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In vitro gene delivery by a novel human calcitonin (hCT)-derived carrier peptide

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Abstract—Gene therapy still awaits a broader application, since safe and efficient gene delivery is a major problem. Also for the investigation of signal transduction and intracellular trafficking, delivery systems for hydrophilic macromolecules that are easy to use are needed. Several peptide-based delivery systems have been developed during the last years. We present here a novel carrier peptide derived from human calcitonin that is capable of transfecting human neuroblastoma cells by complex formation with a plasmid. Because of the peptide's physiological origin, cytotoxic effects are not expected.

In the past years, various different peptide-based gene delivery systems have been established; most of them consist of several components. These multi-component systems usually contain a peptide rich in basic amino acids, for example polylysine, a targeting sequence for tissue or cell specificity, a lytic sequence to promote endosomal release of the plasmid/oligonucleotides and a nuclear localization sequence (NLS).[1–4] Some other cell-penetrating peptides (CPPs) like the HIV-glycoprotein 41-derived peptide named MPG[5,6] or a HIV-Tat protein-derived peptide[7] have also been shown to be of use for the delivery of oligonucleotides or macromolecular DNA. While the CPPs transportan and penetratin have been reported to transport covalently bound peptide nucleic acids across cellular membranes,[8] the loss of the membrane translocation ability after binding to large double-stranded DNA molecules has been reported for penetratin.[9]

A balance between compacting the nucleic acids and stabilizing the complex on the one hand and ensuring sufficient intracellular release and nuclear uptake on the other hand must be achieved.[10] However, since some of the used peptides are of viral origin[11] they might provoke immunogenic reactions and peptides containing many basic amino acids are known to cause histamine release.[12] Therefore, alternatives for in vivo applications are needed.

Human calcitonin (hCT) is a 32 amino acid peptide hormone secreted by the C-cells of the thyroid gland. Physiologically it is involved in the regulation of the calcium homeostasis, therapeutically it is applied in hypercalcemia, osteoporosis and other bone related diseases like Paget's disease and severe bone pain. Interestingly, this relatively large peptide can cross the nasal epithelium, since the application as nasal spray is as effective as iv injection.[13] Structure–activity studies have already shown that calcitonin fragments like hCT(9–32) are still able to enter cells, while the receptor-activating N-terminal part of the molecule is not required.[14,15] Thus, with regard to future therapeutic applications, hormone side-effects are not expected. hCT(9–32) has already been shown to transport fluorophores or chemotherapeutics,[14] but even large hydrophilic molecules like the enhanced green fluorescent protein (EGFP) across cellular membranes when covalently linked to its cargo.[16] Here, we present the first studies on the in vitro-transfection of neuroblastoma cells SK-N-MC with an hCT-derived peptide using non-covalent linkage. The nuclear localization sequence (NLS) of simian virus (SV) 40 large T antigen was introduced as side chain, in order to promote complex formation with the plasmid and ensure nuclear delivery of the DNA. A plasmid encoding for EGFP was chosen as cargo to allow fast and direct monitoring of the transfection efficiency.

The peptides were synthesized according to the Fmoc-strategy by automated multiple solid phase peptide synthesis using a robot system (Syro, MultiSynTech,

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Germany).[17] Fmoc-protected amino acids, 1-hydroxybenzotriazole (HOBt) and 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy (Rink amide) resin were obtained from NovaBiochem. Double coupling procedures were performed with a 10-fold excess, diisopropylcarbodiimide/HOBt activation and a coupling time of 40 min. For the introduction of the side chain into the branched peptide (see Fig. 1), the Lys[18] carried a Dde-protection group, which can be selectively removed by repeated treatment with 2% hydrazine in *N*,*N*-dimethylformamide.[18] The synthesis of the peptide was continued via the ε-amino group of this lysine, while the peptides N-terminal leucine was Boc-protected. The peptide amide was cleaved from the resin with 1 mL trifluoroacetic acid (TFA)/thiocresol/thioanisol (Fluka) 90:5:5 for 3 h at room temperature. The peptide was precipitated from cold diethyl ether, collected by centrifugation and subsequently lyophilized. The peptides were purified by preparative HPLC (Waters, Millipore RCM 25×10 column) using a linear gradient from 10 to 60% B in A over 50 min (A = 0.1% TFA in water, B = 0.08% TFA in acetonitrile). Product identity has been confirmed by analytical HPLC (Merck Hitachi LaChrom, Vydac C18 column) and matrix assisted laser desorption ionization (MALDI) mass spectrometry (Voyager DE-RP, PerSeptive). For hCT(9–32), a mass of 2608.7 Da was found (calcd mass 2608.3 Da) and a retention time of 14.03 min, for the long linear peptide the mass was 4246.9 Da (calcd 4247.9 Da) and the retention time 16.08 min and the branched peptide was found at 4250.1 Da (calcd 4247.9 Da) and 15.23 min.

