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Application of poly(2-(dimethylamino)ethyl methacrylate)-based polyplexes for gene transfer into human ovarian carcinoma cells

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Abstract

Previously, attempts were made in our laboratory to transfect human ovarian cancer (OVCAR-3) cells, growing in the peritoneal cavity of nude mice, by intraperitoneal administration of poly(2-(dimethylamino)ethyl methacrylate) (pDMAEMA)-based polyplexes. However, hardly any transfection of the OVCAR-3 cells was observed. The aim of the present study was to examine whether pDMAEMA-polyplexes can transfect OVCAR-3 cells in vivo at DNA doses much higher than used previously [J. Gene Med. 1 (1999) 156–158]. We also explored a specific targeting strategy based on the use of folic acid (FA) as a targeting ligand directed against the folate receptor overexpressed on OVCAR-3 cells. Luciferase expression by OVCAR-3 cells mediated by pDMAEMA-based polyplexes was evaluated in the mouse i.p. OVCAR-3 xenograft model of ovarian cancer. By virtue of new formulation options, we were able to administer polyplex dispersions into OVCAR-3 bearing mice at much larger doses (75–120 μg DNA) than used previously (15 μg). The feasibility of folate-mediated targeting of the polyplexes was studied after coupling of FA to preformed polyplexes with poly(ethylene glycol) (PEG) as a spacer. Intraperitoneal administration of naked pLuc plasmid did not result in significant gene expression by the tumor cells. Administration of uncoated, positively charged pDMAEMA-based polyplexes at a DNA dose of 75–120 μg yielded significant transfection activity. However, also considerable gene expression was observed in non-target cells. To avoid transfection of non-target cells, an active targeting strategy based on the use of FA was studied. At a dose of 75 μg DNA (N/P 5), the folate-targeting approach yielded about 10-fold lower luciferase transfection levels in organs lined by the mesenthelial layer. This beneficial site-avoidance effect was achieved without compromising the degree of tumor cell transfection. Successful transfection of OVCAR-3 cells growing in the peritoneal cavity of nude mice can be achieved by i.p. administration of polyplexes at doses between 75 and 120 μg DNA. It was further demonstrated

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that active targeting of polyplexes to OVCAR-3 cells growing in the peritoneal cavity of mice is a realistic possibility to avoid transfection of non-target cells.

Keywords: Ovarian carcinoma cells; Intraperitoneal administration; Folate-targeting; Poly(2-(dimethylamino)ethyl methacrylate); Tumor targeting; Polyplex; Gene delivery

1. Introduction

Regional administration has proven to be a viable option for the local gene therapy of several diseases including certain cancers and cystic fibrosis. Ovarian cancer is a potentially fatal human gynecological malignancy, which is confined to the peritoneal cavity throughout most of its clinical lifetime. Therefore, it is an attractive candidate for regional cancer gene therapy. Literature reports on the use of non-viral gene delivery systems for intraperitoneal gene therapy of ovarian cancer are scarce (Aoki et al., 2001; Horton et al., 1999; Kikuchi et al., 1999; Lanuti et al., 2000; Namiki et al., 1998).

Our group has focused on the development of the cationic polymer poly(2-(dimethylamino)ethyl methacrylate) (pDMAEMA) as a non-viral vector. We have shown previously that the pDMAEMA polymer is able to condense plasmid DNA into small complexes. The size and the zeta potential were shown to be dependent on the polymer/DNA ratio, and to be major determinants for the transfection efficiency in vitro (Cherng et al., 1996). The highest transfection activity in monkey kidney cells (COS-7) and human ovarian carcinoma cells (OVCAR-3) was achieved using polyplexes with a positive zeta potential and approximately 150 nm in size (Van de Wetering et al., 1998). Fluorescence activated cell-sorting (FACS) analyses with fluorescently labeled polyplexes showed that a positive zeta potential of the polyplexes facilitated cellular interaction. Confocal laser fluorescence microscopy data showed that cellular internalization of the positively charged polyplexes occurred after cell binding (Zuidam et al., 2000).

