Changes in Morphology, Cytoskeleton, and Substrate Dependence of Proliferation after Transfection of Immortalized Rat Embryonic Fibroblasts with E7 Gene of Type 16 Human Papilloma Virus

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 127, No. 1, pp. 109-113, January 1999
Original article submitted March 30, 1998

Transfection of rat embryonic fibroblasts with E7 gene of type 16 human papilloma virus changed the cytoskeleton and cell-cell and cell-matrix interactions in two clones of transformed cells. Cell morphology and substrate-dependent proliferation were also changed.

Key Words: *human papilloma virus; transformation; actin; fibronectin*

Transformation is a multistaged genetically determined process of cell change with a number of characteristic features. Phenotypic signs of transformation vary and depend on the inducing agent, cell type, and differentiation stage. The disturbance of substrate dependence of proliferation occurs in cultured transformed cells. Other phenotypic signs of transformation are: 1) decrease in the number of actin microfilament bundles (stress-fibrills) which participate in the formation of focal contacts, i.e., local zones of cell adhesion to the extracellular matrix; 2) tyrosine hyperphosphorilation of proteins involved in focal contacts, which inhibits formation of these contacts and decreases adhesion [5,7,8]; 3) changes in the extracellular matrix structure and in fibronectin (FN) as one of its components [9]. There is structural and functional interaction between the extracellular matrix and the cytoskeleton of an attached cell. Disturbances of this interaction in transformed (malignant) cells potentiate invasion [7,9].

Cells transformed by E7 gene of type 16 human papilloma virus (E7 HPV16) are a convenient model for studying phenotypic characteristics of transformation. The E7 gene immortalizes primary human keratinocytes and transforms fibroblasts of permanent rodent lines when cotransfected with *ras* gene [6].

The properties of the transformed clones obtained by transfection of rat embryonic fibroblasts (EF) with E7 HPV16 gene have been examined. Cytogenetic analysis of these clones revealed clone expansion of the cells with specific chromosome transformations [11].

The aim of the present study was to investigate cell morphology, organization of actin cytoskeleton and FN of extracellular matrix, growth characteristics and substrate dependence of proliferation in immortalized cell clone (IE5) and in trF8 and trB4 clones transformed by E7 HPV16 gene.

METHODS

Parental line of immortalized rat EF was obtained from primary culture transfected with plasmids containing T-antigen polyoma virus gene [3]. IE5 clone was isolated from this line by the final dilution technique. By the start of the present study the virus gene in IE5 clone was lost, as revealed by blot-hybridization technique. IE5 clone was transfected with the E7 HPV16 gene [11]. The transformed clones were grown on selective medium containing geneticin (400 mg/ml,

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0007-4888/99/0001-0099 $22.00

Gibco). Further culturing of all clones was conducted in DMEM (Dulbecco's minimal essential medium) containing 10% fetal calf serum.

To examine growth characteristics of the clones the cells were seeded in 24-well culture plates (bottom area 2 cm^2, plating density of 10^4 cells/ml suspension). Proliferation rate was estimated daily by trypsinization of the cells and counting them in Goryaev chamber. Cloning efficiency was determined in semiliquid DMEM medium with 1.2 % methylcellulose (Lawson). The cells were maintained in an incubator with automatic injection of 5% CO_2. Fourteen days after seeding the colonies were counted under a preparation microscope.

Immunofluorescent staining of fixed 2-day-old monolayer cultures on coverslips was used for the examination of actin cytoskeleton and matrix FN [2]. Actin microfilaments were stained with rhodamine-conjugated phalloidin (Sigma). Monospecific polyclonal antibodies against FN were prepared as described [4]. TRITC-conjugated antiserum against rabbit immunoglobulins (Sigma) was used as secondary antibodies. The cells were examined using a with water immersion objective (magnification 50) under a Leitz ARISTOPLAN fluorescent microscope. Two-day-old cultures were also examined by scanning electron microscopy. The cultures were processed as described [1].

RESULTS

Transfection of immortalized rat EF with E7 HPV16 gene and subsequent cloning of geneticin-resistant cells yielded 7 clones. Clones trF8 and trB4, which differ in morphology and growth character, were selected [11].

