In vivo gene electroinjection and expression in rat liver

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Abstract In vivo targeted gene transfer by non-viral vectors is subjected to anatomical constraints depending on the route of administration. Transfection efficiency and gene expression in vivo using non-viral vectors is also relatively low. We report that in vivo electropermeabilization of the liver tissue of rats in the presence of genes encoding luciferase or β-galactosidase resulted in the strong expression of these genetic markers in rat liver cells. About 30–40% of the rat liver cells electroporated expressed the β-galactosidase genetic marker 48 h after electroporation. The marker expression was also detected at least 21 days after transfection at about 5% of the level 48 h after electroporation. The results indicate that gene transfer by electroporation in vivo may avoid anatomical constraints and low transfection efficiency.

Key words: Electroporation; Gene transfer; Rat liver; Luciferase; Efficiency, expression

1. Introduction

Nonviral delivery of DNA in vivo is a subject of intensive study. With the goal of targeted gene delivery, anionic liposomes were first used [1,2]. Low transfection efficiency and transient gene expression characterized these first attempts; nevertheless, they indicated the feasibility of targeted DNA delivery to specific liver cells following i.v. liposome injection [2]. Targeting of liposomes with monoclonal antibodies directed against surface antigens of hepatoma cells proved quite successful in vitro, but expression of genes encapsulated in these liposomes is not very efficient [3]. The introduction of cationic liposomes has increased the efficiency of DNA delivery and demonstrated enhanced gene expression in vitro [4], and recently, in vivo [5,6]. Even if cationic liposomes could be efficiently targeted in vivo to specific cells, they would be subjected, like all types of liposomes, to the usual anatomical constraints depending on the route of administration [7]. A method of efficient DNA delivery to any specific tissue in vivo, overriding the anatomical constraints, might lead to useful therapeutical applications of gene transfer. In 1982, Neumann et al. [8] demonstrated that in vitro electroporation of cells in the presence of plasmid DNA resulted in DNA transfer and expression. Since then, this method has become a widely used technique for in vitro transfection [9]. The mechanism of DNA transfer, using this approach, is based on formation of membrane pores followed by DNA electrophoresis into the cells [10].

Electroporation has been used in vivo, in animals and patients [11–13], primarily to introduce anti-tumor drugs into cancer cells. Given the in vitro efficiency of this method of gene transfer, it appeared as a potentially efficient approach to target in vivo transfection. Using the liver of anesthetized rats as a model organ for gene delivery and expression, we investigated the applicability of electroinjection to the liver tissues for in vivo gene therapy.

2. Materials and methods

2.1. Plasmids

The expressing vector pSV-β-galactosidase carrying the β-gal gene under the control of SV40 promoter was purchased from Promega (Madison, WI).

2.2. Plasmid expressing firefly luciferase

The *Bam*HI-*Xho*I fragment carrying the Luc coding sequence from pGEM-Luc (Promega) was cloned into the pRc/CMV plasmid (Invitrogen, San Diego, CA) between corresponding restriction sites, under the control of the CMV promoter.

2.3. Electrode and electric pulse delivery

Electric pulses were delivered using a rectangular direct current (DC) generator (T820; BTX, Inc.; San Diego, CA) and a switch box (195-7460; BTX, Inc., San Diego, CA). Six pulses were delivered to each animal at a rate of one pulse per s. Each pulse was 99 μs in duration, and an electric field strength of 1000 V/cm was used unless otherwise specified. Pulses were administered to the tissue using a needle-array electrode (878-2a; BTX, Inc., San Diego, CA). Each array consisted of a cylindrical epoxy body with a circumferentially arranged set of six 28 gauge stainless-steel acupuncture needles protruding from one end. The needles were equispaced around a 1 cm diameter circle; each needle had an independent electrical connection. The switch box and needle array were used to deliver pulses from the DC generator. Pulses were monitored using a digital storage oscilloscope (PM3375; Philips, Eindhoven, The Netherlands). Pulse data were transferred, using an IEEE 488 interface bus and custom-written software, to a personal computer for analysis.

