# Human platelets exclusively bind oxidized low density lipoprotein showing no specificity for acetylated low density lipoprotein

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**Abstract The widely studied macrophage scavenger receptor system is known to bind both acetylated low density lipoprotein and oxidized low density lipoprotein. Although only the latter ligand has been shown to occur in vivo, acetylated low density lipoprotein is often used to evaluate the contribution of scavenger receptors to different (patho)physiologic processes, assuming that all existing subtypes of scavenger receptors recognise both lipoproteins. In the present work, we identify human platelets as the first natural cell type to bind oxidized low density lipoprotein without showing specificity for acetylated low density lipoprotein. Consequently, platelets possess exclusive receptor(s) for oxidized low density lipoprotein distinct from the 'classical' scavenger receptor AI/AII. From the data presented in this work, we conclude that the class B scavenger receptor CD36 (GPIV) is responsible for this exclusive oxidized low density lipoprotein binding.**



*Key words:* CD36; Oxidized low density lipoprotein; Scavenger receptor; Platelet

## 1. Introduction

Today, the term scavenger receptor (SR) is synonymous for receptors that are able to bind chemically modified low density lipoprotein (LDL).

The first modified LDLs that were shown to specifically bind to the macrophage SR system were acetylated LDLs (AcLDLs). Macrophages incubated with AcLDL undergo characteristic morphological changes due to the unrestricted uptake of lipoproteins, thereby developing a similarity with a certain type of cell typical of atherosclerotic lesions, the so-called foam cell. This is why from an early stage, SRs have been attributed a possible role in the clinical manifestation of atherosclerosis.

The finding that LDL develops specificity for the SR system when subjected to oxidative stress was an extremely important one. It marked the identification of an in vivo ligand of this receptor, able to induce the same morphological changes in target cells as AcLDL.

However, a more detailed examination of the interaction between acetylated and oxidized LDL with macrophage membrane receptors revealed an unequal level of competition between AcLDL and oxidized (Ox) LDL. This seemed to indicate the existence of different types of SRs, some of which are able to exclusively bind the respective lipoproteins [1,2].

In fact, a number of different SRs could be identified in the past few years. On the basis of a systematic classification in mammalian cells, there has been differentiation into class A (consisting of SR-AI and SR-AII) and class B SRs (consisting of SR-BI and CD36) (for review see [3]). Further SRs have been identified recently, which have not yet been classified according to this system [4–6].

A common feature of all SRs is their ability to bind an unusually broad array of ligands. Nevertheless, as was initially assumed, differences exist between the respective SRs with regard to their particular ligand specificity.

Paradoxically, the very finding that there is non-reciprocal cross competition between OxLDL and AcLDL that led to the speculation that different types of SRs exist, might, at least in part, be explained as a consequence of properties of the receptors themselves [7] and/or the extent of oxidative modification of the lipoproteins [8].

A whole range of (patho)physiological functions can be attributed to the SRs thus far discovered. Their most prominent feature is their involvement in the atherosclerotic process due to their specificity for OxLDL. On the other hand, SR-BI fulfills an important protective function due to its experimentally verified central role in reverse cholesterol transport [9,10].

Among all experimentally verified functions of SRs, the binding and phagocytosis of apoptotic cells is of particular interest, as there is evidence that tends to suggest that this physiologically most important process is mediated by an exclusive OxLDL receptor [11,12].

However, up to the present day, there has been no unequivocal identification of such a receptor in vivo. The process of this identification is complicated by the fact that there is still no knowledge of a 'natural' cell type (i.e. one that has not been genetically manipulated) which exclusively binds OxLDL but not AcLDL. One is therefore dependent on expression cloning strategies when examining this question although such strategies seem to result in widely ranging and contradictory results when approaching this very problem.

Furthermore, as AcLDL is often used as an inhibitor to evaluate the contribution of SRs to various macrophage functions, the identification of an exclusive OxLDL receptor would be of pivotal importance, as some of the results obtained with AcLDL would then need to be reassessed.

