monoclonal antibody PAb100 (29). Cells were counted-stained using FITC-conjugated rabbit antimouse IgG (Miles Laboratories, Elkhart, IN).

Chromosome studies. Actively growing cultures were refed with fresh medium, and 24 h later the cells were removed from the surface of the flask using 0.04% (wt/vol) trypsin in Puck's EDTA solution (30). Spindle inhibitors such as Colcemid or Velban were not used in these preparations to avoid the excessive chromosome contractions seen with these cells. Cells were then collected by centrifugation at 125 ×*g* for 10 min and resuspended in a hypotonic solution of 0.2% (wt/vol) sodium citrate and 0.2% (wt/vol) potassium chloride for 20 min. After centrifugation, the cells were fixed in ice cold methanol:glacial acetic acid (3:1). After two changes of the fixative, the cell suspension was dropped onto wet slides and dried with gentle blowing. Slides were either stained with 2% (vol/vol) Giemsa (Harleco) in Gurr's buffer at pH 6.8 for approximately 3 min and examined for chromosome number and aberrations or stained with Giemsa-trypsin (31) to produce banded preparations for examination of chromosome translocations.

RESULTS

The first proliferative life history of BFA-39e is represented in Fig. 1 *A*, (*circles*). Proliferation was vigorous up to Week 11, with the cultures

accumulating an average of 4.1 population doublings per week. Proliferation gradually decreased between Weeks 11 and 17, and cultures were refed at Week 18 because the cells were not confluent. Cultures were subcultivated at Week 19 and then refed for the next 3 consecutive weeks due to subconfluency. At Week 23, dense colonies or foci of smaller-appearing cells were evident, with approximately 200 foci in each of quadruplicate T-75 flasks. These cultures proliferated slowly for the next 6 wk and were subcultured biweekly with weekly refeedings. Between Weeks 29 and 32, cultures were refed weekly due to slowed proliferation. At Week 33, foci of cells had appeared again, very similar to those observed at Week 23. Attempts to subculture these cultures failed because the cells would not detach from the flasks with our standard trypsin-EDTA solution treatment regimen, and some flasks of cells were discarded due to this failure. The remaining flasks were refed weekly up to Week 36, whereupon there was a concerted effort to detach the cells with trypsin-EDTA solution as described in Materials and Methods. This procedure took approximately 2.5 h and succeeded in detaching about 80% of the cells. The released cells were reseeded into a fresh T-75 flask. Thereafter, the cultures were no longer resistant to trypsin-EDTA solution treatment, and cultures were subcultivated weekly between Weeks 37 and 43 using a seeding inoculum of 2×10^4 cells/cm^2. After Week 43, cultures were subcultivated biweekly using our normal seeding inoculum of 1×10^4 cells/cm^2 with weekly refeedings and accumulated an average of 4.9 population doublings at each subcultivation. The cultures have been maintained in our laboratory for more than 22 months and have reached CPDL 244 and show no indication of losing proliferative activity. At confluency, cultures have approximately 30% of their cells floating in the medium; therefore, these population doubling values are probably an underestimate.

Autoradiographic detection of [^{3}H]thymidine incorporation into the nuclei of cells during a 30 h period after subcultivation (Fig. 1 *B*) during this first life history demonstrated active proliferation early in the culture life history [80 to 90% labeled nuclei (PLN)] and cessation of proliferation between Weeks 12 and 16 in a substantial proportion of the cells (40 to 45 PLN). By Week 23, the PLN rose to 83 and remained high thereafter.

Figure 2 shows photomicrographs of BFA-39e cells during this first life history at different time periods. The majority of cells at Week 23 (Fig. 2 *B*) were much larger than cells earlier in the life history (Fig. 2 *A*) and had a morphology typical of senescent cells, including vacuolization of the cytoplasm. The first appearance of these large cells coincided with the decline in PLN and a causal relationship is likely. Among these apparently senescent cells, however, were the dense colonies containing smaller cells (Fig. 2 *C*), some of which were in mitosis. By Week 43, the cells had become somewhat more spindle shaped than their pretransformant counterparts (*see* Fig. 2 *D*, *A*).

