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Targeting of liposomes to liver cells in vivo.

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GENERAL INTRODUCTION

Targeting concept

Targeting of drugs in medical practice has been recognized long ago by Paul Ehrlich as a useful means to reduce unwanted side-effects of therapeutic treatment (1). In drug targeting the aim is to specifically direct drugs in the body to cells on which they should act, without interference with other cells. In general the targeting concept includes three components: a drug, a carrier for the drug and a homing device. A variety of carriers has been proposed, of which macromolecules, erythrocytes, liposomes, nanoparticles, microemulsions and albumin spheres (2) have been studied. The homing device can be constructed of molecules which are able to recognize determinants on the target cell's surface. For this purpose immunoglobulins, glycoproteins, glycolipids, polypeptides and antigens (2) have been studied. For use in vivo the carrier-homing device combinations have to be non-toxic and biodegradable without giving rise to formation of toxic degradation products and they should preferably be applicable for a large variety of drugs including both hydrophobic and hydrophilic derivatives. Upon reaching the target cells by means of the carrier-homing device combination, the drug may exert its action in two possible ways. Firstly, it may gradually be released from the carrier bound on or near the surface of the target cells leading to a high local drug concentration for a prolonged period of time and secondly, the carrier with the drug may be internalized by the target cells giving rise to a high intracellular drug concentration. The first mechanism can be considered an example of the well-known pharmacological principle of "sustained drug release", but at the cellular level. For the second mechanism to become operative it is important to know the mechanism of uptake of the drug-carrier complex and its implications for intracellular release of the drug. At the moment one of the combinations under study is the system in which liposomes serve as carriers and glycolipids, immunoglobulins or glycoproteins are used as homing devices (3, 4).

In this thesis the experimental part is focused on liposomes with and without glycolipids. By the incorporation of naturally occurring glycolipids (lactosylceramide, chapter I, II) or a synthetic one (tris-galchol, chapter III) into liposomes efforts were undertaken to target the liposomes to the parenchymal cells of the liver, the hepatocytes. Furthermore, attention was paid to the question whether the liposomes do become internalized and if so, by what mechanism (chapter IV) and finally, the influence of the composition of the (non-glyco-)lipids in the liposomal membranes on the targeting to hepatocytes was studied (chapter V).

In the following sections of this introduction it will be explained or discussed

- what liposomes are and how they are prepared;
- what the fate of a liposome could be once it has reached the plasma membrane of a cell, in particular the hepatocyte;
- why the hepatocytes were chosen as a target, and
- what the implication of the liver anatomy is for the availability of liposomes to the various cell types constituting this organ.

Liposomes

Liposomes are artificial lipid vesicles with diameters ranging from 25 nm to several microns. They consist of one or more lipid bilayers enclosing a similar number of aqueous compartments. The number of lipid bilayers per vesicle and the diameter can be varied by applying different preparation techniques (5) and thus liposomes can be divided into three main classes:

a. small unilamellar vesicles (SUV) with a diameter in the range of 25-70 nm and consisting of one lipid bilayer;
b. large unilamellar vesicles (LUV) also bounded by only one lipid bilayer but with diameters ranging from 100 to 400 nm;
c. multilamellar vesicles (MLV), composed of two or more concentric bilayers and ranging in diameter from 200 nm to several microns.

For reasons which will be made clear in the section on liver anatomy, we chose to use SUV for the investigations presented in this thesis. The preparation of such vesicles will briefly be described. When phospholipids are dispersed in an aqueous solution, multilamellar vesicles are spontaneously formed with a mean diameter in the order of microns. By ultrasonic irradiation (sonication) their size can be reduced and, ultimately, small unilamellar vesicles are formed. When a drug is mixed with the lipids or dissolved in the buffer before sonication it will either be incorporated into the lipid bilayer (in case of a lipophilic drug) or it will be trapped in the aqueous compartment of the vesicles (in case of a hydrophilic drug). Gel filtration of the preparation upon sonication can separate any remaining larger vesicles and free drug from the SUV (Fig. 1). By pooling the SUV-containing fractions a SUV preparation without contaminating larger vesicles or free drug can be obtained. Since this thesis mainly deals with the in vivo behavior of liposomes

after intravenous injection, the possible influence of blood on the integrity of liposomes has to be taken into consideration.