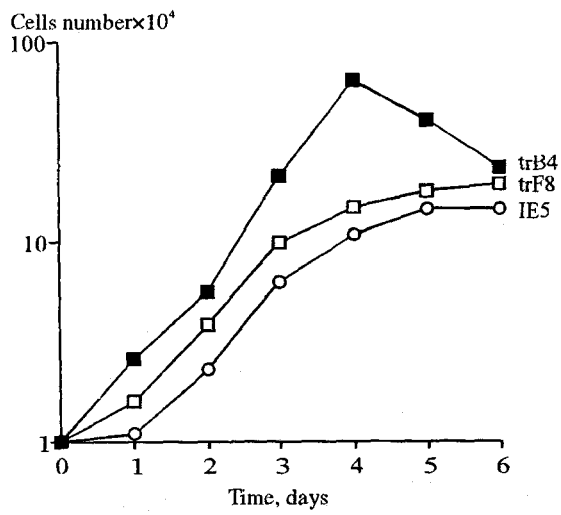

Fig. 1. Growth of the initial (IE5) and two transformed (trF8 and trB4) clones of rat embryonic fibroblasts.

Examination of the growth pattern of the initial (immortalized, IE5) and two transformed clones revealed the following features: 1) shorter lag-period in trB4 clone compared with two other clones (Fig. 1, trB4); 2) contact inhibition of proliferation in trF8 clone (Fig. 1, trF8), i.e., the cells attain the maximum density that inhibits subsequent proliferation; 3) stationary growth period in trF8 clone and its absence in trB4 clone, in which detachment was observed on the 5th or 6th day of monolayer culturing. Light microscopic examination revealed areas of multilayer growth in a monolayer culture of trB4 clone. The differences between two transformed clones were revealed in substrate-independent cloning in a semilsolid medium. Cloning effectiveness in methylcellulose was two times higher for trB4 than for trF8 clone (7.8 and 4.2 % respectively). The occurrence of colonies 80 μ in diameter and greater in trB4 clone was twice as that in trF8 clone (1.4 and 0.7 % respectively).

A comparison between parental IE5 clone and transformed clones under a light microscope showed that immortalized cells were flattened, and had standard fibroblast-like polarized shape with wide lamelloplasm. The cells of trF8 clone were less flattened, while trB4 cells practically did not flatten and had various numbers of short processes. These changes in cell morphology were confirmed by scanning electron microscopy. The surface of IE5 cells was even, and cell morphology was similar to that of primary culture (Fig. 2, *a*). The surface of trF8 cells was even or contained small microvilli and folds (Fig. 2, *b*), the cells looked slightly shrinked and deprived of lamelloplasm. The morphology of trB4 cells was similar to that described for trF8 clone; in dense regions of the monolayer the cells overlapped each other (Fig. 2, *c*).

Staining of actin filaments in IE5 cells revealed long stress-fibrills (actin bundles) crossing the cell parallel to its long axis, the lamella were weakly stained (Fig. 3, *a*). The cells of the transformed clone trF8 had well-developed system of stress fibers located parallel to the long axis of the cells (Fig. 3, *b*). In the cells of trB4 clone thin actin bundles were not oriented parallel to the long axis and were observed in the processes with intensely stained terminal ruffles (Fig. 3, *c*).

In subconfluent cultures of immortalized cells thick fibronectin (FN) fibers formed massive network with chaotic orientation of fibers(Fig. 3, *d*). In transformed trF8 cells FN fibers were thinner than in the immortalized cells; in dense cultures these fibers formed a thick chaotic network (Fig. 3, *e*). In trB4 cells FN was organized into small spots and short thin fibers (Fig. 3, *f*). The network was observed only in the multilayer areas.

Thus, clones of rat EF with different degree of transformation were obtained: 1) immortalized cells

