In vitro interaction of the photoactive anticancer porphyrin derivative photofrin II with low density lipoprotein, and its delivery to cultured human fibroblasts

C. Candide, P. Morlière+, J.C. Mazière, S. Goldstein, R. Santus°, L. Dubertret+, J.P. Reyftmann° and J. Polonovski

Laboratoire de Biochimie & CNRS UA 524, Faculté de Médecine St. Antoine, 27 rue Chaligny, 75012 Paris, +Laboratoire de Recherche Dermatologique, Hôpital H. Mondor, 94010 Creteil and *°Laboratoire de Physico-Chimie de l'Adaptation Biologique & CNRS UA 481, Muséum d'Histoire Naturelle, 43 rue Cuvier, 75231 Paris, France*

Received 26 June 1986; revised version received 21 August 1986

Low density lipoprotein (LDL) doped with the anticancer mixture of hematoporphyrin derivatives Photofrin II (P2) competes with native LDL for binding to fibroblast receptors, despite a slight increase in the negative net charge related to the presence of acidic residues of porphyrins. P2 delivery to fibroblasts can be achieved by LDL, HDL3 or albumin doped with P2 (LDL-P2, HDL-P2 or A-P2, respectively). P2 delivery to cells assessed by fluorescence measurement, is much more efficient, at low protein concentrations (10–20 μg/ml) by LDL-P2 than by HDL-P2 or A-P2. Moreover, P2 delivery to cells by LDL-P2 as a function of protein concentration is a saturable process, whereas P2 delivery by HDL-P2 or A-P2 is a linear process. Finally, reduction of the LDL-receptor number by preincubation of fibroblasts in medium supplemented with lipoproteins results in a decrease of P2 delivery by LDL-P2. These results suggest a special role of the LDL-receptor pathway in P2 delivery to cells and could be of interest in cancer phototherapy by porphyrins.

Photodynamic therapy *Photofrin II* *Porphyrin* *LDL* *(Human fibroblast)*

1. INTRODUCTION

Protoporphyrin and hematoporphyrin have been demonstrated to bind to albumin and lipoproteins [1,2], mainly low density lipoproteins (LDL) and high density lipoproteins (HDL). The LDL uptake by cells is achieved by receptor-mediated endocytosis, which results in LDL-component delivery to the lysosomal compartment [3,4]. Several authors pointed out an increase in LDL catabolism by transformed cells [5–7], and it has thus been suggested to use LDL as a carrier for lipophilic antitumoral drugs [8,9]. Hematoporphyrin derivatives have been proposed for tumor diagnosis and phototherapy [10,11]. Among them, photofrin II (P2) is a recently developed mixture which includes the main active component [12]. Due to poor intrinsic solubility in water, it forms high molecular mass aggregates in aqueous solutions. It should therefore exhibit a rather good affinity for lipophilic environments encountered in LDL. Since data on its pharmacokinetics using lipoproteins as carriers are not yet available, we investigated the interaction of P2 with LDL and its consequences on LDL binding to human fibroblasts by competition with native LDL. We also compared the delivery of P2 to cells by various serum proteins doped with P2: albumin (A-P2), LDL (LDL-P2), and HDL3 (HDL-P2).

2. MATERIALS AND METHODS

2.1. *Cell culture*

Human foetal lung fibroblasts (strain MRC5) were purchased from Biomérieux (France), and maintained in Ham F10 medium (Flow) sup-

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00145793/86/$3.50

plemented with 10% foetal calf serum (Gibco), in 25 cm^2 Falcon flasks, at 37°C, in a 5% CO_2 humidified atmosphere.

2.2. *Preparation of LDL and HDL3*

HDL3 and LDL were prepared from normal human serum by the method of Havel et al. [13]. LDL and HDL3 were the 1.024–1.050 and the 1.125–1.21 fractions, respectively.

2.3. *Labeling of LDL*

LDL was labeled according to Bilheimer et al. [14], using ^{125}I-Na (Amersham, 13–17 mCi/μg). The specific radioactivity was about 250–350 cpm/ng of LDL protein. Protein determination was done according to Lowry et al. [15].