In liposomes consisting of one species of phospholipids only, the lipid bilayer can undergo a temperature induced transition from a gel phase to a liquid crystalline phase. In the presence of plasma, vesicle integrity is most severely endangered when the liposomal lipids are near or at the gel to liquid crystalline phase transition temperature (T_C). Under these conditions the liposomal membrane, particulary of SUV, is extremely susceptible to the destructive action of high-density lipoproteins (6), but also above T_C SUV are sensitive to these plasma effects. Thus, phospholipids such as distearoylphosphatidylcholine (DSPC) with a T_C of 56°C have been used to prepare relatively plasma-resistant liposomes (7). Also cholesterol has been found to protect liposomes from the solubilizing effects of the plasma lipoproteins (8). Cholesterol tends to tighten the packing of phospholipid molecules in a bilayer due to strong interactions with these lipids; incorporation of 33 mol% of cholesterol abolishes the phase transition in a phospholipid bilayer and thus the extreme sensitivity of liposomes to plasma influences at the T_C. Cholesterol also reduces the exchange of phospholipids between vesicles and lipoproteins; particulary in case of sphingomyelin as phospholipid cholesterol effectively surpresses this phospholipid exchange (8). Thus radioactive-labeled sphingomyelin is quite suitable as a lipid label for cholesterol-containing liposomes in circulation. In our experiments however, we mainly used the aqueous space marker (^{3}H-)inulin as a liposomal label. Any inulin (a poly-fructose molecule with a M_r of $\sim$5000) released from liposomes in circulation will quickly be excreted by the kidneys. Because inulin can not be metabolized, the measurement of (^{3}H-)label in a cell or organ yields a good indication of the amount of accumulated liposomes.

When liposomes have reached the target cells they may adsorb to the cell's surface and the following events may take place:

a. during adsorption, either reversible or irreversible, lipid constituents may be exchanged between the liposomes and the cells;
b. the peripheral liposomal membrane may fuse with the plasma membrane leading to a release of vesicle content into the cell's cytoplasm;
c. the liposomes may be taken up by t[...] docytic process, delivering the li[...] lysosomal system.

This last event is generally thought [...] prominent role in the uptake of liposo[...] cells (9, 10, 11); therefore this proc[...] described in more detail.

Endocytosis

Endocytosis is the process of inter[...] tracellular material by invagination [...] membrane and the subsequent f[...] cytoplasmic vesicle. This vesicle has [...] ferent names such as phagosome (12)[...] coated vesicle (14) or primary food vac[...] ding on the cell type studied and o[...] docytosis involved. Classically, endo[...] divided into two types: phagocytosis [...] ternalization of particles larger than [...] micron in diameter, and pinocytosis d[...] take of all smaller substrates, varyin[...] particles such as lipoproteins an[...] molecular weight solutes and to the s[...] Phagocytosis always involves adsorp[...] cle to the plasma membrane prior to i[...] take by pinocytosis can also be adsor[...] (adsorptive pinocytosis), but it may al[...] internalization of fluid with solutes (f[...] Adsorptive endocytic processes di[...] kinetics, whereas uptake by fluid pinc[...] proportional to the concentration o[...] sorptive pinocytosis may be precede[...] binding of the substance to the cell su[...] volve interaction with highly specifi[...] (receptors) on the cell membr[...] mechanism is known as receptor med[...] For example, polypeptide hormon[...] growth factor (16) and insulin (17), pl[...] such as low density lipoprotein (18), [...] (19) and asialoglycoproteins (20) are [...] up by receptor-mediated endocytosis [...] fate of receptors and substrates [...] depends on the cell type and the nat[...] For instance, when the F_C-receptor, [...] surface of macrophages, is occupie[...] munocomplex) both receptor and li[...] in the lysosomes, whereas interna[...] receptors are recycled back to the [...] (21). Receptor recycling also occu[...] transferrin receptor (22) but in th[...] (transferrin) is recycled together wit[...] to the cell surface while the transport[...]

