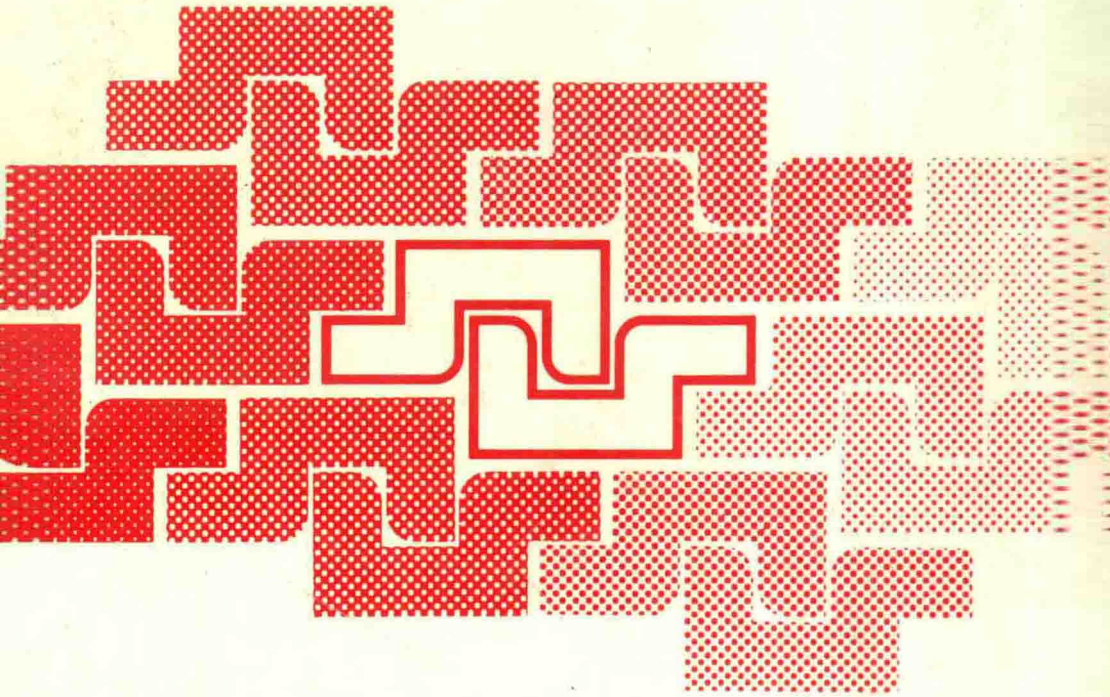

Targeting of Drugs

Strategies for Gene Constructs and Delivery

Edited by
Gregory Gregoriadis
Brenda McCormack

IOS Press
Ohmsha

NATO Science Series

Series A: Life Sciences - Vol. 323

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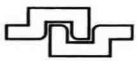

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Preface

The NATO Advanced Studies Institute (ASI) series "Targeting of Drugs" was originated in 1981. It is now a major international forum, held every two years in Greece, in which the present and the future of this important area of research in drug delivery is discussed in great depth. Previous ASIs of the series (1–9) dealt with drug carriers of natural and synthetic origin, their interactions with the biological milieu, ways by which milieu interference is circumvented, approaches to carrier design or modification that contribute to optimal carrier function, strategies for gene and oligonucleotide delivery in therapy and, more recently, with "stealth" therapeutic systems. The present book contains the proceedings of the 10th NATO ASI, "Targeting of Drugs: Strategies for Gene Constructs and Delivery", held in Marathon during 24 June–5 July 1999. As the title implies, the book deals with a variety of viral and non-viral vectors for the effective delivery of genes to target cells, the problems associated with such vectors and ways of circumventing them.

We express our appreciation to Mrs. Concha Perring for her assistance with the organization of the ASI. The ASI was held under the sponsorship of NATO Scientific Affairs Division and supported by The Liposome Company (Princeton, NJ), Gene Medicine (The Woodlands, Texas), SmithKline Beecham, Alza Group (Palo Alto), Merck and Co. Inc. (Rahway, NJ) and Lipoxen Ltd (London).

