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Modified microplex vector enhances transfection of cells in culture while maintaining tumour-selective gene delivery in-vivo

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Abstract

A non-commercial liposome (dimethyl dioctadecyl ammonium bromide:dioleoyl phosphatidylethanolamine) was compared with a commercial variety (Lipofectamine) for transfection of cultured rat adenocarcinoma cells and in an in-vivo kidney tumour model. Transfection of the cells in culture and in tumours in-vivo was variable with both types of liposomes. A high-dose microplex (lipoplex–microsphere) vector enhanced liposome-mediated transfection of cells in culture. When these high-dose microplexes were tested in-vivo, they were better than both microspherical and liposomal delivery modes in terms of transgene expression levels and the tumour-to-normal tissue ratio of gene delivery. Microplexes have been demonstrated to be capable of not only selective delivery of plasmids to solid tumours, but also of increasing transfection in cell culture, a finding that may be used in ex-vivo transfection studies. It is hypothesized that microspheres anchored the combination vector closer to the cultured cells, allowing attached liposomes to gain easier access into cells. In-vivo, microspheres permitted the microplexes to selectively deliver their genetic payload within the tumour tissue, from where the action of cationic liposomes on cellular membranes facilitated increased access of plasmids into the cytosol of target cells.

Introduction

Cationic liposomes are without the risks commonly associated with viral gene delivery, but are limited by indiscriminate systemic distribution when delivered in-vivo (Zhu et al 1993; Lew et al 1995), and rapid uptake by cells of the reticuloendothelial system (Lasic 1996). This highlights the need for the ability to target cationic liposomes to the tumour site, since such selective removal depletes the reservoir of liposomes in the bloodstream and consequently the entry of therapeutic genes into target cells. In contrast, microspheres, commonly used for delivery of anticancer agents, can be targeted by the blood flow to a particular organ or tissue type such as a tumour if delivered into an artery afferent to the target site (reviewed in Dass & Burton 1999).

In tumour tissue, compression and occlusion of blood vessels facilitates entrapment of incoming microspheres by capillary embolism. Such an approach has been employed by our group (Gray et al 1989; Campbell et al 2001) and others (Stubbs et al 2001) to selectively deliver radioactive microspheres for treatment of liver metastases in clinical studies. Lodged spheres release radioactive energy in the tumour vicinity for destruction of cancerous cells. No side-effects due to tissue embolism were noted in any of these studies, mainly owing to the fact that a limited number of spheres were administered to patients. However, the inability of microspheres to enter cells limits their applicability for transfer of genetic medicine, which needs to be delivered directly into tumour cells and, in most circumstances, further into the nucleus. Combination of these vascular selective agents with an agent capable of delivering genes into cells may enhance tumour gene therapy.

In light of the above issues, a novel vector was developed and tested in-house, comprising the commercial liposome formulation, Lipofectamine, being bound to polystyrene divinylbenzene (PDB) ion-exchange microspheres for the formulation of microplexes (Dass et al 1999, 2000). In the present study, a higher loading of lipoplexes

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was carried out and the ability of this modified microplex vehicle to enhance transfection of a transplantable rat cancer cell line in culture and to selectively deliver a chloramphenicol acetyltransferase (CAT) reporter plasmid to tumours was tested in-vivo.

Materials and Methods

Materials

The pCMV-CAT plasmid was kindly donated by Dr Robert Debs of the Cancer Research Institute, University of California, USA. pCMV-CAT is 4233 bp in length and contains the *CAT* gene under the control of a cytomegalovirus (CMV) promoter. Approval for the procedures used in Dark Agouti rats was obtained from the Charles Sturt University Animal Care and Ethics Committee. Nembutal (pentobarbitone sodium; Boehringer Ingelheim, Artarmon, NSW, Australia) was used for rat anaesthesia. CSU-SA1, a rat salivary adenocarcinoma cell line, has been used in other in-vivo studies (Esdale et al 1997; Walker et al 1998, 2002; Dass et al 2000).

