Transfection of human hepatoma HepG2 cells with METAFECTENE PRO

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The ability to introduce nucleic acids into cells has enabled the advancement of our knowledge of genetic regulation and protein function within eukaryotic cells, tissues and organisms. However efficient DNA delivery and reproducible transfections with minimal toxicity is a critical prerequisite for any transfection experiment. In our laboratory we perform research on human cancer cell lines with the aim of analising the effect of new proapoptotic anticancer drugs and understanding their molecular mechanism of action. One of the cancer cell lines studied in our laboratory is the human hepatoma HepG2 cells. Hepatocellular carcinoma has been reported as one of the most frequent and malignant diseases worldwide. Although there are various treatments for hepatocellular carcinoma, the efficacy is rather limited mainly because of its high rate of recurrence and strong tendency of metastasis. Recently, to transfect human hepatoma HepG2 cells, we have employed lipofectamine 2000 with good results (Int J Oncol. 2008, 32:177-84). In the present study, to transfect human hepatoblastoma HepG2 cell line with a DNA plasmid expressing Akt1-myc tagged protein, we employed, in comparison, two transfection agents, Lipofectamine 2000 and Metafectene Pro. Here we demonstrated that Metafectene Pro is able to efficiently transfect HepG2 cells with low cytotoxic effect and good experimental reproducibility. The results also suggest that Metafectene Pro is superior to Lipofectamine 2000 in the transfection of Akt1, suggesting metafectene as an excellent candidate as transfection agent.

MATERIALS AND METHODS

Materials

Heat-inactivated fetal bovine serum (FBS), RPMI-1640, L-Glutamine (200 mM), penicillin/streptomycin (10,000 IU/ml and 10,000 mg/ml, respectively), 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide (MTT), were purchased from Sigma (St. Louis, MO); Metafectene Pro, a polycationic liposomal transfection reagent, was kindly provided by Biontex Laboratories GmbH (Munich, Germany). Lipofectamine 2000 was purchased from Invitrogen; The plasmid pUSEAkt1 encoding Myc-tagged Akt1 protein plasmid was obtained from Upstate Biotechnology (Lake Placid, NY).

Cell culture.

Human hepatoma HepG cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% sodium pyruvate and 2mM L-glutamine, penicillin (100 U/ml), streptomycin (100 µg/ml). Cells were maintained at 37 °C in a saturating humidity atmosphere containing 95% air and 5% CO_2.

Transient transfections.

Cells ($2x10^5$cells/well) were seeded into six-well plates (35-mm) and cultured in RPMI 1640 medium supplemented with 10% FBS for 24 h to reach approximately 60-80% confluence before transfection**.** For each transfection, aliquots of Metafectene Pro (1,5µl-9µl) were diluted in 100 µl of serum- and antibiotic-free RPMI medium; in a separate tube, 2 µg pUSEAkt1 or mock plasmid was diluted in 100 µl RPMI. Each concentration of Metafectene Pro was combined with 100 µl of the plasmid solution, gently mixed, and incubated for 20 min at room temperature, to allow the production of DNA plasmid-Metafectene Pro complexes. Cells were pre-washed with PBS and then incubated at room temperature for 5 h with DNA-Metafectene Pro complexes, or with Metafectene Pro alone (mock) in 1 ml serum free RPMI. The reaction was stopped replacing the culture medium with RPMI + 20% FBS and transfection efficacy was evaluated after 48 hours by western blotting analysis. To test the efficiency transfection, all DNA delivery experiments were designed to compare Metafectene Pro with Lipofectamine 2000 (Invitrogen), another optimal transfection reagent frequently used in our laboratory according to manufacturer's protocol.

Cell Morphology

Cell morphology was assessed by examining the cells without fixation under an inverted Leica microscope equipped with an automatic photomicrograph system.

MTT colorimetric survival assay.

The effect of of Metafectene Pro and Lipofectamine 2000 on cell viability was determined by 3-[4,5-dimethylthiazolyl-2] 2,5-diphenyltetrazolium bromide (MTT) assay. For the experiments, cells were seeded on 96-well plates at 60-70% confluence. Twenty-four hours after seeding, cells were transfected as described in the previous section and, thereafter, incubated for 48 h before performing the MTT assay. Cell survival was estimated as a percentage of the value of the vehicle-treated control. Dye absorbance in viable cells was measured at 570 nm, with 630 nm as a reference wavelength. Each experimental condition was repeated at least in quadruplicate wells in each experiment. In some wells trypan blue was used to count viable cells with comparable results.

