J. Pharm. Pharmacol. 1999, 51: 755–771
Received November 16, 1998
Accepted January 22, 1999

Lipoplexes and Tumours. A Review

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Genotherapy for Solid Tumours

Within a decade genotherapy (gene therapy) for tumours has gained rapid momentum largely due to the sobering fact that only 50% of all cancers can be treated with the conventional treatment regimes of surgery, chemotherapy and radiotherapy, (or any combination thereof). Genotherapy refers to the administration of genes (or other nucleic acid strands) to cancer cells to cause a cytotoxic effect. Interestingly, whilst genotherapy is used against cardiovascular disease, arthritis, neurodegenerative disorders and other genetic or acquired diseases, more than 50% of all current clinical gene therapy trials are for cancer.

In the context of cancer, there are various interpretations of the term 'genotherapy'. Genotherapy commonly relates to the delivery of a wild type tumour suppressor gene, such as p53, into a cancer that is mutated at that locus (this is now known as 'gene replacement therapy'). In a broader context, genotherapy includes the use of antisense molecules to down-regulate the expression of an oncogene, for example c-myc, linked to either the initiation or progression of the tumour. In another form, genotherapy may include enhancement of the immune system by tagging cancer cells for autoimmune destruction or vaccination whereby the body itself produces antigens that induce an anticancer immune response. This is currently how most cancers earmarked for genotherapy are treated. Transfection of prokaryotic genes that metabolize a harmless chemical into a toxic product is yet another way of eradicating tumour masses in animal models and is currently being evaluated in clinical trials. This branch of genotherapy is presently the second largest form in the clinical scenario.

Whilst this review discusses the relevance of liposomal genotherapy for solid tumours with either tumour suppressor genes or delivery of antisense oligonucleotides, the technology may also be used, with appropriate modifications, for the metabolic toxic products (reviewed in Connors (1995)) and immune-based cancer gene therapies (reviewed in Zier et al (1996)), as these also involve delivery of therapeutic nucleic acid strands.

In addition, whilst genotherapy may be performed ex-vivo, such as in blood cell disorders like adenosine deaminase deficiency (severe combined immunodeficiency disease), in-vivo genotherapy is the main focus for this review. This form refers to the extraction of cells from a patient before genetic manipulation of the cells in culture, and then the readministration of these attenuated cells into the same patient. Ex-vivo gene shuttling may be performed using biological (e.g. retroviral), chemical (e.g. calcium phosphate precipitation) or physical techniques (e.g. electroporation) and since 1996, results of early clinical studies have been appearing in the literature. However, for in-vivo delivery, both physical and chemical techniques have serious shortcomings. The major form of in-vivo gene transfer relies on viral vectors, the drawbacks of which will be discussed below. Liposomal transfer, the second choice, is a safer alternative and has great potential if certain hurdles are overcome. These issues will also be dealt with herein.

For genotherapy to be performed and evaluated, an appropriate animal model is necessary. Characterization of the molecular cause of the cancer is the initial step. Once it is known which tumour suppressor gene is inundated or which proto-oncogene has been activated to an oncogene, gene delivery may then be performed. Importantly, a model has to be chosen that fits as close as possible to clinical tumours. In other words, models should not be chosen just because they are more amenable or responsive to therapy, or treatment started before the tumour has been given an appropriate interval in which it can establish itself in the new host. The tumour burdens in animal models are usually below one million cells whilst those encountered clinically comprise 100 million to 10 billion cells (Hanania et al 1995). In any case, choice of one model from various available ones may only be representative of a small subset of human tumours. Thus, emphasis has to be placed on emulating the clinical scenario as closely as possible.

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In cell culture, delivery of tumour suppressor genes such as the Wilms tumour suppressor gene (Kudoh et al 1995), adenomatous polyposis coli gene (Morin et al 1996) and p53 gene (Lesoon-Wood et al 1995) have resulted in either a mitotic arrest in tumour cells or apoptosis. In-vivo studies with transplantable tumours in animals show that p53 tumour suppressor genotherapy leads to regression of tumours lacking normal p53 function (Lesoon-Wood et al 1995; Nguyen et al 1996; Overholt et al 1997). All the above studies utilized viral transduction or were limited in that either the tumours were very small when exposed to the gene or the gene vectors were injected directly into the tumours which were positioned near the skin for ease of access. However, regardless of the listed deficiencies, importantly, these studies did show that genotherapy was not a distant hypothetical notion as it once may have seemed.

Lipoplexes and Tumour Genotherapy

Anionic nucleic acids initially bind to the surface of cationic liposomes eventually forming multilamellar lipid–DNA complexes (Radler et al 1997). DNA persists glued to lipidic molecules with a lipid bilayer surrounding the compacted nucleolipidic particles in one of several different moieties: a cylindrical form, where the DNA is coated by a curved lipid bilayer; a flat lamellar form, where DNA is sandwiched between lipid layers (Dan 1998); and a form where DNA is condensed as parallel helices between lipid bilayers (Battersby et al 1998). These discrepant observations may be attributed to the lipidic formulation of the vesicles, the manner in which the complexes were formed, and the technique used to treat and visualize these complexes. Whilst most observations of lipoplexes are made using electron microscopy, erythrosine dye, which has affinity for the cationic lipids, may be employed to enhance visibility of complexes under the light microscope (Dass et al 1996a). In addition to electrostatic attraction, hydrophobic interactions are believed to aid complex formation between lipids and nucleic acids (Wong et al 1996). These highly compact complexes are referred to as 'lipoplexes'.