Lipofectamine, a 3:1 w/w ratio of polycationic 2,3-dioleyloxy-*N*-[2(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate and dioleoyl phosphatidylethanolamine (DOPE), was sourced from Gibco BRL (Bethesda, MA, USA). For preparation of liposomes in the laboratory, dimethyl dioctadecyl ammonium bromide (DDAB) and DOPE were obtained from Sigma (Castle Hill, NSW, Australia). The DC protein assay kit was supplied by Bio-Rad (Regents Park, NSW, Australia), and the CAT assay kit was from Promega (Annandale, NSW, Australia). Chloramphenicol, D-threo-[1,2-^{14}C] was from ICN Pharmaceuticals (Irvine, CA, USA) and erythrosine dye from Merck (Darmstadt, Germany). The Aminex A27 PDB microsphere (Bio-Rad, Richmond, CA, USA) is an 8% cross-linked anion exchanger with a diameter of 15 ± 2 μm, and acetate as the exchangeable ion. Aminex 50W-X4 (Bio-Rad) is a 4% cross-linked cation-exchange resin formulated from PDB with particle diameters of 32.5 ± 2.5 μm. Sulfonic acid functional groups bind sodium exchange ions in place. For sizing of liposomes, 0.2, 0.6 and 3.0 μm polycarbonate membranes were obtained from Poretics (Livermore, CA, USA).

Developed procedures

Liposomes were prepared and complexes visualized with erythrosine dye as described previously (Dass et al 1999, 2002). Transfection in cell culture and in-vivo, cell protein extraction and CAT activity assay of cell and tissue lysates were performed as described previously (Dass et al 2000, 2002).

Preparation of combination vector

For the in-vivo study, 12 μg pCMV-CAT was complexed to 250 μg Lipofectamine in a total volume of 150 μL at laboratory temperature (22°C) for 5 min. Following this, 50W-X4 microspheres (7.25×10^5 spheres) were added to the mixture and the volume was adjusted to 300 μL. Microspheres were slurried with the plasmid–liposome complexes at laboratory temperature for 30 min. The suspensions were centrifuged (11 300 *g*, 5 s) and pelletted microspheres were washed twice with 50 μL water. After a final centrifugation to remove as much water as possible, microspheres loaded with plasmid-laden liposomes were introduced into rats as described below. For the in-vitro study, 6 μg plasmids were mixed with 150 μg Lipofectamine, and these complexes were bound to 4.35×10^5 50W-X4 microspheres.

Preparation of other vectors

For in-vivo microspherical delivery, pCMV-CAT (12 μg) was added to 7.25×10^5 A27 microspheres in a total volume of 250 μL and slurried for 30 min. Following this, microspheres with bound plasmids were centrifuged and washed with two changes of 50 μL water. The volume was adjusted to 300 μL and spheres were administered to rats. For the in-vitro study, 4.35×10^5 A27 microspheres were loaded with 6 μg plasmids. This number of spheres was chosen as it lies within the range of microspheres delivered to Dark Agouti rats by other researchers (approx. 8.0×10^5 spheres/animal; Anderson et al 1991; Napoli et al 1992).

For in-vivo liposomal delivery, pCMV-CAT (12 μg) was added to 250 μg Lipofectamine in a total volume of 300 μL and the mixture made homogeneous by gentle pipetting. For in-vitro delivery, 150 μL Lipofectamine was complexed with 6 μg plasmids. The mixtures were incubated at laboratory temperature for at least 30 min before use. For free plasmid delivery, 12 μg of pCMV-CAT was diluted in water to a final volume of 300 μL. For the in-vitro study, 6 μg plasmids was suspended in 300 μL water.

Statistics

For cell culture and in-vivo data, data from different treatments were analysed using normal probability plotting (Smith 1993). All samples were normally distributed, and the differences between the means of two treatments (one pair at a time) were tested using Student's *t*-test after determining whether samples had similar variances using the F_{max}-test.