Van de Wetering et al. (1999) attempted to transfect human ovarian cancer (OVCAR-3) cells, growing in the peritoneal cavity of nude mice, by intraperitoneal administration of pDMAEMA-based polyplexes. However, hardly any transfection of the OVCAR-3 cells was observed. In the latter study, relatively low DNA doses were used (lower than 15 μg DNA per mouse), because of the inability to prepare stable polyplex dispersions with relatively high DNA concentrations. Aggregation phenomena prevented the use of dispersions with a DNA concentration higher than 40 μg/ml. Later, Cherng et al. (1999) described a procedure to prepare highly concentrated polyplex dispersions containing a DNA concentration up to 150 μg/ml.

The aim of the present study was to examine in the mouse i.p. xenograft model of ovarian cancer whether pDMAEMA-based polyplexes can transfect OVCAR-3 cells at DNA doses much higher than used previously. The results point to successful transfection of the OVCAR-3 cells growing in the peritoneal cavity. However, also considerable gene expression was observed in non-target cells present in organs lined by the peritoneal mesothelial layer. Therefore, to reduce expression by non-target cells, we also explored an active targeting strategy based on the coupling of folate as targeting ligand directed against the folate receptor overexpressed on OVCAR-3 cells.

2. Materials and methods

2.1. Materials

The cationic polymer poly(2-(dimethylamino)ethyl methacrylate) was synthesized as described before (Van de Wetering et al., 1997). The weight average molecular weight (M_w) and number average molecular weight (M_n) of pDMAEMA were determined by gel permeation chromatography (GPC). The used batch had a M_w of 1.6×10^6 and a M_n of 5.7×10^4 g/mol. 1,2-Dioleyl-3-trimethylammonium-propane (DOTAP) was purchased from Avanti Polar Lipids (Alabaster, USA). TBoc-NH-PEG-CO_2-*N*-hydroxysuccinimide

ester (NHS-PEG) were obtained from Shearwater Corporation (Huntsville, AL, USA). Preparation of pDMAEMA-co-NAPMAm and folate-conjugated *N*-hydroxysuccinimidyl ester (FA-PEG-NHS) was according to van Steenis et al. (2003). Plasmids containing the luciferase gene under control of the CMV-promoter (pLuc) were grown, isolated and purified by In Vivogen, Inc, (San Diego, CA, USA). All other reagents were of the highest grade available.

2.2. Polyplex formation

For in vivo experiments, polyplexes were prepared according to Cherng et al. (1999), except that when NHS-activated succinimidyl esters were used, a Hepes buffer pH 7.4 was used to facilitate the coupling of the NHS-activated succinimidyl esters. In short, polyplexes were prepared by diluting pLuc plasmid DNA (100 μl, maximally 1.5 mg/ml) in acetate buffer, pH 5.7, to which 500 μl of 40% sucrose was added. To this solution 400 μl of polymer was added and mixed. The N/P ratio (which is defined as the ratio between polymeric nitrogen residues and DNA phosphate groups) was 5. DOTAP liposomes were prepared essentially as described by Song et al. (1997). Appropriate amounts of DOTAP lipid were dissolved in chloroform in a round-bottomed flask. The solvent was evaporated under reduced pressure in a rotary evaporator and further dried under nitrogen. The dried lipids were hydrated in 5 mM Tris Buffer pH 7.4 for 2 h after which the liposomes were extruded through two stacked polycarbonate 100 nm membranes (Poretics Corp. Livermore, CA, USA) and stored as a 20 mM suspension. Complexes of DOTAP and pLuc plasmid were prepared by mixing equivalent volumes of DNA solution and lipid dispersion, followed by thorough mixing. After 30 min at room temperature, all complexes were used for in vivo studies or were further modified with FA-PEG-NHS conjugate.