Plasmid length does not influence binding to the liposomes but mass does (Maccarone et al 1992; Dass 1998). In addition, binding is stable for at least 24 h post-complexing of nucleic acid to vesicles. The ratio of DNA to liposome may be determined either by radiolabelling the nucleic acid with ^{32}P, enzymatic degradation of non-bound DNA and subsequent column chromatography (Schaefer-Ridder et al 1982), or by using an ultracentrifugation method for separating uncomplexed DNA from bound (New 1994b; Dass 1998).

A common molecule used in cationic liposome synthesis is the neutral lipid dioleoylphosphatidyl ethanolamine. The role of dioleoylphosphatidyl ethanolamine is to facilitate membrane fusion or aid in the destabilization of the plasmalemma or endosome (Felgner et al 1994). In addition, helper lipids such as dioleoylphosphatidyl ethanolamine are required to stabilize the cationic liposome suspension as cationic lipids repel each other (Zuidam & Barenholz 1998). Liposomes formulated without neutral lipid have inferior rates of transfection (Lasic & Pearlman 1996), whilst varying rates of transfection may result from varying ratios of cationic : neutral lipid used to formulate the liposomes (Farhood 1994). Success of cationic liposome-mediated DNA transfer is, however, dependent on numerous factors, some of which are

Table 1. Factors influencing the success of cationic liposome-mediated DNA transfer.

Factor	Reference
Cell type	Hernandez et al 1997
Whether the culture is primary or subcultures of the primary	Harrison et al 1995
Stage of cell in the growth cycle	Pickering et al 1994
Cell seeding density	Lascombe et al 1996
DNA to liposome ratio	Song & Liu 1998
Chemical treatment of liposomes before addition of DNA	Kariko et al 1998
The type and concentration of salts and biomolecules present in the liposome–DNA mixing medium	Fasbender et al 1995
The constitution of the cell culture medium	Escriou et al 1998
Size of the liposome–DNA complexes	Kawaura et al 1999
Dilution of liposome–DNA complex suspension	Staggs et al 1996
Time lipoplexes are allowed to mature before addition to cells	Yang & Huang 1998
Time liposome–DNA complexes are incubated with cells	Zabner et al 1995
The lipid components making up the vesicles	Dass et al 1997a
Stabilization of liposomes with non-lipidic agents such as polyethylene glycol	Meyer et al 1998
Presence of fusogenic viral coat proteins such as those derived from the Sendai virus	Namoto et al 1998

Table 2. Routes of administration of lipoplexes in-vivo.

Route	Species	Reference
Intravenous	Mouse	Stewart et al 1992;Zhu et al 1993;Lesoon-Wood et al 1995;Liu et al 1995, 1997;Parker et al 1995;Thierrv et al 1995;Clarke et al 1996;Hong et al 1997;Xu et al 1997;Bei et al 1998;Song & Liu 1998
	Rat	Leibiger et al 1991;Tsan et al 1995
	Rabbit	Nabel et al 1992;Conary et al 1994;Canonico et al 1994
	Monkey	Parker et al 1995
	Pig	Nabel et al 1992
Intra-arterial	Man	Nabel et al 1994
	Rat	Dass et al 1997a; 1998; Dass unpublished results;Schmid et al 1998
	Rabbit	Leclerc et al 1992;Nabel et al 1992;Losordo et al 1994;Takeshita et al 1994
	Pig	Nabel et al 1990, 1992, 1993a, b, c
	Dog	Lim et al 1991;Chapman et al 1992
Intraperitoneal	Mouse	Philip et al 1993;Namiki et al 1998
Intraductal injection into the pancreas	Rat	Schmid et al 1998
Intraluminal injection into bladder	Mouse	Sugimura et al 1997
Intraluminal injection into oesophagus	Rat	Schmid et al 1997
Intramural injection into oesophagus	Rat	Schmid et al 1997
Intranasal topical application	Man	Caplen et al 1995
	Mouse	Wheeler et al 1996
Aerosol inhalation	Man	Middleton et al 1994
	Rabbit	Canonico et al 1994
	Mouse	McLachlan et al 1995
Tracheal insufflation	Rat	Tsan et al 1995
Direct injection into airway	Rat	Hazinski et al 1991;Logan et al 1995
	Mouse	Yoshimura et al 1992;Wheeler et al 1996
Direct injection into brain	Mouse	Roessler & Davidson 1994;Mizuguchi et al 1997
Direct injection into kidney	Rat	Tomita et al 1992
Direct injection into liver	Mouse	Parker et al 1995
Direct injection into skeletal muscle	Mouse	Malone et al 1994;Bei et al 1998
Direct intratumoral injection	Man	Nabel et al 1993d, 1996;Pauli et al 1998
	Mouse	Stewart et al 1992;Son & Huang 1994, 1996;Takakuwa et al 1997;He et al 1998
	Rat	Nomura et al 1997;Yanase et al 1998; Burton unpublished results
Administered as eye droplets	Rat	Matsuo et al 1996
Administered as an enema preparation	Mouse	Eldin & Hargest 1997

listed in Table 1. This plethora of factors may explain the inherent variability of lipofection (lipoplex-mediated transfection) especially in-vivo (Wheeler et al 1996; Dass et al 1997a). Nevertheless, the fact that lipoplexes may be administered in-vivo via various routes (Table 2) highlights the versatility of these gene transfer vehicles.