Results

Preliminary cell culture testing and in-vivo evaluation of liposomes

In this study using CSU-SA1 rat salivary adenocarcinoma cells, Lipofectamine gave the best transfection results, with CAT activity being 3.5-fold greater than free delivery, and 2.1-fold better than delivery on DDAB:DOPE liposomes (Table 1). DDAB:DOPE liposomes gave 4.0-fold better transfection than delivery of free plasmids. The results were not significantly different between the three modes of

Table 1 Comparison of transfection efficiency between liposomes.

Delivery mode	CAT activity ($\times 10^{-8}$ units/cell)
Free	2.3 (0)
DDAB:DOPE	9.2 (2.9)
Lipofectamine	19.5 (6.6)

Means and standard deviations (in parentheses) of chloramphenicol acetyltransferase (CAT) activity from quadruplicate analyses are shown. DDAB, dimethyl dioctadecyl ammonium bromide; DOPE, dioleoyl phosphatidylethanolamine.

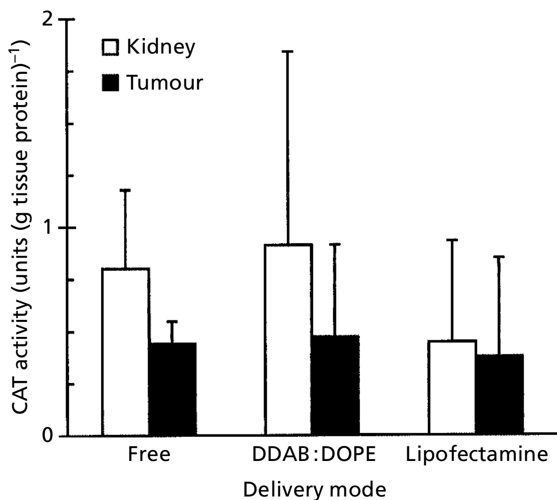

Figure 1 The aorta of rats was temporarily clipped below the left renal artery and then 300 μL of one of the following treatments was administered: sterile water (control); 12 μg pCMV-CAT in saline (free); 12 μg pCMV-CAT/DDAB:DOPE liposomes; or Lipofectamine reagent. Treated animals were killed 48 h after surgery and the kidneys and tumours harvested, proteins extracted, and chloramphenicol acetyltransferase (CAT) activity assayed. Means and standard deviations from four tumours and kidneys are depicted for each treatment.

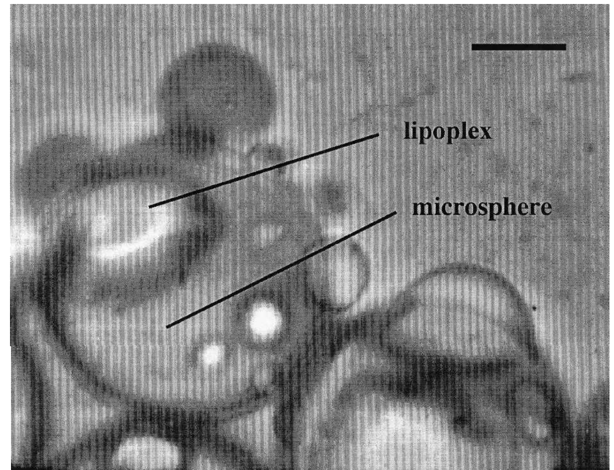

Figure 2 Suspensions of the combination vector (300 μL) were mixed with an equal volume of 0.1 g L^{-1} erythrosine dye and observed under the light microscope. Shown are several microspheres with lipoplexes ranging in size up to 10 μm attached to their surfaces. At least 20 different fields of triplicate microplex batches were analysed. Scale bar = 10 μm.

Table 2 Comparison of transfection efficiency/cytotoxicity between delivery modes.

Delivery mode	CAT activity (units (mg cell protein)$^{-1}$)	Cell viability (%)
Free	4.8 (1.5)	81 (4)
Microsphere	0.5 (0.3)	82 (4)
Lipofectamine	8.1 (1.3)	69 (7)
Combination	12.3 (1.2)	78 (5)

Means and standard deviations (in parentheses) of chloramphenicol acetyltransferase (CAT) activity and cell viability from quadruplicate analyses are shown.

delivery as the variability within the liposomal groups was large ($P > 0.01$).