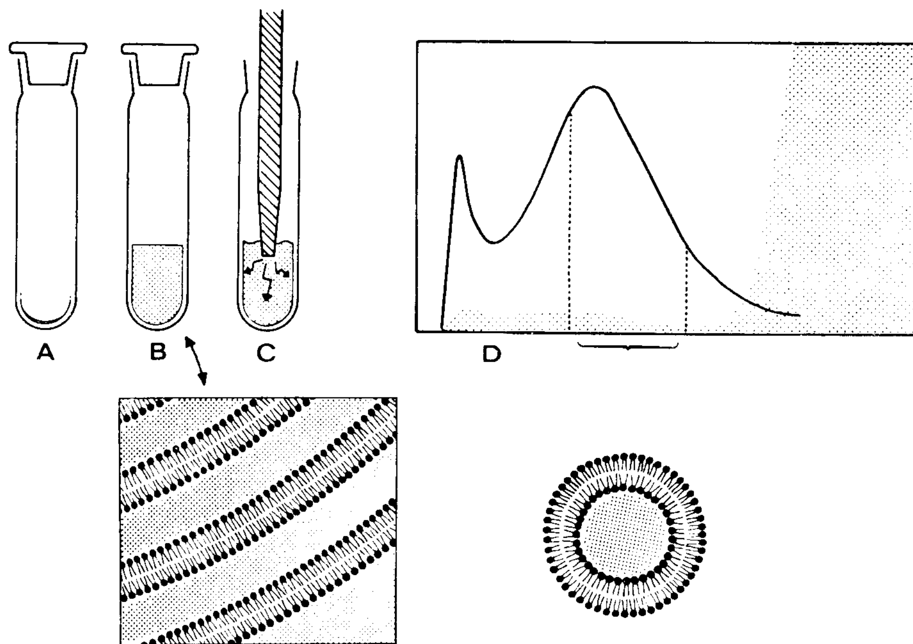

Fig. 1. Preparation of small unilamellar liposomes.
Lipids are thoroughly dried under vacuum. To the resulting lipid film (A) the aqueous medium containing the solute to be trapped (:::) is added. By dispersion of the lipids large multilamellar liposomes are formed (B). Ultrasonic irradiation by means of a probe sonifier (C) results in break down of the multilamellar structures and the formation of small unilamellar liposomes. By gel filtration the liposomal suspension is freed of remaining multilamellar liposomes and unentrapped solute (D).

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c. the liposomes may be taken up by the cells by an endocytic process, delivering the liposomes to the lysosomal system.

This last event is generally thought to play the most prominent role in the uptake of liposomal contents by cells (9, 10, 11); therefore this process will now be described in more detail.