Mechanisms for Lipoplex Entry into Cells

The events that occur between initial contact of liposomes with blood and eventual drug liberation from the carrier and drug action depend on the type of preparation utilized and the physiological state of the animal (Gregoriadis 1985). Immediately following injection, liposomes are tagged by plasma high-density lipoproteins (HDLs). This results in transfer of liposomal phospholipid molecules to HDL followed by disruption of the liposome membrane, which results in a leakage of contents into the circulation (Gregoriadis & McCormack 1993).

In-vitro studies reveal that proteins make liposomes more permeable and modify their cell-associating mechanism (Felgner & Holm 1989). Such interaction is by way of electrostatic, hydrophobic and van der Waals interactions of the vesicles with plasma proteins (Lasic 1996). In addition to HDL and low-density lipoproteins, albumin, α- and β-globulins, clotting factors, fibronectin, complement components and immunoglobulin G (IgG) have all been suggested as adsorbing proteins (Juliano 1989). Some of these proteins, such as C-reactive protein and fibronectin, increase the opsonization of liposomes since these proteins have specific receptor sites on macrophages.

The lipoplex gains entry into cells by endocytosis (Kawaura et al 1999), probably encapsulated within an anionic endosomal vesicle (Koltover et al 1998). The lipoplex destabilizes the endosomal lipidic

layer components, before anionic lipids laterally diffuse into the complex and form a charge neutral ion pair with the cationic lipids (Xu & Szoka 1996). This results in displacement of the DNA from the cationic lipid and release of the DNA into the cytoplasm. Such displacement has been demonstrated in in-vitro studies using anionic liposomes (Harvie et al 1998).

The cationic liposome–DNA complex must possess an overall positive charge to enable the complex to interact with the anionic cellular surface in a sequence of events culminating in fusion (Fasbender et al 1995). It is believed that negatively-charged sialic acid residues on cellular surfaces attract the positive lipids on the liposome surface. In cases where phosphatidyl ethanolamine is used as the neutral lipid component, fusion ensues when phosphatidyl ethanolamine is destabilized, in turn destabilizing the plasma membrane (Duzgunes et al 1989). A similar electrostatic attraction is believed to be responsible for transfection of cells with nonlipid cationic polymers (called 'polyplexes' when complexed with plasmids).

There exist alternative explanations for the mechanism of entry of liposomes into the cellular interior. One model is that of destabilization of both carrier and cellular membranes upon contact whereby liposomal contents diffuse into the cell (Gregoriadis 1985). Finally, liposome–DNA complexes have also been observed to directly penetrate the plasma membrane into the cytoplasm (Singhal & Huang 1994). Regardless of the mechanism of delivery, liposomes have been used successfully as carriers for a wide range of agents into cells.

Liposomes, at various stages of destruction, are removed by fixed macrophages of the spleen and liver (Nicolau et al 1983). In the spleen, removal of liposomes may also occur through filtration (Moghimi et al 1991). Liposomes may also interact with macrophages of other tissues such as the lung (Perry & Martin 1995) and circulating monocytes to a lesser extent (Mizushima et al 1994). As mentioned above, liposomes interacting with phagocytic cells are taken up by endocytosis forming endosomes that mature into lysosomes. This lysosomotropic action of liposomes facilitates access of drugs to cellular organelles and the nucleus.

Rationale for Using Lipoplexes

Table 3 lists the advantages liposomal delivery of DNA has over other methods such as viral transfer. Like any drug, the safety of cationic liposomes has been monitored in clinical studies (Nabel et al 1993d, 1994; Middleton et al 1994; Caplen et al 1995). For instance, clinical studies using lipoplexes to deliver cystic fibrosis transmembrane regulator (CFTR) cDNA to the nasal epithelium showed no treatment-related local or general adverse reactions and significant small but transient correction of the ion transport defect (Coutelle & Williamson 1996). However, it would be premature to state that generally cationic liposomes are safe for use in-vivo as clinical studies carried out hitherto have involved relatively few patients per study. It is known that antisense strands introduced free of lipidic carriers enter mammalian cells in cell culture (Walker et al 1996) and in-vivo (Walker et al 1995). The major problem of using phosphodiester oligodeoxynucleotides (ODNS) is that they are degraded within 2 h in serum-containing medium (Eder et al 1991). Thus, to avoid gross ODN degradation in-vivo, pluronic gels (Simonset al 1992) or implantable osmotic pumps are employed (Walker et al 1998). In addition, ODN strands are made resistant to enzymatic attack by synthesizing analogues such as phosphorothioate or methyl phosphonate ODNs (reviewed in Walker et al (1997)). Liposomes minimize enzymatic degradation of ODNs whilst boosting their uptake into cells (Tari et al 1996; Gokhale et al 1997).

Plasmids may also be delivered bound to ion-exchange microspheres to minimize degradation

Table 3. Advantages of using liposomes for gene delivery.