Gregory Gregoriadis
Brenda McCormack

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Targeted Gene Delivery: Variations on a Common Theme

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Abstract. Targeted gene delivery is presently one of the major fields of gene therapy research. It aims to enhance the efficiency of transgene delivery and to avoid unwanted gene transfer to other organs or cells. However, this goal can be approached in a variety of different ways. Targeting to a particular organ or tissue system can be achieved by the choice of route of application *in vivo*. Specific vectors or cell recognising ligands can direct gene delivery towards a specific cell type or subset of cells in an organ or tissue. After cell entry targeting of the transferred gene to the nucleus is a major task, and finally increasing interest is given to procedures to target the specific gene of interest either directly or by transcription regulation.

1. Introduction

Most researchers working on gene therapy will agree that targeted gene delivery is presently one of the major issues of research in this field (For reviews see [1, 2]). This results from the need to enhance the efficiency of transgene delivery and to avoid unwanted gene transfer to other organs or cells, which will, at best, lead to the loss of vector but may also cause adverse effects. However, from the perspective of individual researchers this goal may have a completely different meaning or at least each of them may approach this problem in a different way. This is a reflection of the still very immature state of our science in which every step from delivering the transgene to a cell, to having it expressed in the tissue of interest is subject to many hurdles and hindrances.

Targeting to a particular organ or tissue system can already be achieved simply by the route of application *in vivo*. By use of specific vectors or cell-recognising ligands gene delivery can be directed towards a specific cell type or a particular subset of cells in an organ or tissue. Once inside the cell targeting to the nucleus becomes a major issue and finally increasing interest is devoted to procedures to target the specific gene of interest in specific cells either by transcription regulation or even more directly by manipulation at the gene locus itself.

This introductory lecture intends to give an overview of these different approaches to gene targeting and to discuss the progress and problems involved in each of them.

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This introductory lecture intends to give an overview of these different approaches to gene targeting and to discuss the progress and problems involved in each of them.

2. Targeting by Route of Application

As already pointed out, topical application can be the first and very effective step of targeting. It is based on the simple notion that the closer one can get to a target, the better the chance may be to deliver the therapeutic gene without loss or gene transfer into tissues one does not want to hit. Unfortunately this simple concept is often not as straight forward as it sounds, it requires some basic knowledge of the anatomy and physiology of the target organ and it can sometimes be technically quite demanding.

The easiest approach of this kind would of course be direct injection into the site of interest. This has been applied to the brain, different muscles, the gut, the bladder the gall bladder and biliary duct and to the pleural and peritoneal cavities. But unless a cavity or a very specific region of restricted size is targeted, e.g. a tumour, the lack of vector spread will often be limiting.

Because of out interest in cystic fibrosis (CF) we have used retrograde biliary catheterisation to reach the biliary tract which is an important site of pathology in CF. This can be performed in humans endoscopically either from the gut or by transabdominal keyhole surgery using fibre-optics and puncture of the bile duct. In the mouse, obviously, laparotomy has to be performed for the instillation for biliary canulation Using this route we have applied adenovirus to achieved primarily liver infection or fairly selective gene transfer to the biliary epithelia by complexing the virus with cationic polymers [3].

An other organ we are particularly interested in with respect to cystic fibrosis is the lung, or more precisely the bronchial epithelia and the submucosal glands of the bronchi, but not the lung alveoli. The commonly used access to the bronchial epithelia is of course the airway route by which the bronchial epithelia have been reached using adenoviral as well as liposome vectors in animal models and humans. However, several phase one clinical trials with different vector systems have demonstrated disappointing inefficiency. This is probably due to the poor accessibility of the mucous covered brushborder of these cells as well as because of a lack of receptors for adenovirus and non-targeted liposome vectors on the apical surface of the airway epithelia. Despite nice pictures of β-galactosidase-blue lungs after intravenous vector delivery, this is certainly not the way to get to the bronchial epithelium, because it will lead predominantly to gene delivery to the alveoli. An alternative approach may be to access the bronchial epithelia from its vascular side. The bronchial epithelia are served from the arterial side by the Aa. bronchiales branching off the aorta and would require substantial technical skills to be reached by catheterisation.