For the in-vivo study where the CAT plasmids were delivered free, or with either DDAB:DOPE liposomes or Lipofectamine liposomes, CAT expression in tumours was always less than in kidney parenchyma for all delivery modes (Figure 1). However, transfection of tumours by the three modes (two liposomes and free plasmids) were not significantly different ($P > 0.05$). Lipofection variability within the two liposomal groups was again large.

Modified microplex formulation and cell culture testing

Figure 2 depicts the microplex vector formulated with a higher loading of lipoplex complexes, made from Lipofectamine containing 12 μg of CAT plasmid DNA rather than the previously tested 10 μg (Dass et al 2000). At this loading, the entire mass of lipoplexes was loaded onto microspheres as determined with radioactively labelled plasmid DNA. The erythrosine dye staining allows clear visualization of the loaded Lipofectamine vesicles docked onto the PDB microspheres. The attached complexes were usually from 2 to 15 μm across when attached to microspheres. These complexes would represent a bridging of adjacent plasmid–liposome complexes as suggested from earlier findings (Dass et al 1999). In that study, complexes over the range of 0.5 μm to approximately 10 μm were visible. Complexes smaller than 0.5 μm would not be resolved by light microscopy. DDAB:DOPE lipoplexes were unable to load onto microspheres as has been noted previously (Dass et al 1999).

CAT expression in cultured tumour cells with the combination vector was 23.5-fold ($P < 0.05$) compared with microspherical delivery, 2.6-fold compared with free delivery, and 1.5-fold compared with Lipofectamine delivery (Table 2). This represented an improvement over the com-

monly used transfection reagent Lipofectamine when it is used alone. Free and Lipofectamine transfections were significantly greater ($P < 0.005$) than microspherical delivery, whereas delivery using Lipofectamine was not significantly different compared with free delivery. The viability of cells after transfection with different delivery modes were not significantly different from each other. The lower viability of CSU-SA1 cells with Lipofectamine has been noted previously (Dass et al 2002).

In-vivo delivery with combination vector

As shown in Figure 3, in tumours, the greatest amount of expression ($P < 0.005$) was achieved using the combination vector; 3.2-fold compared with Lipofectamine delivery, and 1.9-fold compared with microspherical delivery. Owing to a large variability in data, the results for tumour transfection between free, microspherical and Lipofectamine deliveries failed to be significantly different ($P > 0.00$). Only delivery with the combination vector was significantly different to the other modalities ($P < 0.005$). Expression of the *CAT* gene in the kidneys was in general not significantly different between all delivery modes ($P > 0.05$).

Tumour-to-normal tissue (normal tissue being kidney) *CAT* expression ratios was 2:1 for the combination vector. The tumour-to-normal tissue ratios for the other three delivery modes failed to be significantly different ($P > 0.05$). Additionally, the liver was chosen for analysis since it is the major organ within the reticuloendothelial system that is associated with the removal of foreign particulate material from the bloodstream. Again, only the microplex delivery managed to exhibit significant difference between tumour-to-liver ratio (2:1, $P < 0.005$). As for the kidney, expression of the *CAT* gene in the liver was in general not significantly different between all delivery modes ($P > 0.05$).