2.3. Surface modification of preformed polyplexes

PEGylation of polyplexes was performed by adding DMSO-solutions of either NHS-PEG-FA (52 mg/ml, 13.5 mM) or NHS-PEG (40 mg/ml, 11.8 mM) to the polyplexes, and incubating these for 1 h at room temperature yielding targeted PEG-polyplexes and non-targeted PEG-polyplexes, respectively (van Steenis et al., 2003). The required amount of PEG was determined by taking into account the amount of primary amines of pDMAEMA-*co*-NAPMAm (DMAEMA/NAPMAm ratio: 3/2 (mol/mol)), corresponding with a molecular weight of 467 g/mol per primary amine. A molar ratio of 1.5 between NHS-PEG(-FA) and NH_2 was used throughout the study.

2.4. Gene transfer in vivo

Experiments with non-targeted polyplexes were performed using Adult female NMRI nude/nude mice (6–8 weeks old) which were obtained from a specific pathogen free colony maintained at the Animal Facility of the University of Nijmegen, The Netherlands. Experiments with targeted polyplexes were performed using athymic BALB/c mice (6–8 weeks old) which were obtained from Harlan. Tumor growth and morphology in these mice was comparable to the growth and morphology in NMRI nude/nude mice (data not shown). The animals were housed in plastic cages and received standard food (Hope Farms, Woerden, The Netherlands) and water ad libitum. The human ovarian cancer cell line NIH:OVCAR-3 was propagated intraperitonally as described earlier (Pizer et al., 1996; Daoud, 1994). At 3–5 week intervals, cells were harvested from the donor animals by rinsing the peritoneal cavity with RPMI medium without supplements. Cells were centrifuged and resuspended in RPMI medium. Mice were injected with OVCAR-3 tumor cells (1×10^7) intraperitoneally in PBS in a volume of 0.5 ml. The tumor cells were allowed to grow for 4 days. The mice were injected intraperitoneally with 800 μl of DNA complexes at the indicated N/P ratios and DNA concentrations. After 24 h, mice were killed by CO_2 asphyxiation, and the peritoneum was rinsed twice with 5 ml of cold PBS to harvest peritoneal cells. Subsequently, the cells were pelleted by centrifugation and resuspended in a known volume of PBS. A 50 μl sample was taken and 900 μl of water and 50 μl of Zap-Oglobin-II was added. The cells were lysed by osmotic shock and nuclei were counted by microscopical inspection to determine the number of cells. The lungs, heart, liver, spleen, kidneys, colon, uterus, diaphragm, connective tissue, and abdominal muscle were dissected. Organs and pelleted tumor cells were frozen at −80 °C. The next day, organs were defrosted, and then kept on ice. After weighing, all organs were homogenized for 20 s

on ice in 0.5 ml of reporter lysis buffer using a tissue homogenizer (Ika Labortechnik, Dijkstra Vereenigde B.V., Lelystad, The Netherlands), except for the liver, which was homogenized in 1 ml of lysis buffer. Then, the resulting tissue homogenates were left on ice for 1 h, vortexed for 20 s and subsequently centrifuged at 13,000 × *g* for 10 min. Luciferase activity of each sample was measured by mixing 100 μl of luciferase assay reagent with 20 μl of supernatant using a Berthold 9507 Luminometer (EG&G Benelux BV, Breda, the Netherlands). Relative light units (RLU) were measured for 10 s at room temperature. Data were corrected for luciferase background levels in non-treated animals. Results were statistically evaluated by the Student's *t*-test.

3. Results and discussion

Intraperitoneal administration of therapeutic DNA delivery systems represents a rational strategy that might contribute to the therapy of ovarian cancer. Earlier in our laboratory, an unsuccessful attempt was made in a xenograft model of ovarian carcinoma (OVCAR-3) to transfect tumor cells growing intraperitoneally by i.p. injection of pDMAEMA-based polyplexes (Van de Wetering et al., 1999). A number of suggestions were given to explain the negligible in vivo transfection of OVCAR-3 cells. Firstly, aggregate formation induced by the cationic plasmid/polymer particles might have hampered the transfection activity. Secondly, as the OVCAR-3 cells tend to grow in clusters in the ascites fluid, the clustering of cells might have reduced the accessibility of the tumor cells to the complexes. Thirdly, the presence of destabilizing factors in the ascites fluid might have played an inhibitory role. For example, it was demonstrated that the presence of ascites fluid in cell culture medium induced a strong negative effect on the capability of pDMAEMA-based polyplexes to transfect OVCAR-3 cells in vitro.