Administration through the blood stream is certainly the most universal route of delivery and should in theory allow to get very close to the cells of interest, which are generally only separated by a few cell layers of the capillary endothelium, basal membrane and connective tissues. This route is used by many viruses, but we should not forget that they are applying an additional mechanism which we are deliberately avoiding in our viral and non viral vectors, namely replication and virus spread originating from only a few initially infected cells. In addition dilution and cellular and humoural immune-reactions are most effective in removing the vector system before it can reach the target cells. Therefore, bringing the vector as close as possible to the vascular site serving a particular organ and getting the relatively large viral and nonviral constructs at a distinct anatomical location out of the capillary bed and through the interstitial cell layers to the parenchyma of the target organ is certainly a major task for this approach to gene delivery.

It is quite astonishing that the liver can be reached relatively efficiently by peripheral intravenous injection, since as already mentioned the vector will first go to the lung where it will be squeezed through the capillary system of the alveoli before being distributed with the arterial blood stream all over the body, passing through the capillary bed of the different organs. Only the relatively small proportion of arterial blood that goes directly to the liver through the hepatic artery

and the venous blood emerging from the capillary system of the alimentary organs and gathering in the portal vein will then reach the hepatocytes. That this route is usually quite effective for gene delivery to the liver is probably due to the very intimate contact of hepatocytes with the sinusoid blood stream which is a unique situation. Direct application to the liver would need access to the portal vein or the hepatic artery, both requiring major surgery involving opening of the abdomen. However, in fetal life the umbilical vein which comes from the placenta, and is accessible by minimally invasive ultrasound guided percutaneous puncture, divides into two branches of which one leads about 50% of the blood stream directly into the portal vein while the other half bypasses the liver through the ductus venosus. In adulthood, therefore, attempts can be made to reopen the atrophic umbilical vein and obtain direct and repeated access to the liver without repeated surgery. Targeting of the CNS from the vascular side is severely hindered by the well known blood brain barrier. Retrograde transport from the peripheral nerves after intra muscular application may be more successful.

3. Cell Targeting by Use of Specific Ligands

In addition to such mechanical approaches, vector targeting by use of cell specific ligands is one of the most hopeful and intensively worked on concepts to achieve selective gene delivery to the tissue of interest. Natural examples of this approach are found in the mechanisms of virus tropism. Retroviruses owe their tropism to the specific receptor binding domain in the surface subunit (SU) of the envelop protein (env). This binds to specific cell receptors and induces conformational changes in the transmembrane subunit (TM) which trigger fusion between the viral and cellular lipid bilayers. The receptor binding domain of the murine ecotropic retrovirus recognises for instance an amino-acid transporter present on the surface of mouse cells but not on cells from other species, whereas a ubiquitous phosphate receptor is responsible for cell attachment of amphotropic retroviruses. Changing these natural tropisms has been achieved by exchange of the envelope genes in the packaging cell lines. This procedure called peudotyping has for instance been used to provide the classical Mouse Malony retrovirus (MoMLV) genome with the Vesicular Stomatitis Virus (VSV) envelope which is more stable and has a broadened cell tropism including human cells [4]. The logical extension of this concept was then to engineer the viral env gene to express nonviral cell targeting ligands in the envelop protein.

Significant progress in cellular targeting by use of cell binding ligands was made by a reductionalist approach pioneered by George and Cathrine Wu using lactose to target the liver specific asialogycoprotein receptor of hepatocytes which are the only cells in body carrying this receptor [5]. The lactose ligand can be chemically linked to polylysine which binds DNA through electrostatic interactions between its positive charges and the negatively charged phosphate backbone of DNA, usually an expression plasmid. Specific receptor ligand interaction of such polyplexes results in cell binding and internalisation of the condensed DNA complexes via clathrin coated pits and endocytosis. Examples of other constructs based on the same principle are the transferrin polyplexes developed by the group of Birnstiel, Wagner and Cotton [6], the polymorphic immunoglobulin receptor targeting construct using an immunoglobulin Fab fragment to target bronchial epithelia from the laboratories of Ferkol, Perales and Davies [7] and more recently targeting of neuronal cells using the tetanus toxin C-fragment by work in my laboratory in collaboration with Neil Fairweather [8] as well as integrin targeting constructs based on peptide sequences of which RGD is a prototype developed first in my laboratory by Steven Hart and Richard Harbottle [9, 10]. This peptide consist of an integrin targeting peptide moiety, the cyclic RGD peptide, in fusion with an oligo-lysine moiety which binds and condenses the DNA making it compact enough for cell entry and protecting it against DNAse.

