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COMMENTARY

RECEPTOR-MEDIATED ENDOCYTOSIS OF MACROMOLECULAR CONJUGATES IN SELECTIVE DRUG DELIVERY

SANDIP K. BASU*
Institute of Microbial Technology, Chandigarh-160 036, India

Selective delivery of therapeutic agents to specific cell types, first conceptualized by Paul Ehrlich in 1906, continues to be an elusive objective despite considerable interest in the subject evinced by academic researchers as well as pharmaceutical industries. The literature on the subject is vast, and several comprehensive recent reviews and monographs are available [1–3]. Accordingly, in this article, we will concentrate only on the recent approaches to deliver therapeutic agents to specific cell types using the exquisite specificity and efficiency of receptor-mediated endocytosis of soluble macromolecules.

Receptor-mediated endocytosis: an overview

Biochemical, ultrastructural and genetic approaches in a number of systems during the last two decades have provided remarkable insights into the process of receptor-mediated endocytosis which serves to selectively retrieve and assimilate various macromolecules from the extracellular milieu with high efficiency for a variety of purposes [4–6]. The efficiency of this process results from: (i) the ability of specific cell surface receptors to bind specific ligands to the cell surface with high affinity, and (ii) rapid internalization of the ligand–receptor complexes through "coated pits." Coated pits are the sites where intensive vesicularization of the plasma membrane occurs leading to formation of coated vesicles which carry the ligand–receptor complexes into the cells. Movement of receptors into the coated pits could be spontaneous or ligand-induced. It appears that trapping of receptors into coated pits is determined by their cytoplasmic domains recognized by 100/50/16 kD clathrin-associated proteins, resulting in attachment of clathrin to the membrane [7]. A single tyrosine residue in the cytoplasmic domains of receptors seems critical for their spontaneous localization in coated pits [8, 9]. Receptors which localize in coated pits only after ligand binding (e.g. transferrin, epidermal growth factor, macrophage Fc receptor) may have structural features in their cytoplasmic domains different from those discussed above [10]. However, once in the coated pits, the receptors (with or without bound ligand) seem destined for internalization through the formation of coated vesicles. The coated vesicles, in turn, give rise to an assortment of acidic endocytotic vesicles where sorting of the receptor–ligand complexes takes place. Depending on the nature of the receptor, the receptor–ligand complexes are committed to follow one or the other of the following four pathways which lead to distinct consequences for the receptor and the ligand:

(1) Receptors recycled, ligands degraded, e.g. low density lipoprotein [11], mannose-, mannose-6-phosphate or galactose-terminal oligosaccharides/glycoproteins [12–14].
(2) Both the receptors and the ligands recycled, e.g. transferrin [15], major histocompatibility antigens [16].
(3) Receptors degraded, ligands degraded, e.g. macrophage Fc receptors [10], epidermal growth factor [17].
(4) Receptors degraded, ligands transported across the cell, e.g. immunoglobulin A and immunoglobulin M [18].

The various signals that regulate the sorting of receptors through the pathways outlined above are beginning to be understood as receptor structures are being worked out revealing that receptors contain multiple functional domains which allow them to interact with ligands and other macromolecules for translocation to various sites within the cell or even across the cell.

Receptor-mediated endocytosis: characteristics important for drug delivery

As the intricacies of the process of receptor-mediated endocytosis unfolded over the last decade, crucial advantages that the process offered to achieve site specific drug delivery began to be apparent and exploited. These advantages accrued from: (i) high affinity of the receptor for ligand permitting effective sequestration of the ligand from low concentrations in the medium, (ii) rapid recycling of the appropriate receptor molecules permitting building up of relatively high intracellular ligand concentrations, (iii) expression of specific receptors only on certain cell types permitting design of cell type specific drug delivery systems, and (iv) specificity as to intracellular compartmentalization of the ligand depending on the nature of the receptor–ligand duo (e.g. low density lipoproteins are degraded in lysosomes, transferrin is transported out of the cell for repeated

* Correspondence: Dr Sandip K. Basu, Institute of Microbial Technology, Sector 39-A, Chandigarh-160 036, India.

use, nerve growth factor localizes in the nucleus whereas some toxins become localized in cytosol).