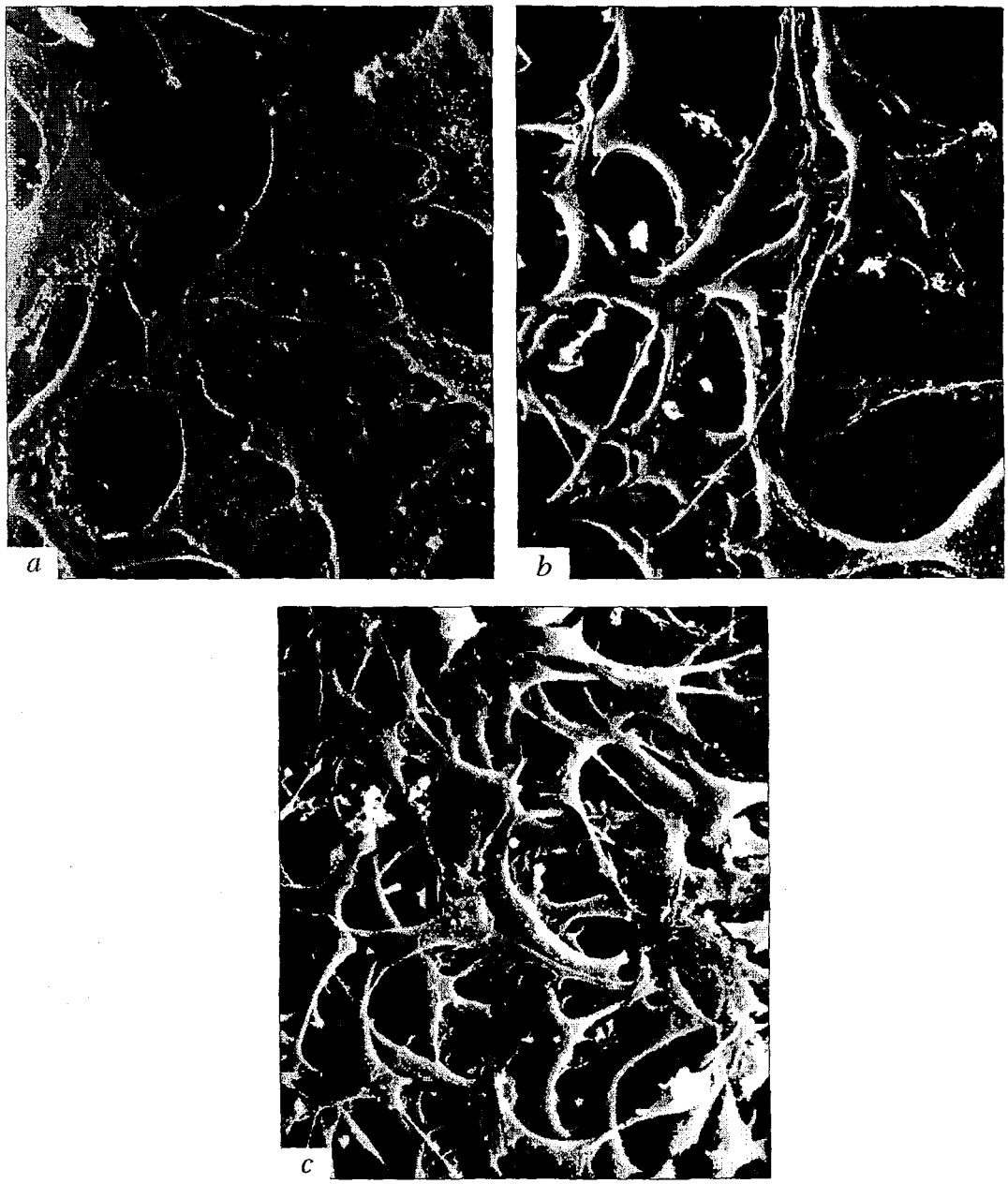

Fig. 2. Scanning electron microscopy of monolayer fibroblast cultures (×640). *a*) immortalized fibroblasts, IE5 clone; *b*) transformed cells of trF8 clone; *c*) transformed cells of trB4 clone.

(IE5 clone) characterized by minimal transformation; 2) trF8 clone with the features of normal phenotype (topoinhibition at increasing cell density) but tumorigenic [8] and characterized by proliferation independent of growth factors and substrate; 3) trB4 clone characterized by the most pronounced phenotypic transformation features such as the ability to form multilayer growth centers in a monolayer, independence of growth factors, density and substrate proliferation, and tumorigenicity [11]. Higher efficiency of cell cloning in semisolid medium for trB4 cells and their ability to form larger colonies in comparison with trF8 cells indicate higher degree transformation of trB4 clone to the malignant phenotype. An increase in the degree of transformation is accompanied by a decrease in the degree of cell flattening and by changes in the cytoskeleton organization (the ability to form actin bundles decreases and the orientation parallel to the long cell axis disappears). These changes in the cytoskeleton in the immortalized and transformed cultures as well as changes in the cell-matrix interactions (progressive loss of FN from the surface of trF8 and trB4 cells point to the transforming activity of E7 HPV16 gene.