A potential negative factor not discussed in the paper by Van de Wetering et al. (1999) is that relatively low doses of plasmid DNA (lower than 15 μg per mouse) were used. Higher DNA doses could not be administered due to the occurrence of aggregation in the dispersion at DNA concentrations over 40 μg/ml. Obviously, if one would be able to administer the polyplex system at a much higher DNA dose, negative interference by one or more of the postulated inactivation mechanisms might be overcome. Cherng et al. (1999) gained insight into the formulation parameters affecting the aggregation tendency of pDMAEMA-polyplex dispersions. At pH 5.7, in media with a low salt concentration (20 mM acetate) and containing 20% sucrose, small sized polyplexes could be formed at relatively high DNA concentrations (i.e. up to 150 μg/ml). More recently, we confirmed the critical role of salt ions by showing that also salt-free media like 5% glucose can be used to obtain polyplex dispersions with a high DNA concentration. By virtue of these new formulation options, we were able to inject polyplex dispersions into OVCAR-3 bearing mice at much larger DNA doses than used previously (Van de Wetering et al., 1999). The in vivo tumor cell transfection results after i.p. administration of the polyplex dispersions at plasmid DNA doses ranging from 75 to 120 μg DNA are shown in Fig. 1. The transfection activity of pDMAEMA-based polyplex systems was also compared with the activity produced by a lipoplex system based on the frequently used cationic lipid 1,2-dioleyl-3-trimethylammonium-propane. In line with expectations, administration of naked pLuc plasmid did not result in any significant gene expression. Administration of pDMAEMA-based polyplexes at a DNA dose of 120 μg and an N/P ratio of 15 resulted in low, but detectable gene expression. In comparison, i.p. injection of DOTAP-based lipoplexes at the same DNA dose and N/P ratio yielded a 60-fold higher level of gene expression. Lowering the DNA dose to 75 μg yielded a 6-fold enhancement of the transfection activity of pDMAEMA-based polyplexes. Lowering the DNA dose to 75 μg and the N/P ratio to 5 yielded a 520-fold enhancement of the transfection activity of pDMAEMA-based polyplexes. The increased transfection efficiency observed upon lowering the DNA dose and N/P ratio might relate to decreased toxicity of the polyplex system under these experimental conditions.

These results are in line with those of our group (Cherng et al., 1996). A bell-shaped curve was obtained when an increasing number of polyplex-particles was added to in vitro cultured cells at fixed N/P ratio. Obviously, the transfection increases with a larger number of particles internalized. However, when too much particles are entering the cells, the transfection is decreasing due to toxicity of the polymer.