The knowledge-base in the area of receptor-mediated endocytosis is being applied towards the quest for the ideal target specific drug of the future which should have at least three elements:

(1) An element for specific recognition by the target cell,
(2) An element which would elicit the pharmacological action only at the target site, and
(3) An element designed to facilitate crossing of anatomic barriers to the target cell.

Target cells: recognition elements

Two distinct types of recognition elements on the surface of target cells are current favourites in selective drug delivery. These are:

(1) Antibodies against cell surface antigens, and
(2) Receptors on the cell surface which transport a variety of substances for various purposes.

Antibody-mediated drug delivery. In principle, it is possible to raise extremely specific antibodies which recognize antigens present on specific cell types. Therefore, cytotoxic moieties conjugated to such antibodies should be delivered specifically to the selected cell type. Immunoconjugates containing potent toxins [19], cytotoxic drugs [20, 21] and radioisotopes are being used extensively in investigations for cancer chemotherapy, and some such conjugates have reached preclinical and clinical trial stages [21, 22]. The initial euphoria that accompanied the introduction of the technique for the production of monoclonal antibodies has waned considerably because, although monoclonal antibodies can direct immunoconjugates with a high degree of selectivity to the target cells, the limitations imposed by the number of cell-surface antigens, the number of drug molecules attachable to the antibody as well as the poor efficiency of internalization of such conjugates do not permit accumulation of pharmacologically effective intracellular concentrations of the drug in many cases. Attempts are being made to overcome this problem by delivering molecules which act catalytically rather than stoichiometrically in disrupting important cellular reactions, or by delivering potent radionuclides which can destroy the cells after binding to the cell surface. A recent addition to this genre is enzyme-immunoconjugates containing (i) an oxidase and/or a peroxidase system which generates cytocidal amounts of H_2O_2 and reactive halogenated products at the target cells [23], or (ii) enzymic activation of a relatively non-toxic prodrug in the vicinity of the target cells as demonstrated by the efficacy of antibody-alkaline phosphatase conjugates in enhancing the toxicity of etoposide phosphate both *in vitro* and *in vivo* [24]. The area of immunoconjugates in cancer is in a state of rapid ferment, and innovative approaches continue to emanate. One such approach consists of preparing heterobifunctional antibody duplexes in which one antibody recognises an element on the tumour cell while the other antibody binds specifically to the antigen receptor complexes on T cells which in turn become activated and lyse the tumour cell [25]. A variation of this theme in which the recognition function provided by the antibody to the tumour cells is replaced by a hormone or autocrine growth factor needed by the tumour cell has shown promise in the case of human melanoma [26]. Recently, delivery of non-cytotoxic differentiation inducers to tumour cells via antibody-mediated targeting achieved differential elimination of neoplastic cells, suggesting a new therapeutic modality for treatment of neoplasias susceptible to such agents, e.g. leukemia and lymphoma [27]. A novel application of immunoconjugates has been the delivery of plasminogen activators specifically to blood clots in which fibrin-specific monoclonal antibodies were coupled to urokinase [28]. Subsequently, this group has also produced a hybrid protein by recombinant DNA techniques by fusing the appropriate domains of the fibrin-specific monoclonal antibody with the DNA sequences coding for the plasminogen activator function [29].

Monoclonal antibodies in drug delivery applications can be considered to be a generalized method of seeking receptors capable of efficient internalization for which natural ligands are yet to be identified. Thus, monoclonal antibody-mediated targeting is the most versatile and general modality of selective drug delivery at present. As the repertoire of such antibodies recognized by specific cell types is enlarged, it should be possible to overcome the disadvantages of cross-reactivity with non-target cells and poor efficiency of internalization by target cells. Potential immunogenicity of the immunoconjugates *in vivo* might be eliminated when techniques of producing homologous human monoclonal antibodies become established.