Biodegradable and non-cytotoxic
Formulated from relatively inexpensive chemicals
Preparation does not involve biologically hazardous procedures as in retroviral production
Can be made from a wide array of components and thus may possess various properties
May be formulated with various methods
DNA is bound without chemical modification on the lipid bilayer
Small size and lipidic nature allows entry into cellular and intracellular sites
Size alteration enables control over clearance rates from the bloodstream
Can be prepared with a relatively high DNA/lipid ratio
Restrict access of carried materials to the external milieu providing protection against enzymatic degradation

(Dass et al 1996b), with the added advantage that microspheres are capable of selectively transporting genes to the tumour microvascular bed (Dass et al 1997b). This selective delivery is due to the tortuous nature of these beds causing the luminal diameter of the microvessels to decrease until they are completely occluded (reviewed in Dass et al (1997c)). Furthermore, the combination of lipoplexes with microspheres as a single gene-transfer vehicle increases targeting of plasmids to tumours in-vivo as well as enhancing the quantity of genes reaching the tumour cells (Dass unpublished results). This vehicle, termed 'microplex', harnesses the advantage of microspheres (selective delivery into tumour microvacsular bed) and lipoplexes (intracellular delivery of plasmids) into a single vehicle.

When introduced intravascularly, free plasmids are degraded with a half-life of less than 10 min (Kawabata et al 1995). Most of the intravenously infected plasmids end up in the liver, in non-parenchymal cells, most likely Kupffer cells (Emlen et al 1988). Since clearance from the bloodstream occurs rapidly, to be unaccounted for solely by degradation inside liver cells, a surface DNAse on liver cells is postulated to be responsible. As the injection was intravenously administered in the study carried out by Kawabata et al (1995), a high dose of plasmids was found in the lung within 15 min of injection, tapering off thereafter. As in the liver, a consistent amount of plasmids was noted in the spleen lasting up to 3 h in a separate study (Tsumita & Iwanaga 1963).

To minimize degradation, plasmid DNA may be directly injected into tissues such as skeletal muscle or even into tumours (Table 2). However, degradation of DNA is inevitable and expression of the foreign gene is limited to the site of injection (Hickman et al 1994; Ledley 1995). An interesting alternative is to implant plasmid DNA pellets into the muscle (Wolff et al 1991; Jiao et al 1992). Multiple pellets are implanted and transfection efficiency is greater than injections of plasmids in solution. Importantly, whether given by injection or implanted within the muscle tissue, large amounts of plasmid DNA cause no immunological responses to either the DNA or associated nuclear proteins (Parker et al 1995). An interesting, albeit highly variable finding is that naked plasmids delivered without complexation to liposomes proffer rates of transfection comparable with lipoplexes (reviewed in Felgner (1997)). Nevertheless, liposomal complexation has been noted to increase the residence time of plasmids in tumours (Nomura et al 1997). Whether intravascularly-delivered plasmids are also retained differentially is not known since Nomura et al (1997) administered the plasmids intratumorally.

For conventional drugs, release when these lipid carriers are destabilized allows access of the anticancer chemicals to neighbouring cells via tiny gap junctions. This intercellular transfer is only pertinent to a certain percentage of cancers (Blaese 1997). In contrast, in the context of gene delivery, destabilization of liposomes within a cell automatically exposes the nucleic acid strands to rapid degradation by endogenous nucleases. Thus, the percentage of genetic material traversing from one cell to the other without the protection of a carrier is expected to be minuscule.

The alternative to liposomal delivery of genes is viral vectors. The two major forms of these biological agents are retroviral and adenoviral vectors. These vectors are considered the method of choice to efficiently and stably transfect mammalian cells, including cancer cells. However, the field of viral gene transfer has undergone major reviews due to numerable factors such as the production of viral vectors being both slow and costly; existence of an inherent risk of the viral vector recombining with endogenous virions; viral vectors being extremely labile in-vivo due to inactivation by the complement cascade and other components of the immune system (Curiel et al 1996); retroviral vectors inducing novel tumours in animal studies via insertional mutagenesis (Anderson et al 1993); adenoviral transfer of genes into patients (Boucher 1996) leading to inflammation in different organs such as lungs, liver and brain; adenoviral vectors showing high discrepancies in gene transfer between species and even different tissues within the same species (Grubb et al 1994); retroviral vectors currently in use capable of transfecting dividing cells only (Miller et al 1990); and viruses being limited in the size of the foreign gene carried (Curiel et al 1996).

To address these deficiencies, current research seeks to enhance the targeting ability of viral vectors and reduce their undesired side effects. Perhaps, the major advantage of using liposomal delivery of genes is that only the expression plasmid is delivered instead of strands of contaminating DNA or other sequences that may cause unwanted effects in the patient. Whilst immunologic recognition disallows re-administration of viral vectors, lipoplexes may be re-administered. This is quite appropriate since over the past five or so years, physicians have come to realise that for many of the diseases amenable to genotherapy, repeated administration rather than a one-time dose may be necessary (Felgner 1997). Recently, researchers have ventured to use cationic liposomes to increase

the efficiencies with which viral vectors deliver genes intracellularly (Sharma et al 1997; Swaney et al 1997; Dietz & Vuk-Pavlovic 1998; Porter et al 1998). It has been postulated that these viral–lipoplex complexes augment attachment of viral particles to the cell membrane (Hodgson & Solaiman 1996) and that entry into cells occurs via endocytosis (Meunier-Durmort et al 1997).