Acridine Orange (A/O)-Ethidium Bromide (EB) staining.

Morphological evidence of apoptosis was also obtained by means of AO/EB staining. Monolayer cultures in 96-well plates were employed for these studies. After transfection and successive 48 h incubation, the incubation medium was removed and the cells were rinsed and treated with a solution composed of AO/EB (100 μg/ml PBS of each dye). Cells were then observed with fluorescent microscope. The emission wavelengths were set on 525 and 610 nm.

Cell Cycle analysis by flow cytometry.

After transfection and successive 48 h incubation, cells were harvested and centrifuged. Then, the cells were fixed with cold ethanol, resuspended in hypotonic solution containing 10 μg/ml propidium iodide, 0.1% sodium citrate, 0.01% Nonidet P-40 and 100 μg/ml RNase A and incubated 1 h at room temperature. Cell cycle phase distribution was evaluated by an Epics XL flow cytometer (Beckman Coulter) using Expo32 software. The percentage of cells in the subdiploid region was considered as an index of apoptosis.

Western blot analysis.

After transfection and successive 48 h incubation, cells were harvested, washed with PBS and incubated for 20 min in ice-cold lysis buffer containing protease inhibitor cocktail. Then, cells were sonicated three times for 10 sec and the protein content evaluated by Bradford method. Equal amounts of protein samples (40 μg/lane) were subjected to SDS polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane to detect the overexpression of Akt1 or Myc-tag at NH2 terminus of the protein. Membranes were stripped and re-probed with antibody to actin to ascertain equal protein loading. Bands were quantified by densitometric analysis using Image SMX software, and the data were normalized to actin values.

RESULTS

Here we have set up experiments to investigate transfection efficacy and to identify possible cytotoxicity or other undesirable side effects induced by the transfection reagent Metafectene Pro on human hepatoblastoma HepG2 cells. In addition, the efficiency of Metafectene Pro was tested to evaluate whether it is advantageous over Lipofectamine 2000, optimal transfection reagent usually employed in our laboratory according to the manufacturer's recommendations.

To this purpose, we used pUSEAkt1, a plasmid encoding a myc-tagged Akt1 protein. Metafectene Pro or Lipofectamine 2000 were complexed with the Myc-tagged Akt1 expressing plasmid as described in Methods. Cells were either mock transfected or transfected with Metafectene Pro or Lipofectamine 2000 complexed with the Myc-tagged Akt1. Transfection efficiency was evaluated comparing myc-tagged Akt1 expression by western blotting analysis 48 hours after transfection. Fig. 1A shows that, as expected, untrasfected parental HepG2 cells, employed as a control, and the mock transfected cells evidenced only a 60-kDa band, related to a normal size Akt1, whereas Akt1-transfected HepG2 cells expressed an additional band with a slower electrophoretic mobility correspondent to myc tagged Akt1 protein. As the figure shows, increasing concentration of Metafectene Pro resulted in increasing efficacy of transfection. The expression of this recombinant protein was much higher in Metafectene Pro-transfected cells than Lipofectamine 2000, indicating Metafectene Pro as an optimal transfectant agent in delivering DNA plasmid in human hepatoblastoma HepG2 cells. After considering the efficacy of Metafectene Pro in performing transfection, we investigated the possible side effects induced by the transfection agents.

In Fig 1B, cell viability, was determined by MTT colorimetric assay to measure possible cytotoxic effect of Lipofectamine 2000 and various Metafectene Pro concentrations. The figure demonstrates that, similarly to Lipofectamine 2000 (which was employed at the concentration suggested by the manufacturer) concentration of Metafectene Pro varying between 1,5 and 4,5 µl caused little loss of viability (10-20%).