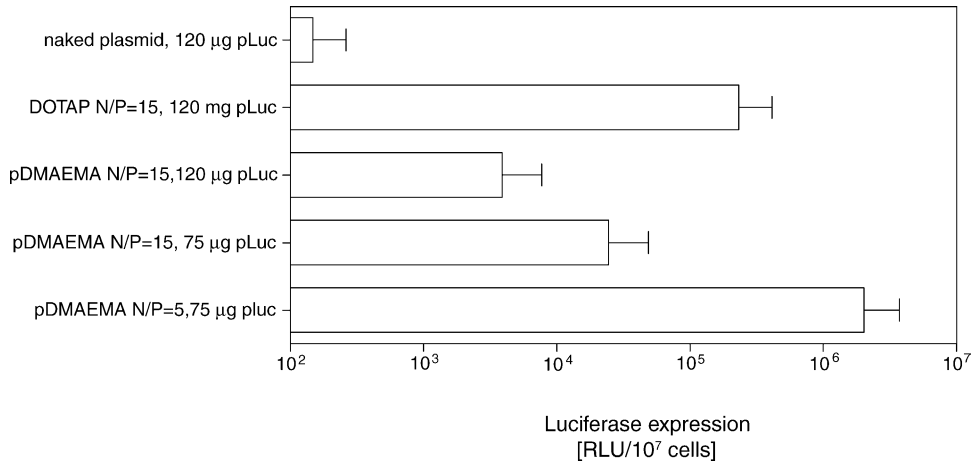

Fig. 1. Transfection of OVCAR-3 cells growing in the peritoneal cavity after i.p. administration of pDMAEMA-polyplexes and DOTAP lipoplexes. Data represent the mean ± S.E.M. ($n = 3$).

Fig. 2 shows that i.p. injection of pDMAEMA-based polyplexes not only leads to transfection of the cells present in the peritoneal fluid. Relatively high gene expression was also observed in several non-target tissues, including the spleen, diaphragm, uterus and abdominal muscles. Low, but still significant gene expression was found in the liver, lungs, heart, colon, mesenteria, small intestine, kidneys and connective tissue. In line with the tumor cell transfection results, pDMAEMA-based complexes injected at the 75 μg DNA dose and N/P ratio of 5 yielded higher transfection levels than pDMAEMA-based polyplexes injected at the 120 μg DNA dose and N/P ratio of 15. At the 120 μg DNA dose and N/P ratio of 15, DOTAP-based complexes yielded similar levels of transfection in of non-target tissues when compared to pDMAEMA-based polyplexes of the same dose and N/P ratio. The expression associated with the investigated organs after

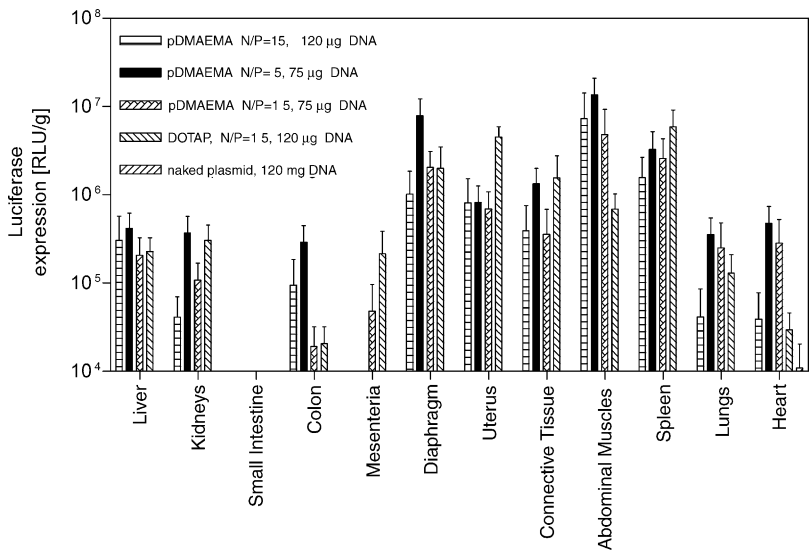

Fig. 2. Distribution of luciferase gene expression after i.p. administration of pDMAEMA-polyplexes and DOTAP lipoplexes. Data represent the mean ± S.E.M. ($n = 3$).

intraperitoneal administration of the transfection systems could very well be due to transfection of the mesothelial layer lining the peritoneal cavity and covering the tissues examined. Transfection of the mesothelial layer has also been observed after intraperitoneal injection of viral vectors (Setoguchi et al., 1994).