In a second life history examination of BFA-39e, the pattern of proliferation was virtually the same, except that cultures did not regain active proliferative activity until Week 48 (Fig. 1 *A*, *triangles*). Although F12 medium was used in this second life history instead of MEM, we do not attribute the later emergence of renewed proliferation to the use of a different medium. The cultures of the second life history were also resistant to

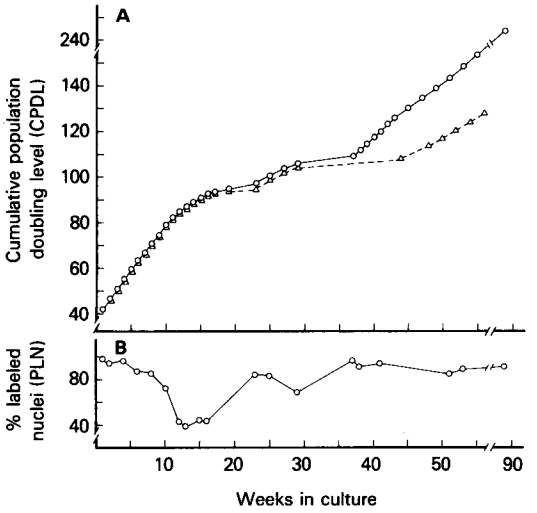

FIG. 1. Life history growth kinetics of bovine vascular endothelial cell clone BFA-39e. *A*, CPDL as a function of the weeks in culture. Data are presented for the first life history (—○—) and the second life history (---△---). Data for the first 40 CPDLs are unavailable due to the doublings required for cloning. In the second life history, the cultures were not subcultured after the formation of the foci and rebirth of proliferative activity (Week 54) because spontaneous transformation was already apparent. *B*, Proliferating cells as a function of weeks in culture. The percentage of proliferating or rapidly dividing cells during the first life history was estimated by the percentage of cells able to incorporate [^{3}H]thymidine into their nuclei during a 30 h labeling period as described in the text. Each point is the average of duplicate determinations that agreed within 5%.

trypsin-EDTA solution treatment for a short time, and the concerted effort needed to subculture the cells was not applied until after a longer refeeding time.

Another cloned line of bovine fetal aortic endothelial cells cultured in our laboratory, BFA-39b, had a finite life span of 85 and 90 CPDL, respectively, on two separate life span determinations (not shown). These cultures showed no indication of transformation even after refeeding the cultures in one case for 26 consecutive wk after their defined life span had ended.

Because the transforming virus SV40 is commonly used in our laboratory and is a possible cell contaminant, we assayed for T-antigen expression. Indirect immunofluorescence revealed no SV40 T-antigen in the nuclei of cells from cultures of BFA-39e at Weeks 5, 23, 25, 47, and 89, ruling out the possibility that SV40 contamination accounted for the transformation (not shown). The SV40-transformed cell lines BFA-39e-VA1, BFA-39b-VA1, and BFA-39b-VA2 derived in our laboratory (Gorman, manuscript in preparation) served as positive controls for T-antigen expression.

Karyotype analysis of BFA-39e during the first life history before and after transformation confirmed its bovine origin and revealed that the transformants have an altered karyotype. At Week 4 of the life history, the modal chromosome number of a 50 cell sample was 60, the diploid chromosome number for bovine cells (32). Two percent of the cells were polyploid and only a single cell in more than 50 cells viewed had one Robertsonian translocation. At Week 66 of the life history, analysis of 100 Giemsa-stained cells revealed a range of 37 to over 100 chromosomes per cell. The modal chromosome number was 45, and 35% of the cells were polyploid. Examination of acute unstable aberrations of a 50 cell sample showed that 38% of the cells had chromatid and chromosome breaks with one chromatid interchange. Each of 50 cells studied after staining by the Giemsa-trypsin banding method exhibited several Robertsonian translocations, which are formed by centric fusions between two chromosomes with terminal or near terminal centromeres. Banded karyotypes prepared on 10 cells did not reveal any consistent abnormality; however, Chromosome 5 was involved in a variety of abnormalities in 7 of the 10 cells examined. A representative karyotype is seen in Fig. 3. Chromosome abnormalities are consistent with the transformed state (3).