2.4. *Loading albumin, HDL3 and LDL with P2*

P2 (Photofrin Medical Inc., Raritan, NJ) was added as concentrated (2.5 mg/ml) aqueous solution to diluted proteins, to achieve the same concentration of P2 in the incubation medium (13.7 μg/ml). The protein concentrations were such that their ratio corresponded to their ratio in plasma: thus, the solution to be doped contained 0.2 mg/ml LDL or HDL3, and 5 mg/ml bovine serum albumin (Sigma). Proteins were diluted in 0.1 M Tris buffer, 0.15 M NaCl, pH 7.4. These conditions have been chosen after preliminary experiments in order to obtain measurable fluorescence intensities. P2-loaded proteins were prepared just before use.

2.5. *Competition between LDL-P2 and iodinated LDL*

Fibroblasts were seeded at 25000/cm^2 in 35 mm Petri dishes, and grown for 48 h in Ham F10 medium supplemented with 10% foetal calf serum. The medium was then replaced by fresh lipoprotein-deficient medium (Ham F10 + 2% Ultroser G, IBF), for maximal receptor expression. After 24 h, cells were washed and incubated for 1 h at 4°C with 10 μg/ml ^{125}I-LDL in the absence or presence of unlabeled native LDL or LDL-P2 at various concentrations, in 0.1 M Tris/0.15 M NaCl buffer, pH 7.4. After incubation, cells were washed 4 times with the same buffer, harvested with rubber policemen, centrifuged, and the radioactivity in the pellet measured using a Packard gamma counter. Protein determination was done on the same sample, and results are expressed in % of control (^{125}I-LDL alone).

2.6. *P2 delivery to cells by A-P2, HDL-P2 and LDL-P2*

After a 24 h preincubation in Ham F10 medium + 2% Ultroser G, cells were washed, and then incubated for 1 h at 37°C with P2-loaded proteins at various concentrations, in 1 ml of 0.1 M Tris buffer/0.15 M NaCl, pH 7.4. In experiments designed for the study of the effect of serum lipoproteins on P2 delivery to cells, cultures were preincubated for 24 h either in Ham F10 medium supplemented with 10% foetal calf serum, or in Ham F10 medium supplemented with 2% Ultroser G. After incubation with P2-doped proteins, cells were washed 4 times and harvested with rubber policemen. After centrifugation, the fluorescence of the pellet was measured with a Perkin Elmer LS5 spectrofluorometer. The excitation and emission wavelengths were 405 and 632 nm, respectively. Results are expressed in arbitrary units (fluorescence intensity/μg of cell protein).

3. RESULTS AND DISCUSSION

As seen in fig.1 (inset), aqueous P2 (1.8 μg/ml) exhibited a fluorescence peak at 613 nm. Addition of LDL to the solution increased the fluorescence intensity, and induced a red shift of the emission maximum to 627 nm. Fig.1 also displays the increase in fluorescence intensity of P2 as a function of LDL concentration. The fluorescence intensity increased with LDL concentration, reaching a plateau at 0.38 mg/ml. This can be interpreted as the result of the incorporation and monomerisation of the porphyrin aggregates into the LDL. From the intercept between the plateau level and the initial slope of the fluorescence intensity graph, it is evaluated that 140 μg LDL (protein weight) can incorporate up to 1.8 μg P2, e.g. 1 LDL molecule for about 130 porphyrin rings (600 Da = average molecular mass of a porphyrin ring). Under the same conditions [2], one LDL molecule can only bind 50 molecules of the closely related protoporphyrin, responsible for skin photosensitivity in porphyria patients.

