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BBA Report

In vivo electroporation and stable transformation of skin cells of newborn mice by plasmid DNA

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The skin cells of newborn mice were stably transformed in vivo with the aid of electroporation. The plasmid DNA was introduced subcutaneously followed by high-voltage pulses applied to the skin pleat. NEO-resistant colonies were found in primary cell cultures obtained from the treated skin. The experiments show that in vivo electroporation can be used for the introduction of plasmid DNA into skin cells of mouse.

The search for clinically applicable gene transfer has so far been centered around genetic transfection of target cells in vitro followed by their implantation in vivo [1]. A substitution mutation has been introduced into the *c-abl* locus of murine embryonic stem cells by homologous recombination, and these cells have been injected to the blastocysts of mice. The chimeric mice were obtained which could transmit the mutation in *c-abl* locus to progeny [2]. Bone marrow cells infected in vitro by retrovirally delivered transgenes can restore enzymic activities for months after repopulation of bone marrow in mice [3], monkeys [4] and sheep [5]. Some cell types can generate desirable products for as long as several weeks after in vitro transfection followed by delivery into the organism in microcarrier-attached form. Among them are rat hepatocytes [6] and human keratynocytes [7]. Normal rat primary hepatocyte culture could be seeded into the network of the vascularized fibers and implanted near the liver of the rat lacking the enzyme conjugating bilirubin. The decreased level of bilirubin in the blood of recipient rats could be maintained at least 181 days in the presence of viable hepatocytes and neovascular structure [8].

The direct introduction of a gene transfer vector into a target organ in vivo is a way to avoid manipulations with the cells outside the organism. Several procedures have been reported that permit in vivo transfection. For example, in vivo liposome-mediated gene delivery into the vein of rat has led to the expression of foreign insulin I and preproinsulin genes in recipient rats [9,10].

The injection of calcium phosphate-precipitated plasmid into rat liver and spleen, or introduction of protein-coated plasmid into the portal vein resulted in transient gene expression in liver [11]. Chloramphenicol acetyltransferase (CAT) gene was expressed transiently in the liver and spleen of newborn rats after intraperitoneal injection of calcium phosphate-precipitated plasmid DNA [12]. Recently, it was demonstrated that foreign DNA expressed in the muscles of mouse after simple microinjection [13].

In the current study, we demonstrate that plasmid DNA introduced into skin cells of newborn mice in vivo with the aid of electric pulses can yield stably transformed mouse fibroblasts.

In our experiments we used (a) pSV3neo plasmid [14] containing neomycin-resistance gene (NEO-R) under control of SV40 early promoter and the gene for SV40 T-large antigen, which could transform primary rodent cells [21] and (b) pHEB4 plasmid containing the E1A region of Adenovirus 2, which could immortalize primary rodent cells [15]. The purpose of mixing pSV3neo and pHEB4 plasmids together was to minimize the difficulties in proving transfection of foreign DNA by phenotype of clones.

The mixture of the two supercoiled plasmid DNA's was introduced subcutaneously to newborn CBA mice 1–3 days of age. After an adsorption period (10–60 min), the pleat of skin was exposed to two high-voltage pulses in opposite polarities using a special device (Fig. 1; Table I). Electric contact between electrodes and skin

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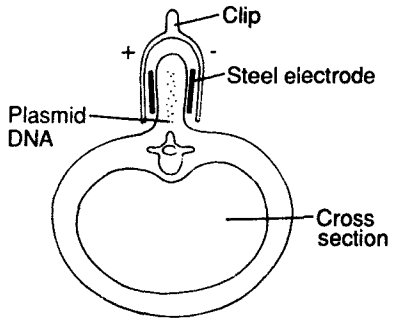

Fig. 1. Scheme of cross section of the body of mouse at the time of in vivo electroporation. Special device for in vivo electroporation was laboratory built. Two flat stainless steel electrodes of about 2 cm^2 mounted on the plastic clip were connected to high-voltage pulse generator. The generator has 8 μF storage capacitor which can be charged up to 1 kV. Electric pulses up to 400 V did not cause visible skin damages. Starting from pulses of 600 V, necrotic damages of skin had appeared which were relieved during the next 2 weeks without affecting viability of newborn mice.

was facilitated by conductive grease (30–50% glycerol in 1 × PBS). Viability of mice was 100% in all experiments.