2.4. DNA electroinjection

Male Sprague Dawley rats (Harlan, Indianapolis, IN) were weighed on a digital top loading scale. A 3 mg/kg dose of atropine sulfate (WAB10125, The Butler Co., Columbus, OH) was administered subcutaneously into the left flank using a 0.5 inch, 30 gauge needle. 10 min later a 45 mg/kg dose of sodium pentobarbital (WAB10505, The Butler Co.) was injected into the peritoneal cavity using a 0.5 inch, 30 gauge needle. After the animals were completely anesthetized, the liver was exposed by making a transverse incision starting from the midsagittal position, approx. 1 cm caudal to the xiphoid process, extending 3–4 cm toward the dorsal surface of the rat. The median lobe

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Abbreviations: β-gal, β-galactosidase; Luc, firefly lucirefase; C12 FDG, 5-dodecanoylamino-fluorescein di-β-galactopyranoside

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(both halves) of the liver was exposed by drawing it out of the incision taking precautions not to cause any tissue damage.

Gene transfer was performed by injecting the desired quantity of gene (luciferase or β-galactosidase) in 100 μl of normal saline just beneath the surface of the right median lobe. The entire dose was injected at a single location in the center of the lobe. A period of 1.5 min was allowed to pass in order to allow dissemination of the gene. Next, the electrode was placed into the tissue. Great care was taken to insert the electrode needles so that their annular pattern completely surrounded the injection point. Pulses, in the form of a rotating electric field, were administered immediately after the electrode was placed. The location of the electrode was marked using sutures in order to allow the treatment area to be later identified for biopsy. Animals were closed following gene transfer by suturing the abdominal muscles together and then stapling the skin incision.

Expression was determined in tissue samples from the treatment sites. These samples were taken by first killing the animals by delivering a 100 mg/kg dose of sodium pentobarbital. Then, the right median lobe was exposed as described above. Next, the treatment area was identified (to the extent possible) using the sutures that remained in the lobe to mark the location. Finally, an 11 mm diameter biopsy punch was used to remove a core (full thickness) of tissue that was encompassed by the needle array. Samples for luciferase expression were frozen rapidly by emersion into liquid nitrogen and analyzed approx. 12 h after biopsy. Samples for β-galactosidase expression were stored at 4°C in phospate-buffered saline for 12 h before analysis.

2.5. *Luciferase expression*

Luciferase expression was measured using a TD20E luminometer (Turner Design, Sunnyvale, CA) and a luciferase detection kit with Co A-luciferin as substrate (Promega, Madison, WI). The level of luciferase expression was quantified using commercial firefly luciferase (Sigma, St. Louis, MO). Freshly prepared enzyme solution was aliquoted and stored at −86°C. Serial dilutions of the enzyme were prepared from a frozen aliquot and stored on ice during each experiment with liver tissue. The level of luminescence corresponding to the known activity of luciferase in serial dilutions was measured before and after each experiment to compensate for the possible denaturation of the enzyme. Additionally, serial dilutions of the enzyme were prepared in the tissue homogenate of normal rat liver in order to compensate for the adsorption of the enzyme and its proteolytic degradation by the tissue homogenate. Neither significant adsorption nor degradation/inactivation of luciferase was detected in these experiments.

2.6. *Flow cytometric analysis of β-gal expression*

The rat liver was perfused with PBS, and the electroinjected liver tissue (about 0.5 g) was removed. The tissue was dissociated in ice-cold PBS with syringe needles and washed with PBS. 5 ml of collagenase solution (100 μg/ml in PBS) was added to the tissue sample for 30 min at 37°C on a rotating platform. The suspension was cooled on ice and carefully pipetted 2–3 times using a wide-tip pipette to disconnect the cells from the stroma. The cell suspension was filtered through cotton wool to remove macroscopic debris and cell aggregates. Cells were washed twice with PBS using centrifugation at $250\times g$ for 10 min and resuspended in DMEM supplemented with 10% FCS (10^6 cells/ml).

