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Gene transfer mediated by α_2-macroglobulin

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ABSTRACT

α_2-Macroglobulin covalently linked to poly(L)-lysine can be used as a vehicle for receptor-mediated gene transfer. This modified α_2-macroglobulin maintains its ability to bind to the α_2-macroglobulin receptor, and was shown to introduce a luciferase reporter gene plasmid into HepG2 human hepatoma cells *in vitro*. The α_2-macroglobulin receptor is a very large and multifunctional cell surface receptor, whose rapid and efficient internalization rate makes it attractive for gene therapy, e.g. for hepatic gene targeting via injection into the portal vein.

More than 12 years ago Cheng *et al.* (1) published a method for the covalent coupling of DNA to α_2-macroglobulin, formulating the concept of receptor-mediated gene transfer. However, the authors did not report gene transfer into cells. In the meantime this concept has been further investigated, and several strategies have been developed to introduce DNA into cells using receptor-mediated endocytosis pathways. The proposed gene delivery systems include complexing the DNA with cell-surface receptor-binding sugars (2), transferrin (3), asialoglycoprotein (4), bacterial proteins such as internalin or invasin (5), integrin binding peptides (6), adenoviral coat protein (7), immunoglobulins (8) or insulin (9), which all work well at least *in vitro*. Ligand–DNA complexes are formed through electrostatic interactions between a positively charged polycation, usually poly(L)-lysine, which is covalently linked to the receptor-binding ligand, and the negatively charged phosphate residues in the DNA backbone. The efficiency of gene expression achieved by these approaches depends on a variety of factors, including the size of the DNA–ligand complex and the rate of ligand uptake into the target cells (10).

The α_2-macroglobulin receptor/low density lipoprotein receptor-related protein (α_2-MR/LRP) is unique in its ability to bind and endocytose a variety of very different ligands such as lipoprotein remnants, cytokines/growth factors, and proteases (in complex with α_2-macroglobulin) as well as plasminogen activators complexed with inhibitors. It is a very large cell surface receptor (mol. wt 600 kDa) that internalizes its ligands *in vivo* within minutes (11,12). This receptor is present predominantly, but not exclusively, in human liver and intestine, however its expression is strongly restricted to certain cell types, e.g. hepatocytes, smooth muscle cells, neurons and fibroblasts (13). Here we report for the first time gene delivery and expression by means of α_2-macroglobulin.

Poly(L)-lysine (SIGMA, Deisenhofen) with an average chain length of 450 lysine residues was coupled to purified human α_2-macroglobulin (Biomac, Leipzig) through disulfide bonds after modification with the cross-linking reagent *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP; Pierce, Rockford). After dialysis against PBS in order to remove unreacted SPDP and low molecular weight reaction products the modified poly(L)-lysine was allowed to react with α_2-macroglobulin in the presence of methylamine (200 mM) for 2 h at 22°C. On average 3 moles poly(L)-lysine were attached to 1 mol α_2-macroglobulin as calculated by measuring the remaining free SH-groups in the protein as previously described (15). Afterwards NaCl was added to a final concentration of 1 M and the reaction volume was adjusted to 10 ml. The α_2-macroglobulin-poly(L)-lysine conjugate was separated from non-coupled poly(L)-lysine on a Zn-chelate sepharose column using 0.1 M sodium phosphate washing buffer, pH 7.0, containing 1 M NaCl, and eluted with 50 mM EDTA in 0.1 M sodium phosphate buffer, pH 7.0. The conjugate solution was concentrated by dialysis against 40% PEG-6000 (SERVA, Heidelberg) and finally dialyzed against HEPES-buffered saline (150 mM NaCl, 10 mM HEPES, pH 7.5). This modified α_2-macroglobulin maintains its ability to bind to the α_2-macro-