Competent *Escherichia coli* DH5α cells (pretreated with rubidium chloride) were subjected to a heat shock transformation to incorporate the plasmid pEGFP N1 (Clontech), which carries a kanamycin-resistence gene.[19] Cells were plated and grown over night on agar-plates that contained kanamycin. A single clone was picked and incubated at 37 °C over night in 200 mL LB-medium with 0.6 mL kanamycin. Cells were collected by centrifugation and a High Purity Maxiprep was carried out as described in the manufacturer's protocol (Gibco). Plasmid concentration was determined by gel electrophoresis in comparison to a DNA standard ladder (Gibco, High DNA Mass Ladder).

Neuroblastoma cells SK-N-MC (obtained from ATCC) were grown in Minimal Essential Medium (MEM, Gibco) supplemented with 10% fetal calf serum (FCS), 4 mM glutamine, 1% non essential amino acids and 1 mM sodium pyruvate. CHO (chinese hamster ovary, obtained from ATCC) cells were grown in DMEM-NUT (Gibco) with 10% FCS. The cells were grown to confluency at 37 °C and 5% CO_2. After removal of the growth medium, the cells were washed with Dulbecco's PBS (phosphate buffered saline; Gibco). Following incubation with PBS containing 0.02% ethylenediaminetetraacetic acid for 3 min the cells were detached by mechanical agitation and suspended in new medium.[20]

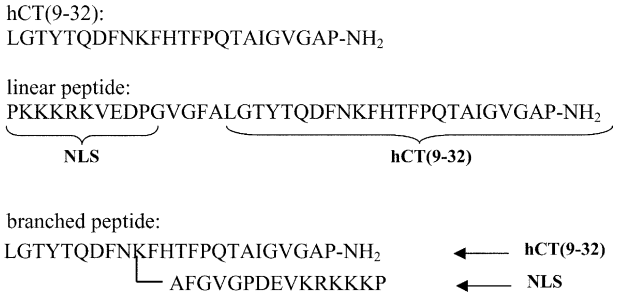

Figure 1. Different hCT-derived peptides based on the hCT-fragment hCT(9–32), the longer peptides carry an NLS derived from SV 40 large T antigen.

Cells were seeded in 96 well plates in a density of 10,000 or 20,000 cells per well and grown for 2–3 days until cell confluency was about 60–70%. The plasmid preparation (1 μg/μL) was diluted in OptiMEM (Gibco) to obtain a final concentration of 0.5 or 1 μg in 75 μL, the peptide solution was set to contain 10 or 20 μg in 75 μL in OptiMEM. 75 μL of both plasmid and peptide solution were mixed and incubated for 1 h at room temperature to allow complex formation. Even after centrifugation, no precipitation was observed. Meanwhile the cells had been washed once and pre-incubated for 30 min in 200 μL OptiMEM. After removal of the OptiMEM the cells were incubated with the plasmid-peptide solution for 5 h at 37 °C. Then the incubation solution was replaced by normal growth medium and the cells were grown over night. Next day plasmid incorporation and expression of EGFP was monitored by fluorescence microscopy. In addition, one well was always transfected using Lipofectamine™ (Gibco), a commercial liposomal transfection agent, in a previously established protocol (1.5 μL Lipofectamine, 0.5 μL plasmid)[21] while one well was always treated with pure plasmid DNA (1 μg).

Fluorescence microscopy was performed using a Zeiss Axiovert 25 inverted microscope, excitation light was set at 485 nm and the GFP-emission filter was used. Pictures were taken with an AxioCamMRm digital camera using the AxioVision software.