Expression of β-gal in the electroinjected liver cells was detected using an ImaGene Green Gene Expression Kit (Molecular Probes, Eugene, OR) according to the protocol of the manufacturer. Briefly, cells were treated with chloroquine for 30 min, washed with DMEM by centrifugation and resuspended in DMEM supplemented with 10% FCS. The lipophilic β-gal substrate, 5-dodecanoylaminofluorescein di-β-galactopyranoside (C_{12}FDG), was added at a final concentration of 60 μM, and cells were incubated for 1 h at 37°C. Cells were then washed once with PBS and analyzed by flow cytometry. The assays were made using an Epics Elite flow cytometer (Coulter, Hialeah, FL).

2.7. *Cytochemical assay of β-galactosidase*

Tissue specimens were removed from animals and immediately stored at 4°C. Within 1 h, specimens were embedded in tissue freze medium (Triangle Biomedical Science, Durham, NC) and frozen in liquid nitrogen. Several (3–5) frozen sections (7 μm thick) were cut from each sample. After cutting, each section was fixed for 15 min in 2.5% glutaraldehyde and then washed three times in PBS (Mediatech, Washington, DC). Specimens were then incubated at 37°C in the presence of 0.2% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-GAL) for 24 h. Then, each specimen was washed again with PBS and viewed using light microscopy.

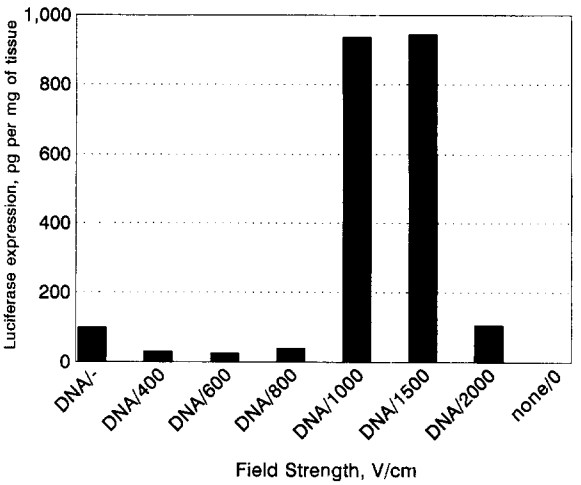

Fig. 1. Field strength dependence of luciferase expression in rat liver after electroinjection of 100 μg of DNA expressing luciferase. Anesthetized rats were incised and both halves of the median lobe were exposed (see Section 2). 100 μl of DNA in 100 μl of saline were injected beneath the surface of the median lobe at a single location. 1.5 min after injection the electrodes were placed on the tissue and 6 pulses of 99 μs duration were administered in the form of a rotating electric field. Expression was assayed in tissue samples taken by first killing the animals 48 h after the procedure. Expression was measured by luminescence emission in the presence of CoA-luciferin as substrate (see Section 2).

3. Results

Expression of firefly luciferase (Luc) and of β-galactosidase in the electroinjected liver tissue was used to evaluate the efficiency of intracellular DNA delivery. Electroinjection of plasmid DNA in the liver resulted in strong enzyme expression 48 h later. Optimal gene expression was achieved at field strengths between 1.0 and 1.5 kV/cm (Fig. 1). Electric field strengths below 1 kV/cm were insufficient for the permeabilization of liver cells. Field strengths higher than 1.5 kV/cm resulted in a strong decrease of the detected Luc expression, probably as a result of inefficient heat dissipation in the liver and necrosis of the electroinjected tissue. The level of expression in electroinjected cells was probably higher than that we report, because all measurements were performed in liver tissue homogenates from an area larger than the electroinjected region. Accurate location of the electroinjected area was limited by the fact that electrode marks on the tissue disappear within a few days. The level of luciferase activity in tissue reached a maximal value at 48 h, followed by a decrease to less than 5% of the peak activity during the first week. This level of expression remained stable during the next two weeks (Fig. 2). DNA injected in the liver with electroporation was also expressed albeit much more weakly; within 14 days, expression could not be reliably detected in non-electroporated tissue.

