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Review Article

Vehicles for oligonucleotide delivery to tumours

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Abstract

The vasculature of a tumour provides the most effective route by which neoplastic cells may be reached and eradicated by drugs. The fact that a tumour's vasculature is relatively more permeable than healthy host tissue should enable selective delivery of drugs to tumour tissue. Such delivery is relevant to carrier-mediated delivery of genetic medicine to tumours. This review discusses the potential of delivering therapeutic oligonucleotides (ONs) to tumours using cationic liposomes and cyclodextrins (CyDs), and the major hindrances posed by the tumour itself on such delivery. Cationic liposomes are generally 100–200 nm in diameter, whereas CyDs typically span 1.5 nm across. Cationic liposomes have been used for the introduction of nucleic acids into mammalian cells for more than a decade. CyD molecules are routinely used as agents that engender cholesterol efflux from lipid-laden cells, thus having an efficacious potential in the management of atherosclerosis. A recent trend is to employ these oligosaccharide molecules for delivering nucleic acids in cells both in-vitro and in-vivo. Comparisons are made with other ON delivery agents, such as porphyrin derivatives (< 1 nm), branched chain dendrimers (≈ 10 nm), polyethylenimine polymers (≈ 10 nm), nanoparticles (20–1000 nm) and microspheres (> 1 μm), in the context of delivery to solid tumours. A discourse on how the chemical and physical properties of these carriers may affect the uptake of ONs into cells, particularly in-vivo, forms a major basis of this review.

Cancer genotherapy

Results appearing in the mid-1990s from clinical trials with the various forms of genotherapeutic approaches, including those for cancer (Table 1), highlighted major shortcomings of anticancer genotherapy. Nevertheless, a closer examination of the clinical results, primarily in patients with very advanced cancers that were refractory to conventional therapies, revealed that genotherapy had the potential to be effective in inducing tumour regression with a concomitant acceptable low toxicity as attested to by the fact that 50% of all gene therapy clinical trials to date have been for cancer. One aspect of genotherapy, which remains to be adequately addressed and which has traditionally plagued other forms of therapies such as radiotherapy, is the targeting of genetic medicine to neoplastic tissue. Not only does targeting genetic medicine to tumours prevent side-effects to normal cells, it maximizes dosage to their cancerous counterparts. The prospects for targeting oligonucleotides (ONs) to tumours via the vascular route are discussed below. However, a prior look at the ability of ON molecules to enter cultured cells with and without these carriers is needed to lay the groundwork for the subsequent discussions.

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Table 1 Genotherapeutic constructs used against cancer.

Non-oligonucleotide approach
Tumour suppressor gene into tumour
Suicide gene into tumour
"Tag" gene encoding a foreign antigen into tumour to enhance immunological recognition
Cytokine gene into immune cells to enhance antitumour activity
Gene encoding the multidrug resistance protein into non-tumour cells for protection
Oligonucleotide approach
Antisense strands that specifically block expression of oncogene
Ribozymes that cleave oncogenic RNA
Deoxyribozymes that cleave oncogenic RNA

Cell culture studies with oligonucleotides

Since the first documented use of phosphorothioate ONs (PS-ONs) for HIV-1 replication (Matsukura et al 1987), various new chemistries for ONs have emerged. This is largely owing to concerns about PS-ONs exhibiting non-sequence-specific effects in cells (Yaswen et al 1993; Jansen et al 1995; Rockwell et al 1997; Agarwal 1999) In-vivo usage of PS-ONs is also problematic (reviewed in Crooke 1998; Flanagan 1998; Sun et al 2000). Each ON sequence, including its concentration, size and chemistry must be evaluated empirically to determine the most effective carrier for the greatest activity in a given cell type. Numerous types of transfection reagents are currently available for ON transfection, with carriers such as DOTAP cationic lipid formulation and Superfect branched dendrimer reagent being common choices for ON transfection.

Several studies have revealed that ONs are taken up by cells via adsorptive endocytosis, pinocytosis or a combination of both mechanisms (Stein et al 1993; Beltinger et al 1995). The mechanism of entry is speculated to depend on ON chemistry; nonionic ONs, such as methylphosphonates, enter cells by passive diffusion in a concentration-dependent manner (Regnier et al 2000), whereas ionic ONs, such as unmodified phosphodiesters and phosphorothioates, enter via receptor-mediated endocytosis (Loke et al 1989; Yakubov et al 1989). Zelphati and Szoka (1996a) showed that ON–cationic lipid complexes are endocytosed, mainly by uncoated vesicles. They demonstrated that release of the ON does not require acidification of the vesicles and that release takes place in the early stages of endocytosis, probably at the early endosome stage where vesicle pH ranges from 6.3 and 6.8 (reviewed in Mellman 1996). If ON channelling through the classical pathway of endocytosis occurs, these early endosomes transit very rapidly and transform into late endosomes within 2–3 min. Once within lysosomes, the quantity of intact ONs is expected to drop dramatically.

Wrobel and Collins (1995) found that destabilization of cationic liposomes occurs early in the endocytic pathway. In addition to being found in endosomal structures, ONs may also be present free within the cytoplasm and once within the nucleus, they tend to preferentially localize to the euchromatin–heterochromatin interface (Beltinger et al 1995) or predominantly associate with euchromatin (Zamecnik et al 1994). It has been estimated that at least 70% of ONs gaining entry to the nucleus are bound to proteins (Geselowitz & Neckers 1992), most likely non-histone proteins (Leonetti et al 1991). One concern is that a negligible quantity of ONs are associated with ribosomes or with endoplasmic reticulum, target sites for mRNA processing.

Recently, Mac-1, a member of a family of leukocyte integrins, has been found to bind to, and internalize, ONs (Benimetskaya et al 1997). Research in this area is still on-going and much has to be done as indicated by documented findings (Beltinger et al 1995; Laktionov et al 1999) revealing the presence of multiple cell surface PS-ON-binding proteins ranging in size from approximately 20 to 143 kD. This uptake of naked nucleic acids may be part of a naturally occurring salvage pathway for acquisition of nucleotides entering the circulation from dying cells and exogenous organisms (Budker et al 2000). ONs bound to the surface of cells undergo rapid efflux when exposed to medium devoid of ONs (Wu-Pong et al 1992). Following rapid efflux, cell-associated ON levels stabilize, indicating that the ON is undergoing neither mass secretion nor transcytosis. ONs already in endosomes may theoretically be trafficked back to the plasma membrane in perinuclear recycling vesicles, with the transit time being approximately 10 min (reviewed in Mellman 1996).

Furthermore, ONs, particularly phosphorothioated sequences, remain largely entrapped in "endosomal-like" vesicles (Bennett et al 1992; Beltinger et al 1995; Zelphati and Szoka 1996a). Mitochondria may be an additional preferential site of ON accumulation in the cytoplasm (Iversen et al 1992). One concern is whether these vesicle-entrapped ON molecules are eventually able to escape the endocytosis pathway and become available for either targeting mRNA strands or entering the nucleus to hybridize with pre-mRNA targets.

Zelphati & Szoka (1996b) proposed that cationic-lipid–ON complexes, after internalization by endocytosis, induce a "flip-flop" of anionic lipids from the cytoplasmic-facing lipids making up the plasma membrane. These anionic lipids are believed to diffuse into

the complex and neutralize the positive charge of the cationic lipids, thus leading to the release of the complexed nucleic acids into the cytoplasm. Those authors also demonstrated that fluorescein-labelled 28-mer all-phosphorothioated ONs entered the nucleus, leaving the rhodamine-labelled phosphatidylethanolamine (PE; from cationic liposomes composed of DOTAP and PE) in punctate cytoplasmic structures. Recently, Marcusson et al (1998), using a DOTAP derivative labelled with a BODIPY fluorescent tag, revealed that the cationic lipid remained in the cytosol after ONs had gained entry into the nucleus of A549 cells.

With cationic lipid-based reagents, decrease in viability has been proposed to be owing to binding of these positively charged lipids to anionic lipids in cells. For instance, binding of reagent cationic lipids to anionic lipids such as cardiolipin, the major anionic lipid in mitochondrial membrane, may severely compromise the metabolic pathways of the cell. Polymers such as Superfect, on the other hand, may have a different effect once released from endocytotic vesicles owing to their different chemical compositions.