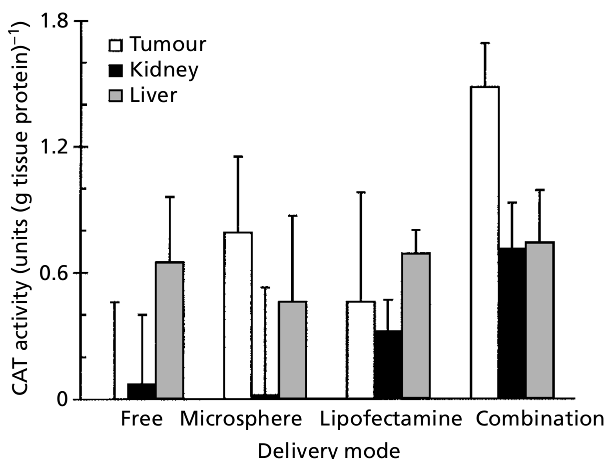

Figure 3 The aorta of rats was temporarily clipped below the left renal artery and then 300 μL of one of the following treatments was administered: sterile water (control); 12 μg pCMV-CAT in saline (free); 12 μg pCMV-CAT/A27TM microspheres; 12 μg pCMV-CAT/Lipofectamine; or 12 μg pCMV-CAT/microplex. Treated animals were killed 48 h after surgery and the kidneys, tumours and livers harvested, proteins extracted, and chloramphenicol acetyltransferase (CAT) activity assayed. Means and standard deviations from eight tumours and kidneys and four liver tissues are depicted for each treatment.

Discussion

In the present study, inferior in-vitro transfection was achieved with free DNA. This could be owing to both lower rates of entry of plasmids into cells as well as enzymatic degradation. These complications may be overcome by using liposomes as demonstrated in earlier studies (Bertling et al 1991; Alexakis et al 1995). However, transfection of CSU-SA1 cells in culture with liposomes (lipofection) in the present study was associated with a high level of variability. This is not surprising because of the multiplicity of factors that may have different degrees of influence on the ability of lipofection reagents to facilitate entry of nucleic acid constructs into cells (reviewed in Dass 2002b).

When compared in-vivo using the model kidney tumour system developed in-house, the variability in lipofection was again noted with both in-house and commercial liposomes. Such variability of lipofection in-vivo is not uncommon (Wheeler et al 1996; Dunlap et al 1997). As delivery by the various modes in the present study was done identically via the aorta, variability may be explained by the plethora of events that occur between initial contact of liposomes with blood and eventual plasmid release from the carrier, steps that may largely depend on the inherent physiological state of the animal. Although it could be argued that statistical differences between the delivery modes may reach significance if more animals had been used, similar numbers of animals (3–5) have commonly been used in previous gene delivery studies (Tsan et al 1995; Mathiowitz et al 1997; Miyoshi et al 1997).

In a previous study, Lipofectamine bound the most number of pCMV-CAT compared with Lipofectin, Lipofectace and liposomes formulated in the laboratory using DDAB:DOPE (in mass ratios of 1:2, 1:1 and 2:1) and DDAB:phosphatidylcholine (in mass ratios of 1:2, 1:1 and 2:1) (Dass et al 1999). Furthermore, Lipofectamine released the most number of plasmids in an in-vitro release study and was demonstrated to be the best transfection agent in cell culture. Thus, Lipofectamine was chosen here for the construction of the combination vector. It is worth noting that the vector developed and tested in previous studies by our group (Dass et al 1999, 2000) (termed "microplexes") was loaded with less lipoplexes (10 vs 12 μg) compared with the combination vector used in the present study.

PDB ion-exchange microspheres were chosen in the present study for comparison since they have been used for delivery of anticancer drugs to tumours without complications in both animals and humans (reviewed in Dass & Burton 1999). The number (Anderson et al 1991; Napoli et al 1992) and size (Meade et al 1987; Anderson et al 1991) of microspheres used in the present study have been demonstrated to successfully entrap in the tumour microvas-

culature of animals. Although novel nanospheres have been developed and tested for the ability to ferry nucleic acids in-vivo using such biocompatible matrices as poly (DL-lactide-co-glycolide; PLGA) polymer (Cohen et al 2000), alginate (Aynié et al 1999), gelatin (Leong et al 1998) or polyalkylcyanoacrylate (Fattal et al 1998), the size of these submicron particles will lead to systemic distribution of spheres after the first tissue pass and hence detract from their ability to selectively entrap in the aberrant microvasculature of the tumour. In other words, the tumour tissue residence time of these spheres will be severely limited.