Gene transfer using in vivo electroporation strongly depended on the amount of DNA injected into the tissue (Fig. 3). There was an apparent sharp maximum for marker expression at 25 μg of plasmid DNA per tissue injection. This un-

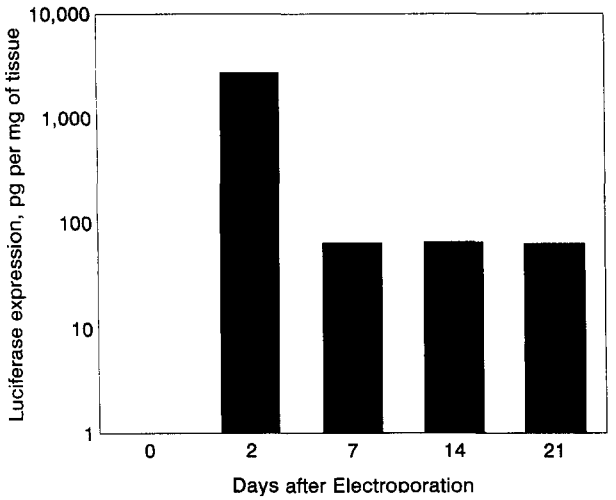

Fig. 2. Time course of luciferase expression in rat liver after electroinjection of 100 μg of DNA containing the luciferase gene. Procedures as above. Field strength=1 kV/cm. Expression measured by luminescence.

expected dose dependence in vivo is different from in vitro electroporation, in which the DNA dose dependence exhibits saturation [15].

One important advantage of electroporation in vitro is the high efficiency of intracellular DNA delivery. In order to quantitate the expression efficiency of the in vivo procedure, the liver tissue was electroinjected with the β-gal gene, and hepatocytes were isolated from the dissected liver tissue after collagenase treatment [2]. The isolated cells were treated with chloroquine, to block lysosome activity, followed by incubation with the lipophilic β-gal substrate C_{12}FDG (5-dodecanoylamino fluorescein di-β-galacto pyranoside).

The activity of the reporter gene was assayed using flow cytometry and histochemical staining. Cells isolated from electroporated liver tissue in the absence of the β-gal reporter gene showed no β-gal activity whereas the cell suspension prepared from β-gal-transfected liver contain over 30% β-gal-positive cells (Fig. 4). The actual number of efficiently

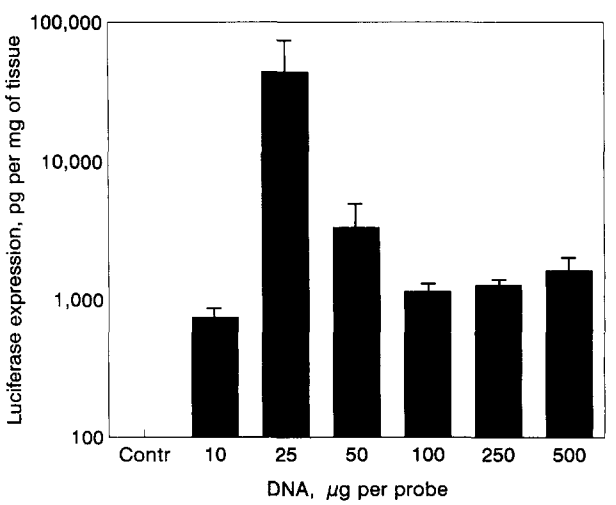

Fig. 3. Dose dependence of luciferase expression in rat livers after electroinjection of different amounts of DNA expressing luciferase. The field strength of pulses was 1 kV/cm. Procedures as above.

