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TRANSFORMATION OF HUMAN CORNEAL ENDOTHELIAL CELLS BY MICROINJECTION OF ONCOGENES

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Human corneal endothelial cells (HCEC) normally form a monolayer covering the posterior surface of the cornea. HCEC are highly specialized cells with as yet unexplained mechanisms of differentiation and regulation of their functional activity and also an unexplained origin. The main function of HCEC is to maintain the water and electrolyte balance of the corneal stroma. Disturbance of the function of HCEC usually leads to edema and opacity of the cornea, resulting in loss of sight.

The properties of the corneal endothelium have been studied mainly on cultures of animal (rabbit, bovine) cells, and in vitro studies of HCEC have been limited to the search for methods of obtaining cultures of these cells, for they possess low proliferative activity and are unable to grow in culture in the absence of stimulating factors [12, 13]. By a combination of methods already developed it is possible to isolate and cultivate HCEC for a long time (up to 50-fold doubling of the population), while preserving their basic morphological characteristics [8]. Cells in culture can be used to compare preparations for use in ophthalmology, and also to reconstruct the injured corneal endothelium. Since the cells to be studied may change their properties while in culture, the need arises to obtain primary cell lines with a stable phenotype.

Combinations of oncogenes of adenovirus (regions E1a and E1b) and cellular origin, differing in their neoplastic transforming efficiency, have been used as transforming agents [6, 10]. It has been shown that microinjection of the E1 region of the strongly oncogenic type 7 simian adenovirus (SA 7) leads to the immortalization and neoplastic transformation of mammalian embryonic kidney cells [5]. The same region of the genome of the nononcogenic human type 5 adenovirus (Ad 5), while preserving the ability to transform and immortalize primary cells, does not induce tumor formation in the experimental animals [3]. The Ha-ras oncogene, in combination with the E1a region of the adenovirus, can induce effective immortalization and transformation of primary cells [9].

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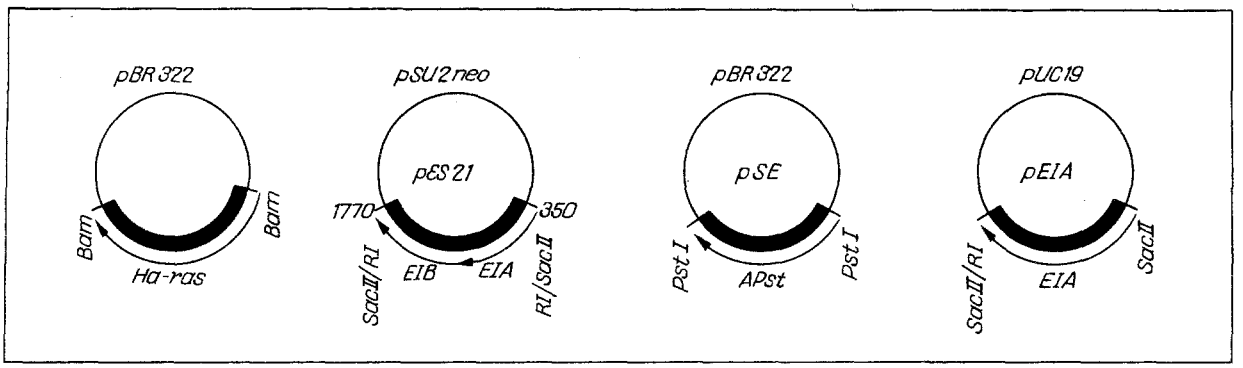

Fig. 1. Structure of recombinant plasmids. Thin line — plasmid sequences; bold line — cloned oncogenes. Numbers indicate position of nucleotides in Ad5 genome. Fragment APst I of SA7 DNA cloned with the use of a connector. The detailed structure of a recombinant plasmid with the Ha-ras oncogenes was published in [6].

The aim of this investigation was to immortalize embryonic HCEC by transfection with various oncogenes, and then to obtain cell lines capable of unlimited passages and preserving the phenotypic characteristics of primary corneal endothelial cells.