Radiolabelling of ON molecules is expected to have a much lesser effect on the overall chemical structure of the strand, unlike both the FITC and TAMRA labels, which are functional groups in their own right. However, although fluorescent tags have been speculated to have an effect on uptake of ONs, such an effect has not been found with tags such as FITC and Cy3.18 (Zhao et al 1993) and Bodipy (Noonberg et al 1993). However, FITC has been found to attenuate the efficacy of antisense ONs in cell culture (Lappalainen et al 1994). However, with radiolabelled ONs, removal of the ^{32}P label from the 5′-terminus of the ON sequence, presumably by phosphatases (Shaw et al 1991; Benimetskaya et al 1999) and transfer to the nuclei may also be possible. In fact, it has been shown that phosphomonoesterase readily catalyses the removal of ^{32}P label from the 5′ position of ONs and the label rapidly incorporated into higher molecular weight nucleic acids (Saison-Behmoaras et al 1991).

Using confocal microscopy, several studies have revealed that when cells are incubated with free or complexed fluorescent-dye-labelled ON, the majority of the fluorescent-labelled ON is located in punctate (presumably endosomal) structures in cultured cells (Benimetskaya et al 1998; Conrad et al 1998; Zelphati et al 1998; Delong et al 1999; Dheur et al 1999; Pichon et al 1999; Santiago et al 1999) and within cytoplasm when administered in-vivo (Litzinger et al 1996; Zhao et al 1998; Alvarez-Salas et al 1999; White et al 1999). When administered in-vivo, akin to plasmid DNA, naked ONs enter cells via three possible mechanisms (reviewed in Budker et al 2000), which include large membrane disruption (caused by mechanical force owing to injection), transient large membrane pores (that arise either naturally or from the injection procedure), and transient small pores ($\approx$ 10-nm diameter pores caused by the injection procedure).

Importance of nuclear entry of oligonucleotides: potential and practice

The use of either microinjection (Chin et al 1990; Leonetti et al 1991; Clarenc et al 1993; Fisher et al 1993; Wagner et al 1993) or electroporation (Bergan et al 1993, 1996) favours accumulation of ONs in the nucleus. Chemical procedures, such as the use of cationic lipid reagents (Zelphati & Szoka 1996a, b), streptolysin-O-mediated plasma membrane permeabilization (Spiller & Tidd 1995), an amphiphilic anionic peptide (Pichon et al 1997) or cationic porphyrin derivative (Takle et al 1997; Benimetskaya et al 1998; Flynn et al 1999), also result in the ONs being introduced directly into the cytosol, from where they migrate within minutes to the nucleus.

Zelphati & Szoka (1996a) demonstrated that there was no effect by Brefeldin A on movement of PS-ONs from the cytoplasm to the nucleus. They also found that nuclear delivery of phosphorothioated ONs is energy-dependent, and is inhibited by cytochalasin B (depolymerizes microfilaments of actin and blocks uncoated-pit-mediated endocytosis) and *N*-ethylmaleimide (blocks fusion between endosomes). Beltinger et al (1995) had reported that PS-ON entry into nuclei may be independent of the nucleoporins and, as such, was perhaps diffusion-driven. It has been shown that mitosis increases the uptake of nucleic acids into nuclei (Krieg et al 1991; Noonberg et al 1993; Zhao et al 1993; Mortimer et al 1999). This could inherently be useful for cells undergoing rapid cell proliferation and membrane turnover such as many types of cancer cells (reviewed in Liang et al 1999).

Entry of chemical entities into the nucleus via passive diffusion occurs through a 9–10-nm diameter, 100-nm length nuclear pore complex (NPC) (Feldherr et al 1984; Pouton 1998; Luo & Saltzman 2000), which can dilate up to 26 nm (several million Daltons in weight) when a nuclear localization signal is coupled to the particles being studied (Dworetzky & Feldherr 1988; Dworetzky et al 1988). Entry of ONs up to 50-mer in length should take 2 min to reach equilibrium, assuming simple diffusion (Childs 1999). The NPC allows for both passive diffusion and active transport, with most macromolecules moving across through a gated channel by

signal- and energy-dependent mechanisms (Melchior & Gerace 1995).

Görlich & Mattaj (1996) estimated that 100 ribosomal proteins are imported and three ribosomal subunits are exported by each NPC every minute. This rapid turnover of ribosomes suggests that the nuclear transport machinery has a great capacity for importing macromolecules and that the potential for active transport of nucleic acids is considerable, once proper ways of trafficking ONs through the NPC are realized. In addition, the NPC undergoes distinct changes in its substructure to accommodate nucleocytoplasmic transport (reviewed in Stoffler et al 1999). Further, mRNA is transported to the cytoplasm as RNA–protein (RNP) complexes (Izaurralde & Mattaj 1995; Nakielny et al 1997). These carrier proteins are rapidly and continuously shuttled in and out of the nucleus (Piñol-Roma & Dreyfuss 1992; Lee et al 1996). Perhaps nuclear entry of ONs is linked to such carrier proteins (i.e. "piggy-backing" from the cytosol), or small carriers such as cyclodextrins (CyDs) or dendrimers may allow entry through mimicking this protein-linked transport (albeit in the opposite direction). Such suggestions have been made elsewhere (Cao et al 2000).

Entry of ONs into the nucleus appears to be cell-type-specific as primary keratinocytes rapidly and efficiently accumulate ONs into nuclei even when incubated with naked ONs (Noonberg et al 1993; Nestle et al 1994). This discrepancy between keratinocytes and other cell types has been attributed, at least partly, to the relative abundance of ON-binding proteins present on the plasma membrane of the keratinocytes (Laktionov et al 1999). However, primary keratinocytes do not always internalize ONs into nucleus efficiently and uptake may be dependent on monolayer confluency and differentiation state (White et al 1999) and passage number (Laktionov et al 1999) of cells.

A similar finding has been documented for the entry of plasmids into cultured myotubes (Wolff et al 1992; Dowty et al 1995) when other cell types are relatively hard to transfect. An assessment of the ability of ONs to enter myotube cells may lead to interesting findings. In any case, both skin and muscle are sites that interest gene therapists. Transfection of naked nucleic acids into myotubes and even hepatocytes in-vivo (Budker et al 1996; Zhang et al 1997, 1999; Liu et al 1999a, b), is attributed to the hydrostatic pressure caused by the injection. Furthermore, kidney, spleen and other organs can also take up naked plasmids administered intravascularly or intraparenchymally (Budker et al 2000).

The requirement for nuclear entry of ON constructs depends on the type of target: pre-mRNA or mRNA sequences. Nuclear entry of therapeutic ONs may allow greater efficacy since, theoretically, the target mRNA sequences will be sequestered and/or cleaved before reaching the cytoplasm to generate multiple copies of its gene product. The technology for targeting ONs to nuclei by attachment of nuclear localization peptide sequences at 3′ and 5′ ends, although possible, is still in the developmental phase (Tetzlaff et al 1998; de la Torre et al 1999). Finally, the use of cationic liposomes for transfection of tumour cells in-vivo results in efficient nuclear delivery of ONs with ensuing efficacy (Endo et al 2000).

Potential for delivery of oligonucleotides to the tumour via the vasculature

Although there are various mechanisms for targeting ONs to tumours, administration of these constructs through an artery feeding a tumour facilitates localization to the tumour. Administration through this route depends on an exploitation of vascular differences between tumour and normal tissue. These differences include the increased permeability of tumour vessels, tortuosity and disarray of tumour vessels and occlusion of tumour blood vessel lumens (reviewed in Dass et al 1998). In fact, the more rapidly growing and poorly differentiated a tumour, the more chaotic its vasculature.

Increased permeability may be attributed to factors secreted by the tumour cells inducing up-regulation of vesiculo–vacuolar organelles in the tumour compared with normal tissue venules (Kohn et al 1992; Qu-Hong et al 1995). Vesiculo-vacuolar organelles are present in all venules, but are concentrated at the tumour–host interface. These transendothelial channels ferry complexes as large as 50 nm in size within 10 s of delivery into the vessel. Even though the tumour to normal ratio of vesiculo-vacuolar organelles is not different in and around the tumour vicinity, there is 4-fold permeability in the tumour venules and this has been attributed to an up-regulation of the function of these vesicles in tumour venules. Increased permeability may also be owing to the presence of an increased frequency of pores along capillaries of tumour beds (Wu et al 1993; Yuan 1998). These capillaries and newly formed larger vessels supplying the tumour tend to have incomplete (discontinuous) basement membranes, which may be attributed to the rapid and aberrant growth of the cancer cells. To augment the efficacy of a drug that is delivered to the tumour via the vasculature, tumour vasculature may be rendered more permeable by the use of hyperthermia (Schuster et al 1995; Hauck et al 1997; Kong et al 2000)

or biomolecules such as VEGF or nitric oxide (reviewed in Dass & Su 2000).