Fig.2 displays the electrophoretic profile of native LDL and LDL-P2. The cationic mobility of LDL-P2 is slightly increased as compared to native

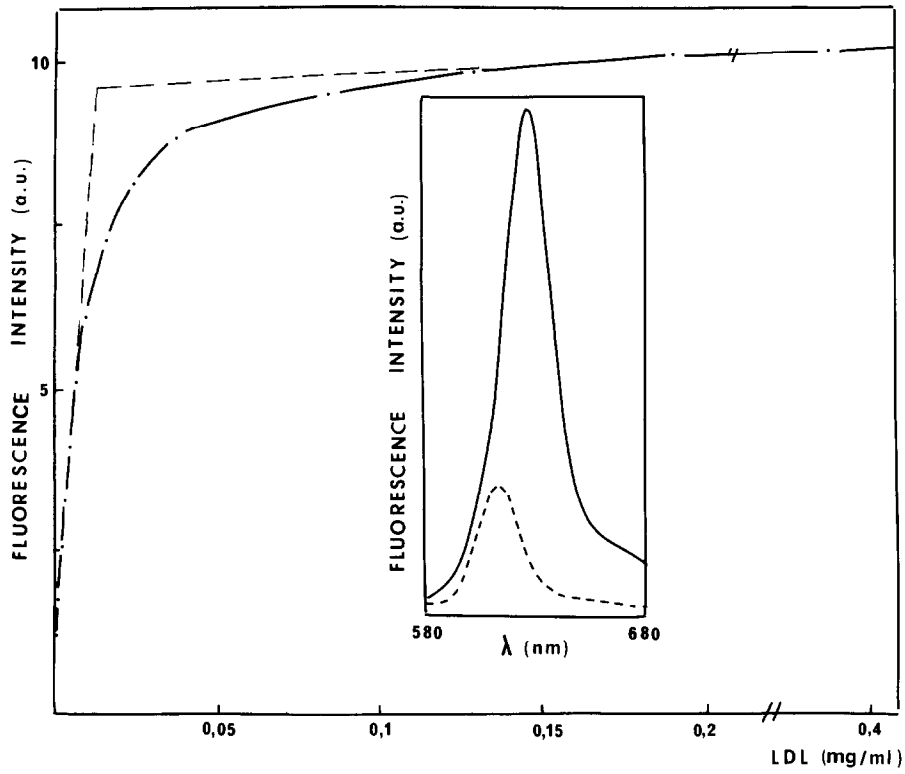

Fig.1. Fluorescence intensity of a 1.8 μg/ml solution of Photofrin II (excitation and emission wavelengths: 500 and 628 nm, respectively) as a function of the LDL concentration in saline phosphate buffer. Inset: emission fluorescence spectrum of 1.8 μg/ml Photofrin II in the absence of LDL (– – –) and in the presence of 0.38 mg/ml LDL (——).

LDL (+ 3 mm). This increase in the negative net charge of LDL is probably due to the presence of acidic (acetic, propionic) residues of the porphyrin.

It was of interest to investigate if this increase in the negative charge of the LDL could modify the interaction of the lipoprotein with its specific receptor at the cell surface, since chemical modifications of LDL such as acetylation have been reported to strongly decrease LDL binding to fibroblasts [16,17]. Fig.3 shows that there was no significant difference in the ability of native or P2-doped LDL to compete with ^{125}I-LDL.

The delivery of P2 to MRC5 fibroblasts by LDL-P2, HDL-P2 and A-P2 as a function of the carrier protein concentration is shown in fig.4. Clearly a plateau was obtained for about 30–40 μg/ml of LDL-P2, whereas the fluorescence increased quite linearly with carrier protein concentration for HDL-P2 or A-P2. Furthermore, the fluorescence recovered in fibroblasts was markedly higher from LDL-P2 than from HDL-P2 or A-P2, in the range of 5–25 μg/ml. At 10 μg/ml, LDL-P2 was about 3-fold more efficient than HDL-P2. The efficiency of HDL-P2 was comparable to that of LDL-P2 only at very high concentrations (above

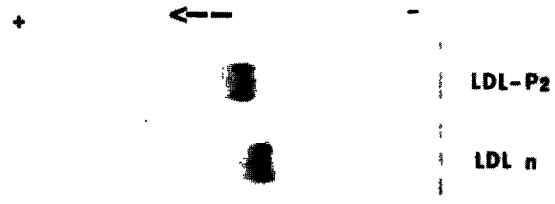

Fig.2. Agarose gel electrophoresis of native LDL and LDL-P2 (Universal Electrophoresis Film Agarose 1%, Corning; staining: Red Oil O). LDL-P2 exhibits an increased cationic mobility (about + 3 mm as compared to native LDL).