At least 300 clinical trials for genotherapy involving more than 3000 patients have been carried out since the first landmark trial in 1990. Apart from cancer, these therapies are against diseases of an acquired nature such as rheumatoid arthritis, infectious diseases such as HIV-1 infection and hereditary diseases such as cystic fibrosis. Of these, at least a third used lipoplexes for gene transfer. This was second only to retroviral transduction. Thus, in future, provided that the current trend persists, greater utility of lipoplexes in the clinical scenario is envisaged.

Disadvantages of Liposomes in General

In most cases, uptake of liposomes by the mononuclear phagocytic system is undesirable, since it limits the carried substance to the mononuclear phagocytic system and lowers its delivery to the tumour site (Robert & Gianni 1993). However, blocking the mononuclear phagocytic system by pre-dosing with agents such as doxorubicin (Longman et al 1995) or with a preliminary dose of liposomes (Proffitt et al 1983) alleviates this problem. This pre-dosing effect is only a partial solution as the phagocytic cells of the liver have an upper limit to the amount of liposomes they may ingest (Hwang et al 1987).

Interaction of liposomes with plasma components, including opsonins, mediates rapid uptake by the Kupffer cells of the liver, which consequently minimizes uptake by target cells (Litzinger et al 1996). Another drawback of liposomes is their instability. They have to be prepared relatively fresh before administration, although different formulations possess variant shelf-lives. Cationic liposome complexation of plasmids results in a heterogeneous population of complexes with various shapes and sizes (Dass 1998; Hirsch-Lerner & Barenholz 1998) which may disallow efficient transfer of nucleic acid into cells. Prolonged storage may lead to aggregation of the complexes, which, as mentioned above, may limit the usefulness of a preparation.

Once liposomes are delivered, they can neither be removed nor their drug-release kinetics manipulated. This contrasts various implantable drug delivery systems, which can be removed and reimplanted; the drug delivery of these systems can be altered externally. In addition, when delivered into the vascular system, liposomes tend to disperse to all regions of the circulation rather than concentrating at a particular region (Zhu et al 1993; Dass et al 1997a). This contrasts with other DNA carriers such as microspheres (Dass et al 1996b; DeCruz et al 1996) that are able to target the vascular bed of a tumour (Codde et al 1993; Dass 1998).

Cationic liposomes have the drawback of some cytotoxicity at high doses (Felgner & Holm 1989) or at high liposome:DNA ratios (Schreier et al 1997). Incubation of cationic liposomes in plasma formed a clot-like mass and increased the turbidity of the usually clear stroma (Lasic 1996). Haemolysis of erythrocytes was noted when incubated with cationic liposomes made from dioleyloxypropyl trimethylammonium chloride or stearylamine, but not with 2,3-dihexadecyloxylpropyl-*N*,*N*,*N*-trimethylammonium chloride (BisHOP). This effect is probably due to the ability of liposomes to strip cholesterol from the erythrocyte membranes, thereby causing membrane disruption. It is important to note that haemolysis ceases when liposomes are first mixed with DNA to form lipoplexes before exposure to erythrocytes (Schreier et al 1997).

Thus, toxicity varies with formulation (Filion & Phillips 1997; Kao et al 1999) and even cell type (Singhal & Huang 1994). Even though there have been reports of toxicity of cationic liposomes in cell culture (Behr 1994; Fasbender et al 1995), in-vivo studies rule out any sort of toxicity (Logan et al 1993; Zhu et al 1993). It has been noted that tumour cells in-vivo are more sensitive to liposomal cytotoxicity than surrounding normal parenchymal cells (Burton unpublished results). This finding, if duplicated in other studies, will provide further backing for the use of lipoplexes for tumour gene therapy. In any case, cytotoxicity is believed to be due to the interaction of the cationic lipids with cell organelle membranes, specifically the anionic lipids making up these membranes (Xu & Szoka 1996). For instance, in mitochondrial membranes, cardiolipin is the major anionic lipid and interaction of this lipid with cationic species would prove detrimental to the basic energy reactions of the cell.

Even under optimal conditions however, the efficiency of in-vivo lipofection is low (0·3–5%) (Colledge & Evans 1995; Egilmez et al 1996). Nevertheless, even at the lowest efficiency in cell culture, liposome transfection is comparatively better than the more conventional calcium

phosphate precipitation method (Lascombe et al 1996). One reason for the low efficiency compared with viral vectors is the low rate of transfer of intact plasmids from the endosomes into the cytoplasm (Liang & Hughes 1998). Movement of DNA from the cytoplasm to the nucleus is a second form of limitation for successful transfection (Zabner et al 1995). Moreover, akin to other gene transfer devices, lipofection is quite variable especially in-vivo (Fasbender et al 1995; Dass et al 1997a).

Delivering transcription factors together with the therapeutic gene on liposomes which allow gene expression independent of the host transcriptional machinery in the nucleus makes nuclear entry unnecessary (Gao & Huang 1993). Due to a limited lifespan of the transcriptional polymerase within the cytoplasm, a novel expression system that also encodes the polymerase under its own promoter (so-called autogene system) has been successfully deployed using cationic liposomes (Singhal & Huang 1994). In any case, a transient system using recombinant plasmids will decrease in time since cell division will dilute the episomal DNA into the daughter cells. Even though integration of plasmid DNA into host chromosomes has been noted at low frequencies in cell culture (Scangos et al 1981; Gareis et al 1991), this has not been noted in-vivo (Ledley 1995).