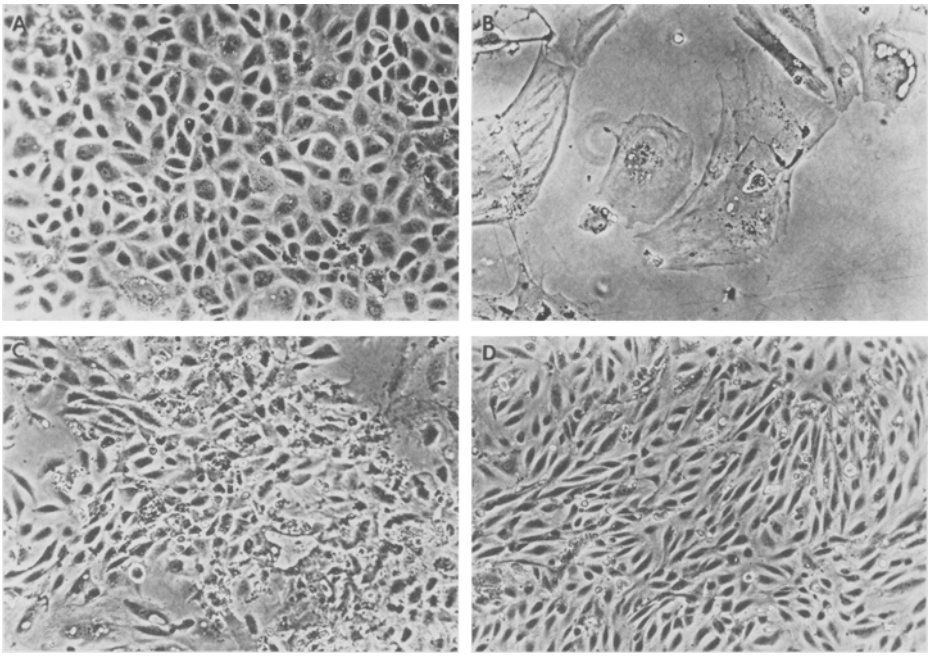

FIG. 2. Phase contrast photomicrographs of BFA-39e during the first life history described in the text. Cells ×115. *A*, Cells at Week 6 (pretransformation) with cobblestonelike morphology; *B*, Large senescent-appearing cells at Week 23; *C*, Focus or colony of smaller cells at Week 23 occupying most of the field of view and found in the same flask as the apparently senescent cells of *B*; *D*, Cells at Week 43 (transformants) showing slightly altered morphology as compared to those in *A*.

Discussion

We report here the spontaneous transformation of a cloned line of normal diploid bovine vascular endothelial cells, BFA-39e. The only previous report of bovine vascular endothelial cell lines with indefinite life spans describes clonal lines passaged for over 390 population doublings in medium containing exogenous fibroblast growth factor (FGF) (17,18). These lines were reported to maintain a diploid karyotype and require FGF for continued proliferation. Cell Line BFA-39e, however, has undergone extensive chromosome alterations and does not require exogenous FGF for proliferation. Additionally, Cell Line BFA-39e exhibited a significant decline in proliferation (senescence) before the appearance of transformants, whereas such a decline was not reported for the FGF-requiring lines (17,18).

The spontaneous transformation of Cell Line BFA-39e does, however, resemble the establishment of Cell Line MDBK, a spontaneously transformed line derived from normal adult bovine