Endocytosis

Endocytosis is the process of internalization of extracellular material by invagination of the plasmamembrane and the subsequent formation of a cytoplasmic vesicle. This vesicle has been given different names such as phagosome (12), pinosome (13), coated vesicle (14) or primary food vacuole (15), depending on the cell type studied and on the type of endocytosis involved. Classically, endocytosis has been divided into two types: phagocytosis, covering the internalization of particles larger than a few tenths of a micron in diameter, and pinocytosis describing the uptake of all smaller substrates, varying from colloidal particles such as lipoproteins and SUV to low-molecular weight solutes and to the surrounding fluid. Phagocytosis always involves adsorption of the particle to the plasma membrane prior to invagination. Uptake by pinocytosis can also be adsorptive in character (adsorptive pinocytosis), but it may also merely involve internalization of fluid with solutes (fluid pinocytosis). Adsorptive endocytic processes display saturation kinetics, whereas uptake by fluid pinocytosis is linearly proportional to the concentration of the solute. Adsorptive pinocytosis may be preceded by non-specific binding of the substance to the cell surface or it may involve interaction with highly specific recognition sites (receptors) on the cell membrane; this latter mechanism is known as receptor mediated endocytosis. For example, polypeptide hormones like epidermal growth factor (16) and insulin (17), plasma lipoproteins such as low density lipoprotein (18), α_2-macroglobulin (19) and asialoglycoproteins (20) are known to be taken up by receptor-mediated endocytosis. The intracellular fate of receptors and substrates thus internalized depends on the cell type and the nature of the ligand. For instance, when the F_C-receptor, expressed on the surface of macrophages, is occupied by a ligand (immunocomplex) both receptor and ligand are degraded in the lysosomes, whereas internalized unoccupied receptors are recycled back to the plasma membrane (21). Receptor recycling also occurs in case of the transferrin receptor (22) but in this case the ligand (transferrin) is recycled together with its receptor back to the cell surface while the transported iron is delivered intracellularly. The asialoglycoprotein (ASGP) receptor, after internalization, is uncoupled from its ligand before the lysosomes are reached, and is recycled to the cell surface, whereas its ligand ultimately is degraded in the lysosomes (23). Some aspects of the overall uptake mechanism are shared by different ligands, but the ligand-receptor combination is thought to dictate which divergent path is taken once they are inside the cell. The ASGP receptor, which is abundant on the plasma membrane of hepatocytes, binds ligands with terminal non-reducing galactose residues. Liposomes which expose such galactose residues, it was reasoned, could specifically be directed towards the hepatocytes.

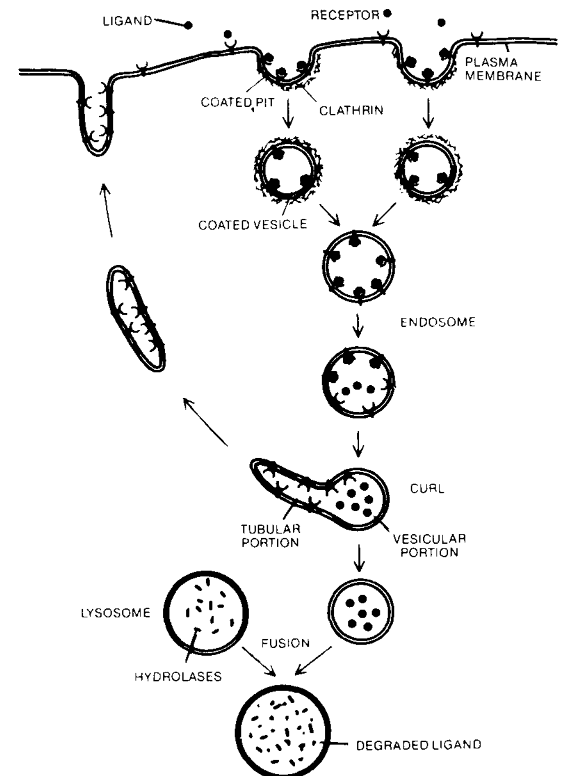

Fig. 2. Pathway of receptors and ligands as determined for galactose terminal glycoproteins.
Ligands bind to receptors diffusely and then collect in coated pits, which invaginate and are internalized as coated vesicles whose fusion gives rise to endosomes and then to a CURL. In the acidic CURL-environment ligand is dissociated from receptors. Ligand accumulates in the vesicular lumen of the CURL and the receptors are concentrated in the membrane of an attached tubular structure, which then becomes separated from the CURL. The vesicular part moves deeper into the cell and fuses with a lysosome, to which it delivers the ligand for degradation. The membranous tubular structure is thought to recycle receptors to the plasma membrane.
(From Dautry, A. & Lodish, H.F., Scientific Am. (1984), 250(5), 48-54)