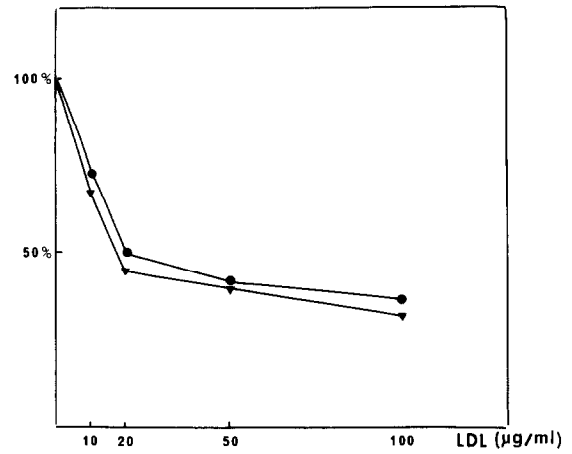

Fig.3. Competition between ^{125}I-LDL and native LDL (▼) or P2-doped LDL (●). Cells were preincubated for 24 h in Ham F10 medium supplemented with 2% Ultroser G, washed, and LDL binding measured as described in section 2 with ^{125}I-LDL (10 µg/ml) in the absence or presence of native or P2-doped LDL. Results are expressed in % of control (^{125}I-LDL alone). 100%: 118 ± 15 ng LDL bound/mg of cell protein; mean of 3 experimental values.

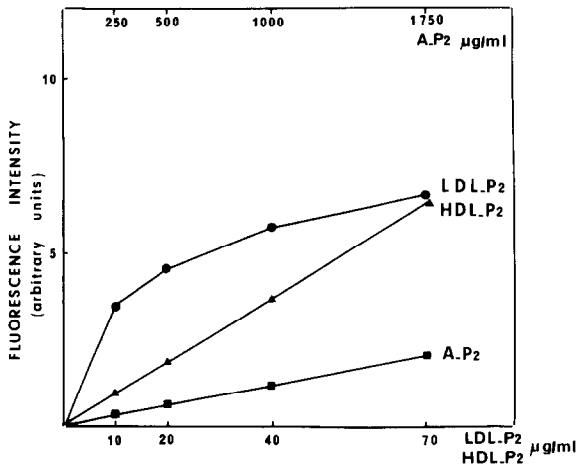

Fig.4. P2 delivery to MRC5 fibroblasts by LDL-P2, HDL-P2 or A-P2. Cells were preincubated for 24 h in Ham F10 medium supplemented with 2% Ultroser G, for maximal expression of LDL-receptors, then washed, and incubated for 1 h at 37°C in 0.1 M Tris/0.15 M NaCl, pH 7.4, buffer with P2-loaded proteins at various concentrations. P2 delivery to cells was assessed by fluorescence measurement. Results are expressed in arbitrary units (fluorescence intensity/mg cell protein). Lower scale: LDL-P2 (●) or HDL-P2 (▲). Upper scale: A-P2 (■). Mean of 3 experiments.

50 µg/ml). A-P2 was much less effective, as 10 µg/ml of LDL-P2 gave about a 5-fold higher fluorescence than 250 µg/ml of A-P2.

The influence of the preincubation of cells in the absence or presence of lipoproteins has also been studied (fig.5). The fluorescence recovered from LDL-P2 in fibroblasts preincubated for 24 h in a lipoprotein-deficient medium was about two-fold higher than that of fibroblasts preincubated in medium supplemented with 10% foetal calf serum.

From these results, it can be concluded that:

(i) P2 binds to LDL, probably by insertion into the external leaflet of the lipoprotein, as suggested by the increase in negative net charge of LDL-P2 as compared to native LDL. It is worthy of note that the binding of P2 to LDL does not significantly alter the ability of LDL to be recognized by its specific receptor at the cell surface, despite this slight increase in the negative net charge of the lipoprotein.

