Transformation of Rat Cerebral Endothelial Cells by Rous Sarcoma Virus

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ABSTRACT Rat cerebral microvascular endothelial cells were infected with Schmidt-Ruppin Rous sarcoma virus-strain D (SR-RSV-D), an avian retrovirus. A single focus of transformed cells was isolated and the resultant cell line designated RCE-T1. The specificity for SR-RSV-D transformation was determined by virus rescue assay and demonstration of virus-specific antigens. RCE-T1 cells are virogenic when fused with chicken embryo fibroblasts (CEF) and do not produce infectious virus as demonstrated by the absence of detectable virus in culture fluid from these cells alone. Studies using an enzyme-linked immunosorbent assay (ELISA) for avian retrovirus-coded internal proteins show that RSV-transformed endothelial cells contain mainly p27 and react to some extent to p19 and p15 viral antigens. These data demonstrate conclusively that the transformation event was indeed due to SR-RSV-D. In addition, chromosome analysis confirmed these cells to be of rat origin.

RSV-transformed endothelial cells express the typical array of transformation-related properties such as anchorage-independent cell growth in soft agar, decreased cell adhesiveness, ability to grow in low serum, and capability of producing tumors in newborn rats. Demonstration of differentiated endothelial characteristics included positive immunofluorescent staining for factor VIII antigen and angiotensin-converting enzyme and histochemical localization of γ-glutamyl transpeptidase activity. This cell line should provide a useful model to study not only specialized biochemical and other functional characteristics of cerebrovascular endothelium but also the cellular mechanisms that involve the transition from normal to neoplastic expression.

Attempts to transfer normal differentiated tissues from an in vivo environment to defined culture conditions in vitro are often frustrating. In vitro, normal differentiated cells often fail to proliferate or cease to express differentiated cell properties beyond one or a few passages. We recently reported the establishment and maintenance of long-term primary endothelial cell cultures derived from rat cerebral microvessels (1). Although important information can be obtained from such cells maintained in primary culture, the ability to form subcultures and to obtain large numbers of cells while maintaining the differentiated characteristics of interest would obviously facilitate biochemical studies, as well as studies pertaining to cell growth properties and requirements. One approach for circumventing these limitations in culture is viral-induced cell transformation with a well characterized oncogenic virus.

While in vitro transformation of cells by DNA- and RNA-containing tumor viruses has been widely documented, reports on the successful transformation of vascular endothelial cells have been limited. Gimbrone and Fareed (2) described the successful transformation of human umbilical cord endothelial cells with SV40 viral DNA, but these transformed cells failed to routinely express normal differentiated endothelial cell properties. Rous sarcoma virus (RSV), an avian retrovirus, is capable of transforming a wide variety of differentiated cells of various species (3–9) and may even stimulate growth of differentiated cells under normal culture conditions (10–12). These observations prompted us to use the Schmidt-Ruppin-D strain of RSV, which can transform mammalian cells (13–15), as a transforming agent for rat cerebral endothelial cells in culture.

We report a number of features of an RSV-transformed
cell line, derived from a single focus of transformed primary rat cerebral endothelial cells, including the expression of differentiated characteristics.

MATERIALS AND METHODS

Virus

The Schmidt-Ruppin strain of Rous sarcoma virus (SR-RSV-D) was originally obtained from Dr. Peter K. Vogt and was recloned twice by single-focus isolation and passaged at a low multiplicity of infection in chick embryo fibroblast (CEF) cells. The focus-forming titer was determined by a standard focus assay for RSV (16) with 2 μg/ml of polybrene incorporated into the medium during infection (17).

Cell Cultures and Transformation Procedures

Primary CEF of the C/E genotype were grown from a single White leghorn embryo as described by Dougerty et al. (18). These cells were avian retrovirus group specific (gs) antigen negative and chick helper factor (chf) negative when tested according to published criteria (19). All cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. We prepared primary rat cerebral microvascular endothelial cells and characterized as reported previously (1). We used morphologically homogeneous populations of endothelial cells in these studies. Primary cultures were maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 20% fetal calf serum (FCS) (Grand Island Biological Company, Grand Island, NY), 25 μg/ml gentamicin and 150 μg/ml endothelial cell growth supplement (ECGS) (Collaborative Research Inc., Waltham, MA).