In Fig 1C morphology analysis under light microscopy is reported. The figure shows that none of the transfection conditions caused a reduction of total amounts of cells, when compared to control cells. We, then, performed analysis to evaluate whether the loss of viability induced by transfectant agents was correlated with the induction of apoptosis. As chromatin condensation and nuclear fragmentation remain the hallmarks of apoptotic cells, apoptosis was assessed by nucleic acid staining with the complementary, semiquantitative fluorescent dye technique AO/EB staining (Fig. 2A). The figure shows the absence of typical apoptotic nuclei exhibiting highly fluorescent condensed chromatin.

In Fig 2B cell cycle analysis, performed to examine any change in cell cycle distribution, demonstrates no changes in cell cycle distribution.

CONCLUSIONS

Metafectene Pro successfully transfected human hepatoma HepG2 cells with pUSEAkt1 plasmid, providing a good compromise between transfection efficiency, low toxicity and optimal experimental reproducibility. The results also suggest that the efficacy is even better than that obtained with Lipofectamine 2000.

FIGURES

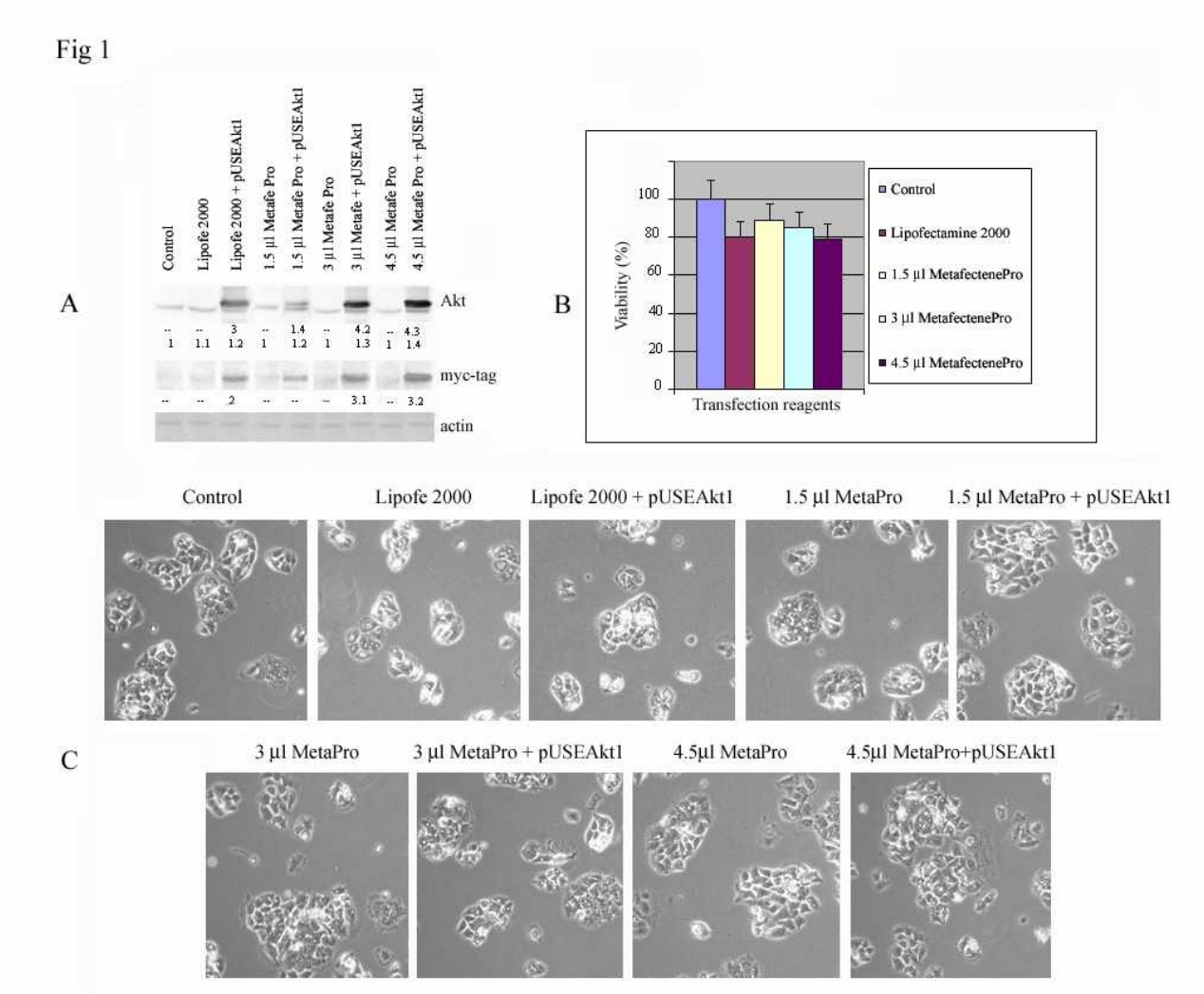