For CHO cells only a minor degree of successful transfection could be detected under the tested conditions with the branched peptide, only a small number of cells showed green fluorescence after excitation. In contrast, the CHO cells treated with Lipofectamine™ showed a high level of EGFP-expression (data not shown). However, for neuroblastoma cells SK-N-MC, opposite results were obtained. The previously optimized transfection protocol with Lipofectamine™ resulted only in a low EGFP-expression (see Fig. 2C), whereas the hCT-derived branched peptide (see Fig. 2A and B) achieved a good transfection rate. With a ratio of 20 μg peptide per 1 μg plasmid (which corresponds to a peptide/plasmid ratio of 14,000:1) optimal results were obtained (Fig. 2B), a high degree of cells emitting green fluorescence was observed, while 10 μg peptide per 1 μg plasmid (peptide/plasmid ratio 7000:1) resulted in less fluorescent cells (Fig. 2A). In the control well containing 1 μg pure plasmid without transfecting reagent, no EGFP-expression could be detected, demonstrating that the plasmid itself does not sufficiently reach the cell interior (Fig. 2D). Also the shorter peptide hCT(9–32) (see Fig. 2E) and the linear NLS-containing peptide (data not

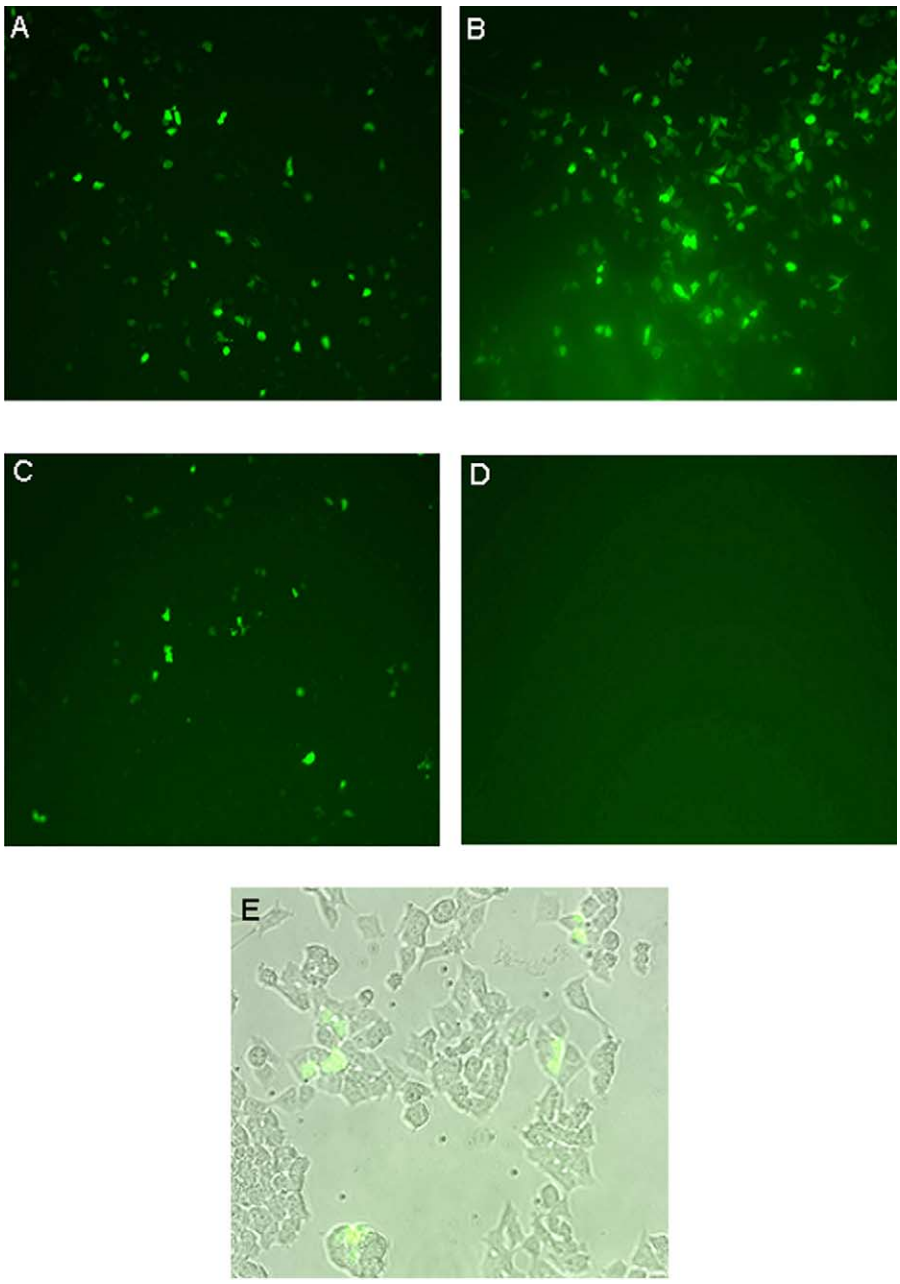

Figure 2. (A) SK-N-MC cells transfected with the branched hCT-derived peptide in a mass ratio peptide to plasmid 10:1 and 20:1 (B), respectively. (C) SK-N-MC cells transfected with Lipofectamine™; (D) SK-N-MC cells treated with pure DNA. (E) Overlay of fluorescent picture and picture from light microscope. SK-N-MC cells transfected with hCT(9–32) and pEGFP.

shown, comparable to the short peptide hCT(9–32) as shown in Fig. 2E) led to some expression of EGFP when used under the described conditions, however to a smaller extent than the branched hCT-derived peptide. So obviously the branched structure is more suitable for the complex formation with the plasmid DNA. Cell viability was not affected under the used concentrations (as assessed by trypane blue exclusion), demonstrating that the peptides are not cytotoxic under the used conditions.

These results demonstrate that a single hCT-derived peptide can efficiently transfect neuroblastoma cells SK-N-MC. For other cell lines like CHO cells the protocol has to be optimized, in preliminary studies comparing the efficiency of several CPPs, also established cell-penetrating peptides like penetratin, transportan or HIV-Tat(43-60) did not lead to higher transfection rates in CHO cells. In SK-N-MC cells the expression of EGFP (the chosen marker for successful transfection) after transfection with the hCT-derived branched peptide was even higher than with a liposomal formulation. However, the various hCT-derived peptides differ in their effectivity. Whereas the hCT(9–32) contains presumably not enough basic amino acids for the complex formation with the DNA, the lower activity of the linear long peptide in contrast to the branched peptide demonstrates the importance of the spatial orientation.