## 2. Materials and methods

Carrier-free $Na^{125}I$ was purchased from ICN (Costa Mesa, CA,

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*Abbreviations:* AcLDL, acetylated LDL; OxLDL, oxidized LDL; PBS, phosphate-buffered saline; LDL, low density lipoprotein; M-BSA, maleylated bovine serum albumin; mAb, monoclonal antibody; SR, scavenger receptor; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; f.c., final concentration


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USA). PBS, cell culture media and sera were from BioWhittaker Europe (Verviers, Belgium). Cell culture plastic ware was from Greiner (Frickenhausen, Germany). Monoclonal antibody (mAb) FA6.152 directed against the OxLDL binding domain of human CD36 was from Immunotech (Marseille, France). IV.3, a mAb against human Fc gamma RII (CD32) was purchased as Fab fragments from Medarex (Annandale, France). Both mAbs were purchased azide-free. poH.4, a polyclonal antibody against platelet GPIIb/IIIa was previously prepared as described [13]. M-BSA was prepared as originally described [14].

*2.1. Lipoproteins*

Extreme care was taken throughout all experimental procedures to avoid inadvertent oxidation of LDL. All steps were performed at 4°C and EDTA was added to all solutions (except preceding copper oxidation), resulting in a final concentration (f.c.) of 100 μM to prevent oxidation by trace metal ions. Lipoproteins were used immediately after preparation. Native LDL was rebuffered immediately before use (PBS, 100 μM EDTA). Unless otherwise stated, rebuffering was performed by gel filtration (Econo-Pac 10DG Columns, Bio-Rad, CA, USA).

Concentrations of lipoproteins are expressed in terms of their protein content as determined by the Lowry method [15].

*2.2. Preparation of native LDL*

LDL was isolated from normal human plasma (anti-coagulated with 1/10 volume of 3.8% (w/v) trisodium citrate) immediately after blood sampling by sequential centrifugation at $100\,000 \times g$ in the density range $d = 1.019$–$1.063$ g/ml [16] in the presence of 100 μM EDTA. After flotation, the LDL was filtered (0.45 μm).

*2.3. Acetylation of LDL*

Acetylation was performed by the method of Basu et al. [17] on ice. The reaction mixture was rebuffered after 1 h by gel filtration.

*2.4. Oxidation of LDL*

After exhaustive dialysis of LDL against PBS to remove EDTA, LDL (at a concentration of 200–250 μg/ml) was incubated with $CuSO_4$ (5 μmol/l f.c.) at 37°C in borate buffer (0.1 M, pH = 7.2). After 24 h, the incubation was stopped and the lipoproteins were concentrated by the use of Ultrafree-15 with a MW cut-off of 100 000 (Millipore, Vienna, Austria) to a protein concentration of approximately 2000 μg/ml. Subsequently, OxLDL was rebuffered (PBS, 0.1 M, 100 μM EDTA).

*2.5. Radioiodination*

Radioiodination of OxLDL was performed by the Iodo Bead method using *N*-chloro-benzenesulfonamide-conjugated polystyrene beads (Pierce, Rockford, IL, USA) following the manufacturers instructions. The specific radioactivity of the labelled OxLDL species ranged from 150 to 250 cpm/ng protein.

*2.6. Isolation of human platelets*

Human platelets were isolated from freshly drawn blood as described previously [18]. Briefly, blood was anti-coagulated with 1/10 volume of 3.8% (w/v) trisodium citrate and centrifuged immediately at $120 \times g$ for 20 min to yield platelet rich plasma. Prostacyclin ($PGI_2$) was added at f.c. of 25 μg/l. Platelets were pelleted at $800 \times g$ for 5 min and resuspended with Tyrode buffer without $Ca^{2+}$ (NaCl 137 mM, KCl 2.7 mM, $NaHCO_3$ 11.9 mM, $MgCl_2$ 1.0 mM, $NaH_2PO_4$ 0.42 mM, D-glucose 5.5 mM, human serum albumin 3.5 g/l, $PGI_2$ 25 μg/l, pH 6.5). This washing procedure was repeated twice and the final resuspension of the platelet pellet was performed with Tyrode buffer containing 2 mM $CaCl_2$ (Tyrode-$Ca^{2+}$), pH 7.35, without $PGI_2$. After isolation, the platelet suspension was immediately chilled on ice for at least 30 min before performing the binding assays.

