New Trends in Photobiology (Invited Review) The role of the low density lipoprotein receptor pathway in the delivery of lipophilic photosensitizers in the photodynamic therapy of tumours

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Abstract

Lipoproteins are now recognized as major blood carriers of many hydrophobic porphyrins and related chromophores which are being investigated as possible photosensitizers in the photodynamic therapy of tumours. *In vitro* and *in vivo* studies have demonstrated the role of the low density lipoprotein (LDL) receptor pathway in the delivery of photosensitizers to tumour cells and its importance in porphyrin accumulation by tumours. Lysosomes, which are involved in the cellular processing of LDL, are important intracellular targets in the LDL–porphyrin-induced phototoxicity. The use of the LDL receptor pathway as a tool for enhancing the selectivity of photosensitizer delivery to tumour cells appears to be a promising field of research in the photodynamic therapy of tumours.

1. Introduction

Twelve years after the article of Diamond *et al.* [1] on the possible use of the photodynamic effect induced by porphyrins to cure cancers, it was still assumed that albumin and haemopexin were the most important carriers of the tetrapyrrolic photosensitizers in the blood. This assumption, clearly put forward in ref. 2, was based on papers which appeared in the 1970s describing the strong interaction of albumin with porphyrins encountered in the porphyria diseases (for a review of these previous studies, see ref. 3).

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A dramatic change in this belief occurred in 1984 when our group in Paris and G. Jori's group in Padova described the strong binding of hydrophobic (protoporphyrin) or moderately hydrophobic (haematoporphyrin, HP) porphyrins to human blood lipoproteins [4, 5].

2. The transport of porphyrins by lipoproteins

In ref. 4, we reported that low density lipoproteins (LDLs) and high density lipoproteins (HDLs) had the same binding capacity for HP, but HDLs, which are at the highest concentration in blood, retain more than half the amount of HP that can be bound to albumin, whereas the HDL concentration is only about 5% of that of albumin. Similar data were obtained by Jori *et al.* [5] with blood from cancer patients who were given 5 mg HP kg^{-1} body weight intravenously. In their study of the time dependence of the HP content of ultracentrifugally separated lipoprotein fractions of the same patients, these workers found that, after 48 h, HDLs, LDLs and very low density lipoproteins (VLDLs) still contained HP. This is a rather intriguing result since the catabolism of VLDLs occurs with a half-time of about 30 min. Thus all the VLDLs which originally contained HP at the time of injection have disappeared after 48 h. The presence of HP in VLDLs a long time after injection may be related to the clearance of the HP accumulated in the liver (since this tissue preferentially retains HP and other porphyrins (see below)). Another explanation may be that a redistribution of the circulating HP occurs between the different plasma protein fractions. Indeed, when HP-loaded LDLs or HDLs are incubated *in vitro* with human serum, the photosensitizer becomes gradually redistributed among the various serum lipoproteins [6].

Although somewhat questioned at the beginning [7], these results provided an important clue for the understanding of the accumulation of porphyrins by tumours. Indeed, previous studies from several groups had demonstrated that LDL processing was generally increased in tumour cells as compared with their normal counterparts [8–10]. It could therefore be expected that porphyrin-loaded LDL might interact with some selectivity with tumour cells in a tumour-bearing patient, provided that the porphyrin-loaded LDLs were still able to be recognized by the apo B/E receptor. Indeed, our study on the interaction of Photofrin II (P2)-loaded LDLs with cultured human fibroblasts demonstrated that the solubilization of P2 into LDLs (one LDL can incorporate 130 porphyrin rings of P2) did not alter their ability to recognize their specific membrane receptors despite a slight increase in the negative net charge of the lipoprotein [11].

The pioneering studies demonstrating the strong affinity of HP or haematoporphyrin derivative (HPD) for lipoproteins [4, 5] stimulated further studies on the characterization of the physicochemical parameter of the associations between lipoproteins and porphyrins [12] and other photodynamic therapy (PDT)-related chromophores. According to some workers, the lipoprotein transport is mainly governed by the hydrophilic or lipophilic

character of the photosensitizers. As a rule [6], hydrophilic photosensitizers (HP, tetrasulphonated porphyrins and phthalocyanines) are transported by albumin and globulins. However, more hydrophobic photosensitizers such as HP oligomers, porphyrin esters, monosulphonated or unsubstituted phthalocyanines are preferentially solubilized by lipoproteins. Moan and coworkers [13] arrived at the same conclusions for the solubilization of photosensitizers in serum. However, their study suggested that factors other than hydrophobicity could play a role in porphyrin binding to LDL. Thus an asymmetric charge distribution such as in tetraphenylporphine sulphonate 2a (TPPS2a) favours the solubilization of porphyrins in LDLs. They ascribed this enhanced binding to a high affinity for the lipid–water interface. Such an explanation, together with a preferential interaction of the photosensitizer with the apolipoprotein B100, may explain, in the case of P2, the two-step solubilization process observed by Candide *et al.* [14] in LDLs.