Nevertheless, as shown in Tables 1 and 2 , liposomes are capable of delivering plasmid DNA into the nucleus both in cell culture and in-vivo. It is believed that once in the nucleus, plasmid DNA is unravelled from the lipidic carrier as a result of competition from the mass of chromatin DNA for the cationic lipids (Behr 1994). Moreover, inclusion of nuclear targeting proteins such as the high-mobility group 1 peptide with the genetic load facilitates movement of the foreign nucleic acid molecules past the nuclear membrane once the liposomes are degraded in the cytoplasm even in non-dividing cells (Yamada et al 1995). Nuclear delivery is three- to tenfold when DNA is introduced with high-mobility group 1 (Kato et al 1991).

Biological and Physiological Considerations for Liposomal Gene Transfer into Tumours

Tumours are generally characterized by wide inter-endothelial gaps, large number of fenestrae and transendothelial channels formed by vesicles, and discontinuous or absent basement membrane (reviewed in Murray & Carmichael (1995); Dass et al 1998). In other words, fenestrated or discontinuous capillaries may be found in the same tumour. The vesicles are present in capillaries and facilitate transport by transcytosis (shuttling back and forth) or fusing to form transendothelial channels. In venules and vein-like vessels at the solid tumour–host interface, the vesicles join up with vacuoles to form aggregates known as vesiculo-vacuolar organelles, which form the hyperpermeable regions of a solid tumour vasculature (Kohn et al 1992).

In many types of tumours, the vascular bed is well-developed, in some cases, better than normal tissues (Jain 1996). For such tumours, liposomal delivery of genetic material may hold great promise (Lasic 1996). Large liposomes are retained in the first capillary bed they encounter. Therefore, use of cationic large unilamellar vesicles rather than intermediate lamellar vesicles may be more feasible. In addition, targeting may be achieved by delivering the therapeutic DNA as close as possible to the site via a catheter (Shi et al 1994; Dass et al 1997a,b; Dass 1998). This would ensure a maximum dose of therapeutic DNA since enzymatic degradation, interaction of the nucleic acid with the biological surroundings such as the vessel wall, and dilution in the blood would be minimized.

The tumour vasculature is more permeable compared with normal tissue vasculature (Sands et al 1985). In addition, neovascularization of tumours usually leads to newly formed vessels that are leaky due to weak basement membranes (Liotta et al 1976). Tumour cells in culture and in-vivo secrete a factor (vascular permeability factor) that increases the permeability of tumour vessels and aids in the accumulation of excess fluid commonly associated with tumours (Brock et al 1991). Apart from vascular permeability factor, others factors inducing vascular permeability in tumours include nitric oxide (Doi et al 1996), tumour necrosis factor α (Ettinghausen et al 1988) and interleukin-2 (Ettinghausen et al 1988). Increased permeability would also facilitate movement of lipoplexes from vessels into tumour cells.

Using flow cytometry, fluorescently labelled plasmid delivered by cationic liposomes was detected in the cytoplasm after 10-min incubation and increased with continued exposure, but at a decreasing rate, up to 36 h within cells (Tseng et al 1996). After 36 h, on average each cell contained approximately 10 000 plasmid molecules. All cells participated in transfection equally (Felgner et al 1987; Leventis & Silvius 1990), and entry of DNA–liposome complex was independent of cell cycle stage. However, contradictory results have been attained.

For instance, Pickering et al (1994) and Takeshita et al (1992) found that cells in culture that were liposomally transfected were dividing at a faster

rate compared with the normal mitotic period. These results were duplicated in-vivo (Pickering et al 1994). Thus, tumours, having an inherent expedited cell cycle, would theoretically be more amenable to gene delivery via liposomes than normal cells. Interestingly, a recent study (Thurston et al 1998) has shown that angiogenic vascular endothelial cells in murine tumours internalized lipoplexes at a greater rate than anionic, neutral or sterically-stabilized liposomes. Such selectivity in uptake should render it possible to target tumour vascular endothelial cells with antiangiogenic genes (or antisense strands). If lipoplexes are targeted to the tumour vascular bed using such devices as microspheres (Dass et al 1997b; Dass 1998), then side effects of therapeutic genes such as p53 will be reduced. Such selective delivery is important particularly when dealing with the vascular system (Folkman 1998). Once the blood supply to a tumour is curbed or completely inhibited, the tumour itself should be eradicated.

It has also been noted that uptake of antisense strands is much faster in leukaemic human cell lines than in normal cells from the same patient (Calabretta et al 1991; Zhao et al 1996). Normal brain cells of rats do not permit entry of plasmids as much as brain tumour (glioma) cells (Nishi et al 1996). A transplanted tumour line in the kidneys of rats showed greater expression of a foreign gene than the normal kidney parenchyma (Dass et al 1997a, b; Dass 1998). Such a mechanism may exist because of the greater division rate of mutated cells. Alternatively, it may be explained by a more demanding blood supply to the tumour. Regardless of the mechanism, these findings have great implications for vascular-based gene delivery to solid tumours.