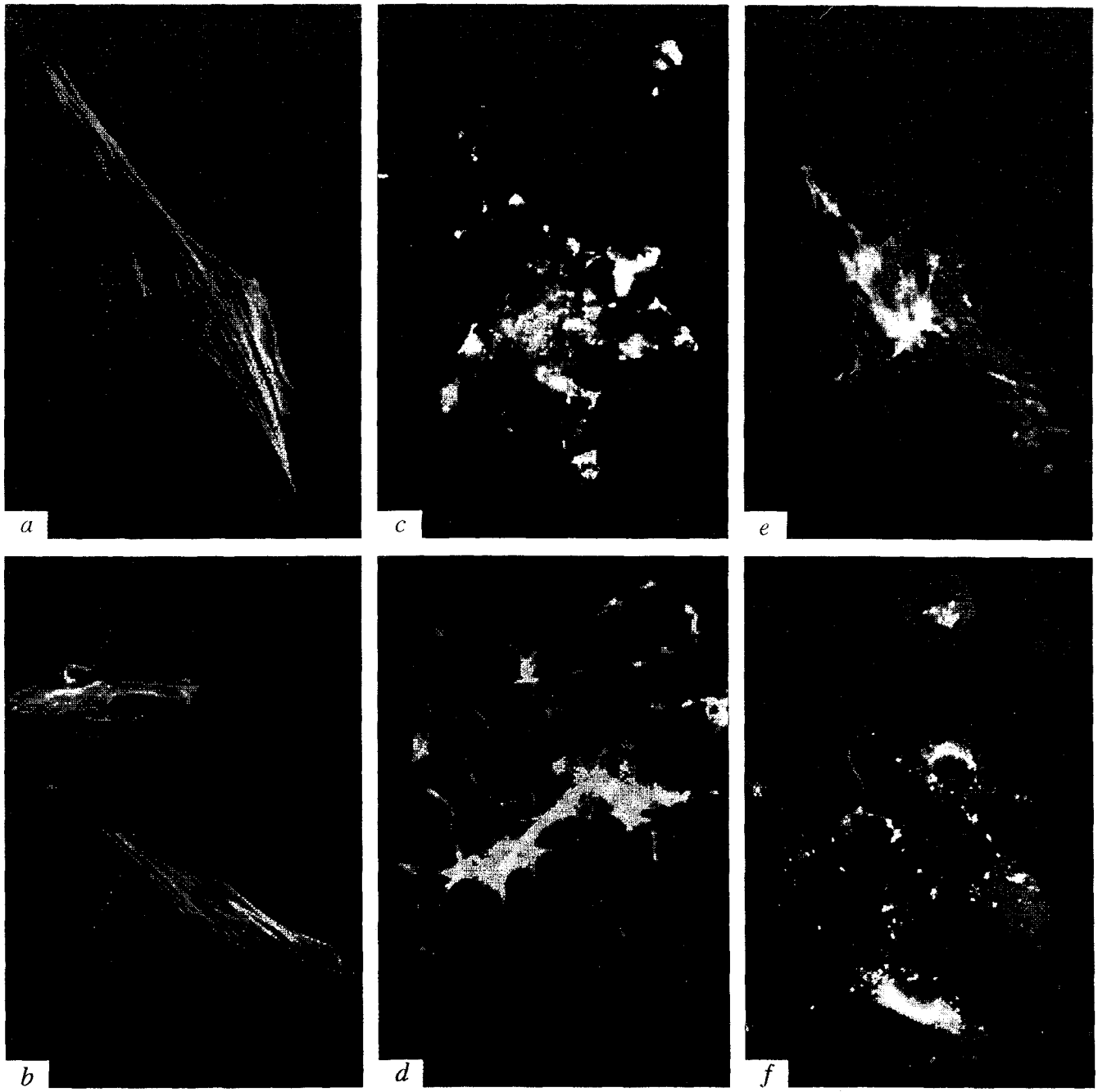

Fig. 3. Immunocytochemical determination of actin (*a-c*) and fibronectin (*d-f*) expression in fibroblast monolayer cultures. Immortalized fibroblasts (*a, d*), transformed cells of trF8 (*b, e*) and trB4 (*c, f*) clones.

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