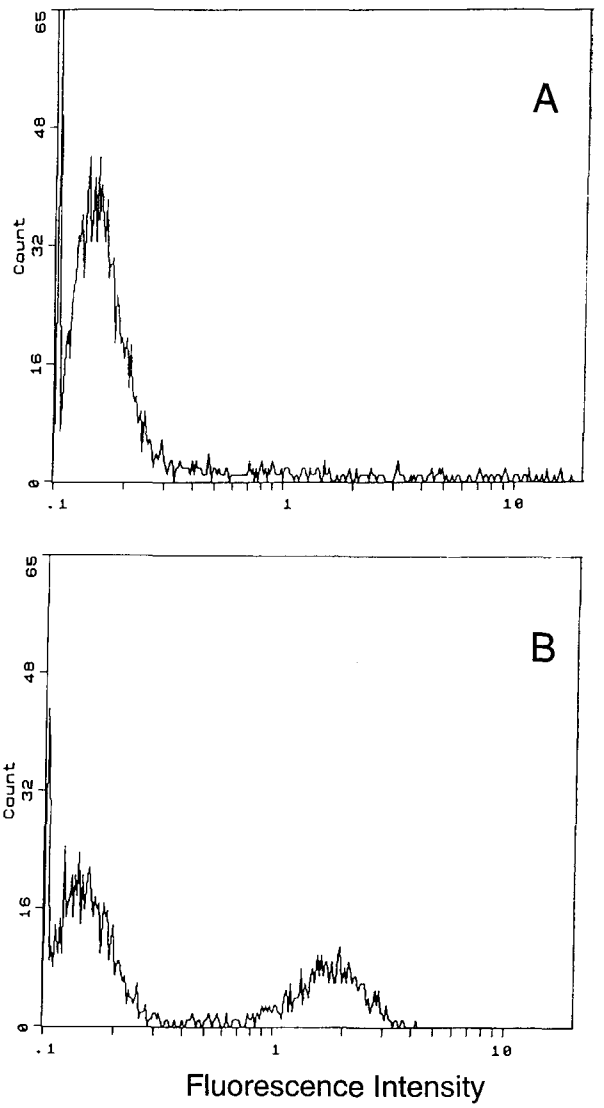

Fig. 4. Flow cytometric assay of β-galactosidase expression in rat livers after administration of 25 μg DNA. Expression was detected using an Ima-Gene Green Gene Expression Kit (Molecular Probes, Eugene, OR). The lipophilic β-gal substrate C12 FDG (5-dodecanoylaminofluorescein di-β-galactopyranoside) was added to the cell suspension as in Section 2. Flow diagrams were obtained using an Epics Coulter cytofluorometer. (A) 25 μg of DNA encoding β-galactosidase injected without electroporation. (B) 25 μg of DNA encoding β-galactosidase injected followed by electroporation. Field strength = 1.0 kV/cm (see Section 2).

transfected cells in the electroinjected area might be significantly higher due to technical difficulties in locating the transfected tissue.

Histochemical staining of the electroporated liver tissue yielded a similar calculated efficiency for in vivo transfection of hepatocytes: 30–35% of hepatocytes in the electroinjected area demonstrated β-gal activity (results not shown). This efficiency of transfection was identical to the best transfection obtained in vitro and was significantly higher than any in vivo transfection reported to date [14].

4. Discussion

The results presented demonstrate that in vivo electropora-

tion provides an efficient approach for tissue-targeted local gene expression with a relatively high efficiency of tranfection. The total number of transfected cells in our experiments is limited only by the geometry of the applied electrical field. The control experiments also confirmed the reports that direct hepatic DNA injection results in the detectable level of gene expression. However, electroporation appeared significantly more efficient than gene transfer by simple injection, the levels of expression being much higher and more stable with time.

Histological examination and histochemical β-gal staining of electroinjected liver tissue suggested that electroinjection with field strengths between 1.0 and 1.5 kV/cm does not produce any tissue damage, and expressing cells seem to be broadly and randomly distributed within the electroinjected organ area. This broad distribution of marker expression is different from expression reported after simple DNA injection, in which gene transfer and expression occurred only along the track of the injection needle [14].

Efficient gene transfer and expression in relatively extended macroscopic organ areas makes in vivo electroinjection an attractive approach for local gene cancer therapy [14]. Our results suggest that in vivo electroporation may be effective in transfecting a sufficient proportion of cells in tumors accessible to the electrodes to be of therapeutic interest [16].

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