The ASGP receptors are probably randomly distributed in the plasma membrane of the hepatocytes (24). After binding of a ligand the receptor-ligand complex moves to a so-called coated pit (14), a specialized region of the plasma membrane, of which the name is derived from the fuzzy coat that decorates its cytoplasmic surface. This coat is formed of clathrin (25, 26), a protein which structurally supports (the formation of) the invaginations of the plasma membrane. In the coated pits microaggregation, and thus concentration, of the receptors takes place (24). Internalization is completed by closure of the coated pit, giving rise to the formation of a coated vesicle (14). The coated vesicle looses its coat of clathrin molecules and the remaining smooth endocytic vesicle fuses with or converts into a so-called compartment of uncoupling receptor and ligand (CURL) (27, 28). Probably due to acidification of this compartment the receptor and ligand dissociate (29), followed by spatial segregation. The receptors are transported back to the cell surface where they are reutilized and the vesicles containing the ligands fuse with primary lysosomes (23) where degradation takes place (Fig. 2).

Liver

The liver is an important organ involved in the maintenance of homeostasis in the body. It performs a scala of functions including a regulatory role in the metabolism of carbohydrates, proteins and lipids and is involved in detoxification and storage. It regulates blood glucose concentration and synthesizes and eliminates almost all plasma proteins. Participation of the liver in lipid metabolism involves for example the regulation of the blood cholesterol level, the synthesis from carbohydrates of triglycerides for assembly into lipoproteins and the synthesis of apolipoproteins for the transport of triglycerides in the blood; it also synthesizes the phospholipids required for lipoprotein formation and for the production of bile. Most of the metabolic liver functions are performed by the parenchymal liver cell or hepatocyte, the most abundant cell type in the liver. Several pathological conditions are the result of interference with the hepatocyte's metabolism e.g. by parasites or viruses or malignant transformation. These considerations constituted a major argument for choosing the hepatocyte as a target for the delivery of drug-containing liposomes. In addition, the liver is one of the few organs with an architecture allowing liposomes to escape from the vascular system and to reach cells beyond the endothelial barrier. As pointed out by Poste, discussing the problems and opportunities of liposome targeting in vivo (10), liposomes injected intravenously are otherwise unlikely to escape from the continuous capillaries and to reach extravascular tissue.

The features of the hepatic blood circulation and microanatomy that allow liposomes to reach the hepatocytes will now briefly be discussed. The blood supply of the mammalian liver is provided by two afferent vessels: the hepatic artery, which delivers oxygen-rich blood and the portal vein, which transports substances absorbed from the gastrointestinal tract to the liver. Both the hepatic artery and the portal vein have numerous branches within the liver, with successive branches having smaller diameters. The terminal branches are called sinusoids and there venous and arterial blood come together. Extensive exchange of materials and metabolites between blood and liver cells occurs in the sinusoids. After passage through the sinusoids, the blood is collected in branches of the hepatic veins, converging in the vena hepatica, which drains on the inferior vena cava.

In addition to the hepatocytes the liver contains three other cell types: Kupffer cells (liver macrophages), the sinusoidal endothelial cells and the fat-storing cells (30). Each of these cells is found at specific locations in the sinusoid (Fig. 3), which measures about 6-7μ in diameter. The endothelial cells form the lining of the sinusoids, with the Kupffer cells usuall[y] on or imbedded in this endothelial c[...] blood side. The hepatocytes are locate[d] of the endothelial cells. The spac[e] hepatocytes and the endothelial cells is [...] of Disse where the fat-storing cells are [...] dothelial cells are fenestrated i.e. [...] numerous holes or pores allowing th[e] free access to all the cells in the live[r] parenchymal cells. The fenestrations i[n] cells have an average diameter of about [...] any drug carrier to reach the hepatocyt[e] the diameter should therefore be les[s] imately 100 nm.