(ii) P2 delivery to fibroblasts from LDL seems to be mainly achieved by the receptor pathway, as suggested by studies of the carrier-protein concentration and time dependency of the phenomenon, as well as by the study of the influence of prein-

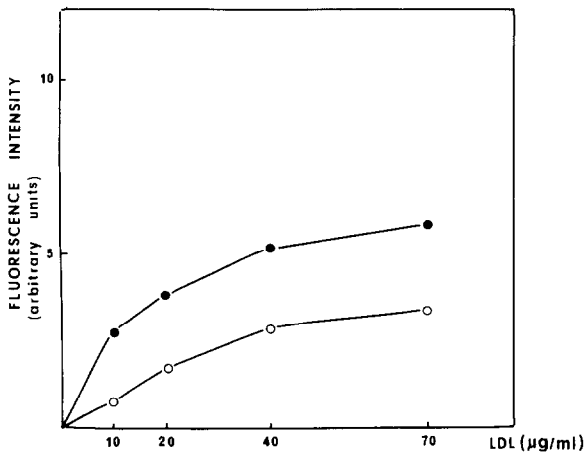

Fig.5. Effect of LDL-receptor expression on P2 delivery to MRC5 fibroblasts. Cells were preincubated for 24 h either in Ham F10 medium supplemented with 2% Ultroser G (●), for maximal expression of LDL receptors, or in Ham F10 medium supplemented with 10% foetal calf serum (○). P2 delivery to cells was assessed by fluorescence measurement. Results are expressed in arbitrary units (fluorescence intensity/mg cell protein). Mean of 3 experiments.

cubation in a medium devoid of lipoproteins. In this latter condition, cells exhibited an increased number of LDL receptors, as demonstrated by Goldstein and Brown [3]. Thus, the rise in LDL-P2 uptake when receptor expression is maximum supports the hypothesis of a role of the receptor pathway. However, it must be considered that a 24 h preincubation of cells in whole medium decreased P2 delivery only 2-fold, whereas in the same conditions the LDL-receptor number, measured with ^{125}I-LDL, was about 4–5-fold reduced. This suggests that, besides the LDL-receptor pathway, P2 may also enter by non-specific mechanisms. A similar observation has been recently reported for the delivery of the anthracyclin derivative AD-32 to white blood cells by LDL [9].

(iii) From the three studied P2 carriers, LDL appears to be the most efficient, especially at low concentrations.

Thus, the LDL-receptor pathway is possibly involved in P2 delivery to cells. This is of interest in view of the fact that several reports pointed out a significant increase in LDL catabolism by the receptor pathway in transformed cells as compared to their normal counterparts [5–7]. The use of LDL as a carrier for other lipophilic antimitotic drugs has been previously suggested, taking advantage of this increased LDL uptake by tumor cells, in order to improve the selectivity of drugs towards tumor cells [18].

It is also of interest to consider that P2 probably reaches the lysosomal compartment by the LDL-receptor pathway, whereas it can be suspected that it is widely distributed among cellular membranes when delivered by albumin or HDL. Experiments are now undertaken to specify the intracellular distribution of P2 delivered by LDL, HDL or albumin by fluorescence microscopy. If it is confirmed that P2 rapidly reaches lysosomes after delivery by LDL-P2, this may be interesting for the strategy of cancer phototherapy. Indeed, irradiation of porphyrins induces the formation of very active oxygen species such as singlet oxygen [19]. This could lead to disruption of lysosomes and release of lysosomal enzymes in the cytosol, with subsequent cytolysis.

Moreover, generation of singlet oxygen by porphyrins can induce oxidation of unsaturated LDL lipids. It is well established that oxidation products of cholesterol such as 25-hydroxycholesterol strongly inhibit endogenous cholesterol biosynthesis [20]. It is possible that this is also true for other lipid oxidation products, which can contribute to decrease tumor cell proliferation. In addition, oxidized LDL has been reported to be cytotoxic towards fibroblasts [21,22]. It thus may be suggested that the LDL-receptor pathway can enhance tumor cell killing by porphyrin derivatives through delayed secondary reactions induced by decomposition of photoperoxides of LDL lipids.

ACKNOWLEDGEMENTS

R.S., P.M. and L.D. thank INSERM for a grant (CRE 852021). R.S. gratefully acknowledges a grant from 'La Fondation pour la Recherche Médicale'.

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