Tumour genotherapy using lipoplexes

Lipoplexes are formed by the interaction of anionic nucleic acids binding to the surface of cationic liposomes eventually forming multilamellar lipid–nucleic acid complexes. In the case of double-stranded DNA, the nucleic acid molecules persist glued to lipidic molecules with a lipid bilayer surrounding the compacted nucleolipidic particles in one of several different moieties (reviewed in Dass & Burton 1999a). These observations may be attributed to the lipidic formulation of the vesicles, the manner in which the complexes were formed, the lipid to nucleic acid ratio, the size of the nucleic acid construct, batch-to-batch variation in reagents, and the technique used to treat and visualize these complexes (reviewed in Dass et al 1997c). In addition to electrostatic attraction, hydrophobic interactions are believed to aid complex formation between lipids and nucleic acids (Wong et al 1996).

Success of cationic liposome-mediated DNA transfer is, however, dependent on numerous factors, one of which is the overall charge of the complexes (reviewed in Dass et al 1997c). Such a 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 stored for as long as a year under water (Cao et al 2000) and may be administered in-vivo via the vascular system (Dass 1998) highlights the usefulness of these vehicles. Other routes of administration of lipoplexes in-vivo are listed in Table 2. Lipoplexes have been shown to enter cells via clathrin-involved endocytosis, becoming entrapped in endosomes, being released from these vesicular structures and entry into the perinuclear area, before being finally taken up into the nucleus (Cao et al 2000). Friend et al (1996) describes vesicular and reticular intranuclear membranes probably resulting from fusion of lipoplexes with the nuclear envelope.

However, one basic problem with lipoplexes is toxicity. This is normally closely associated with the charge ratio of the cationic lipid species in the formulation and the ON. Higher charge ratios are generally more toxic to a variety of cell types, including cancer cell lines. In addition, different reagents have different degrees of toxicity to cells, and toxicity is cell-specific. There are currently in excess of 30 different commercial varieties of cationic liposome formulations available. Because of the toxicity, delivery of lipoplexes has to be as close to the target site as possible to minimize side-effects. Size is another issue, as larger complexes tend to get entrapped in the first microvascular bed encountered. For instance, tail vein injections in mice would lead to entrapment in the lung microvasculature. The issue of non-specific efficacious effects with lipoplexes as well as other cationic polymeric carriers (Bielinska et al 1996; Lambert et al 1998; Xu et al 1998) is an issue that must also be monitored closely.

Considerations for lipoplex transfer into tumours

In many types of tumours, the vascular bed is well developed, in some cases, better than in normal tissues (Jain 1996). For such tumours, liposomal delivery of genetic material may hold great promise, since large liposomes (diam. $>1\ \mu$m) are retained in the first capillary bed they encounter. Such administration must be into an artery upstream of the target site (Dass et al 1997b, 2000). For instance, tail vein injections result in retention of the majority of liposomal material in the lung vasculature. Small vesicles (diam. $\leqslant 200$ nm) escape the capillary bed and are predominantly taken up by the organs of the reticuloendothelial system (RES), the liver and spleen (Liu et al 1992; Litzinger et al 1994). Lipoplexes with diameters ranging from 200–400 nm are taken up rapidly by the liver and spleen when administered via the tail vein in mice (Osaka et al 1996). Upstream intra-arterial delivery of cationic large vesicles (diam. $>1\ \mu$m) rather than intermediate vesicles (between 200 nm and 1 μm diam.) may be more feasible. However, vesicles greater than 400 nm in diameter may not be able to extravasate through the permeable tumour vasculature pores, and thus pass straight through to be taken up by the RES (Juliano & Stamp 1975; Kong et al 2000). Thus, targeting may be achieved by delivering therapeutic genetic constructs as close as possible to the site via a catheter (Dass et al 1997b, 2000; Santiago et al 1999). This would ensure a maximum dose of ONs to the target site 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.

Modification of liposome size enables a liposome technologist to alter the pharmacokinetics of drug delivery in general. Liposome diameter influences plasma half-life (Litzinger et al 1994; Harashima et al 1995) and site of accumulation (Nagayasu et al 1996) in-vivo. Size may also dictate the quantity of drug delivered and release characteristics of drug molecules (Ferdous et al

Table 2 Routes of administration of lipoplexes in-vivo.

Route / Species	References
Intravenous injection	
Mouse	(Stewart et al 1992; Zhu et al 1993; Lesoon-Wood et al 1995; Liu et al 1995, 1999b; Parker et al 1995; Thierry et al 1995; Clarke et al 1996; Osaka et al 1996; Stephan et al 1996; Hong et al 1997; McLean et al 1997; Xu et al 1997, 1999; Mahato et al 1998; Song & Liu 1998; Song et al 1998; Barron et al 1999a, b; Ochiya et al 1999; Anwer et al 2000b, c; Floch et al 2000; Ishiwata et al 2000; Meyer et al 2000; Tu et al 2000)
Rat	(Leibiger et al 1991; Tsan et al 1995; Lee et al 1998; Jeschke et al 1999)
Rabbit	(Nabel et al 1992; Canonico et al 1994; Conary et al 1994)
Monkey	(Parker et al 1995)
Pig	(Nabel et al 1992)
Intra-arterial injection	
Human	(Nabel et al 1994)
Rat	(Dass et al 1997a; Dass 1998, 2000; Lee et al 1998; Schmid et al 1998; Rainov et al 1999)
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; Muller et al 1994; Armeanu et al 2000)
Dog	(Lim et al 1991; Chapman et al 1992)
Peri-arterial (collar) delivery	
Pig	(Pakkanen et al 2000)
Intraperitoneal injection	
Mouse	(Philip et al 1993; Namiki et al 1998)
Intraductal injection into pancreas	
Rat	(Schmid et al 1998)
Intraluminal injection into bladder	
Mouse	(Sugimura et al 1997)
Intraluminal injection into oesophagus	
Rat	(Schmid et al 1997, 1998)
Intramural injection into oesophagus	
Rat	(Schmid et al 1997, 1998)
Intranasal topical application	
Human	(Caplen et al 1995; Hyde et al 2000)
Mouse	(Wheeler et al 1996)
Aerosol droplet inhalation	
Human	(Middleton et al 1994)
Rabbit	(Canonico et al 1994)
Mouse	(McLachlan et al 1995; Densmore et al 1999; Stern et al 2000)
Intratracheal administration	
Mouse	(Yoshimura et al 1992; Wheeler et al 1996; Jiang et al 1998; Zou et al 1998, 2000b)
Rat	(Hazinski et al 1991; Logan et al 1995; Tsan et al 1995; Boncuk et al 1997)
Human	(Gill et al 1997)
Percutaneous injection into lung	
Mouse	(Saito et al 2000)
Percutaneous injection into heart	
Rabbit	(Wright et al 1998)
Direct injection into brain	
Mouse	(Roessler & Davidson 1994; Mizuguchi et al 1997)
Rat	(Imaoka et al 1998)
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; Leong et al 1998)
Rat	(Cohen et al 2000)

(*cont.*)

Table 2 (*Cont.*)

Direct intratumoral injection	
Human	(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; Dunphy et al 1999; Endo et al 2000; Yerulshami et al 2000)
Rat	(Zhu et al 1996; Nomura et al 1997; Yanase et al 1998)
Intravesical instillation into bladder	
Mouse	(Horiguchi et al 2000)
Administered as eye droplets	
Rat	(Matsuo et al 1996)
Microinjection into cochlea	
Guinea-pig	(Wareing et al 1999)
Administered as topical application	
Mouse	(Domashenko et al 2000)
Administered as an enema preparation	
Mouse	(Eldin & Hargest 1997)

1996). Indirectly, by being tied in with release characteristics, the size of liposomes may also affect the toxicity of administered drugs such as Annamycin (Zou et al 1995). These factors, also relevant to delivery of ONs by liposomes, make particle diameter a valuable factor in determining the efficacy of a therapeutic agent used against solid tumours.