In order to find if plasmid DNA was integrated into skin cells, the fibroblast primary cell cultures were obtained from the treated skin and put to selective conditions. Skin samples were removed and treated by collagenase (1 mg/ml) in 1 × PBS at 37°C. After 40 min, the cells were washed twice with 1 × PBS and seeded in 30–40 ml of DMEM medium containing 10% fetal calf serum (FCS; Gibco), 40 μg/ml gentamicin and 1 × Antibiotic-antimycotic (Gibco) into 250 ml plastic bottles (Costar). After 24–48 h antibiotic G418 (Sigma) was added.

After 2–3 weeks of selection clones of stable transformants were found. Thus, in vivo electroporation resulted in NEO-R clone formation. Two controls were performed: (a) the plasmid DNA injected without subsequent electroporation and (b) electric pulses applied without DNA. In both cases no clones were found.

Transformation efficiency was measured by dividing the number of NEO-R colonies by the total number of primary cells attached 16 h after seeding. In four independent experiments transformation efficiency was $(0.2–7.0) \times 10^{-4}$ (Table I).

To prove that clones of transformants were obtained by foreign DNA incorporation, the total cellular DNA from six clones was analysed by the Southern blot method [16]. Total DNA was isolated from 10^7 cells [17]. 10 μg DNA samples were restricted and, after electrophoresis in 1% agarose (Sigma), blotted on Hybond N paper. Hybridization with ^{32}P-labeled DNA probes was performed according to Ref. 18.

It was demonstrated that the total cellular DNA of all the clones has the intact 3.0 kb *Bam*HI fragment containing SV40 early region (Fig. 2a). Fig. 2b shows more definitively the existence of SV40 specific 1.25 kb and 0.56 kb *Hin*dIII-*Pst*I fragments in the DNA of all clones, except clones No. 1 and No. 2 where loss of the 1.25 kb fragment may be due to site rearrangment which did not result in the loss of transformed phenotype. E1A gene of Ad2 was found in the total cellular DNA of one clone (No. 3) from other four clones which implicated co-transfection of pSV3neo and pHEB4 plasmids (Fig. 2c). Low ratio of co-transformation in vivo compared to that in vitro could be due to differences in environment.

Expression of SV40 large T-antigene was found by Northern analyses [19] of the total RNA of the same clones in which a 2.6 kb fragment (Fig. 3) corresponds to the SV40 T-antigen transcript [20].

All the clones grew in low serum medium and they had multilayer phenotype (data not shown), which could reveal transformed phenotype of the clones. Stability of NEO-R phenotype of all 14 clones was established by comparing the clone-forming efficiencies of cells grown with and without antibiotic G418. No significant differences were found (data not shown). Thus, the plasmid DNA persisted in the cells for at least 30 generations without selection.

TABLE I

Conditions of four independent experiments of in vivo electroporation of skin cells of mouse

Mixture of pSV3neo and plasmid DNA's (60 μl in 1 × PBS) was injected subcutaneously to newborn mice. After adsorbtion time, two pulses were applied to skin. Primary culture was obtained 24–168 h later. Transformation efficiency was culculated by dividing the number of NEO-R colonies by number of cells attached to Petri dish 16 h after seeding them in culture.

Clones Nos.	Plasmid DNA		Adsorption time (min)	Volts per cm	Pulse width (μs)	Primary culture (h)	No. of attached cells	Start of G418 (h)	No. of NEO-R colonies	Transformation efficiency
	pSV3neo (μg)	E1A (μg)								
1, 2	12.5	12.5	10–15	400	100	24	$1.0 \cdot 10^4$	48	7	$7.0 \cdot 10^{-4}$
3–8	2.0	10.0	15	600	180	24	$0.8 \cdot 10^5$	48	15	$1.9 \cdot 10^{-4}$
9–11	5.0	10.0	60	400	300	42	$1.0 \cdot 10^6$	48	24	$0.2 \cdot 10^{-4}$
12–14	5.0	10.0	15	400	300	168	$1.0 \cdot 10^5$	24	16	$1.6 \cdot 10^{-4}$