Receptor-mediated drug delivery. Receptor-mediated stratagems for selective drug delivery, of necessity, depend on detailed knowledge about the nature of the ligand, relative distribution of the receptor on various cell types, and the intracellular pathways followed by the receptor–ligand complexes. As a general rule, efficient drug delivery would be possible only through receptor systems that are recycled rapidly and participate in multiple rounds of ligand delivery. Unfortunately, most of the receptor systems for which such information is available at present (e.g. receptors for low-density lipoprotein, transferrin, epidermal growth factor, insulin) are distributed on many cell types or ubiquitously. Attempts have been made to exploit the quantitative differences in the content of such receptors for selective targeting of antineoplastic drugs to cancer cells through these receptors [30, 31]. At present, only hepatocytes and the cells of macrophage lineage seem to possess efficient receptor systems restricted largely to these cell types and amenable to selective drug delivery.

The receptor system recognizing galactose-terminal oligosaccharides/glycoprotreins present exclusively on hepatocytes has been used for delivery of a large variety of pharmacologically active agents as macromolecular drug conjugates [32]. As hepatocytes are primarily affected in a number of disease conditions, selective delivery of appropriate therapeutic agents to hepatocytes would be important in the amelioration of such diseases. These conditions

include: (i) infectious diseases controllable by delivery of antimicrobial agents, e.g. exoerythrocytic stages of malaria and hepatitis, (ii) reduction of the toxicities of drugs (acetaminophen, halothane, isoniazid) and toxins as well as alcohol or solvent-induced liver injury achieved by delivering antidotes, antitoxins and free radical scavengers, (iii) enzyme and receptor deficiency diseases such as Lesch–Nyhan syndrome, Pompe's disease, familial hypercholesterolemia and, galactosemia ameliorated by delivery of appropriate enzymes and ultimately by delivery and expression of the appropriate version of the genetic sequences to correct these deficiencies. Defects in synthesis of important plasma proteins such as α_1-antitrypsin and coagulation factors could also be corrected by gene therapy, and (iv) severe cases of iron overload such as occurs in thalassemia or repeated blood transfusion are better managed by selective delivery of chelating agents to hepatocytes. It has been shown recently that hepatocyte-specific delivery of the antiviral agent vidarabine monophosphate conjugated with lactosaminated serum albumin is superior to the free drug in the treatment of patients with chronic hepatitis B [33].

While selective delivery of therapeutic agents to hepatocytes via receptor-mediated pathways still remains largely limited to *in vitro* studies, considerable success has been achieved in this respect with cells of macrophage lineage. Macrophages serve as the host for a large number of intracellular bacterial, fungal and parasitic pathogens causing diseases which affect millions of people worldwide. These include tuberculosis, leprosy, brucellosis, salmonellosis, syphilis, leishmaniasis, toxoplasmosis, schistosomiasis, histoplasmosis and cryptococcosis. Pathogenesis of several viral diseases frequently involves replication of the virus within macrophages causing diseases such as acquired immunodeficiency syndrome, dengue, yellow fever, stomatitis and encephalitis caused by Herpes Simplex Type 1, genital and disseminated conditions caused by Herpes Simplex Type 2, hepatitis and pneumonia associated with cytomegalovirus infection, measles, and flu-like illness caused by vesicular stomatitis virus. A number of viral diseases of domestic animals also result from the proliferation of these viruses in macrophages, e.g. canine distemper, equine encephalomyelitis and infectious anemia, Japanese encephalitis affecting pigs, and leukoencephalomyelitis, periarthritis and synovitis affecting goats. In addition, human metabolic diseases such as Gaucher's disease, rheumatoid arthritis and neoplastic diseases like histiocytosis X are macrophage-associated disorders. Therefore, drug delivery systems targeted to cells of macrophage lineage are likely to be extremely useful. Targeted drug delivery to macrophages has been the subject of a recent review [34].

At present, three receptor systems are known to occur primarily on macrophages that are being exploited for endocytotic drug delivery purposes. These are: (i) the mannosyl-fucosyl receptor which recognizes mannose- or fucose-terminal oligosaccharides/glycoproteins [12], (ii) the galactosyl particle receptor (different from the galactosyl receptor of hepatocytes) limited to Kupffer cells [31], and (iii) the so-called "scavenger receptor" system which recognizes a range of polyanionic macromolecules including acetylated, maleylated or malondialdehyde-treated proteins, sulfated polysaccharides such as fucoidin or dextran sulfate, and polyinosinic acid [35–37]. All three receptor systems are probably recycled many times resulting in efficient intracellular delivery of the ligand. The mannosyl-fucosyl and galactosyl receptors have thus far been very popular in liposome-mediated drug delivery applications [1, 2]. Scavenger receptors are beginning to find applications in drug delivery (see below).