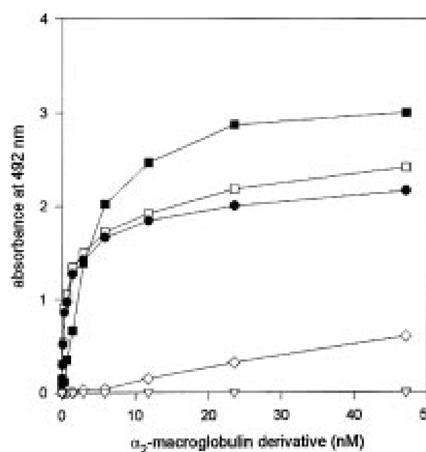

Figure 1. Binding of the α_2-macroglobulin–poly(L)-lysine conjugate to the immobilized α_2-macroglobulin receptor. Titre plates, which had been coated with purified α_2-M/LRP (2 µg/ml) in coating buffer (20 mM HEPES, 5 mM Ca^{2+}, pH 7.5) overnight, were washed and then incubated with α_2-macroglobulin derivatives dissolved in 20 mM HEPES buffer, pH 7.5, containing 5 mM Ca^{2+} and 0.05 % Tween 20, at 37°C for 1 h. After washing, the plates were incubated with rabbit anti-α_2-macroglobulin–horse radish peroxidase-conjugate in the same buffer for 1 h. Finally, the plates were washed and developed using 3,3-diaminobenzidine (SIGMA, Deisenhofen) and H_2O_2. (□) transformed α_2-macroglobulin; (∇) transformed α_2-macroglobulin without immobilized α_2-macroglobulin receptor; (■)α_2-macroglobulin–poly(L)-lysine conjugate; (◊)α_2-macroglobulin–poly(L)-lysine conjugate without immobilized α_2-macroglobulin receptor; (●) as (■), but incubated in the presence of 5 molar excess of free poly(L)-lysine

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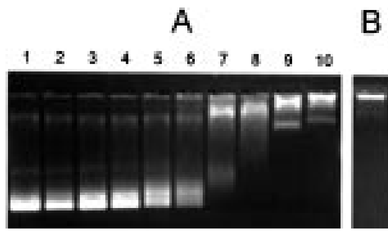

Figure 2. Agarose gel electrophoresis analysis of DNA–α_2-macroglobulin complex formation. Increasing amounts of α_2-macroglobulin–poly(L)-lysine conjugate were added to 0.2 µg pGL2 plasmid DNA in HEPES-buffered saline (150 mM NaCl, 10 mM HEPES, pH 7.5) in a final volume of 25 µl. After incubation for 30 min at room temperature with constant mixing, samples were analysed for retardation of their electrophoretic mobility on a 0.8 % agarose gel. (**A**) Lane 1: 0.2 µg DNA, no α_2-macroglobulin–poly(L)-lysine conjugate; lanes 2–10: 0.2 µg DNA, α_2-macroglobulin–poly(L)-lysine conjugate increasing serially (2×) from 62.5 ng up to 16 µg. (**B**) 0.2 µg DNA, 0.5 µg α_2-macroglobulin–poly(L)-lysine conjugate plus 80 ng free poly(L)-lysine. The experiment was performed by mixing the DNA with the conjugate for 20 min, adding the poly(L)-lysine and mixing again for 30 min.