The preferential solubilization in lipoproteins of benzoporphyrins, a new class of very potent photosensitizers considered for use in the PDT of cancers, has been recently reported by Kessel [15]. In plasma, the monoacid and diacid derivatives bind primarily to HDLs, but significant fractions are also bound to LDLs. These results were confirmed in a subsequent study centred on the monoacid derivative [16].

The above papers suggest that the LDL pathway may be an excellent tool for the delivery of photosensitizers to tumours. Consequently, it appears advisable to develop procedures which can facilitate the incorporation of the photosensitizers into LDLs. Accordingly, the pre-incorporation in dipalmitoyl phosphatidylcholine (DPPC) liposomes enriched with 10%–15% cholesterol strongly enhances the efficiency of the photosensitizer solubilization into the LDL. Such a technique has been successfully applied to HP and zinc phthalocyanines [17–19], and several other photosensitizers [6].

3. Evidence for the involvement of the LDL endocytotic pathway in the photosensitizer uptake by tumours *in vivo*

In the light of the above data on the transport of photosensitizers by lipoproteins, including LDLs, it is predictable that many reports on the distribution and elimination of PDT photosensitizers in experimental tumours can be explained by the involvement of the LDL receptor-mediated endocytotic pathway. The pioneering investigations in this respect were performed by Jori's group in Padova and Kessel in Detroit, who demonstrated that, *in vivo*, lipoproteins are a determinant in the uptake of porphyrins by tumour cells. A pharmacokinetic study involving the delivery of HP incorporated into lipoproteins to tumour-bearing mice unequivocally showed that HP associated with LDLs can be delivered to MS-2 sarcoma grown in Balb/c mice [20]. In an elegant study on mice bearing the Lewis lung tumour, Kessel confirmed the role of lipoproteins in the transport of HPD, and the role of HDLs in its retention. The distribution pattern of HPD was found to

be correlated with the number of LDL receptors in various tissues [21]. However, it should be mentioned that, in his experimental model, the porphyrin uptake by the tumour was only twice that of the skin. As a result, on a therapeutic basis, the destruction of normal tissues by the photodynamic process would be the limiting factor to the effectiveness of the phototherapeutic treatment. The relatively poor selectivity of porphyrins for tumour *vs.* normal tissues must arise from the poor selectivity of porphyrin distribution between plasma proteins after intravenous (i.v.) injection. Albumin and HDLs, two other important blood carriers of PDT porphyrins, are not internalized via specific receptors. Their interaction with cells first results in an exchange of the photosensitizer with the plasma membrane [11], which eventually leads to the unspecific staining of cell membranes and organelles during the intracellular lipid and protein transport and catabolism. The reverse process (*i.e.* intracellular transport of the photosensitizer and/or its degradation products followed by interaction with circulating proteins of the interstitial fluid) might be responsible for the long lasting photosensitivity encountered in PDT patients. It is believed that HDLs are involved in this delayed photosensitivity [22, 23]. If so, we suggest that the oligomeric species which are the main P2 components retained by tumours [24] can be transferred to HDLs during the interaction of this lipoprotein with cells, an essential step in the reverse transport of cholesterol from peripheral tissues [25]. It may also be hypothesized that these oligomeric species, which are very slowly hydrolysed by the liver, can be secreted concomitantly with nascent HDLs over a long period.

It should also be noted, with regard to experiments performed on animal models, that extrapolation of selectivity indexes to humans must be performed with some care, since LDL plasma concentration and/or LDL receptor activity are known to be subject to substantial interspecies variation [26], so that the HPD or P2 distribution in human and animal tissues may not be similar after LDL delivery.

Generally speaking, the uptake of photosensitizers is believed to be controlled by their hydrophobicity and aggregation state [6]. This has been analysed following three different routes.

(i) The "unbound" molecules, which include aggregated HPD components with a serum half-life of about 2 h, can be taken up by macrophages, endothelial and neoplastic cells. Evidence for such mechanisms has been suggested by Bugelski *et al.* [2] who investigated the time-dependent autoradiographic distribution of HPD in normal and tumour tissues of mice bearing the SMT-F mammary carcinoma, and later by Dougherty and Mang [27] in a paper dealing with a tumour removed from a patient. However, in this latter report, Dougherty and Mang pointed to the role of the LDL-driven endocytotic pathway for the HPD retention in the tumour. It must be stressed that the presence of "unbound" aggregated HPD in serum as proposed in refs. 6 and 24 is rather puzzling considering that *in vitro* solubilization of 13 mg ml^{-1} HPD in 400 nM LDL followed by extensive dialysis during 48 h does not produce any material loss [14].