Recently, receptor-mediated endocytotic pathways have been utilized to deliver soluble macromolecular drug conjugates for elimination of intracellular parasites both *in vitro* and *in vivo* [38, 39]. These studies showed that methotrexate conjugated to maleylated bovine serum albumin was taken up and degraded by cultured hamster peritoneal macrophages leading to intracellular release of a pharmacologically active form of methotrexate which effectively destroyed intracellular amastigotes of *Leishmania donovani* and *L. mexicana amazonensis*. The antileishmanial effect of the conjugate could be blocked by excess maleylated bovine serum albumin, lysosomal inhibitors (chloroquine and monensin), and metabolic antagonists of methotrexate (folic and folinic acids). Macrophage-rich tissues, namely liver and spleen, were the primary sites of accumulation of the drug conjugate. The drug conjugate was nearly 100 times as effective as free methotrexate in eliminating intracellular parasites. In an experimental model of cutaneous leishmaniasis in hamsters, the drug conjugate brought about more than a 90% reduction in the size of footpad lesions within 11 days. In contrast, free drug at a similar concentration did not affect lesion size significantly. No antibody response to the drug conjugate was elicited during the period. These studies establish the feasibility of using maleylated albumin as a drug carrier of general utility for delivering pharmacologically active agents to macrophages. Maleylated albumin probably utilizes the "scavenger receptor" system [35–37] to gain access to the interior of cells of macrophage lineage.

The versatility of the above receptor system has also been apparent recently in the selective delivery to macrophages of a lipophilic immunomodulator, muramyl tripeptide phosphatidylethanolamine complexed to acetylated low-density lipoprotein, resulting in the augmentation of their tumoricidal activity [40]. These studies [38–40], taken together, indicate the feasibility of utilizing the scavenger receptor-mediated uptake of macromolecules for delivery of lipophilic as well as water-soluble pharmacologic agents for treating patients with intracellular infections or cancer. While the above regimens would either directly activate macrophages or kill intracellular pathogens residing in macrophages, it should also be possible to suppress the release of certain macrophage-suppressive factors, e.g. prostaglandins, which inhibit the release from lymphocytes of macrophage-activating factors such as interferon γ by macrophage-specific delivery of prostaglandin synthesis inhibitors such as indomethacin [41]. In designing strategies for manipulating the metabolism of macrophages using

receptor-mediated processes, it is important to note that different populations of macrophages differ in expression of various receptors.

Recombinant DNA methodologies are beginning to make important inroads in the field of receptor-mediated drug delivery. A recent example is the use of recombinant CD4-ricin A chain conjugate to specifically eliminate human immunodeficiency virus-infected cells which express the viral envelope glycoprotein gp 120 recognized by CD4 [42]. Another instance of the application of recombinant DNA techniques for receptor-mediated drug delivery is the production of a chimeric diphtheria toxin in which the toxin-receptor binding domain is replaced by the sequence coding for interleukin-2. The resultant fusion protein is taken up only by activated T cells carrying the high-affinity interleukin-2 receptor leading to selective killing of such cells resulting in suppression of T cell-mediated immune response *in vivo* [43].

Elements for pharmacologic action at the target cell

A variety of cytotoxic drugs, toxins, radionuclides, enzymes, immunomodulators and other biological response modifiers have been delivered to neoplastic cells as well as hepatocytes and cells of the monocyte-macrophage system *in vitro* and *in vivo*. In many of these cases, intracellular release of the pharmacologically active moiety was achieved in acidic endocytotic vesicles or lysosomal hydrolysis of the carrier portion. The recent finding that monensin enhances the cytotoxicity of thioether linked immunotoxins, which are likely to be more stable *in vivo*, could provide an important tool in effecting delivery of pharmacologically active agents into cytosol bypassing the lysosomes [44]. It has also been possible to deliver and elicit expression of various genes in the target cells, e.g. hepatocytes in culture [45].