Multidwell dishes containing seven to 10-d-old subconfluent cultures of primary rat cerebral endothelial cells (~5 × 10⁴ - 1 × 10⁵ cells/2.1-cm² well) were infected with SR-RSV-D at a multiplicity of infection of 10-15. After 1 h of absorption at 37°C, the cultures were re-fed with DME plus 20% FCS and 150 μg/ml ECGS. After 5-7 d the culture medium was replaced with medium free of ECGS, and at 3-5-d intervals thereafter, 2 wk after infection, a focus of tightly packed, refractile, polygonal transformed cells was readily detected. These transformed rat cerebral microvascular endothelial cells, designated here as RCE-T1, were subsequently propagated in DME supplemented with 10% FCS and 25 μg/ml gentamicin. The cells were routinely transferred at a split ratio of 1:10 every 3-4 d, adhering the monolayer with 0.25% trypsin-EDTA and counting the dispersed cells in a hemocytometer. The doubling time of the cells was calculated from the plateau level reached in each case.

Characterization Studies

GROWTH AND PLATING EFFICIENCY: Saturation densities and doubling times of RCE-T1 cells were measured at varying passage levels. Cells were seeded in 25-cm² flasks at 1 × 10⁵ cells/flask. The medium was changed at days 3 and 7 of the experiment. On alternating days over a 9-d period, duplicate sets of cultures were trypsinized and the cells were counted in a hemocytometer. The doubling time of the cells was calculated from the exponential part of the growth curve and the saturation density, expressed as number of cells per 25-cm² flask, was calculated from the plateau level reached in each case.

To measure the effects of FCS concentration, the growth of two representative passage levels of RCE-T1 cells was tested in medium containing 0%, 0.5%, 5%, and 10% FCS. RCE-T1 cells were grown from an initial density of 1 × 10⁴ cells/35-mm plastic tissue culture dish (Falcon Plastics, Cockeysville, MD). Cell counts were made at days 1, 2, 3, and 5 by trypsinizing duplicate cultures with trypsin-EDTA and counting the dispersed cells in a hemocytometer.

Cloning efficiency of RCE-T1 cells (P₀) in soft agar was determined by adding various numbers of viable cells (as determined by trypan blue dye exclusion) to 2-ml portions of Eagle's MEM containing 10% tryptose phosphate broth, 10% FCS, 200 mM l-glutamine and 0.35% agar. The mixture was then plated in 60-mm tissue culture dishes containing a previously prepared and hardened base-layer of 8 ml of the same medium and 0.87% agar. Macroscopic colonies of cells were counted after 14 d of incubation at 37°C in a humidified atmosphere of 5% CO₂ in air. The mean of quadruplicate plates from two experiments was used to determine the plating efficiency.

Cloning efficiency in liquid medium with RCE-T1 cells was also determined by seeding plates with 1 and 2 × 10⁵ viable cells in DME with 10% FCS. After 14 d, with periodic medium changes, triplicate cultures were fixed with methanol, stained with May-Grunwald Giemsa, and the number of colonies was determined.

CHROMOSOME ANALYSIS: Control rat cerebral endothelial cells and various passages of RCE-T1 cells were harvested for chromosome analysis. Metaphase and prometaphase spreads were examined using differential ASG and GTG banding techniques by the method described by Sumner et al. (20).

ENZYME-_LINKED IMMUNOSORBENT ASSAY (ELISA) FOR AVIAN RETROVIRUS GS ANTIGEN: Antisera were obtained from hamsters with tumors induced by the Schmidt-Ruppin B strain of Rous sarcoma virus (21). IgG and peroxidase conjugate were prepared as described by Wilson and Nakane (22).