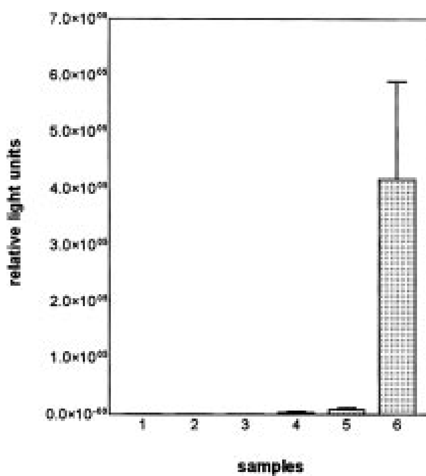

Figure 3. Gene transfer into HepG2 cells. Luciferase reporter gene plasmid pGL2 (6 µg) complexed with 15 µg α_2-macroglobulin–poly(L)-lysine conjugate plus 2.3 µg free poly(L)-lysine with an average chain length of 200 lysine residues were given dropwise to 50 % confluent HepG2 cells (5×10^{-5} cells/well) in DMEM containing 10 % fetal bovine serum, penicillin (100 U/ml), streptomycin (100 U/ml), chloroquine (0.1 mM) and $CaCl_2$ (3.0 mM). After 4 h this medium was replaced with fresh complete DMEM and the cells were further incubated at 37°C for 40 h. Cellular protein was extracted using the Luciferase reporter gene assay (Boehringer, Mannheim), and luciferase activities of three aliquots of cell lysates were measured as Relative Light Units (RLU), standardized per mg of total protein. Each column represents the mean of the RLU values and standard deviation about the mean (n = 3). (1) No DNA, no α_2-macroglobulin, no poly(L)-lysine; (2) naked DNA; (3) as (2), but incubated in the presence of α_2-macroglobulin; (4) DNA complexed with α_2-macroglobulin–poly(L)-lysine conjugate plus free poly(L)-lysine, incubated in the absence of chloroquine; (5) DNA complexed with 5 µg poly(L)-lysine alone; (6) DNA complexed with α_2-macroglobulin–poly(L)-lysine conjugate plus free poly(L)-lysine as described above.

globulin receptor as shown by analysis of its binding to immobilized receptor molecules (Fig. 1). The formation of complexes between the α_2-macroglobulin–poly(L)-lysine conjugate and DNA was monitored by agarose gel electrophoresis of pGL2 plasmid DNA complexed with increasing amounts of the conjugate (Fig. 2A). The DNA was completely retarded at a ratio of 16 µg conjugate to 0.2 µg DNA, representing a molar charge ratio between the conjugate and pGL2 (Promega, Madison) of nearly 40:1. Thus, the binding of the polycation to DNA is destabilized by coupling it to α_2-macroglobulin. This effect of a bulky protein linked to the DNA-binding moiety on the formation of appropriately condensed DNA–ligand complexes is known but poorly understood (10). At the estimated ratio between the conjugate and pGL2 resulting in electroneutrality of the complexes, a unimolecular complex would contain ~300 α_2-macroglobulin molecules. Such a high number of α_2-macroglobulin molecules should not be necessary for receptor-mediated endocytosis, and the large size of the complexes may limit their internalization. Therefore we partially replaced the α_2-macroglobulin–poly(L)-lysine conjugate by free poly(L)-lysine according to Wagner *et al.* (14). Figure 2B shows one of these samples analysed by agarose gel electrophoresis, demonstrating that the DNA is still retarded. An optimised ratio of conjugate, free polycations and DNA was used for the transformation of HepG2 cells, which have been demonstrated before to expose α_2-MR/LRP using the IIF6 monoclonal antibody against the 515 kDa subunit of the receptor molecule. Cellular protein was assayed for luciferase activity after 40 h. Significant differences of luciferase expression were measured relative to poly(L)lysine–DNA complexes lacking the conjugate, naked pGL2 plasmid DNA or DNA incubated in the presence of unmodified transformed α_2-macroglobulin (Fig. 3). The level of luciferase expression was reduced by 150-fold when cells were incubated in the absence of chloroquine, which acts by neutralizing the acidic pH of lysosomes and therefore inhibits the activity of nucleases (10). In the control with poly(L)-lysine alone a concentration was used at which the DNA was completely retarded.

The α_2-macroglobulin-mediated gene transfer, described here, expands the currently available techniques for receptor-mediated gene transfer. α_2-Macroglobulin–DNA complexes may be applicable for hepatic gene targeting via injection into the portal vein or for *ex vivo* transformation of a large variety of cells. Autologous α_2-macroglobulin could be used, which would minimise the possibility of the vehicle being immunogenic *in vivo*.

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