Anatomic barriers to the target cell

In targeting of macromolecular drugs the major anatomic barriers which need to be surmounted are the epithelial and endothelial cells as well as the cells of the reticuloendothelial system. Current knowledge of the biology of the epithelial/endothelial cell is inadequate to arrive at definitive strategies for drug delivery to organs/tissues separated from the circulatory systems by these boundaries. Signals exist on the receptors for transferrin and IgA molecules which ensure their transport across the epithelial cells [15, 18]. It remains to be seen if these signals can be exploited for the specific purpose of crossing these barriers. In recent years, several proteins have been found on enteric bacteria which enable these pathogens to cross intestinal mucosal cells [46, 47]. It may be possible to incorporate these proteins in drug targeting rationales for oral drug delivery. Another formidable task is the delivery of drugs across the blood–brain barrier. A number of strategies to cross this barrier for drug delivery to brain are under active investigation [48]. Carrier-mediated transport systems for neutral amino acids and transcytosis via receptors for insulin, transferrin and insulin-like growth factors appear promising in this regard. Also, the so-called "scavenger receptor" system which recognizes acetylated low-density lipoprotein and is present primarily on cells of macrophage lineage [37] have also been found on the endothelial cells to a limited extent [49]. If this receptor system exists on brain endothelial cells as well, it may be useful in crossing the blood–brain barrier. The observation that cationized proteins could be taken up by cells such as fibroblasts [50, 51] has been applied recently to deliver cationized albumin coupled to β-endorphin and IgG across the blood–brain barrier into the brain interstitial fluid [52, 53]. Chemotherapy of solid tumours is hindered by yet another anatomic barrier, namely the interior of the tumour being largely insulated from the circulatory system, it is difficult for drugs to gain access to these cells. A possible solution to this problem may come when ways to promote transcytosis in such cells or the details of the mode of action of angiogenesis factors which promote formation of microcirculatory systems become available. Angiogenesis factors targeted to tumours by appropriate signals during a chemotherapeutic regimen may be more effective in regression of the tumour as well as contain the risk of metastasis. The problem of containing the uptake of the drug by the cells of the reticuloendoethelial system might be circumvented by depletion or temporary inactivation of macrophages, but the consequences of such interventions may be unacceptably severe in clinical situations. Liposome-encapsulated dichlorodimethylene diphosphonate as a selective eliminator of macrophages seems to hold much promise [54].

Concluding remarks and prospects

Site-specific drug delivery by receptor-mediated endocytotic pathways using soluble macromolecular conjugates appears to be maturing into a rational approach which is likely to lead to clinical applications in the near future. These applications would probably occur faster in the cases where the target cells are of macrophage lineage or hepatocytes because efficient receptor systems largely limited to these cell types are known. These receptor systems recognize relatively simple ligands which are likely to be physiologically compatible. Ability to manipulate even these two cells types would have a major impact in the therapy of a large number of disease states—infectious, metabolic and neoplastic.

Endothelial cells which form the interface between the circulatory system and the various mammalian tissues seem rife for therapeutic manipulations [55]. An even more exciting possibility is the use of genetically manipulated endothelial cells for delivery of functional gene products to correct various genetic defects [56]. Recent successes in the persistent expression of the recombinant β-galactosidase gene *in vivo* by transplanted endothelial cells within the arterial wall in the Yucatan minipig [57] and prosthetic vascular grafts seeded with genetically modified endothelial cells [58] certainly permit optimism in the future of designing delivery systems for treatment of systemic or inherited diseases requiring secretion of gene products in circulation such as vasodilators, angiogenesis factors or antineoplastic agents.

From the perspectives of the late 1980s in the area of receptor-mediated drug delivery, it is tempting to speculate that prospects for the 1990s in this field will feature intensive efforts in: (i) the exploitation of the so-called "homing" receptors on lymphocytes which mediate their tissue localization, (ii) the understanding of the molecular basis of viral tropism which would transform viruses from being tools of the cell biology research to powerful drug carriers, (iii) manipulation of the immune response for therapeutic purposes by selective elimination/activation of the various cell types of the immune system, and (iv) the directed use of migratory cell populations such as monocytes, macrophages, granulocytes, and neutrophils as drug carriers to target sites for a variety of purposes.

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