Such cell specific ligands have also been used to target conventional liposomes to cells and we and others have recently shown that the combination of molecular conjugates with cationic lipids increases transfection above the level of cationic lipid transfection and retains some degree of targeting specificity [11, 12]. Although some success has been achieved *in vivo* the stability of these constructs in the circulation and their final efficiency are still major problems.

This non-viral work has also fed back to the virus world and several approaches have been taken to target viruses with cell specific ligands. One of the first attempts to target retroviruses specifically to hepatocytes was by chemical conjugation of lactose to the retrovirus [13]. Other approaches used antibodies against the SU glycoprotein to modify the virus. More recently insertion of targeting peptide sequences into the SU subunit and exchanging the whole receptor binding domain has been tried. This approach has for instance been used to retarget an avian retrovirus to human cells by insertion of an RGD peptide motif [14]. Although these attempts have shown proof of principle they were associated with loss of virus activity, probably because they misjudged the complex mechanisms this virus uses to enter the cell which involves conformational changes of the env protein after binding to the cell receptor, thereby exposing the fusion peptide moiety and enabling virus entry by fusion with the cell membrane [15].

Similar approaches have also been taken with respect to adenovirus vectors. The cell receptor for this virus resides in the fibre knob while the RGD sequence of the penton base is required for cell entry after binding of the virus. Both Curiel's and Wickam's groups [16, 17] have cloned several peptide ligands among them an RGD sequence into the fibre knob. Unfortunately this approach does not eliminate the adenovirus binding by its so far unknown natural receptor targeting site in the fiber protein. This can, however, be blocked by anti-knob antibodies and David Curiel has developed retargeting strategies mediated by ligands which are covalently bound to such an antibody or its Fab fragment [18, 19].

4. Targeting to the Nucleus

Once inside the cell constructs have to reach the nucleus to become transcribed. Viruses have developed their own very effective mechanisms to either avoid or escape the endosome and to target the nucleus. To help nonviral constructs to survive and eventually escape from the endosome before being destroyed in lysosomes cells have been incubated with chloroquine to buffer the endosomal compartment. Fusogenic peptides, lipids, amphiphiles and even inactivated adenovirus have been added to these constructs to enable them to break out of the endosome. These strategies are usually effective and increase transfection efficiencies by one to two orders of magnitude. However, entering into the nucleus which is probably mediated by binding of the plasmid DNA to nuclear proteins in the cytoplasm [20] is very ineffective and remains probably the major hindrance to non-viral gene transfer. Eukaryotic cells have specifically evolved the nuclear membrane and nuclear pores to separate the cytoplasma and the nucleus and to allow selective transfer between the two compartments. Entry of macromolecules into the nucleus is a very complex energy dependent process. It occurs through the nuclear pore complex (NPC), which is embedded in the nuclear membrane. The NPC consists of about 100 polypeptides and has a relative molecular mass of 125 000K. Cytosolic proteins bearing a classical nuclear localisation signal (NLS) such as that from the SV40 large T-antigen (PKKKRKV) or the two in nucleoplasmin (KRPAAIKKAGQAKKK) are able to enter the nucleus bound to a heterodimer complex consisting of importin α and importin β (also called karyopherin α and β).

The importin heterodimer is formed by association of importin β to the binding domain (IBB) of importin α, a highly basic amino-acid sequence of roughly 40 AS. Importin-α (Mr=60K) recognises the NLS, and importin β (Mr=97K) is responsible for docking on to the NPC and for

translocation into the nucleus. Release of the NLS from the importin complex inside the nucleus is triggered by binding of Ran-GTP to the complex at the Ran binding domain of importin β. The importin complex is then cycled back into the cytoplasm.