It was also demonstrated that the plasmid specific sequences were associated with high-molecular-weight (23 kb) cellular DNA (data not shown), indicative of their integration into the chromosome. However, we could not exclude the possibility that the plasmid DNA

Fig. 2. Southern analysis of the total DNA isolated from the cells of stable transformants. (A) DNA of clones No. 1 to No. 6 restricted with *Bam*HI (lanes 2–7); lane 1 is 1 ng of pSV3neo plasmid DNA restricted with *Bam*HI (100 copies per cell). (B) DNA of clones No. 1 to No. 6 (lanes 1–6) restricted by *Hin*dIII + *Pst*I; lane 7 is the DNA of clone No. 5 restricted with *Hin*dIII; lane 8 is control DNA of primary mouse cells. (C) DNA of clones No. 1, 2, 3, 6 restricted with *Pst*I (lanes 2–5); lane 1 is a 1 ng of pHEB4 DNA restricted with *Pst*I (170 copies per cell); lane 6 is the DNA of mouse primary cells. ^{32}P-labeled DNA of pSV3neo was used as a probe in hybridization with (A) and (B) ($5.0 \cdot 10^8$ cpm per 1 μg of plasmid DNA); and ^{32}P-labeled 1.7 kb fragment containing E1A was used as a probe in hybridization with (C) ($1.0 \cdot 10^9$ cpm per 1 μg of plasmid DNA). Exposition time at −70°C: 5 days (A, B) and 24 h (C); kb, kilobases.

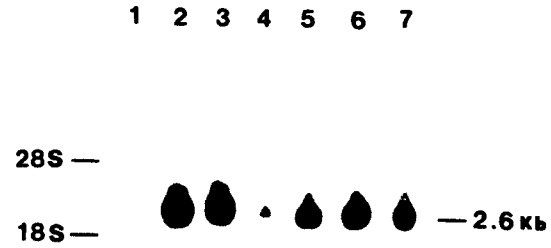

Fig. 3. Northern analysis of the total RNA of clones (No. 1 to No. 6 corresponding to lanes 2–7). Lane 1 is a control for total RNA from embryonic mouse cells. 10 μg of RNA was put on one slot except clone No. 3 (0.7 μg). ^{32}P-labeled DNA of plasmid pSV3neo was used as a probe ($5.0 \cdot 10^8$ cpm per 1 μg of plasmid DNA) and filter was exposed 4 days at −70°C; kb, kilobases.

persisted in the nucleus of the skin cells in an extrachromosomal fashion and integrated into chromosomes only after seeding the skin cells to culture.

No tumors were found in 18 mice during 3 months after in vivo electroporation of their skin using the mixture of the same plasmids. Thus, in vivo electroporation of skin of newborn mice resulted in stable transformation of the skin cells (fibroblasts), which was proved by obtaining the clones of stable NEO-R transformants in vitro. Plasmid DNA was found in the total cellular DNA of all the clones. All clones expessed mRNA specific for SV40 large T-antigen. In electroporation of skin cells of mouse in vivo the most important factor could be voltage applied to skin. Electric pulses less than 200 V did not cause transfection of cells. We did not try more than 600 V. In diaposon at 300–400 V, there was good output of transformants without damage of skin.

The ability of losing contact inhibition and growing in low serum medium confirms expression of SV40 large T-antigen, which can transform the primary rodent cells [21]. SV40 early region could participate in transformation through inactivation of specific cellular proteins, such as retinoblastoma P105 protein, that control cellular proliferation themselves [22]. The skin cells transformed with SV40 early region could have selective advantage and might expand in the treated skin.

Electroporation in vivo could be applied to the investigation of multistep carcenogenesis of the skin cells. This method could have advantage in creating small nests of cells with distinct phenotype over transgenic mice model in which virtually all the cells are expressing the same gene [23]. In vivo electroporation could be used for treatment of pathological conditions resulting from the loss or malfunction of a physiologically im-

portant protein, or for preparative scale production of heterologous proteins in vivo.

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