One limiting factor is that cancerous cells often occupy less than 50% of a tumour volume (Jain 1996). One to ten percent of the volume is made up by the vasculature. The rest of the tumour volume consists predominantly of a collagen-rich matrix, the interstitium. To reach a tumour cell, the active agent must traverse the endothelial barrier and through the often thicker interstitial matrix (O'Connor & Bale 1984). Additionally, in tumours, interstitial pressure is higher than intravascular (Boucher et al 1996). Hence, movement of large molecules such as DNA through vessels occurs mainly by diffusion (Jain 1996). However, in regions of the tumour where interstitial pressure is low, movement of large molecules occurs via convective transport caused by solvent drag. It must be remembered that tumours seen clinically contain well supplied rapidly growing regions interspersed with poorly perfused, often necrotic areas (reviewed in Murray & Carmichael (1995); Dass et al 1998). In solid tumour tissue, blood vessels become tortuous, with variable intercapillary distances and compression and occlusion of lumens. Insufficient perfusion results, eventually, in necrosis in certain areas and also hypoxic areas containing otherwise viable tumour cells. This heterogenicity poses a problem to any sort of drug delivery whether it is from a distance or direct injection into the tumour. Ideally, the drug has to reach the periphery of the tumour, a region that is characterized by vigorous cellular turnover.

Targeting Via Tissue-specific and Inducible Genetic Elements

Apart from attaching monoclonal antibodies or ligands on to the surfaces of liposomes (chemical liposomal modification), researchers have ventured to target specific tissues and cell types by using promoter/enhancer elements that are tissue- or cell-type specific. In other words, targeting may be executed at the nucleic acid level rather than the common protein level. This form of targeting is gaining greater recognition due to the deficiencies of the earlier chemically modified liposomes. Importantly, the plasmids, but not the liposomes used, are modified.

One form of gene targeting is the use of gene regulatory elements, such as promoters and enhancers, that possess cell- or tissue-specific functions or are responsive to external physical factors such as heat or physiological conditions such as low glucose levels in tumours. For instance, lung-specific expression may be attained by linking the regulatory elements for human surfactant proteins A or B to the foreign DNA construct (Smith et al 1994). Liver-specific expression may be obtained by exploiting the phosphoenolpyruvate carboxykinase promoter (Ferkol et al 1993).

The Grp78 gene promoter, inducible by stress conditions such as glucose deprivation, anoxia and acidic pH, characteristics commonly associated with tumours, may also be exploited for tumour specific expression (Gazit et al 1995). Highest expression is found in the centre, where stress would be at its maximal in the tumour. pH-sensitive immunoliposomes, which have successfully targeted genes into mice tumours (Wang & Huang 1987) may be used together with such regulatory elements included in the plasmid DNA to increase specificity of expression. Regulatory elements of genes such as a-foetoprotein, carcinoembryonic antigen and prostate-specific antigen may also be

exploited for enhanced and selective expression in tumours (reviewed in Walther & Stein (1996)).

Another method of limiting expression of the therapeutic gene once it has entered the patient is by including responsive elements within its promoter regions. Even though the gene is administered systemically, expression can be induced selectively using external inducers. One example of this is temperature-responsiveness. Temperatures of 32·5°C are necessary to stimulate the expression of incorporated genes which are otherwise silent at normal body temperature (Lin & Benchimol 1995). Alternatively, the HSP70 (a heat-induced protein) promoter may be linked to a therapeutic gene and expression of the gene effected to a particular site by hyperthermia (Williams & Morimoto 1990). Theoretically, hyperthermic-induced expression should prove doubly lethal as temperatures above 42°C kill cancer cells (Yonezawa et al 1996).

Temporal and spatial limitation of genetic transfer can also be achieved via radiation responsiveness (Weichselbaum et al 1994). One example of a sequence responsive to radiation stimulation and that activates transcription as a result is the nuclear factor kappa B binding sequence (Brach et al 1991). When another inducible transcription activator, elements within the 5′ untranslated region of the early growth response gene, was linked to the tumour necrosis factor-a gene, increased tumour cell death was observed in human xenografts in nude mice (Hallahan et al 1995).

Whilst all these radical ways of limiting gene expression to solid tumours have appeared in a relatively short time, their proper evaluation in animal models is still needed. Studies to date have been carried out by few focussed groups with the appropriate technology. Furthermore, usually small numbers of animals are used to prove the applicability of the new technology and as mentioned above, with a relatively clinically irrelevant tumour model. The effort and time spent for introduction of new ideas to gene therapy has surely outdone the effort and time dedicated to thorough testing of the past techniques for gene transfer. Some techniques never proceed to the in-vivo stage while others seem to become lost within the numerous in-vivo studies done on the one vector, often with minor modifications without proceeding to the clinical stage.

Considerations for Improving Cationic Liposomal Delivery

Commercial preparations of liposomes for gene transfer are intermediate lamellar vesicles (New 1994a). That is, they have average diameters of 100–200 nm (Gibco BRL 1996). Note that other researchers prefer to class them as small unilamellar vesicles (Lasic & Pearlman 1996) or even large unilamellar vesicles (Sugarman & Perez-Soler 1992). Regardless of the class they are assigned to, these liposomes are small enough to extravasate through fenestrated capillaries (New 1994a). Generally, the smaller the size of the liposome, the longer it is retained in the circulation (Sugarman & Perez-Soler 1992). However, once DNA is bound to these vesicles, complex size exceeds that of the free vesicles (Dass et al 1997a; Dass 1998) and thus extravascularization is compromised. Size, however, is a factor that can be controlled through such practices as altering the ratio of DNA to cationic lipid (Dunlap et al 1997).