Figure 1. (A) Comparative analysis of Akt1 expression in Metafectene Pro and Lipofectamine 2000-transfected cells. Cells were transfected with pUSEAkt1 plasmid using Metafectene Pro or Lipofectamine 2000 as described in Methods. After 48 h from transfection, cell lysates were prepared and analyzed by western blotting to assess the expression of Akt1 and myc. Number under the bands represent densitometry values that were calculated on actin loading controls. (B) Cell viability was measured by the MTT colorimetric assay. Results are expressed as percentage alive cells compared to a mock-transfected control. Data represent the mean ± S.D. obtained from triplicate wells, and are representative of two independent experiments. (C) Cell morphology was assessed by examining the cells under an inverted Leica microscope equipped with an automatic photomicrograph system. The results were representative of three independent experiments.

Fig 2

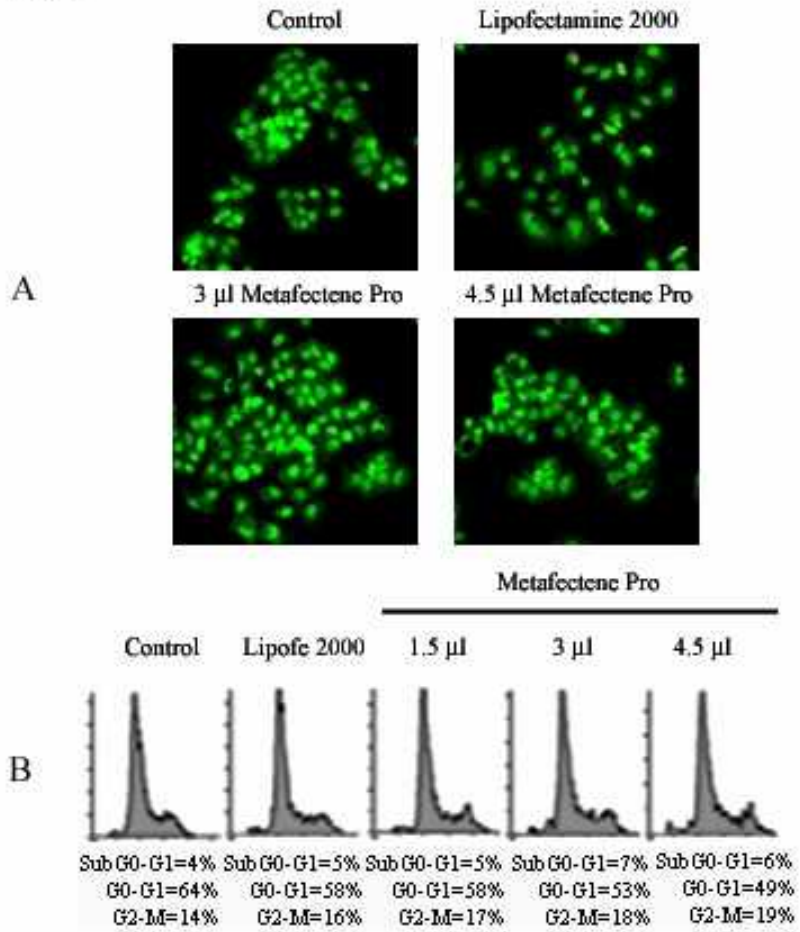

Figure 2. (A) Morphological analysis performed with fluorescenze microscopy. HepG2 cells were treated with Lipofectamine 2000 and various concentration of Metafectene Pro as described in Methods. After 48h incubation cells were stained with acridine orange/ethidium bromide. (B) Flow cytometric analysis of propidium iodide-stained HepG2 cells treated with Lipofectamine 2000 and various concentration of Metafectene Pro as described in Methods. x-axis indicates fluorescence intensity on linear scale and y-axis the number of events. The results were representative of two independent experiments.