Several attempts have been made to adapt mechanisms of protein import or viral entry into the nucleus by adding nuclear localisation peptides to the DNA-complexing constructs or by binding such sequences directly to the DNA. Jon Wolff has covalently attached the NLS, PKKKRKV, to plasmid DNA and has observed nuclear transfer on digitonin-permeised cells but not after microinjection of untreated cells into the cytoplasm. However, a reduction of the load of covalently attached NLS by ligating a small NLS-containing DNA fragment to a luc-expression cassette led to some nuclear entry and expression [21]. As pointed out recently by Jean-Paul Behr's group, any nuclear transfer signal attached to the complexing lipid or amphiphile will be lost by stripping of the DNA prior to nuclear entry while random complexing of such sequences to the DNA may prohibit entry through steric hindrance. Consequently this group has devised an expression plasmid system which contains a mono-molecular ratio of DNA and nuclear targeting ligand covalently linked to each other [22]. This construct transferred into cells by lipo- or polyfection showed a 10 to 1000-fold increase of gene expression compared to constructs not containing the NLS and to constructs containing a mutated NLS without nuclear transfer properties.

5. Targeting Cellular Gene Expression by Transcription Regulation

Transcriptional cell type targeting should allow to restrict gene expression selectively to the cells of interest [23]. This may be of particular importance for expression of toxic proteins e.g. in chemotherapy protocols. By choice of cell specific promotors and construct-specific regulator systems it may become possible to fine tune expression of gene therapy vectors in particular cells. An example for this approach is the use of the tumour cell specific promotor activity of 5'-flanking sequences from the alpha fetoprotein (AFP) gene to transcription-target hepatocellular carcinoma cells. *In vivo* transduction of hepatomas with an adenoviral vector expressing the herpes simplex virus thymidine kinase (HSV-TK) under control of these sequences leads to TK expression and tumour regression after gancyclovir administration only in AFP expressing tumours [24]. The AFP-promotor activity may also serve to illustrate the principle of inducible gene activation, which in this case can be achieved through the glucocorticoid responsiveness of the AFP promotor sequences [25].

An other means by which tissue specificity can be achieved is by linking gene specific promotors to sequences from locus control regions which confer tissue specific gene expression. A classical example of this is the β-globin locus control region LCR which consists of sequences situated 50-60 Kb upstream from the β-globin gene. This sequence appears to be dependent on chromatin structure and may be important for tissue specific expression from integrating vectors. By constructing a vector containing essential sequences from the β-globin LCR linked to a minimal β-globin promotor, high level of regulated tissue specific expression of β-globin was achieved [26]. In principle it should be possible to use this system also for the expression of heterologous genes.

A fully regulatable synthetic transcription system is based on the bacterial tetracyline operon/repressor system in which DNA binding of the repressor to the operon is inhibited by tetracycline, thereby, allowing transcription to proceed. Bujard's group has developed tetracycline repressor systems in which tetracycline, or its more potent variant doxycycline (DOX), induces either the release of the repressor from the Tet Operator DNA (tetOp) or its binding. The latter mutant is called reverse Tet repressor. By fusion of the Tet repressor [27] or the reverse Tet repressor [28] to the activating domain of virion protein 16 (VP16) of herpes simplex virus, tetracycline-regulated transactivators were generated. The reverse tetracycline-regulated

transactivators (rtTA) binds to DNA in the presence of DOX and, therby, induces gene activation. A retroviral vector constructed on the basis of this regulatory principle contains all the components of the rtTA system, a drug selectable marker with the internal ribosome entry site and the gene of interest (GFP). Induction of GFP-fluorescence of two orders of magnitude in retrovirus-infected cells dependent on the amount of tetracycline (DOX) added to the medium was shown by FACS analyses and was demonstrated following oral administration of DOX by GFP expression in infected 208F-cells transplanted into the peritoneal cavity of nude mice [29]. When bound to DNA in the presence of DOX, rtTA stimulates transcription from a promoter sequence containing the tet operator sequences controlling the gene of interest. To overcome residual background activity of this system in the absence of DOX an other fusion protein of the original tet repressor with a promotor silencing sequence has been constructed [30]. For use of this system in a gene therapy context it will be necessary to introduce the genes which constitutively express the regulatory proteins as well as that coding for the therapeutic protein.