Ideally, transfection should be specific, i.e. the transfection should preferentially occur only in the tumor cells. Clearly, as high levels of gene expression are found in many non-target tissues (Fig. 2), the use of a tumor cell targeted system is indicated. To reduce transfection of non-target tissues, non-specific interactions of the cationic polyplexes with anionic structures present on the surface of non-target cells should be minimized. One approach to minimize non-specific interactions is to shield the positive charge of the polyplexes. Shielding can be achieved by covalent attachment of polyethylene glycol (PEG) to the surface of the polyplexes (Ogris et al., 1999; Verbaan et al., 2004). Targeting ligands can be attached to the terminal ends of the PEG-chains to yield specific interactions only with the tumor cells. Targeted gene delivery via the folate receptor is an attractive strategy, as has been recently reviewed by Ward (2000). It is known that in normal non-diseased tissues (like kidneys, placenta and choriod plexus) folate acid receptors are present at only low levels. On malignant cells (including ovarian cancer cells), the folate receptor is often overexpressed (Reddy et al., 2002; Lu et al., 2002). Folate-conjugated lipids were used to achieve targeting of cationic liposomes (Wang et al., 1995), or anionic liposomes containing encapsulated polyplexes (Lee et al., 1996; Reddy et al., 2000). Targeted polyplexes based on poly-L-lysine (Leamon et al., 1999; Mislick et al., 1995) or poly(ethyleneimine) (Guo et al., 1999; Benns et al., 2001; Benns et al., 2002) were prepared by covalent linkage of folate to the polymers. To improve the availability of folate for receptor-binding, attachment to a long (3340 Da) PEG spacer was found necessary for liposomes (Lee et al., 1994), and similar requirements were noted in the case of polyplexes (Leamon et al., 1999). To achieve effective shielding and targeting, we adapted this strategy to our system and modified the surface of the pDMAEMA-based polyplexes with a PEG coating exposing the targeting ligand.

The feasibility of the folate-targeting strategy was evaluated in vitro in our laboratory (van Steenis et al., 2003). Briefly, Van Steenis et al. developed a folate-poly(ethylene glycol) conjugate (FA-PEG-NHS) capable of covalent coupling to primary amines present at the surface of polyplexes after complexation of plasmid DNA with a (DMAEMA-*co*-NAPMAm) copolymer. PEGylation of pDMAEMA-based polyplexes with the FA-PEG-NHS construct led to a sharp decrease of the zetapotential from about 20 mV to about 5 mV. The resulting targeted polyplexes had a mean size of about 120 nm in saline. In this medium, the size of the particles did not change markedly in time demonstrating that colloidally stable particles were formed. Fig. 3 shows that in OVCAR-3 bearing mice the folate-targeting approach indeed yielded about 10-fold lower luciferase transfection levels in organs lined by the mesenthelial layer as compared to uncoated polyplexes administered at the same dose (75 μg DNA, N/P 5). Likely this is due to the steric stabilization effect mediated by the PEG coating on the FA-targeted polyplexes. This beneficial site-avoidance effect was paralleled by a preserved degree of tumor cell transfection ($0.48 \times 10^6 \pm 0.13$ RLU/10^7 cells for the FA-targeted polyplexes versus $2.02 \times 10^6 \pm 1.71$ RLU/10^7 cells for the uncoated polyplexes). It should be noted that the active targeting strategy was carried out with a FA-PEG polyplex formulation which was not optimized regarding the density of the folate ligand on the surface of the targeted polyplexes (Reddy et al., 2002). The in vivo recovery of cells from the peritoneum by peritoneal lavage after i.p. administration of the FA-targeted polyplexes is lower than in the case of the uncoated polyplexes (Table 1), suggesting that transfection mediated by the targeting effect is paralleled by increased toxicity towards the tumor cells.

The present results indicate that specific tumor transfection in vivo is a realistic possibility.

In conclusion, successful transfection of OVCAR-3 cells growing in the peritoneal cavity of nude mice

Table 1
Recovery of OVCAR-3 cells growing in the peritoneal cavity of nude mice after intraperitoneal administration of uncoated and FA-targeted pDMAEMA-based polyplexes

	Recovery of cells [10^7 cells]
FA-targeted	1.2 (±0.8)
uncoated	8.0 (±2.2)

Data represent the mean ± S.E.M. ($n = 3$).