Standard antigen was an extract of purified avian myeloblastosis virus (AMV). Cell extracts from normal rat cerebral endothelial cells and transformed RCE-T1 cells were made by twice freezing and thawing a 50% suspension in phosphate-buffered saline (PBS)-0.05% Tween-80, followed by 20 strokes with a Dounce homogenizer. The preparation was then clarified at 2,000 g for 20 min and the supernatant tested for gs antigen. The double antibody sandwich ELISA described by Clark and Dougerty (23) was used with the following modifications: flat-bottom Linbro microtiter plates (type 76-301-05) were sensitized with 0.15 μg of hamster anti-gs in 100 μl per well of 20 mM PBS (pH 6.8) containing 0.05% Tween-80 (PBS-Tween). Peroxidase-labeled hamster anti-gs IgG (0.75 μg/well) in 25 μl of PBS-Tween was then added. The Trinder reagent (25 mM Phenol, 2 mM 4-amino antipyrine, 0.8 mM H₂O₂) (24) in PBS-Tween at 100 μl/well was used as substrate for the enzyme reaction. The reaction was stopped after 30 min by the addition of 100 μl of 20 mM NaOH, and then the absorbance of each well was read at 492 nm in a Flow Titertek Multispan plate reader.

VIRUS RESCUE ASSAY: CEF and RCE-T1 cells were fused at 38°C by modifications of previously published methods (25-27). Briefly, 8 g of polyethylene glycol (PEG)-6000 (Baker Chemical Co., Sanford, ME) was autoclaved for 15 min. After 3-5 min of cooling, the 10 ml of serum-free DME containing 15% DMSO was added, with stirring. Final concentrations of PEG and DMSO were 44.4% and 8.3%, respectively. CEF and RCE-T1, 1.0 × 10⁴ cells/plate and 5 × 10⁴ cells/ml, respectively, were plated together in 60-mm tissue culture dishes in DME containing 10% FCS and grown for 24 h. The medium was then removed, the cells were washed twice with serum-free DME, and 1 ml of the PEG-DMSO mixture was added to each plate. After 1 min, the PEG-DMSO was removed and the cell sheet was washed four times with serum-free DME, followed by two washes with DME containing 10% FCS. Finally, after the addition of growth medium (DME + 5% FCS) the plates were incubated. Controls consisted of plates with CEF alone and RCE-T1 cells alone. At various intervals the supernatant fluids were removed, filtered through 0.45-μm Millipore filters and stored at -75°C. The supernatants were later assayed on CEF for the presence of infectious virus by the standard focus assay for RSV (16).

HISTOCHEMICAL AND IMMUNOFLOUORESCENT STAINING: Coverslip preparations of RCE-T1 cells were tested for the presence of factor VIII antigen and angiotensin-converting enzyme (ACE) using the indirect immunofluorescence technique as described previously (1). The histochecmic localization of γ-glutamyl-transpeptidase was performed on acetone-fixed coverslip preparations of RCE-T1 cells using γ-glutamyl-4-methoxy-2-naphthylamide as a substrate, according to the method of Deubacht and Cancilla (28).

TUMORIGENICITY IN NEWBORN RATS: Confluent cultures of RCE-T1 cells (P₀) were trypsinized, suspended in medium with 1% FCS, and DMSO were 44.4% and 8.3%, respectively. CEF and RCE-T1, 1.0 × 10⁴ cells/plate and 5 × 10⁴ cells/ml, respectively, were plated together in 60-mm tissue culture dishes in DME containing 10% FCS and grown for 24 h. The medium was then removed, the cells were washed twice with serum-free DME, and 1 ml of the PEG-DMSO mixture was added to each plate. After 1 min, the PEG-DMSO was removed and the cell sheet was washed four times with serum-free DME, followed by two washes with DME containing 10% FCS. Finally, after the addition of growth medium (DME + 5% FCS) the plates were incubated. Controls consisted of plates with CEF alone and RCE-T1 cells alone. At various intervals the supernatant fluids were removed, filtered through 0.45-μm Millipore filters and stored at -75°C. The supernatants were later assayed on CEF for the presence of infectious virus by the standard focus assay for RSV (16).