As stated above, the tumour vasculature is more permeable compared with normal tissue vasculature. In addition, neovascularization of tumours usually lead to newly formed vessels that are leaky owing to fragile basement membranes (Liotta et al 1976). Tumour cells in culture and in-vivo secrete vascular endothelial growth factor (VEGF), which apart from increasing the permeability of tumour vessels, aids in the accumulation of excess fluid commonly associated with tumours (Brock et al 1991). Increased permeability would facilitate movement of lipoplexes from vessels into tumour interstitium. If lipoplexes are targeted to the tumour vascular bed using such devices as microspheres (Dass et al 1996; Dass & Burton 1999b) or microplexes (Dass et al 1999, 2000), then possible side-effects of therapeutic genes (such as *p53*) or antisense strands (such as those against c-*myc*) should be reduced. Highly selective delivery is important particularly when dealing with the vascular system, since generation of new tissue, such as that involved in the menstrual cycle, is dependent on vessel regeneration. Once a tumour's blood supply 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 1996a). Normal brain cells of rats do not permit entry of plasmids to the same degree as brain tumour (glioma) cells (Nishi et al 1996). Greater expression of a foreign gene, when delivered free intra-arterially, occurs in a tumour line transplanted in the kidneys of rats than in the normal kidney parenchyma (Dass et al 1997b, 2000). Such a mechanism may exist because of the greater division rate of mutated cells. Dividing cells undergo breakages in their cellular membranes, thereby allowing nucleic acid constructs easier access to the nucleus. As mentioned above, mitosis increases the uptake of nucleic acids into nuclei (Zhao et al 1993; Mortimer et al 1999) and this could be very pertinent when treating rapidly proliferative cancer 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. Between 1 and 10% of the volume is made up by the vasculature (Jain 1996), and 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. Additionally, in tumours, pressure in the interstitium is greater than within blood vessels (Boucher et al 1996). Hence, movement of large vehicles such as lipoplexes 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 inter-

spersed with poorly perfused, often necrotic areas (reviewed in Murray & Carmichael 1995). 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 heterogeneity poses a problem for any sort of drug delivery whether administered at a distance or by 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. In addition, studies characterizing the permeability of tumour vasculature normally rely on small (1–2 mm) tumours, the cells of which are closer to a feeding vessel than those cells in larger hypoxic tumours (Kong et al 2000).

Thus, there are various limiting factors in the delivery of genotherapeutic agents to solid tumours. Although the efficacy of the vector is dependent on matters such as the expression level in cells or resistance against endonucleases, it is largely reliant on the route of delivery to the tumour. At present, the vascular route promises to be the best mode of administration, since it provides the therapeutic construct access to the rapidly growing regions of the tumour. Certain tumours have been demonstrated to have a pore cut-off size ranging from 200 nm to 1.2 μm in diameter (Hobbs et al 1998), which should allow ease of extravasation of particles as large as the smaller non-aggregated lipoplexes and small microparticles, and most nanoparticles. Some of the problems encountered with the use of cationic liposomes may be overcome with the use of a class of smaller carriers, CyDs.

Cyclodextrins

CyDs are oligosaccharides that are produced by an enzymatic modification of starch. One form, β-CyD, contains seven glucopyranosyl (glucosyl) units linked in a ring formation in the α(1–4) position. Hydrogen and glycoside oxygen atoms are oriented toward the pore, thereby rendering it hydrophobic. β-CyD has the capacity to form inclusion complexes with various molecules, especially bile acids and sterols (Riottot et al 1993; Klein et al 1995). Such binding hinders the absorption of bile acids and sterols from the small intestine. Bonds between host and guest molecules include H-bonds and hydrophobic or ionic interactions between the CyD cavity and the guest molecule (Bolyan & Ganzler 1998). CyDs have diameters of approximately 1.5 nm (compared with 7–12 nm for HDL particles) and, as such, can diffuse more readily through the unstirred water layer and also filter more readily through the glycocalyx that normally restricts access of larger particles from contact areas on the plasma membrane (Davidson et al 1995). CyDs are also quoted as having diameters of less than 1 nm (von Eckardstein 1996).

Chemically modified β-CyDs, such as 2-hydroxypropyl β-CyD (2hpβCyD) and methylated β-CyDs (meβCyD), have enhanced features with regards to solubility, complex formation and toxicity. β-CyDs have a high affinity for sterols compared with other lipids, making these second generation compounds more effective in modifying cholesterol metabolism in-vivo (Riottot et al 1993). Although β-CyD is not hydrolysed by the salivary or pancreatic α-amylase, it is fermented in mammalian large intestine to glucose and oligosaccharides by resident microflora in humans (reviewed in Dass & Jessup 2000). β-CyD is negligibly hydrolysed in, and slightly absorbed from, the stomach and small intestine. After oral administration of β-CyD in humans, only traces are recovered in stools (Flourie et al 1993). In pigs, inclusion of 5 or 10% β-CyD in a cholesterol-enriched diet produces a dose-dependent increase in faecal elimination of bile acids (Ferezou et al 1997).

Potential of cyclodextrins for oligonucleotide delivery in-vivo

Perhaps the major obstacle to in-vivo efficacy of CyD therapy is the localization of the effects of the CyD molecules to the injured site. CyDs, being relatively small in size compared with PLVs or non-adsorbing to surfaces such as that which occurs with apolipoprotein A-I, may be targeted to lesions (in the context of atherosclerosis) by the use of double-balloon catheters or (in the context of cancer) mini-osmotic pumps. Mini-osmotic pumps deliver ON drugs at a constant rate and are available at various flow-rates (Alvarez-Salas et al 1999), but their efficiency is dependent on several factors including temperature (Walker et al 1998) and possibly tissue architecture (Walker 1999). These pumps have been used to enhance the efficacious effect of antisense ONs (Walker et al 1998; Walker 1999). The use of pluronic gels around the injured vessel site may be an additional approach (Santiago et al 1999; Khachigian 2000) for delivery of various anti-atherosclerosis ONs.

In arteries clogged with lipid deposits, localized delivery with these pumps presents a challenge, since an invasive approach into the artery may prove technically difficult and would be theoretically highly dependent on the nature of the lesion, for instance the degree to which

it is calcified. Additionally, delivery into arteries would result in transport of the drug molecules from the injured site. However, a catheter-based intra-arterial approach may be employed against tumours in-vivo (Dass et al 1997a, 2000). In addition, complexes of CyD–ON may be encapsulated in biodegradable microspheres for sustained release in-vivo as has been demonstrated for other CyD-complexed agents (Sinisterra et al 1999). Nevertheless, administration via these pumps ensures a constant supply of CyD molecules to bathe the lesion site.

Recent studies have evaluated the ability of CyDs to deliver genotherapeutic agents, such as plasmids, viral vectors, and ON constructs. CyDs may also be used to enhance delivery of genetic constructs to cells in culture or in-vivo. A recent study by Croyle et al (1998) has demonstrated that certain βCyDs enhance the ability of adenoviral vectors to transduce differentiated Caco-2 cells (a human colorectal carcinoma cell line). In addition, they demonstrated that co-delivery of the vectors did not affect biological activity of the construct, since expression of the delivered gene was observed in the rat jejunum.

CyDs have been used to enhance intracellular delivery of antisense molecules (Habus et al 1995; Zhao et al 1996b). CyDs should avoid two fundamental problems of antisense delivery in-vivo: (1) the limited ability of ONs to extravasate from the bloodstream and traverse cellular membranes; and (2) the high degree of susceptibility of the antisense constructs to endonucleases, which rapidly degrade the oligomers. In most cases, degradation of the phosphodiester bonds between nucleotides renders these drugs useless for therapy. However, stabilization of ONs by certain chemical modifications, such as phosphorothioated backbones (reviewed in Sun et al 2000) or 3′-terminal base inversion (Cairns et al 1999; Sun et al 1999), dramatically increases the biological half-life of these constructs.