EXPERIMENTAL METHOD

Primary HCEC at the 2nd or 3rd passage, used for microinjection, were cultured on gelatin substrate in medium 199 with the addition of nonessential amino acids, 400 μg/ml of human brain extract (HBE), and 20% human serum [8]. Recombinant plasmids from *E. coli*, whose structure is given in Fig. 1, were isolated and purified by the method in [1]. Circular supercoiled or linear forms of plasmids, obtained after hydrolysis by restriction endonucleases, were used. Transfection of the cells by solutions of DNA of plasmids pES 21, pE1A, pES, and Ha-ras was carried out by the method of direct microinjection into the cell nuclei [2]. The plasmid DNAs were dissolved in buffer containing 0.05 M K_2HPO_4, 0.015 M NaH_2PO_4, 0.004 M KH_2PO_4 (pH 7.2). From 100 to 300 equivalent copies of the oncogene were injected into each cell. In each case 200-300 cells were injected. Electrophoresis of the restriction fragments of DNA in agarose gel and hybridization analysis of the cellular DNA, both total and isolated by Hirt's method, were carried out by the method described previously [2]. Cytogenic analysis was performed by the standard method, including hypotonic treatment and Giemsa staining [11]. For the detection of fibronectin, laminin, and entactin in the extracellular matrix, formed by immortalized HCEC, it was treated with the corresponding monoclonal antibodies and developed with PAP (peroxidase — antiperoxidase) reagent [4].

EXPERIMENTAL RESULTS

Transformants were selected in two ways. To select HCEC injected with plasmid pES 21, carrying the bacterial gene of aminoglycoside phosphotransferase, coding resistance to neomycin G 418 (neo^+), DMEM medium with 10% embryonic serum was used, with addition of the antibiotic to a final concentration of 250 μg/ml. Selection was carried out for 14 days. To select all the other types of transformants the cells were cultured in DMEM medium with gradual lowering of the HBE, the embryonic serum (to 5%) and the Ca^{2+} (to 65 mg/ml) concentrations. DMEM medium with 10% embryonic serum and without addition of HBE was used to subculture the transformed cells. In the subsequent experiments three populations of HCEC were used: obtained after microinjection of plasmid pSE (line ECE), of plasmid pES 21 (line SCE), and of a mixture of plasmids pE1A + Ha-ras (line ACE).

The cellular DNA was tested for the presence of sequences of the injected oncogenes by the dot and blot hybridization method after agarose electrophoresis of the restriction fragments of cellular DNA (Fig. 2). The intensity of hybridization is evidence that each transformed cell has at least one copy of the oncogene per genome. The presence of two bands on the autoradiograph of blot hybridization in the Sma I hydrolysate and at least two in the Hind III hydrolysate, and also the different efficacy of their luminescence, indicate either a difference in the integration sites of the injected plasmids in one cell or the presence of clones in the population with oncogenes integrated into different sites of the chromosome. Similar results were obtained during the study of DNA of cells of the ECE and SCE lines.