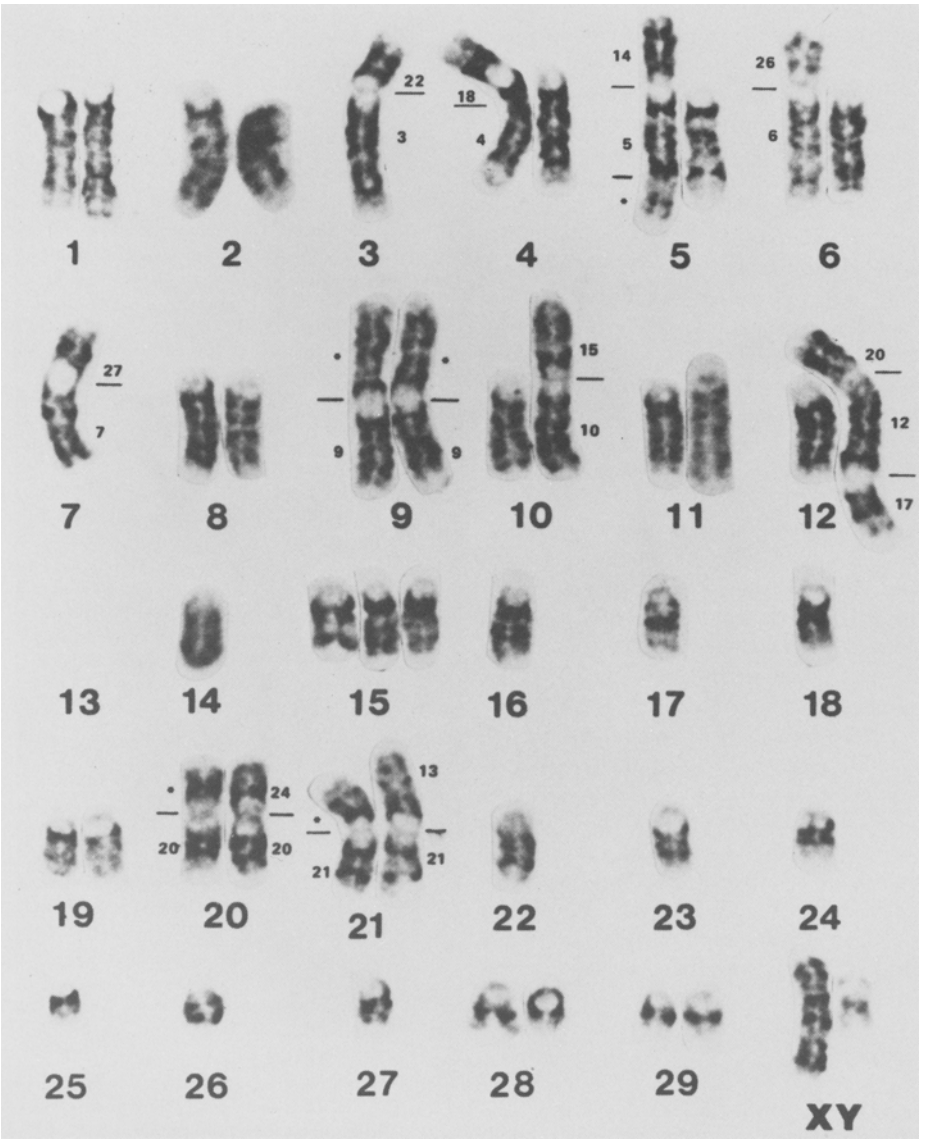

FIG. 3. Giemsa-trypsin banded karyotype of a representative cell from a transformed culture at Week 66; several Robertsonian translocations (Chromosomes 3–7, 9, 10, 20, and 21) and one end-to-centromere translocation (Chromosome 12) are shown. *Asterisks* denote unidentified chromosomes.

kidney (13). Cell Line MDBK exhibited a decrease in growth rate before transformation, required a 60 d refeeding period before active proliferation resumed (13) and possessed an altered karyotype after transformation (33). The spontaneous transformation of bovine turbinate-derived cultures (15) and lens epithelial cells (16) was also accompanied by changes in karyotype, but declines in growth rate before transformation were not reported. The bovine turbinate-derived cultures, however, were maintained for intervals of several weeks and several months with periodic refeedings (15), and a transient alteration in the proliferative status of the cultures could have gone unnoticed.

The difficulty encountered in removing early transformants from the flasks with trypsin-EDTA solution could potentially complicate the detection of spontaneous transformants and their continued proliferation. Initial attempts to subculture early transformants with trypsin-EDTA solution failed, and some flasks of cells were discarded. It was only after exhaustive trypsin-EDTA solution treatment that subculturing was possible, and had the cells failed to be subcultured the transformants may have been lost. Similar observations have been made for chemically induced early transformants of a different cloned cell line, BFA-1c (11), which were also temporarily trypsin resistant.

It should be noted that there is clearly a difference in clonal susceptibility to spontaneous transformation. Another cloned line cultured in our laboratory, BFA-39b, had a finite life span even with a terminal refeeding period of 26 wk, and the finite life span of numerous other cloned cell lines has been well documented (4,10,11,34).

Although the frequency of spontaneous transformation of bovine vascular endothelial cell lines cannot be estimated based on this report, the numerous examples of lines with a finite life span (6–10) demonstrate that the frequency is very low. This detailed description of a spontaeous transformation is important, however, because it serves to make investigators more aware of the possibility and characteristics of spontaneous transformation in these cells.

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