There have been a few documented uses of microspheres for gene delivery in-vivo. For instance, Aggarwal et al (1999) orally administered alginate microspheres containing encapsulated plasmids and noted transgene expression in the intestine, liver and spleen in mice. The route of administration is of importance as well. Lunsford et al (2000) reported that while intramuscular and subcutaneous injections of PLGA microspheres containing plasmid DNA results in persistence of the delivered transgene for 100 days, intravenous injection results in widespread dissemination and long-term persistence in the reticuloendothelial system. The most promising result to date with PLGA microspheres in the context of genetic medicine against cancer is that of Putney et al (1999), who demonstrated that 85–90-μm diameter PLGA spheres containing 15-mer c-*myc* oligonucleotides dosed intravenously resulted in efficacy against both primary tumours as well as metastases in a xenograft model.

It has previously been established that the A27 microsphere may be used to carry plasmid DNA and release it in an intact (Dass et al 1996) and bioactive form (Dass et al 1997). Binding is via an ion-exchange mechanism, which avoids exposure of the nucleic acid strands to adverse conditions such as high temperatures and rapid mechanical stirring speeds used during encapsulation of DNA within microspheres in matrices such as gelatin (Cortesi et al 1994). In another study using spheres with diethyl aminoethyl functional groups, binding via an electrostatic mechanism was shown to be too strong since release and expression of plasmids in cell culture were negligible when compared with delivery using Lipofectin (Bertling et al 1991). Thus, A27, used in the present study, is a better microsphere to use since binding of DNA is a simple one-step procedure, and release of plasmids from the sphere surface does not hinder their expression in target cells. Additionally, these spheres are easy to obtain and obviate the need for chemical expertise for manufacture of delivery spheres for an application akin to the current one. Finally, ion-exchange PDB-based microspheres have been used for delivery of cytotoxic agents to tumours (Napoli et al 1992) and metastases (Esdale et al 1997) in pre-clinical studies.

In the present study, it was shown that by combining cationic liposomes with ion-exchange microspheres, a vector that is capable of increasing the transfection efficiency of liposomes in cell culture is developed. Results showed that the greatest amount of expression in tumour cells was achieved using the combination vector. The vector was 24.6-fold better in transfecting tumour cells compared with microspherical delivery, 2.6-fold better than free delivery and 1.5-fold better compared with liposomal delivery ($P < 0.005$). Free and liposomal deliveries were significantly greater ($P < 0.005$) than microspherical delivery, while delivery on liposomes was not significantly greater than free delivery ($P > 0.005$).

Although plasmids released from ion-exchange microspheres were expressed, levels were lower compared with the other delivery modes as seen earlier (Dass et al 1997). This could be attributed to a slow release of plasmids as demonstrated using in-vitro release analysis (Dass et al 1996). Liposomes enhance transfection compared with free plasmid delivery, but in the present study were inferior to microplexes. This could be attributed to the physical contact of the combination vector with cells. As the attached liposomes are closer to the cells, their entry into these cells would be greater than with liposomes suspended in the culture medium. In an earlier study, greater transfection with microsphere-bound plasmids was attributed to the physical contact of plasmids with target cells (Mathiowitz et al 1997). Recently, Luo & Saltzman (2000) have demonstrated that by increasing the concentration of plasmid DNA at the surface of cultured cells via complexing the nucleic acids with dense silica nanoparticles, a greater rate of transfection was achieved compared with common transfection reagents. The ability of silica nanoparticles for in-vivo gene delivery has not yet been evaluated.

The drawback of expression of the *CAT* gene in the liver with the combination vector may be due to the liposomes detaching in response to ionic exchange and travelling via the bloodstream to this organ. However, the likelihood of the intact combination vehicle travelling via the bloodstream to these organs cannot be ruled out. In a previous study, microspheres (0.1–5 μm in diameter) were noted to travel to the liver from the gastrointestinal tract (Mathiowitz et al 1997). In the present study, retrograde movement of microspheres via the celiac artery may be responsible for the expression of the gene in the liver of treated rats.