As mentioned above, the stability of antisense molecules may be increased by binding these constructs to CyDs such as OHpropylβCyD (Cserhati et al 1996). Whether this correlates to an increased resistance against endonucleases in cultured cells, and ultimately in-vivo, remains to be seen. CyDs such as dimethylβCyD and monoaminoβCyD (MAβCyD) interact to some extent with short (2-mer) phosphorothioate ONs (Bolyan & Ganzler 1998). Results suggest that the amino group on the MAβCyD has a stabilizing effect on the CyD–ON complex via ionic interaction. Binding of ON molecules to other carriers such as cationic liposomes increases stability (Dass & Burton 1999a). When the ionic strength of the solvent is increased, the electrostatic attraction between the amines on CyD molecules and phosphate groups of the antisense molecules are impaired as expected for a nucleic acid carrier operating on an ion-exchange basis (Dass et al 1999a, 2000).

Two- to threefold increase in the cellular uptake of antisense constructs by hydroxyalkylated β-CyDs has been noted in human T cell leukaemia H9 cells (Zhao et al 1995). The increase in antisense uptake observed with the CyD molecules was owing to an increase in uptake and not just binding to the cellular surfaces. Stimulation of the immune system by antisense constructs is quite common and it is believed that such stimulation may be reduced by complexation of the antisense strands with CyDs. In fact, Zhao et al (1996a) proved that CyDs reduced the induction of elevated platelet counts by antisense strands in-vivo. Certain CyDs may also modulate the intracellular distribution and enhance both the stability and activity of antisense molecules (Abdou et al 1997).

Thus, a simultaneous delivery of CyDs and antisense molecules may enhance intracellular delivery. In addition to enhancing membrane penetration, CyD molecules should stabilize the antisense molecules by preventing their hybridization in solution. Such base-pairing would decrease the effectiveness of the ONs. The small size of CyD molecules allows their incorporation into pumps (Sharkey et al 2000), unlike liposome–antisense complexes, which are bulky. In contrast to liposomes, CyDs such as OHpropylβCyD, CEβCyD, trimeβCyD and meβCyD may be concentrated up to >100, 1000, 200 and >750 mg mL^{-1} in water, respectively (Cyclolab 1999). It could prove worthwhile to incorporate the CyD guest drug molecule in polymeric formulations to prolong the release of drug into the target site. Cationic CyDs have also been linearized to produce polymers that bind and transfect 5-kbp plasmid DNA (Gonzalez et al 1999). The polymer–DNA complexes were 100–150 nm in diameter and charge ratios ($+/-$) of 70 did not prove toxic to cells in culture. Most importantly, these complexes failed to show signs of toxicity at polymer doses of 200 mg kg^{-1} administered either intravenously or intraperitoneally in mice. Epa et al (2000) demonstrated that at daily doses of 3 mg kg^{-1}, ON conjugated to hydroxyethyl-β-CyD (HEβCyD, 0.15 mg kg^{-1} per day) administered intraperitoneally to newborn mice for 2 weeks showed no toxic effects, while significant down-regulation of the target gene was noted in the dorsal root ganglia of animals. This backed up the cell culture studies using neuronally differentiated PC12 cells, where an average down-regulation of 40.5% with 1–5% HEβCyD was noted compared with 10.5% with phosphate-buffered

Table 3 Requirements for a vehicle for gene delivery.

Biodegradable and non-cytotoxic when intact and when degraded in-vivo
Non-immunogenic
Relatively inexpensive formulation procedure
Preparation should not involve biohazardous procedures
Possibility of scaling-up formulation process
May be formulated by various methods
Formulation protocol should not alter nucleic acid functionality
Maximal entry into cellular and intracellular sites possible
Control over clearance rates from the bloodstream possible
Can be prepared with a relatively high nucleic acid to lipid ratio
Provides protection against enzymatic degradation

saline control. Thus, not only does complexation of ON molecules with CyDs improve cellular entry, but also covalent attachment of the nucleic acid strands to CyD molecules.

Regardless of the shortcomings of each type of carrier, both lipoplexes and CyD molecules need to possess the desirable attributes for a gene delivery vehicle (listed in Table 3). Lipoplexes are larger in size and therefore are more prone to becoming opsonized and subsequently phagocytosed. On the other hand, CyDs are smaller molecules, and thus may be distributed fairly evenly throughout the vasculature. Such distribution may in fact pose as a side-effect if CyDs have toxic effects. Lipoplexes have been shown to be non-toxic, even in clinical trials, but more studies examining the true toxicity of these vehicles needs to carried out. Cellular uptake of lipoplexes have been demonstrated widely, but that of CyDs has yet to be closely evaluated. Finally, although lipoplexes have been used for at least a decade in-vivo, not much usage in the clinical setting has resulted from all the pre-clinical studies. Gene delivery technologists are now testing novel carriers, such as CyD molecules, and those described below, in the hope of finding an optimal, if not ideal, carrier for therapeutic nucleic acids.

Cyclodextrin-associated toxicity

Before being used clinically, the safety of CyDs has to be monitored initially in cell culture studies and then in animal trials. Since the interaction of CyDs with the plasma membranes may be the initial site of cellular damage, the incubation of erythrocytes with these agents may be a useful bio-indicator system. Among the most powerful solubilizing agents available for most lipophilic compounds are dimeβCyD and trimeβCyD (Cyclolab 1999). These derivatives are cytotoxic, causing tissue irritation and haemolysis in a dose-dependent manner. This behaviour has been directly attributed to the ability of meβCyDs to remove cholesterol from cells. Thus, meβCyDs are rarely used as drug carriers, except in low doses ($\approx$ 0.02 mM) in ophthalmic and nasal formulations (Agu et al 2000).

This cytotoxicity may, however, be used therapeutically. It is known that meβCyDs exhibit the greatest affinity for plasma membrane lipids, especially cholesterol, thus attenuating membrane fluidity at 5 mM/ 2×10^4 cells (Cyclolab 1999; Fukasawa et al 2000). This can perturb the action of membrane-based glycoproteins, which act to restrict drug permeability and result in poor intracellular drug accumulation. Sequestration of membrane cholesterol by meβCyDs causes alteration of membrane fluidity and compromises the action of glycoproteins present on the plasma membrane. This fact is quite important in cancer therapy where certain glycoproteins, such as the multidrug resistance protein (MRP) and P-glycoprotein, actively efflux drugs from aberrant cells. Lipophilic drugs are especially affected.

When βCyD was administered (in the diet) to Fischer 344 rats at concentrations of 0, 2.5 and 5% for more than 2 years, dose-dependent effects on growth were observed in both sexes (Toyoda et al 1997). However, no effects were noted on survival rates and mean survival times. This again may be attributed to the effect CyDs have on plasma membrane cholesterol and no apparent effect on cell cycling. Although the dosages given were about 340 to 400-fold the current human daily intake of the CyD as a food additive and from pharmaceutical usage, no carcinogenic effects were noted.

Differences in the toxicity of CyDs between cells in culture and in-vivo studies have been noted; for example, when the cavity of a CyD molecule is occupied, it is not cytotoxic (Irie & Uekama 1997). This is similar to cationic liposomes, where cationic liposomes not loaded with nucleic acids are more toxic to cells than those complexed to genetic strands. The best approach would be to first expose the CyD, judged by in-vitro tests to be ideal for a certain task, to a variety of mammalian cells in culture. The next step would be to monitor its cytotoxicity in animal studies, deriving important data on the dosage that is safe to use in-vivo. Select organs such as the kidneys would have to be routinely monitored since cholesterol bound to CyDs dissociate and become concentrated in the proximal convoluted tubule when CyDs are administered at doses of 200 mg kg^{-1} (Frijlink et al 1991). The formation of harmful cholesterol microcrystals in the lysosomes of epithelial cells in the renal proximal tubule owing to the transport and disposal by CyD molecules has been noted.

Introducing hydroxypropyl groups on β-CyD molecules has enabled the administration of higher doses of CyDs in-vivo. For instance, intravenous doses of 40 g kg^{-1} in rabbits (Irie et al 1992) and 30 g (total) in humans (Carpenter et al 1995) have been administered with no adverse effects. Nevertheless, caution must be taken since hpβ-CyD has been documented to induce pulmonary oedema in dogs and cause occasional distress or agitation in rabbits (Carpenter et al 1995). It may also cause a breakdown of intracellular organelles, for example by interaction with mitochondrial membrane phospholipids and fatty acids (Sun & Gilboe 1994) at doses as low as 1 mg/10^6 cells (unpublished results).