(ii) Weakly bound photosensitizers such as tetrasulphonated porphyrins or HPD monomers are localized in the vascular stroma. These correspond to the so-called "non-localizing" monomeric fractions of HPD, bound to albumin or globulins [12, 24], reported in studies on the distribution of porphyrins in experimental or human tumours, and not involving the endocytotic pathway [21].

(iii) The strongly bound photosensitizers (HPD oligomers, monosulphonated porphyrins and phthalocyanines) are mainly carried in plasma by lipoproteins. It can thus be assumed that tissue delivery of this type of photosensitizer is achieved at least partially through the LDL receptor pathway. As a consequence, the photosensitizer will be accumulated in tissues having a large number of apo B/E receptors. As tumour cells generally catabolize LDLs at a higher rate than normal cells [8–10], it can be expected that this might result in some degree of selectivity in the uptake of LDL-bound photosensitizers by tumour *vs.* normal tissues. Indeed, the amount of HP in the mouse MS-2 sarcoma was found to be higher after i.v. injection of HP-preloaded LDL as compared with the free drug or with the drug bound to isolated HDLs; furthermore, the localization ratio (tumour *vs.* other tissues) was better using HP-preloaded LDL [28]. Other related studies on the comparison between serum kinetics and tissue distribution of P2 after i.v. or intraperitoneal (i.p.) injection also demonstrated the role of the LDL transport in the photosensitizer delivery to normal mouse tissues [29].

Since liposomes notably enhance the porphyrin solubilization into serum lipoproteins [17–19], it might be anticipated that efficient delivery of photosensitizers to tumours can be achieved using these vehicles. Thus HP is efficiently taken up by experimentally induced pituitary adenoma on female Wistar rats on i.v. injection of the photosensitizer encapsulated into DPPC liposomes [30]. These studies have been recently extended to zinc phthalocyanine [19].

It has recently been published that LDLs can inhibit P2 uptake by cells [31]. This somewhat intriguing assumption is based on the observation that a very efficient solubilization of P2 components in cellular membranes takes place during incubation in pure buffer (absence of serum proteins). This is expected considering the hydrophobicity of the photosensitizer components. Thus in buffer supplemented with LDLs, a reduction of the P2 uptake as compared with that observed in the absence of the lipoprotein is not surprising. However, it must be considered that, *in vivo*, P2 is bound to lipoproteins, and thus data obtained *in vitro* in the absence of lipoproteins have only poor physiological significance. This clearly illustrates the importance of experimental conditions for the interpretation of a result and its extrapolation to living animals or humans.

The correlation between the localizing ability and relative affinity of photosensitizers for LDLs has been questioned by Kongshaug *et al.* [13]. Considering protoporphyrin in particular, they concluded that there was no correlation between the affinity of the photosensitizer for the LDLs and its uptake by the tumour. However, it should be stressed that it is rather difficult

to discuss the tumour localizing capacity of a photosensitizer in the absence of detailed pharmacokinetic data concerning each photosensitizer. Thus protoporphyrin is probably taken up and released very rapidly by tumours. The maximum protoporphyrin concentration is obtained 1 h after injection and efficient photosensitization occurs at this time [32].

Before finishing this section, we wish to emphasize two further points.

(i) The LDL endocytotic pathway results in very rapid delivery of the LDL components to the lysosomal compartment [33]. As a consequence, if we assume that at least part of the porphyrin remains bound to the LDL during its intracellular transport towards lysosomes, the photodynamic effect must result in a rapid alteration of the lysosomal membranes and thus in the release of highly toxic lysosomal hydrolases in the cytosol. We can assume that such a process may play an important role in the photocytotoxic effect of porphyrin-loaded LDLs (see below).

(ii) Another photosensitizer, monoaspartylchlorin e6 (MACE), which is not carried in blood by LDLs but is preferentially bound to albumin and to a lesser extent to HDLs, is accumulated within lysosomes [34, 35]. In this case, MACE, a negatively charged molecule, may enter the lysosomes via the aromatic anion transporters as does, for example, the negatively charged lysosomal vital fluorescent probe Lucifer Yellow [36]. This process also includes pinocytosis and can be inhibited at temperatures below 4 °C, whereas receptor-mediated endocytosis is inhibited below 10 °C. A similar behaviour has been observed for chloro-aluminium sulphonated phthalocyanine [34].