6. Gene Locus Targeting

Obviously , the final goal of targeting would be the direct exchange or exact correction of the gene of interest. Gene locus targeting, would probably be most valuable for the treatment of single gene defects which could be selectively repaired in the endogenous gene. This would restore normal gene function under physiological endogenous control. The technique would be applicable to recessive as well as to dominant disorders. It would only target dysfunctional cells and many present risks connected with gene addition strategies of gene therapy would be avoided (For review see [31]). That this is in principle possible by homologous recombination has been demonstrated by the very successful knockout technology in mice. Because of its very low efficiency of about 10^{-6}-10^{-7} an *in vivo* application has to rely on clonal selection in culture followed by selection of chimeric animals transmitting the targeted gene alteration through the germline. However, using appropriate selection procedures in culture gene locus targeting has been achieved for several human cell lines including primary cells [31]. This technique could therefore in principle be used for *ex vivo* gene correction and readministration of autologous cells for instance to treat haemoglobinopathias. The main problem here would not be the low efficiency of targeting but the availability and *in vitro* expansion of the relevant stem cell populations.

Recently dramatic increases in the efficiency of gene locus targeting have been described by Dieter Gruenert's group reporting correction of 5-25% of the targeted cells by use of small single strand DNA fragments. Even though these smaller molecules can be delivered much more effectively to cells than the usual targeting constructs this efficiency is quite unexpected because of the known inefficiency of homologous recombination using small homologous sequences and the established exponential relationship between the increase of targeting efficiency with increasing length of homology up to about 14kb [32].

Gruenert's group transfected cells originating from a cystic fibrosis patient homocygote for the common 3 base-pair deletion (Delta F508) in exon 10 of the cystic fibrosis transmembrane conductance regulator (CFTR) with about 10^6 molecules per cell of an about 500bp denatured PCR-fragment representing the normal allele of this region. Allele specific PCR into adjacent genomic regions and RT-PCR across exons were employed to show gene repair in a population of cells and single cell patch clamp analysis demonstrated functional correction of 7(3) out of 78 investigated individual cells [33]. More recently Gruenert has used this technique to revert a cell line carrying a 4bp deleted inactive zeocine resistance gene back to zeocine resistance, with a frequency of about 1%. Insertion of the 3bp in the cloned revertants was shown by restriction and Southern analysis of genomic DNA (Gruenert, Am Soc. Gene Therapy Meeting, 1999).

Even more spectacular results of gene locus correction have been reported using an other technique, called chimera directed gene repair which is based on the use of RNA-DNA oligonucleotide hybrids [34, 35]. The paired oligonucleotide containing a single nick and hairpin loops at both ends inserts itself into the target DNA region by base pairing with both strands of this sequence. The site of nucleotide exchange forms a single mismatch on both strands flanked by desoxynucleotides and followed by a stretch 2'O-methylated ribonucleotides on both sides. The inclusion of RNA bases stabilises the hybridisation of the target sequence while their methylation prevents nuclease degradation. The structural distortion of the double strand between oligonucleotide and single mismatch is thought to trigger a repair mechanism to introduce normal base-pairing which, at least in theory, should go in both possible directions. Lipofection was used to deliver the oligonucleotides in first experiments *in vitro* showing correction of the sickle cell mutation [36]. More recently an associated group has used lactosylated PEI to target an RNA-DNA hybrid *in vivo* to the liver liver for introduction of an inactivating point mutation into the factor IX gene of rats [37]. They report a 40% conversion of the target gene and a 25% drop in the functional activity of the Factor IX protein product after two rounds of administration. Unfortunately, this success has not been achieved by several other groups and more basic work is therefore urgently required to understand the molecular mechanisms of this technique in more detail and to define the exact conditions for successful application [35, 38]. However if these technique can be made reproducible in independent laboratories , this will mark a revolutionary milestone in the history of gene therapy.

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