Humans have been noted to tolerate high doses of hpβ-CyD as long as the daily dose is < 16 g orally (reviewed in Irie & Uekama 1997). Above this dose, diarrhoea and soft stools are noted. If a certain CyD is tested to be safe, then it may be used in clinical trials. Otherwise, the CyD may be altered chemically to dilute its toxic effects, or delivered via other agents or devices that limit its exposure to the intended diseased site and avoid or minimize its contact with healthy tissues.

Porphyrin derivatives as oligonucleotide carriers

Benimetskaya et al (1998) proved that at a charge ratio of 0.63 (porphyrin : ON), meso tetramethylpyridyl porphine (TMP)-delivered ON reduced the expression of targeted protein by 80% at an oligomer (20-mer) concentration of 3 μM in certain cell types, such as T24 bladder carcinoma cells, but not in other lines, such as the human prostate cell line LNCaP, which is more responsive to meso tetraanilium porphine (TAP)-mediated uptake of ON molecules (Lebedeva et al 2000). At a 3 : 1 charge ratio, confocal microscopy revealed that the ON was present in virtually all nuclei of T24 cells when delivered with either TMP or TAP. Similar nuclear uptake results were noted by Flynn et al (1999) in HepG2 2.2.15 cells.

In contrast to free ON uptake, which resulted in punctate (presumably endosomal) structures in the cytosol, porphyrin-delivered ON was present diffusely in both cytoplasm and nucleus (Benimetskaya et al 1998; Flynn et al 1999). Electron microscopy studies have revealed that porphyrin–ON complexes are taken up into cells via early endosomes, but that the complex dissociates rapidly, thus precluding formation of punctate vesicular structures (Flynn et al 1999). ON binding to TMP results in a shift in the spectrophotometric maximum from 425 to 440 nm, which renders a simple method for determining the efficiency of binding of ON molecules to these entities (Flynn et al 1999). Although a 1 : 1 binding stoichiometry has been proposed for TMP, at higher concentrations of complexes, aggregation of these complexes occurs probably owing to "bridging" between the complexes.

Protection against S1 nuclease digestion was also offered by TMP (Benimetskaya et al 1998; Flynn et al 1999). One shortcoming of using porphyrin derivatives is that quenching of the fluorescein label on ONs takes place, resulting in erroneously low cellular uptake studies. However, porphyrin-based transfection may be carried out in the presence of serum (Flynn et al 1999), unlike the majority of lipofection reagents. Interestingly, although a concentration of 300 μM TMP alone was not toxic to HepG2 2.2.15 cells at 4 h, it reduced viability to 50% when incubated for 24 h at a final concentration of 20 μM.

Porphyrins have been used for tumour therapy and their tumour cell localization is well known (Tsutsui et al 1975; Megnin et al 1987; Villanueva & Jori 1993). Thus, this class of delivery molecules, which seem to localize to cell nuclei, might be a highly practical mechanism of delivering ON molecules to tumours. Indeed, the hepatocyte-targeting ability of porphyrin molecules (Takle et al 1997; Flynn et al 1999) would make it an ideal candidate for targeting ONs to hepatic tumours and possibly metastatic growths resident in this organ.

Branched polyamidoamine (PAMAM) dendrimers as oligonucleotide carriers

PAMAM ("starburst") dendrimers are a class of polymers in which an amine starting compound is repeatedly substituted at its amino termini resulting in a branched structure. At each round of synthesis, a further layer of branched chains is added (referred to as a "generation"; e.g. a dendrimer with three layers is a generation-3 dendrimer; Tomalia et al 1990). These molecules range in size from 1 to 10 nm and with each generation, a further 1 nm is added to the diameter of the molecule (Bielinska et al 1996). These compounds have a high cationic charge density of primary amino groups on their surfaces. Dendrimers are available in either intact form or a form in which some of the peptide bonds are broken. Dendrimer fracturing (activating) involves heat-treatment of intact dendrimer to cause solvolysis (e.g. in water or butanol) of some peptide bonds at the amide linkages, with an optimal heating period required for optimal transfection efficiency of the dendrimers (Garnett 1999). Increased transfection efficiency with fractured dendrimers is attributed to the increased

flexibility of these molecules, which allows formation of smaller dendrimer–DNA complexes with lower tendency for aggregation (Tang et al 1996).

The first report for gene transfer using these compounds was that by Haensler & Szoka (1993) for cells in culture. They found significant changes in transfection efficiency of plasmids in CV-1 fibroblasts when going from generation-4 to -5 fractured dendrimers. This attenuation in efficiency has been proposed to be owing to a change in the physical shape of the dendrimer–DNA complexes. However, transfection efficiency is reduced by 50% and dendrimer–DNA complexes are slightly toxic to cells, but not as much as other carriers such as polylysine. In any case, cytoxicity may vary from one cell type to another and is less than other reagents such as DEAE-dextran and Lipofectamine (Bielinska et al 1996). As the charge ratio (dendrimer : DNA) of a generation-5 dendrimer is increased from 1 to 4, particle size decreases from > 500 nm to approximately 100 nm (Mumper et al 1995). Dendrimer–plasmid complexes retain the ability to transfect post-lyophilization (Bielinska et al 2000), enabling gene delivery technologists to coat these complexes with, or encapsulate within, matrices such as polylactide co-glycolic acid (PLGA).

Bielinska et al (1996) found that dendrimers were capable of delivering 27-mer unmodified ONs into cells, thereby causing a specific and dose-dependent inhibition of the target gene. Binding of the phosphodiester (PO)-ON molecules to the dendrimers increased their intracellular survival. However, slight cytotoxicity, albeit less than with DEAE-dextran and lipoplexes, was observed with these dendrimer–ON complexes. Dendrimer–ON complexes are stable in 50% serum to variations in pH and ionic strength and result in a 50-fold enhancement of uptake in cells and nuclear entry (Delong et al 1997). Dendrimers form stable complexes with ONs with slight cytotoxicity at 7 nM/2×10^4 cells, but exhibit substantial delivery capacity (Yoo et al 1999). These macromolecular (rather than particulate) complexes were effective in delivering the exogenous nucleic acid to the nucleus in the presence of serum. However, free dendrimer molecules have haemolytic capacity even at concentrations as low as 10 $\mu g\, mL^{-1}$, but this capacity, as well as cytotoxicity in general, is dependent on cell type as well as dendrimer type and generation (Malik et al 2000).

Superfect is a generation-5 activated dendrimer compound that is available commercially and has been utilized for plasmid transfection of cells in culture and, more importantly, in vivo. Superfect was used to transfect interleukin 2 (IL-2) and single chain interleukin 12 (scIL-12) transgenes into NXS2 neuroblastoma cells (Balicki et al 2000). Transfection was capable of inducing an antitumour immune response when these ex-vivo-transfected cells were injected subcutaneously into mice. This polymer has been used for transfection of plasmids into human hepatocellular carcinoma (HCC; Harada et al 2000), Ewing's sarcoma (Maruyama-Tabata et al 2000) and cholangiocarcinoma (Tanaka et al 2000) cell lines, with resultant high expression of transfected transgenes. Although Superfect-complexed plasmids do not exhibit non-specific antitumour effects as noted with lipoplexes (Xu et al 1998), proper evaluation in-vivo needs to be performed.

Free dendrimers are rapidly removed from circulation within 1 h when administered intravenously in rats (Malik et al 2000). When injected intraperitoneally, the dendrimers enter the blood compartment within 1 h and then undergo rapid disappearance from circulation, as for the intravenous administration. When dendrimer–plasmid complexes are delivered using a silastic collar around the carotid artery of rabbits, gene transfer efficiency was greater than with polyethyleneimine (PEI)–plasmid complexes (Turunen et al 1999). Importantly, neither polymers induced an inflammatory response in treated animals. PEI–plasmid complexes are transfected efficiently in tumours when administered slowly using a micropump (Coll et al 1999). However, transfection with these complexes were highly dependent on the location of the tumours, with subcutaneous tumours in the thigh and lung metastatic growths being difficult to transfect.