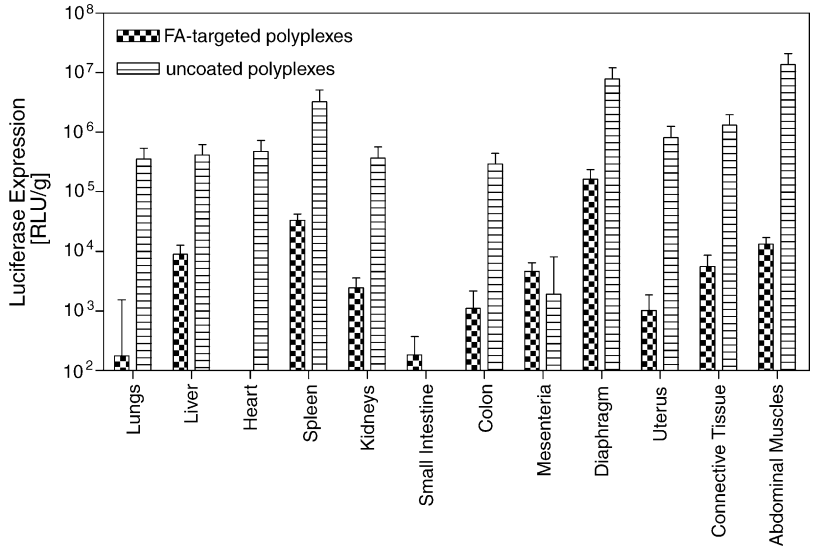

Fig. 3. Distribution of luciferase gene expression after i.p. administration of FA-targeted polyplexes and uncoated polyplexes. Data represent the mean ± S.E.M. ($n = 3$).

can be achieved by i.p. administration of polyplexes at doses much higher than used previously. Moreover, targeting of pDMAEMA-based polyplexes to OVCAR-3 cells is a realistic possibility. Currently, in vivo testing of the FA-targeted polyplexes for the purpose of Gene Directed Enzyme Prodrug Therapy (GDEPT) is ongoing in our laboratory.

References

Aoki, K., et al., 2001. Polyethylenimine-mediated gene transfer into pancreatic tumor dissemination in the murine peritoneal cavity. Gene Ther. 8, 508–514.

Benns, J.M., et al., 2002. Optimization of factors influencing the transfection efficiency of folate-PEG-folate-graft-polyethylenimine. J. Control. Release 79, 255–269.

Benns, J.M., et al., 2001. Folate-PEG-folate-graft-polyethylenimine-based gene delivery. J. Drug Target 9, 123–139.

Cherng, J.Y., et al., 1999. The effect of formulation parameters on the size of poly-((2-dimethylamino)ethyl methacrylate)-plasmid complexes. Eur. J. Pharm. Biopharm. 47, 215–224.

Cherng, J.Y., et al., 1996. Effect of size and serum proteins on transfection efficiency of poly ((2-dimethylamino)ethyl methacrylate)-plasmid nanoparticles. Pharm. Res. 13, 1038–1042.

Daoud, S.S., 1994. Combination chemotherapy of human ovarian xenografts with intraperitoneal liposome-incorporated valinomycin and cis-diamminedichloroplatinum(II). Cancer Chemother. Pharmacol. 33, 307–312.

Guo, W., et al., 1999. Receptor-targeted gene delivery via folate-conjugated polyethylenimine. AAPS PharmSci. 1, E19.

Horton, H.M., et al., 1999. IL-2 plasmid therapy of murine ovarian carcinoma inhibits the growth of tumor ascites and alters its cytokine profile. J. Immunol. 163, 6378–6385.

Kikuchi, A., et al., 1999. Development of novel cationic liposomes for efficient gene transfer into peritoneal disseminated tumor. Hum. Gene. Ther. 10, 947–955.