Figure 1 Phase-contrast micrograph showing a focus of RSV-transformed rat cerebral endothelial cells 12-d postinfection. The transformed cells have a polygonal shape and are closely apposed to each other. Most cells exhibit dense cytoplasm and contain a large nucleus with multiple nucleoli. × 125.
RESULTS

Transformation of Primary Rat Cerebral Endothelial Cells

12 d after subconfluent homogeneous primary endothelial cells derived from rat cerebral microvessels were infected with SR-RSV-D, a single focus of tightly packed, refractile, small epithelioid-like cells was noted (Fig. 1). 7 d later, this population of transformed cells was isolated using a cloning penicylinder and then serially cultured. During this period, both infected and mock-infected control cerebral endothelial cell cultures were maintained in complete medium without ECGS. Foci of transformed cells were never observed in the control culture. Control primary cerebral endothelial cells, without the presence of ECGS, gradually deteriorated and cell death ensued (1). In addition, no contaminating smooth muscle or pericyte outgrowth was observed in control and transformed cultures during the period of observation.

Morphology of the RSV-transformed Cerebral Endothelial Cell Line

Normal rat cerebral endothelial cells in primary culture exhibit density-dependent inhibition of growth and form a single cell thick monolayer with a characteristic "cobblestone" appearance (Fig. 2). The transformed RCE-T1 cells in culture also form a flat, rather homogeneous sheet of polygonal cells closely apposed to one another (Fig. 3). These transformed cells do not form multilayers in the flattened state but, upon reaching confluence, they gradually round up and form grape-like clusters that eventually detach from the culture surface (Figs. 4 and 5).

Growth Behavior of RCE-T1 Cell Line

Fig. 6 represents the growth curve of RCE-T1 cells at various passage levels (10, 40, and 70) maintained in medium with 10% FCS. Population doubling times were 20.4 h (passage 10), 16.2 h (passage 40), and 16.8 h (passage 70) indicat-
Persistence of the SR-RSV-D Genome in RCE-T1 Cells

Both RCE-T1 cells and rat control endothelial cells were tested for the presence of RSV gs antigens by ELISA. The RCE-T1 cell line yielded a positive reaction at a dilution of 1/100 of the 50% cell extract, indicating that the viral gs protein content was \( \sim 10 \text{ ng/mg} \) of wet weight cells. No reaction was detected with extracts of normal control rat cerebral endothelial cells. It should be noted that the antisera used react predominantly with avian retrovirus p27, and to a lesser extent with p19 and p15 viral proteins. Since avian retrovirus-transformed mammalian cells may contain gs antigens (29–31) without the entire genome being present in a rescueable form, fusion experiments were conducted with permissive host cells (i.e., CEF). Focus-forming virus was detectable in fused CEF-RCE-T1 cultures 5 d after PEG-DMSO treatment (Table I). Virus titer increased progressively up to day 8 and declined thereafter, due to aging and overcrowding of the cultures. The absence of detectable virus in supernatant fluids from RCE-T1 cell cultures indicates that these cells are not producing infectious virus. Although serotyping was not performed, the morphology of the foci induced on CEF by the rescued virus released from CEF-RCE-T1 heterokaryons was identical to that of the original SR-RSV-D employed to transform the rat endothelial cells. It is apparent from these results that RCE-T1 cells are virogenic, in that they contain the intact genome SR-RSV-D and that infectious virus is rescueable by fusion with permissive CEF.

Chromosome Studies

Comparison of chromosomal patterns in both RCE-T1 cell preparations at various passage levels (4–12) and control primary rat cerebral endothelial cells exhibited a normal male rat karyotype of 42, xy that is in agreement with published standards and conclusively demonstrated that the transformed cells were of rat origin.
Differentiated Endothelial Properties of RCE-T1 Cells

As previously reported, rat cerebrovascular endothelial cells are capable of retaining morphologic and antigenic characteristics of vascular endothelium in long-term primary cell culture (1). Because a single transformed colony was isolated upon infection of a homogeneous population of cerebral endothelial cells, it was of interest to determine whether or not RSV-transformed endothelial cells were still capable of retaining differentiated endothelial related markers. Fig. 8

TABLE I

| Supernatant from cells | Virus titer* (day) |
|------------------------|--------------------|
| CEF + RCE-T1           | <0.5  2 x 10^2  8.1 x 10^3  8.2 x 10^1  |
| CEF alone*             | <0.5  NT     NT     NT     |
| RCE-T1 alone*          | <0.5  NT     NT     <0.5   |

* Titer on CEF in focus-forming units/milliliter at times shown after cell fusion.