Deoxyribozyme (DNAzyme) molecules (500 μg, 32-mer protected against nuclease activity with an inverted 3′-3′ terminal linkage) targeting the early growth response factor-1 were complexed with Superfect and administered in pluronic gel around the vessel 6 h before and again immediately after balloon injury (Santiago et al 1999). The DNAzyme blocked neointimal thickening 14 days after injury and adventitial delivery. Fluorescent DNAzymes were localized to the rat carotid artery wall post-administration. Superfect-complexed fluorescent DNAzyme molecules were noted to localized predominantly in the nuclei of cultured primary rat aortic smooth muscle cells. Alternatives to the use of pluronic gels for ON delivery to diseased arteries may be catheters and nucleic acid-coated or -impregnated stents (Khachigian 2000; Klugherz et al 2000). In addition, cancer gene delivery technologists can take heart from the fact that controlled release of DNA has been reported for other therapeutic modalities such as suture coatings (Labhasetwar et al 1998; Bonadio et al 1999), bone implants (Labhasetwar et al 1999), tissue-engin-

eering scaffolds (Shea et al 1999) and orally administered vaccines (Roy et al 1999).

Polyethyleneimine (PEI) polymers as oligonucleotide carriers

PEI is a highly branched synthetic polymer produced by the acid-catalysed polymerization of aziridine (reviewed in Anwer et al 2000a). This polymer network is capable of ensnaring nucleic acids and is available commercially in a size range of 22 to 800 kDa. The majority of the amino groups of PEI are uncharged at neutral pH, but following endocytosis of PEI–DNA complexes, PEI can act as a "proton sponge", buffering the endosome to prevent potential degradation of the condensed nucleic acids (Lecocq et al 2000). PEI also causes osmotic swelling and rupture of the endosome at low pH, thus allowing release of nucleic acid into the cytosol (Klemm et al 1998). This vehicle for nucleic acid delivery to cells is available in either of two forms (reviewed in Garnett 1999), branched (only about two-thirds of all nitrogen atoms protonable) or linear (all nitrogen atoms protonable).

As with most vehicles, PEI has been commonly used for transfection of plasmids into cells (Boussif et al 1995, 1996; Abdallah et al 1996) and is quite efficient in transferring plasmids to the nucleus from the cytoplasm (Pollard et al 1998; Godbey et al 1999). There is some indication that PEI–DNA complexes attach to cell surfaces and migrate into clumps before being endocytosed (Godbey et al 1999). Furthermore, PEI enters the nucleus together with its nucleic acid load as ordered structures. Transfection efficiency of PEI also depends on the quantity of plasmids delivered, since a great load of plasmids entering and expressing within the nucleus may cause deleterious effects in cells (Lemkine et al 1999). Complexes may range in size from 40 nm to greater than 1 μm depending on the ionic strength of the suspension buffer (Tang & Szoka 1997; Ogris et al 1998). Although larger diameter particles may transfect better in cultured cells (Ogris et al 1998), because of the effect of gravity (Dass et al 1997a; Dass 1998), smaller particles may prove superior in-vivo against solid tumours, particularly when administered intravascularly.

Within 2 h of incubation, 18-mer unmodified ONs were efficiently delivered to the nuclei of chicken embryo hypothalamic neurons using PEI (Boussif et al 1995). An antisense PS-ON targeted to the mRNA coding for Ca^{2+} channel β subunits, that was delivered with PEI (50 kDa) into peripheral and central neurons in primary culture, caused sequence-specific effects on its target mRNA (Lambert et al 1996). In a more recent study, chimaeric RNA–DNA oligomers complexed with 800 kDa or 25 kDa PEI were efficiently transfected into cultured hepatocytes (Kren et al 1998). Dheur et al (1999) noted that PEI (100 nm average diam. when complexed to 20-mer ONs) was complexed to PO-ONs, the efficacy owing to the nucleic acid strands is increased against cellular target mRNAs. Interestingly, there was no enhancing effect when PEI was used for transfecting PS-ON molecules. Poly(ethylene glycol) (PEG)-conjugated PEI spontaneously forms complexes with 24-mer PS-ON molecules (Vinogradov et al 1998). Although complex formation was just as efficient with unmodified PEI molecules, these complexes rapidly precipitated out of solution. The PEG/PEI–ON complexes were capable of being lyophilized and redissolved and long-term (several months) storage did not result in an attenuation in complex size (32 nm). As these complexes increase in size to 75–103 nm when avidin-biotin-transferrin chains are linked to the PEG corona, complexed ONs, the uptake of which is enhanced by the transferrin complementation, are released intracellularly to act against their target gene (Vinogradov et al 1999).

Lactosylated 25-kDa PEI bound to 20-mer ONs was successfully targeted to the rat liver when administered in-vivo via the tail vein (Kren et al 1998). The authors thus induced site-directed ON-mediated mutagenesis in-vivo of the *factor IX* gene. When PEI–ONs were directly injected into the caudate lobe of the liver, ONs were noted primarily in the nucleus of cells 24 h after administration. Only nuclear presence of ONs was observed in cells farthest away from the needle-track site. Judging from PEI–DNA complexes (Zou et al 2000a), PEI–ON complex size may be varied, possibly allowing selective delivery to tumours. A linear PEI compound was used to deliver plasmids and ONs to the liver of Peking ducks after an intravenous injection of these nucleic acid–PEI complexes (Chemin et al 1998), mimicking the efficient uptake of PEI-complexed nucleic acids into hepatocytes in culture.

Reports on the in-vivo potential of PEI for ON transport are scarce, but some assumptions may be drawn from delivery of plasmid DNA complexes with these vehicles (reviewed in Zou et al 2000a). Goula et al (1998) noted transgene expression in pulmonary cells (alveolar endothelium, squamous and great alveolar epithelial, and septal cells) when PEI-complexed plasmids were injected intravenously in mice. In a more recent examination, transgene expression was noted in lung bronchioli epithelial cells within 2 h of intravenous administration of PEI-complexed plasmids in mice (Goula et al 2000). PEI–plasmid complexes were better

at transfection than dendrimer complexes when targeted to the murine lung via cannula intubation (Rudolph et al 2000).

The issue of toxicity of the delivered PEI–DNA complexes remains to be adequately evaluated. In any case, toxicity owing to PEI may be a minor factor since PEI has been used in the environment for at least 35 years; it has been used for water purification, mineral extraction and shampoos without complications, and has been found to be safe in animal trials (reviewed in Boussif et al 1995). PEI failed to cause pulmonary inflammation when administered intravenously in mice (Goula et al 2000) and periarterially in rabbits (Turunen et al 1999). One other advantage of PEI is its low price compared with other vehicles, such as commercial cationic liposome formulations.

Nanoparticles and microspheres as oligonucleotide carriers

Biodegradable polyalkylcyanoacrylate nanoparticles were used to bind ONs, via electrostatic attraction, and increase both ex-vivo serum stability and cellular uptake (Fattal et al 1998). In-vivo, these particles increased the plasma half-life of the nucleic acids and were targeted to the liver. ON molecules were incubated with sponge-like alginate nanoparticles to allow efficient association of the nucleic acid strands with the polymer matrix by diffusion (Aynié et al 1999). The association increased serum half-life of the ON and, when administered intravenously in mice, they accumulated in the liver, lung and spleen.

As with other carriers, there are numerous reports on the use of nanoparticles for transgene delivery. Truong-le et al (1998) delivered plasmid DNA encapsulated within 200–700-nm gelatin nanoparticles. The release rate of DNA from the matrix was 2.2% (25–30%, w/w, loading) in 10% fetal bovine serum and plasmids were protected against enzymatic digestion for up to 4 h. Transgene expression was enhanced by these spheres when injected intramuscularly in mice. Gelatin and chitosan nanospheres with a diameter range of 200–750 nm were used to encapsulate plasmid DNA (Leong et al 1998). In-vivo administration of these spheres in mice, via intramuscular injection, resulted in greater expression of transgene than when plasmids were delivered either free or complexed with cationic liposomes. At Day 21 after administration, expression of the transgene was significantly greater with nanospheres, suggesting a sustained release system. Cohen et al (2000) encapsulated plasmid DNA in 600-nm diameter 50:50 poly(DL-lactic-co-glycolic acid) (PLGA) particles. Encapsulated plasmid expression was prolonged when compared with naked or lipoplex-complexed plasmid administered intramuscularly when evaluated at 28 days after injection. In-vitro transfection rates with the nanoparticle-encapsulated plasmids were higher than with free plasmids, and released plasmids were visible in the nuclei of cells.