Lanuti, M., et al., 2000. Cationic lipid: bacterial DNA complexes elicit adaptive cellular immunity in murine intraperitoneal tumor models. Cancer Res. 60, 2955–2963.

Leamon, C.P., et al., 1999. Folate copolymer-mediated transfection of cultured cells. Bioconjug. Chem. 10, 947–957.

Lee, R.J., et al., 1996. Folate-targeted, anionic liposome-entrapped polylysine-condensed DNA for tumor cell-specific gene transfer. J. Biol. Chem. 14, 8481–8487.

Lee, R.J., et al., 1994. Delivery of liposomes into cultured KB cells via folate receptor-mediated endocytosis. J. Biol. Chem. 269, 3198–3204.

Lu, Y., et al., 2002. Folate-mediated delivery of macromolecular anticancer therapeutic agents. Adv. Drug Deliv. Rev. 54, 675–693.

Mislick, K.A., et al., 1995. Transfection of folate-polylysine DNA complexes: evidence for lysosomal delivery. Bioconjug. Chem. 6, 512–515.

Namiki, Y., et al., 1998. Gene transduction for disseminated intraperitoneal tumor using cationic liposomes containing non-histone chromatin proteins: cationic liposomal gene therapy of carcinomatosa. Gene Ther. 5, 240–246.

Ogris, M., et al., 1999. PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. Gene Ther. 6, 595–605.

Pizer, E.S., et al., 1996. Inhibition of fatty acid synthesis delays disease progression in a xenograft model of ovarian cancer. Cancer Res. 56, 1189–1193.

Reddy, J.A., et al., 2002. Folate-targeted, cationic liposome-mediated gene transfer into disseminated peritoneal tumors. Gene Ther. 9, 1542–1550.

Reddy, J.A., et al., 2000. Enhanced folate receptor mediated gene therapy using a novel pH-sensitive lipid formulation. J. Control. Release 64, 27–37.

Setoguchi, Y., et al., 1994. Intraperitoneal in vivo gene therapy to deliver alpha 1-antitrypsin to the systemic circulation. Am. J. Respir. Cell Mol. Biol. 10, 369–377.

Song, Y.K., et al., 1997. Characterization of cationic liposome-mediated gene transfer in vivo by intravenous administration. Hum. Gene Ther. 8, 1585–1594.

Van de Wetering, P., et al., 1998. 2-(Dimethylamino)ethyl methacrylate based (co)polymers as gene transfer agents. J. Control. Release 53, 145–153.

Van de Wetering, P., et al., 1997. Relation between transfection efficiency and cytotoxicity of poly(2-dimethylamino)ethyl methacrylate)/plasmid complexes. J. Control. Release 49, 59–69.

Van de Wetering, P., et al., 1999. Comparative transfection studies of human ovarian carcinoma cells in vitro, ex vivo and in vivo with poly(2-(dimethylamino)ethyl methacrylate)-based polyplexes. J. Gene Med. 1, 156–165.

van Steenis, J.H., et al., 2003. Preparation and characterization of folate-targeted pEG-coated pDMAEMA-based polyplexes. J. Control. Release 87, 167–176.

Verbaan, F.J., et al., 2004. Steric stabilization of poly(2-(dimethylamino)ethyl methacrylate)-based polyplexes mediates prolonged circulation and tumor targeting in mice. J. Gene Med. 6, 64–75.

Wang, S., et al., 1995. Delivery of antisense oligodeoxyribonucleotides against the human epidermal growth factor receptor into cultured KB cells with liposomes conjugated to folate via polyethylene glycol. Proc. Natl. Acad. Sci. U.S.A. 92, 3318–3322.

Ward, C.M., 2000. Folate-targeted non-viral DNA vectors for cancer gene therapy. Curr. Opin. Mol. Ther. 2, 182–187.

Zuidam, N.J., et al., 2000. Effects of physicochemical characteristics of poly(2-(dimethylamino)ethyl methacrylate)-based polyplexes on cellular association and internalization. J. Drug Target 8, 51–66.