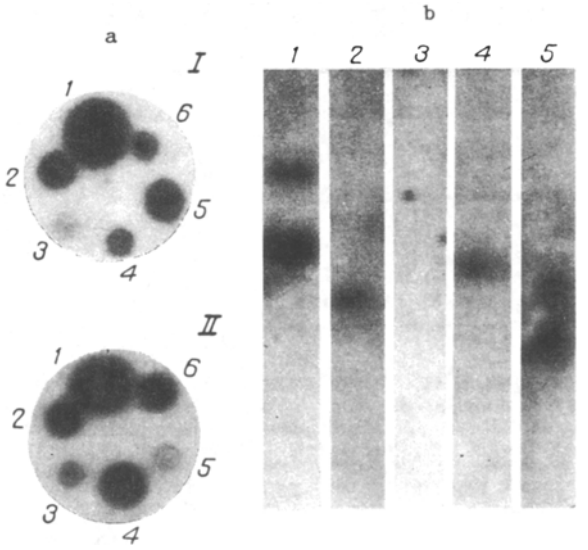

Fig. 2. Autoradiograph of hybridization of total cellular DNA of immortalized lines ACE, ECE$, and SCE. a) Dot hybridization: I) DNA of plasmid pSE, $\times 10^{-4}$, 10^{-5}, and 10^{-6} μg (1-3, respectively), DNA from SCE, ECE, and ACE cells (4-6, respectively); II) DNA of plasmid pES 21, $\times$ 10^{-4}, 10^{-5}, and 10^{-6} μg (1-3, respectively), DNA from SCE, ECE, and ACE cells (4-6, respectively). In center — DNA from primary HCEC. Application of 10 μg cellular DNA to dot; b) Southern blot hybridization: 1 and 5) DNA from ACE cells, 2 and 4) DNA of plasmid pE1A, 3) DNA from HCEC. DNA preparations were hydrolyzed with Sma I(1-3) and Hind III (4, 5). Electrophoresis in 0.7% agarose gel. Sma I fragment of plasmid pE1A, measuring about 800 base pairs, was not tested under these conditions.

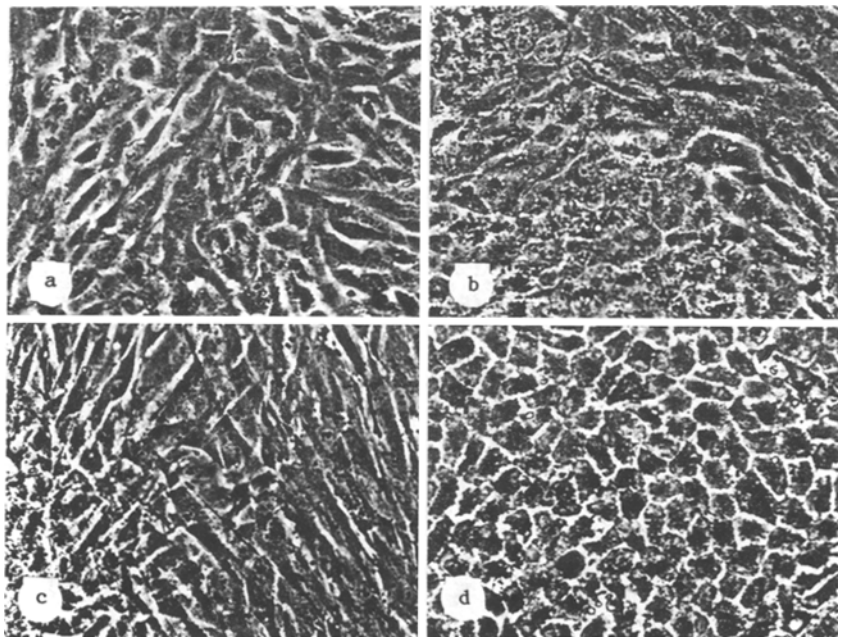

Fig. 3. Morphology of immortalized HCEC in monolayer. a) Line ACE, b) ECE, c) SCE, d) primary HCEC. Phase-contrast microscopy. 300×.

TABLE 1. Growth Characteristics of Immortalized and Primary HCEC

Parameter	ACE	ECE	SCE	HCEC
Clonogenicity	40	92	28	—
Doubling time, h	26—30	16—20	23—27	20—25
Final density (cells/cm^2)	10^5	$1{,}5 \cdot 10^5$	$0{,}8 \cdot 10^5$	$(1—5) \cdot 10^5$

Legend. Clonogenicity determined with the use of DMEM medium with 15% embryonic serum and 30% conditioned medium.

Cells of the three lines obtained in a monolayer differed in their morphological features from the primary HCEC. The cells were elongated in shape, some were partly creeping one over the other, and there were areas of stratified growth (Fig. 3). The stability of the monolayer was low, and 5-7 days after growth became confluent the cells began to separate from the substrate. According to the characteristics studied, cells of the lines now obtained were closely similar to the primary HCEC (Table 1). Of the differences that were found, ability to proliferate without stimulation by endothelial cell growth factors from HBE must be noted. We showed by enzyme immunoassay that the immortalized cells, like the primary HCEC, can form an extracellular matrix, of which laminin, entactin, and fibronectin are components.

The results published in this paper show that, first, immortalization of HCEC takes place in response to injection of oncogenes, differing in their nature and transforming powers, into the cell nuclei. Second, analysis of the properties of the cells during more than 50 passages reveals no change in their phenotype during long-term culture. Third, the fact that immortalized cells preserve the properties of the primary HCEC indicates that there are good prospects for the use of this technique in order to obtain stable cell lines producing biologically active substances.

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