Similar to nanospheres, microspheres are one class of vehicles gaining greater recognition as a potential targeting mechanism in the field of gene delivery. Prior to 1997, there were limited reports of such usage of microspheres in the literature (Dass 1998). Mathiowitz et al (1997) demonstrated that polyanhydride microspheres could be used to deliver plasmid DNA encoding β-galactosidase into rats via the oral cavity. Encapsulation of plasmids within the microspheres allowed greater expression of the gene in both the small intestine and the liver. Increased expression may be attributed to delayed release of DNA owing to a slow dissolution of sphere matrix, increased DNA uptake into cells owing to physical contact of microspheres with target cells, and resistance proffered by complexation of DNA with the microsphere matrix. However, transfection was not reproducible in the small number of animals studied. Importantly, polyanhydride did not cause an immunological reaction with mammalian tissue as seen earlier (Menei et al 1994; Edwards et al 1997).

A 15-mer phosphorothioated c-*myc* antisense ON was complexed with zinc and encapsulated in injectable biodegradable 50:50 PLGA microspheres (Putney et al 1999). Microspheres were 85–90 μm in diameter and released ONs for up to 9 days in-vitro. When administered intravenously, the microencapsulated ON was more effective as shown by reduced tumour growth in nude mice, a decrease in the number of metastases, reduced target gene expression, and overall increased survival in treated tumour-bearing animals. PLGA is non-immunogenic and biocompatible (Lee et al 1997), is chemically inert and so does not react with the encapsulated drug agent, and permits sustained release of drugs in-vivo (Putney 1998).

Mice administered alginate microspheres with encapsulated plasmids expressed the transgene in their intestines, livers and spleens (Aggarwal et al 1999). Encapsulated plasmids in PLGA microspheres were protected from enzymatic degradation and were maintained in the supercoiled form (Capan et al 1999). Whereas intramuscular and subcutaneous injections of PLGA microspheres encapsulating plasmid DNA result in persistence of the delivered transgene for 100 days after injection, when administered intravenously, widespread dissemination and long-term persistence in the lymphoid

organs and the RES system occurs (Lunsford et al 2000). Importantly, the ability to influence the release rate of encapsulated nucleic acids from the PLGA matrix by altering the PLGA formulation and polymer concentration (Tinsley-Bown et al 2000) is a desirable trait for gene delivery.

Little else has been reported for ON delivery with microspheres; however, there are various studies on plasmid DNA delivery using these particles. Cationic polystyrene microspheres (15 and 30 μM) were shown to bind plasmids ranging in size from 4.2 to 7.3 kb (Dass et al 1996, 1999, 2000). Plasmids were eluted in physiological saline and were determined to retain their biological activity. Anionic polystyrene microspheres (30 μM) were shown to bind cationic liposomes (Dass 1998; Dass et al 1999) and lipoplexes (Dass et al 1999). The latter entities were coined "microplexes" and were composed of plasmid–cationic–liposome complexes bound to anionic PDB microspheres. In cell culture, microplexes demonstrate greatest transfection capability, whereas in-vivo, the targeting potential to solid tumours exceeded that of microsphere alone or lipoplexes (Dass 1998; Dass et al 2000). This targeting potential is owing to the selective entrapment of microspheres in the tumour microvasculature, which then enables localized release of lipoplexes into the tumour interstitium. These lipoplexes enter tumour cells and deliver their nucleic acid load into the cytosol, from where the plasmids are believed to enter the nucleus and undergo expression of the transgene. The use of microplexes in targeting ONs to tumours theoretically holds promise and needs evaluation in proper neoplastic models.

Major hindrances to antitumour oligonucleotide therapy and strategies to overcome this

In general, a major concern in targeting therapeutic agents to tumours is the inherent heterogeneity of tumours. Superimposed on this is the fact that the vascular supply to a tumour is not consistent throughout. There are sites that are well-vascularized, whereas other regions tend to be poorly supplied with a vascular bed. Delivery of an agent into the feeding artery would not equate to reaching all areas of the tumour equally in a lot of cases.

Another form of hindrance is the biological and physiological barriers to intravascular delivery. Firstly, an anticancer agent that is administered via the blood supply encounters the vastness of the vascular space and the possibility of non-specific interactions with the numerous components in blood, including opsonizing proteins and lipoproteins. Although gross systemic distribution may be counteracted by delivery through an artery upstream of a tumour, additional mechanisms of tumour cell-specific targeting are needed to enhance the targeting effect. Secondly, the agent has to extravasate into the appropriate region. Here, exploitation of the tumour vascular bed abnormalities (e.g. greater permeability) may aid selective delivery into the tumour tissue.

Perhaps the greatest obstruction is transport through interstitial space. Owing to a reduced lymphatic fluid drainage in most tumours, fluid pressure tends to build up, and thus further extravasation of fluid and carried agents is hindered. Necrotic regions within a tumour would act to trap and rapidly degrade genetic medicine owing to cellular nucleases and other enzymes being released from dying cells. Finally, entry of genetic constructs into tumour cells is necessary for the therapeutic effect. Furthermore, in the case of therapeutic genes, the construct has to gain entry into the nucleus for the transcriptional process to set in motion expression of the therapeutic protein. Failure to satisfy any of these steps equates into a non-therapeutic result.

There are properties that the gene vector has to have as it travels towards the tumour via the bloodstream (if delivered in that way). Even if delivered into the tumour directly, these features are desirable. Firstly, the agent must be resistant against metabolism and degradation. Secondly, it should avoid non-specific binding to proteins and other biomolecules. For instance, binding of proteins to the surface of liposomes affects their transfection rate. Finally, the vector must not elicit an immunologic response as has been noted with certain viral vectors. Lipoplexes have commonly been used for transfection of cells in culture, but problems such as lack of targeting hinder their efficient usage in-vivo. Regional delivery into the tumour vasculature proves to be the best route of administration of these complexes.

CyDs have not been tested extensively in-vivo with ONs. Because of their small size, these carriers should extravasate the tumour microvessels, but systemic distribution after the first pass may be a problem. Porphyrins have been noted to deliver ONs into the nucleus of various cell types in culture, but need to be tested in appropriate tumour models. The effects of both CyDs and porphyrins when complexed with ON molecules need to be assessed, as previous studies in-vivo with these agents have only been with free (unoccupied, unassociated) molecules. Other polymeric carriers, such as linear PEI and branched radiating dendrimers, allow entry into the nucleus, but more studies are required in-

vivo. The effect of the polycationic nature of these agents may prove more toxic in-vivo than carriers based on monocationic chemical entities. Nanoparticles and microspheres show that particle-based delivery of ONs is highly feasible, especially if the particle size is used to enhance tumour-selective delivery. The use of biocompatible particles is essential and the trend is sure to be driven largely by available clinically proven matrices such as PLGA. Perhaps, the greatest cellular uptake in-vivo may be achieved by encapsulating small carrier-complexed ON molecules into particles in nanoparticles or microspheres. This should enable targeting to the aberrant tumour microvasculature and facilitate the entry of ONs into target cancerous cells. Finally, although not discussed in this review, the efficacy of ONs transported by these carriers against metastases needs to be evaluated.

Summary

The relatively permeable vasculature of the solid tumour has been exploited for selective delivery of chemo-, radio- and genotherapeutic agents to a tumour. In addition, the tortuous and disarrayed nature of a tumour's microvasculature may allow selective lodgment of chemo-, radio- and genotherapeutic carriers such as lipoplexes. Lipoplexes enhance the intracellular delivery of genetic medicine. However, there are various hindrances to optimal delivery of lipoplexes to in-vivo target sites such as tumours. These hindrances may be overcome by novel small-diameter drug carriers, such as CyDs, porphyrin derivatives or branched dendrimers. ONs may also be targeted using larger diameter carriers, such as nanospheres or microspheres. A closer examination of the tumour's physical composition and the various barriers to reaching a neoplasm is essential for the design of better mechanisms for enhancing the efficacy of ONs. A combination of the positive attributes of the various particles into one carrier may facilitate greater delivery of ONs to tumours.

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