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Letter to the Editor

IMMORTALIZATION OF HUMAN EPIDERMAL KERATINOCYTES BY THE RECOMBINANT SV40 ADENOVIRUS VECTOR

Dear Editor:

A method for culturing human epidermal keratinocytes in the low calcium defined medium has allowed access to study the growth regulation and differentiation of human keratinocytes *in vitro* (3,7). These cells could not be maintained for a long time because of their definite life spans. Transfection procedures with plasmid DNA containing the SV40 early gene have been utilized to immortalize human keratinocytes (2,6). However, the efficiency of immortalization by DNA transfection was very low. To increase the efficiency, we attempted to introduce the SV40 early gene into human keratinocytes through an adenovirus vector by using alternative procedures, infection and electroporation. We indicate here that the introduction of the recombinant SV40-adenovirus, ori$^-$ (8), by each procedure showed significantly higher efficiencies in gene introduction and immortalization of human keratinocytes compared with DNA transfection.

To examine the efficiency of introduction, transient expression of SV40 T antigen was examined 48 h after introduction by indirect immunoperoxidase staining. The number of molecules of the virus vector or pUCori$^-$ DNA (1) introduced was roughly calculated at 5×10^9 (*at 50 PFU per cell*) or 1.5×10^{12}, respectively. We estimated that one PFU was equivalent to 100 particles of adenovirus and that the molecular weight of pUCori$^-$ DNA was 4×10^6 dalton. An excess of molecules of pUCori$^-$ DNA over that of the virus vector was introduced into the cells under our experimental conditions. The results are summarized in Table 1. This clearly indicated that the ori vector was able to efficiently introduce the SV40 early gene into human keratinocytes when compared with DNA transfection. The number of T antigen-positive cells increased as the multiplicity of ori$^-$ infection was lowered to 5 PFU per cell, suggesting that the virus vector might have a cytotoxic effect on the cells when introduced at a higher multiplicity. Electroporation showed higher efficiency in the introduction of the virus vector than infection, although approximately 60% of the cells died. The virus vector might be able to enter human keratinocytes through adenovirus receptors on the surface of the cell membrane or phagocytic action of the cells, although the exact mechanism of virus internalization is not known.

It was difficult to quantitatively compare the efficiency of colony formation of keratinocytes after introduction of either ori$^-$ or plasmid DNA because unintroduced normal keratinocytes formed colonies when cultured in KGM. It has been demonstrated that keratinocytes transformed by human papilloma virus (HPV) were resistant to terminal cell differentiation by treatment with serum and calcium and formed colonies in the presence of them (4). Furthermore, some SV40-transformed cell lines grew in the presence of serum and a high calcium concentration (5). Therefore, we attempted to examine the efficiency of serum/calcium-resistant colony formation after introduction of either ori$^-$ or pUCori$^-$ DNA according to the experimental conditions described by Schlegel et al. (4) with slight modifications. Table 1 shows that the virus vector formed more serum/calcium-resistant colonies of keratinocytes than the plasmid DNA did. Under these experimental conditions, no colonies were detected in cultures of cells electroporated with plasmid DNA. In

TABLE 1

EFFICIENCIES IN INTRODUCTION OF THE SV40 EARLY GENE INTO HUMAN EPIDERMAL KERATINOCYTES AND FORMATION OF SERUM/CALCIUM-RESISTANT COLONIES

Methods of DNA Introduction		Transient[a] Expression (%)	Number of Serum/Calcium[b] Resistant Colonies
pUCori$^-$ DNA electroporation		<1	0, 0
	m.o.i.[c]		
ori$^-$ infection	5	32, 43	10
	50	27, 38	15
	500	12, 19, 20	1
ori$^-$ electroporation	5	91, 93	8
	50	83, 86	8
	500	35, 58, 74, 82	4

[a] Preparation of primarily cultured human keratinocytes was performed according to the method described (3). The cells cultured in KGM (Clonetics Corp.) *containing 0.15 mM calcium were subcultured* by EDTA-trypsin. Secondarily cultured cells were harvested by EDTA-trypsin, washed with KGM supplemented with 10% FCS and washed twice with KGM. To give the indicated multiplicities of infection, 1×10^6 cells were mixed in 0.5 ml of Dulbecco's modified minimum essential medium (DMEM) containing various amounts of virus stock. For infection, the mixture was incubated for 2 h at 37° C and then washed with PBS(−). An aliquot of the cells was *cultured in KGM at 37° C in a 5% CO_2 incubator. For electroporation, the* mixture was kept for 5 min on ice and then electrically pulsed under conditions of 1,180 μF and 500 V/cm. After incubation on ice for 10 min, the cells were washed with cold PBS(−) and cultured in KGM at 37° C. When DNA transfection was performed, 1×10^6 cells were mixed with 10 μg of pUCori$^-$ DNA in 0.5 ml of DMEM and kept for 5 min on ice. Electrical pulsation and subsequent cultures were performed by the same procedures *as described above. The cells were fixed with acetone 48 h after introduction*, and stained for SV40 T antigen by the indirect immuno-peroxidase technique using mouse monoclonal antibody against SV40 T antigen and horseradish peroxidase conjugated goat anti-mouse IgG. Percentages of the T antigen-positive cells were calculated. At least 200 cells were examined in each experiment.

[b] The SV40 early gene was introduced into 1×10^6 keratinocytes as *described above, aliquots of the cells* (1.4×10^5 cells) *were cultured in* KGM in 60 mm dishes at 37° C for 2 days. Then, the medium was changed and the cells were maintained in DMEM supplemented with 10% FCS. The number of colonies proliferating for more than 3 weeks was counted.

[c] Multiplicities of infection.

cultures of cells either infected or electroporated with ori$^-$, 8–15 colonies were developed with introduction of multiplicities of 5 and 50 PFU per cell.

We examined the efficiency of immortalization by ori$^-$ or pUCori$^-$ DNA. 1×10^6 cells were cultured in KGM at 37° C after electroporation with ori$^-$ at 5 PFU per cell or 10 μg of pUCori$^-$ DNA. When the cells were subconfluent, one-fifth of the cells were subcultured by trypsin-EDTA treatment. Expression of T antigen was monitored during each passage. After several passages, more than 90% of the cells electroporated with ori$^-$ were positive for T antigen. The cells grew for more than 150 doubling populations (more than 30 passages), and at that time almost all of the cells expressed T antigen. No apparent senescence or crisis of the bulk of the cells was observed as passages progressed although microscopic observations suggested that only a small population of the cells underwent terminal differentiation and stopped growing. In contrast, keratinocytes electroporated with plasmid DNA or untreated cells stopped growing with a pattern of senescence after several passages under these conditions. We regarded the cells which grew for more than 150 doubling populations as immortalized. The immortalized cells expressed SV40 T antigen and still formed a cobblestone monolayer similar to normal keratinocytes. The results suggested that introduction of the virus vector caused more efficient immortalization of human keratinocytes than that of the plasmid DNA.

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