The inability of liposomes to target tumour tissue has been noted in other studies (Zhu et al 1993; Lew et al 1995). Furthermore, interaction of liposomes with plasma components, including opsonins, inhibits cellular uptake of the injected liposomes and is believed to mediate rapid uptake by Kupffer cells of the liver. However, liposomes aid entry of DNA into the nucleus by fusion with the nuclear envelope, creating vesicular and reticular intranuclear membranes (Friend et al 1996). There are numerous in-vivo studies documenting use of these important carriers for genetic medicine via various routes (reviewed in Dass 2002c).

The basis of enhanced delivery mediated by the combination vector may be outlined as follows: (i) microspheres administered upstream of the tumour into an artery deliver the carried liposomes at close proximity to the intended site; (ii) attached liposomes are then released from the microspheres due to the action of competing ions present in the tumour microvasculature; (iii) released liposomes extravasate into the tumour interstitium; (iv) liposomes gain entry into cells reliant on the endocytic processes of the cell and or fusion with the cellular lipid bilayer for uptake; and

(v) endosomal processes separate cationic lipids from the plasmids allowing the nucleic acids to gain entry into the target cell nucleus. As stated above, liposomes may also aid in nuclear entry of plasmids and may in fact offer a certain degree of protection against enzymatic degradation of the ferried nucleic acids.

Entry into cells is dependent on the numbers of plasmids delivered at the target site. The combination vehicle enables more plasmids to reach tumour cells by protection via complexation (Dass et al 2000), by selectively lodging in tumour microvasculature (reviewed in Dass & Burton 1999), and by enabling a physical contact of the delivery vector with the microvessels of the tumour. The third issue has been bolstered by the present findings that in cell culture, microplexes aid in the transfection of cells most probably by increasing contact between cells and lipoplexes. Such a vector may be used for in-vivo targeting of genotherapeutic agents to diseased sites such as tumours as well as enhancing the transfection efficiency of cells such as leukaemic cells ex-vivo.

Not addressed in this study is the possibility of targeting tumour vascular endothelial cells with anti-angiogenic or antivascular agents such as antisense strands against vascular endothelial growth factor, or chemotherapeutic agents such as vinblastine with the combination approach. Since the microplexes would reach vascular endothelial cells first, and since these cells endocytose lipoplexes readily in-vivo, such a selective delivery approach may hold some promise against solid tumours (reviewed in Dass 2002a). Selective delivery using microplexes would also avoid the issue of non-specific effects of anti-angiogenic genetic medicine to processes such as wound healing and the menstruation cycle.

Substitution of Lipofectamine with laboratory-formulated vesicles may significantly reduce the cost of manufacture (Dass et al 2002) of such targeting devices. Since selective delivery using microspherical agents has been performed by our group (Gray et al 1989; Campbell et al 2001) and others (Stubbs et al 2001) for delivering radioactive microspheres for treatment of liver metastases in clinical studies, this may be the platform for further testing the delivery of genetic medicine using microplexes against hepatic metastases that lack expression of a tumour suppressor gene such as *p53* (gene therapy) or overexpress an oncogene such as *bcl-2* (gene knockdown therapy). It is our belief that microplexes may be adapted to ferry other potentially therapeutic nucleic acid constructs such as ribozymes, antisense oligonucleotides, deoxyribozymes and double-stranded RNA molecules. It can be further hypothesized that microplexes may prove quite beneficial for selective delivery of genotherapeutic constructs against other common tumours such as those of the brain, breast and prostate.

Conclusion

The novel vector developed in the present project has the ability to enhance lipofection of mammalian cells ex-vivo as well as being able to target genes to solid tumours in-vivo. The microplex vector is non-replicating and is designed from plasmids, liposomes and microspheres, all of which have been proven safe in clinical trials. Since three different components are required, it is possible that by altering the physical/chemical characteristics of the vector and hence its targeting potential, one may attain better results in the future for various uses.

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