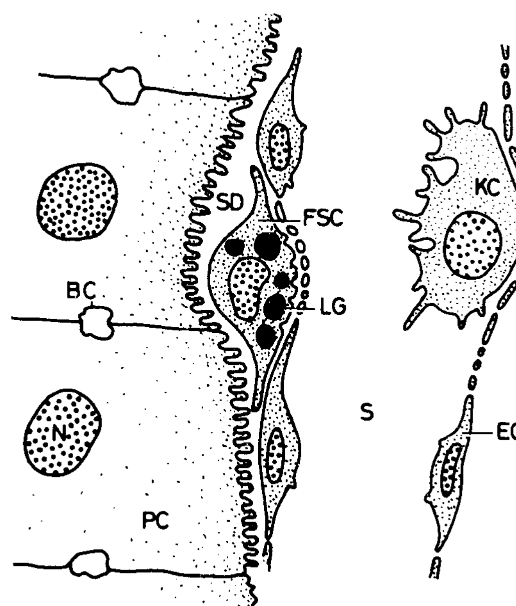

Fig. 3. Scheme of a liver sinusoid.
Endothelial cell (EC); Kupffer cell (KC); parenchymal cell (PC); space of Disse (SD); Fat-Storing Cell (FSC); lipid granule (LG); bile canaliculus (BC); nucleus (N).

Aim of the thesis

The experiments described in this t[...] at defining optimal conditions for lip[o...] drug targeting to the parenchymal cell [...] In view of the micro-anatomy of the liv[er] chosen for this purpose were of the s[...] type (SUV) to allow free passage t[...] dothelial fenestrations. Variations in li[...] showed that hepatic uptake as well [...] disposition of an intravenously in[...] liposomes is dependent on the liposo[...] the type of phospholipid used. In o[...] distribution in favor of the hepat[...] galactose-carrying ligands were incor[...] liposomal membrane to allow specifi[...] the hepatic asialoglycoprotein recept[...] present on the plasma membrane of t[...]

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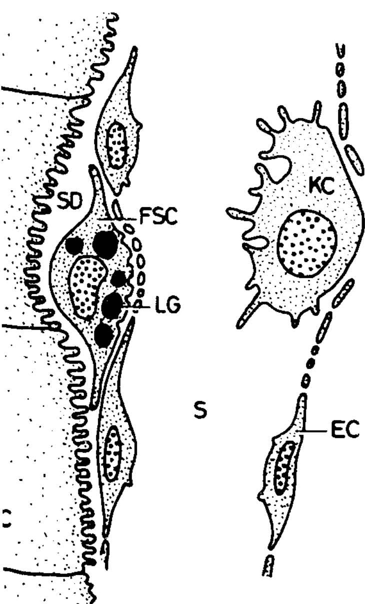

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sinusoids, with the Kupffer cells usually lying anchored on or imbedded in this endothelial cell lining at the blood side. The hepatocytes are located perisinusoidal of the endothelial cells. The space between the hepatocytes and the endothelial cells is called the space of Disse where the fat-storing cells are located. The endothelial cells are fenestrated i.e. they contain numerous holes or pores allowing the blood plasma free access to all the cells in the liver including the parenchymal cells. The fenestrations in the endothelial cells have an average diameter of about 100 nm (31). For any drug carrier to reach the hepatocytes via the blood, the diameter should therefore be less than approximately 100 nm.

Aim of the thesis

The experiments described in this thesis were aimed at defining optimal conditions for liposome-mediated drug targeting to the parenchymal cell of the (rat) liver. In view of the micro-anatomy of the liver the liposomes chosen for this purpose were of the small unilamellar type (SUV) to allow free passage through the endothelial fenestrations. Variations in lipid composition showed that hepatic uptake as well as intrahepatic disposition of an intravenously injected dose of liposomes is dependent on the liposomal charge and the type of phospholipid used. In order to shift the distribution in favor of the hepatocytes, specific galactose-carrying ligands were incorporated into the liposomal membrane to allow specific recognition by the hepatic asialoglycoprotein receptor, known to be present on the plasma membrane of the hepatocytes.

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