Sterically stabilized liposomes with prolonged circulation times are found to have enhanced uptake in solid tumours (Longman et al 1995). For tumours situated in regions inaccessible to normal routes of administration, prolonging circulatory times for cationic liposomes may prove beneficial. However, as DNA binds to the surface of liposomes as opposed to being encapsulated, stabilization with steric barriers such as PEG derivatives (for example, PEG–phosphatidyl ethanolamine) may not prove to be feasible as poor subsequent binding of DNA is anticipated (Wong et al 1996).

The greatest advantage in steric stabilization is that it reduces the binding of plasma proteins to liposomal surfaces. Akin to sterical stabilization of liposomes with either PEG or gangliosides, binding of DNA on to the cationic liposome surface may in fact deter subsequent attachment of plasma proteins and hence reduce opsonization of vesicles. Attachment of plasmids to cationic vesicles is a saturable event, more cationic charge being neutralized with additional DNA binding (Radler et al 1997; Dass 1998). Such reduction of opsonization by complexation may occur in-vivo but needs evaluation.

Apart from steric stabilization, uptake by the mononuclear phagocytic system may be decreased by blocking uptake with doxorubicin (Longman et al 1995). Blocking may also be carried out with a preliminary dose of liposomes as mentioned above (Proffitt et al 1983). Blocking may be explained by the saturability of phagocytic cells of the liver by the amount of liposomes that they ingest (Hwang et al 1987). Although blocking has been evaluated in conjunction with steric liposomes and even conventional ones, its effect on cationic liposomal DNA delivery is yet to be examined.

It has been found that phosphatidyl ethanolamine distributes itself with a bias for the inner layer of

the liposomal coat in small unilamellar vesicles when the molar ratio of phosphatidyl ethanolamine approaches 50% (Nordlund et al 1981). As it is known that phosphatidyl ethanolamine strongly promotes fusion of vesicles with the cellular membrane (Felgner et al 1994), it would be interesting to see how such manipulation of molar ratio of phosphatidyl ethanolamine to cationic lipid affects transfection rate in an in-vivo tumour model.

A traditional form of gene delivery technique, known as 'electroporation' is commonly used for ex-vivo delivery of DNA into mammalian cells. Drugs such as bleomycin (Domenge et al 1996; Heller et al 1996) have reached clinical trials with this sort of therapy, called 'electrochemotherapy' when applied in-vivo. In both the above studies, treatment seemed to be highly applicable to highly vascularized, rapidly growing nodules, with larger nodules regressing to a lesser degree than smaller ones. It has been found that injection of plasmids into the carotid artery of rats combined with application of an electric field into tumours using electrodes leads to highly efficient transfection (Nishi et al 1996). Importantly, expression of the foreign gene was limited to the region around the electrodes. Plasmids were not able to enter tumour cells without electroporation. It would be worthwhile to evaluate the combined effect of electroporation and cationic liposomes on transfection.

It is known that cationic liposome–DNA binding is rapid and that the bond is fairly stable. This suggests the availability of numerous charges on both the liposome surface and the copies of DNA in solution and the avidity of the electrostatic attraction. By adding other sorts of lipids, such as those used in pH- or thermo-sensitive liposomes (reviewed in Dass et al (1997d)) with a reduction in the amount of cationic lipid, the transfectability of tumours, and in fact other genetic disorders, may be increased. Whilst the cationic lipid binds the DNA, and the phosphatidyl ethanolamine destabilizes the cellular membrane, the third lipid would ensure that as much DNA as possible is released intact from the endosome and reaches the target cell nucleus. Few studies have ventured to combine cationic lipids with other novel lipids in the one liposome (Mazur et al 1994; Budker et al 1996). More studies are warranted in this area.

Summary

The need for genotherapy to refocus its attention on to laboratory evaluation of better methods rather than proceeding to the clinic with semi-apt tools for genetic transfer has been highlighted in clinical study reports documented to date. Quintessential for tumour genotherapy is the ability to target abnormal cells, hence reducing exposure of normal cells to genetic material whilst maximizing gene dosage to tumour cells. This becomes increasingly important as genotherapy establishes itself in the clinic alongside the older modes of treatment. This review has discussed the applicability of lipoplexes for genotherapy of solid tumours. Lipoplexes have been used extensively for gene transfer into cells, such as cancerous cells, deficient for a certain gene product. While cationic liposomes have many advantages over other forms of delivery mechanisms, several problems hinder their use in-vivo. A closer examination of the physical limitations of current lipoplex preparations, the development and testing of novel formulations, combined with more attention to the cellular processes of cell membrane breaching and nuclear entry, may enhance gene delivery. Essential for tumour genotherapy is the ability to target these lipoplexes into tumour sites whilst reducing gene dosage to other normal tissues. Development of a better lipofection agent may indeed require a collaboration of the fields of physiology, cell biology, molecular biology, biochemistry, chemistry and membrane physics.

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