4. Cell photosensitization after delivery of the photosensitizer via the LDL pathway

Recent studies from our laboratory have shown that, in human SV40-transformed Wi26-VA4 cultured fibroblasts, the endoplasmic reticulum is altered in addition to the plasma membrane by P2-induced photosensitization after LDL delivery [37], as demonstrated by the strong, light-dependent decrease in the acyl coenzyme A:cholesterol-*o*-acyltransferase activity. In mouse L cell fibroblasts, we have shown by microspectrofluorometry that P2 delivered by human LDLs (which are bound to the transformed murine cells) induces multifocal photobiological effects, including the formation of lipofuscin-like pigments and the permeation of lysosomes [38]. The destabilization of the lysosomal membrane was studied using a synthetic fluorogenic substrate of acidic proteases which does not enter lysosomes but remains located in the cytosol ((CBZ-Ile-Pro-Arg-NH_2)$_2$ Rhodamine 110 (BZIPAR). Thus the substrate hydrolysis demonstrates the rupture of the lysosomal membrane and the release of lysosomal enzymes in the cytosol. Moreover, treatment of cells with chloroquine, which blocks the lysosomal hydrolysis of LDLs, results in a strong reduction of BZIPAR hydrolysis, suggesting that the porphyrin-loaded LDL degradation is an important step in the destabilization of the lysosomal membrane on irradiation. It was thus tempting to take

advantage of this phenomenon by enhancing porphyrin accumulation in the lysosomal compartment. With this aim in mind, we synthesized a new photosensitizer in which a quinoline side-chain similar to the lysosomotropic agent chloroquine was grafted on tetraphenylporphine [39]. This new photosensitizer was effectively located in lysosomes as assessed by microspectrofluorometry, and its *in vitro* photocytotoxicity appeared to be at least similar to that of P2 after delivery via LDLs [39].

These conclusions were supported by *in vivo* studies. Thus 600–800 nm irradiation of Balb/c mice bearing a transplanted MS-2 fibrosarcoma at 24 h after injection of HP solubilized in buffer (HP-buf), LDLs (HP-L) or liposomes (HP-Lip) led to tumour necrosis according to two distinct mechanisms [40]:

(i) HP-buf induced tumour necrosis via vascular damage;

(ii) HP-L and HP-Lip caused direct tumour cell killing with ultrastructural damage at the lysosomal and mitochondrial levels.

In a recent report on the evaluation of the efficiency of animal tumour cure by PDT with zinc phthalocyanines, Reddi *et al.* [19] presented evidence for excellent phototherapeutic efficiency at drug doses as low as 0.07–0.35 mg kg^{-1} after delivery with DPPC liposomes which specifically interact, as seen above, with lipoproteins including LDLs. To our knowledge, these reports are the only examples in support of the phototherapeutic efficiency of photosensitizers after delivery via the LDL receptor-mediated endocytotic pathway.

Finally, if the results given above [40] suggest that, in mice, direct tumour destruction can result from photosensitization by HP-loaded LDL, the hypothesis of a possible role of porphyrin-loaded LDL in the destruction of the tumour microvasculature can also be raised. Although originally it was supposed that direct cell killing was the dominant cause of tumour necrosis following PDT, blood vessel damage was also noted [2]. General agreement is now emerging as to the important role played by damage to tumour blood vessels in the tumour necrosis induced by HPD [24, 41, 42]. Although the direct exchange mechanism between the HDL- or albumin-bound photosensitizer and the vessel wall certainly occurs, the LDL receptor pathway may also be involved since endothelial cells are known to possess LDL receptors [43]. Moreover, if we consider that porphyrin-loaded LDLs, which may remain in the tumour microcirculation at the time of irradiation, are strongly oxidized on singlet oxygen generation [14, 44], and that oxidized LDLs become cytotoxic towards endothelial cells [45, 46], we can suggest that such a process may also contribute to the tumour microvasculature destruction.

5. Conclusions

The papers reviewed in this article demonstrate that the receptor-mediated endocytosis of LDLs may be, together with other pathways, an important

determinant of the photosensitizer delivery and localization in tumours. As a result, it may be an important tool for enhancing the selectivity of the PDT given the large increase in apo B/E receptors in tumour cells as compared with normal tissues and the intense vascularization of tumours whose endothelial cells also express the apo B/E receptor. It can be anticipated that the delivery of new lipophilic, far-red-absorbing PDT photosensitizers (*e.g.* chlorins, phthalocyanines, pheophytins) may take advantage of the efficiency of the LDL receptor pathway. Moreover, the emergence of the lysosome as an important organelle for the processing of photosensitizers bound to LDLs and/or as a key intracellular target in LDL–porphyrin-induced photocytotoxicity is promising and must be investigated in the near future.

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