While some oligonucleotides have been reported to reach their target site by themselves and their cellular

delivery only slightly enhanced by cell-penetrating peptides (regardless whether there was covalent binding or complex formation),[22] for other oligonucleotides a significantly improved cellular uptake after conjugation to cell-penetrating peptides has been observed.[23] In our experimental setup the naked DNA molecules were not able to sufficiently reach the cell nuclei, since no expression of EGFP was found.

The exact mechanism, how hCT-derived carrier peptides translocate across cellular membranes, is not yet completely understood. Endocytic processes are involved, this is also demonstrated by the fact that fluorescent-labeled hCT-derived peptides [both hCT(9–32) and the branched peptide] are not internalized at 4 °C; additionally, it seems that several membrane proteins (but not the so far known hCT receptors) participate in the internalization process (manuscript in preparation). However, the expression of EGFP clearly demonstrates the successful delivery of the plasmid into the nucleus, hence, the plasmid is not entrapped in endosomal compartments.

Thus, this hCT-derived carrier peptide offers several advantages. Since only a single peptide is needed, the preparation of the peptide–DNA complex is easy and convenient, no incompatibilities were observed. Covalent linkage between the peptide and its cargo is not necessary. Both in the described studies, and in experiments with the fluorescent-labeled peptide (up to a concentration of 100 μM) no cytotoxic effects could be observed. In preliminary studies comparing the transfection efficiency of various CPPs, the hCT-derived branched peptide achieved a higher transfection rate than transportan or penetratin. Transportan however caused considerable cytotoxicity in the tested SK-N-MC cells. While the MPG-peptide was approximately as effective as the hCT-derived peptide, the HIV-Tat-derived peptide led to slightly higher transfection rates (as assessed in fluorescence microscopy).

Although the in vivo stability and efficiency has to be assessed in animal studies, this hCT-derived peptide still shows promising potential as gene delivery agent.

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