Tumor Formation

All seven newborn rats injected with RCE-T1 cells developed numerous intraperitoneal neoplastic nodules within 15–20 d postinjection and were most numerous and largest over the peritoneal surface of the diaphragm and the adjacent peritoneum. Most of the tumor tissue grew as well-circumscribed nonencapsulated nodules, with only some of them showing local invasion of adjacent fibrovascular adipose tissue, hepatic and pancreatic parenchyma. Extensive bilateral pulmonary metastases were also noted in two of the rats. The morphology of tumors induced by the RCE-T1 cell is shown in Fig. 11. The predominant histologic pattern of these tumors was consistent with that of a poorly differentiated spindle cell sarcoma frequently displaying well-developed storiform areas. The highly elongated cells were arranged in semiparallel or disorderly arrays. Mitotic figures were common. Multifocal areas of necrosis were present in most nodules and markedly prominent in the pulmonary metastases.

DISCUSSION

As reported previously, long-term primary cerebrovascular endothelial cell cultures have been maintained under restricted culture conditions that required high serum concentration and medium supplemented with ECGS. Confluent cultures exhibited contact inhibition of growth, expressed normal differentiated properties, but lacked the capacity for further proliferation and subculture (1). In another study, Gimbrone and Fareed (2) described the first successful transformation of human vascular endothelium with SV40 virus. However, the resultant cell lines exhibited altered morphology and loss of endothelial-related properties (2).

The present study describes the successful transformation of rat cerebral microvascular endothelial cells with an RNA tumor virus and, to our knowledge, the first establishment of a continuous cell line of RSV-transformed endothelial cells. Although this line, designated RCE-T1, expresses typical transformation-related properties (growth in soft agar, tumorigenicity), it still is capable of retaining normal differentiated endothelial cell characteristics. In vitro virus-induced transformation allowed us to circumvent the culture limitations of primary cultures. Moreover, the occurrence of a single, well-isolated focus of transformed cells was of great advantage, in that the resultant population can be considered to likely be a clonal isolate derived from infection of a single target cell.
chicken embryo fibroblasts to release SR-RSV-D virus clearly indicate that the transformation event was virus-specific. In addition, cytogenetic analysis demonstrated that these transformed cells are of rat origin.

Virus-induced transformation results in a number of phenotypic changes that are collectively termed "the transformed phenotype." The alterations include changes in cell shape, decreased cell adhesiveness, lack of density-dependent inhibition of growth, ability to grow in vitro in low serum concentrations, and tumorigenicity (38, 39). RCE-T1 cells possess all of these stable transformation properties and also have the ability to produce colonies in soft agar, whereas normal cells fail to form colonies. The acquisition of this property of anchorage independence of growth has been shown to correlate well with tumorigenicity (40). RCE-T1 cells inoculated into newborn rats induced tumors that are consistent with the histopathological features of sarcomas.

RCE-T1 cells are an exception to the general finding that differentiated cellular functions often are not expressed following viral transformation. For example, cells of mesodermal origin transformed with RSV usually fail to express normal differentiated functions (8, 41). While the shutoff of these so-called "luxury" molecules can be circumvented when cells are transformed by temperature-sensitive (ts) mutants of RSV and then grown at the nonpermissive temperature (5-7), RCE-T1 cells that were transformed by wild-type RSV and are, within the passage levels studied (4-70), an exception to this rule. Both factor VIII antigen and angiotensin converting enzyme activity were demonstrated in these cells by indirect immunofluorescence. However, it should be mentioned that the expression of these endothelial-related markers in RCE-T1 cells could not be demonstrated at higher passage levels (>70) where marked morphologic alterations also occurred. We recently isolated several morphologic variants from the parent RCE-T1 cell line as well as possible revertants. Examination of these cloned populations for retention of endothelial-related functions and expression of transformation-related properties is currently underway.

In summary, the results show that it is possible to transform rat cerebral endothelial cells with RSV and that these cells retain some of the specific differentiated properties of the original cells in culture. This cell line should then be useful for studying specialized biochemical and functional characteristics of cerebrovascular endothelium. In addition, the RCE-T1 cell line may provide a model to study the mechanism of cell transformation from normal to neoplastic expression.

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