*2.7. Cell culture*

Murine macrophage J774A.1 and human monocytic THP-1 cells were obtained from the European Collection of Cell Cultures (ECACC) and cultured in the recommended media (DMEM and RPMI 1640, respectively, as basal media) in the presence of penicillin (100 U/ml) and streptomycin (100 μg/ml) under the conditions given by ECACC.

Before performing binding studies, THP-1 was differentiated for three days by use of 64 nM phorbol myristate acetate (Sigma) as described [19].

*2.8. Binding studies*

To make the obtained data comparable between platelets and the monocyte macrophage cell lines, all binding studies were performed in PBS (with 2 mM $CaCl_2$ added, pH = 7.35) for 3 h on ice. Albumin was present throughout the incubation procedure at a concentration of 0.1%. After incubation with the labelled OxLDL, platelet suspensions were centrifuged through a prechilled 1:3 dilution of Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) in saline. The supernatants (representing the unbound OxLDL fraction) were aspirated and the tips containing the pellets (representing the bound OxLDL fraction) were cut off using a hot wire [18].

J774A.1 and THP-1 cells were incubated with lipoproteins in 24 wells under the above described conditions. Supernatants were aspirated and collected. The cell monolayer was washed three times and the washing fluids were pooled with the previously aspirated supernatants. Cells were lysed using 0.2 M NaOH.

Bound and unbound fractions were counted in a Cobra II gamma counter (Canberra Industries, Meriden, CT, USA).

Non-specific binding was determined by adding a 20-fold excess of unlabelled OxLDL to the incubation mixture and was usually between 10 and 20% of the total binding.

Trichloroacetic acid-soluble degradation products were assessed as previously described [20]

## 3. Results and discussion

The aim of this study was to compare the behavior of AcLDL and OxLDL when they were binding to human platelets. As there are no data available about the existence of AcLDL binding receptors on the platelet membrane and as human platelets bear two receptors (namely CD36 and CD32) that have been described as potentially exclusive OxLDL receptors in transfected cells [19,21], we speculated about a different binding behavior of OxLDL and AcLDL to human platelets compared to other cell types.

In general, macrophages are considered the 'classic' SR-bearing cell type and are a recognized model for the examination of modified LDL binding.

For this reason, results gained using human platelets were compared with those from similar experiments using the murine macrophage cell line J774A.1, as J774A.1 is probably the best studied cell line in terms of its interaction with modified LDL.

Binding experiments reveal a specific binding of both OxLDL and AcLDL to J774A.1 macrophages with a $K_d$ of $1.95 \pm 0.17$ μg/ml and $2.88 \pm 0.6$ μg/ml, respectively. Analogous studies carried out on human platelets are also able to demonstrate a saturable, high affinity binding of OxLDL to this cell type, which is in accordance with previously published data [19,22].

The affinity of the observed OxLDL binding to human platelets ($K_d = 4.17 \pm 0.8$ μg/ml) is within the range of that obtained with J774A.1 cells. Approximately $3674 \pm 460$ binding sites per platelet can be found and the data gathered tend to indicate a single class of receptors (not shown). However, this does not preclude the existence of several types of OxLDL binding receptors as virtually all presently known SRs display a similar degree of affinity to modified LDL.

In marked contrast to results attained using macrophages, no specific binding of AcLDL to platelets can be observed, a clear indication of the absence of 'classic' SRs (SR-AI/AII) in the platelet membrane.

Accordingly, AcLDL, as shown in Fig. 1, is not able to

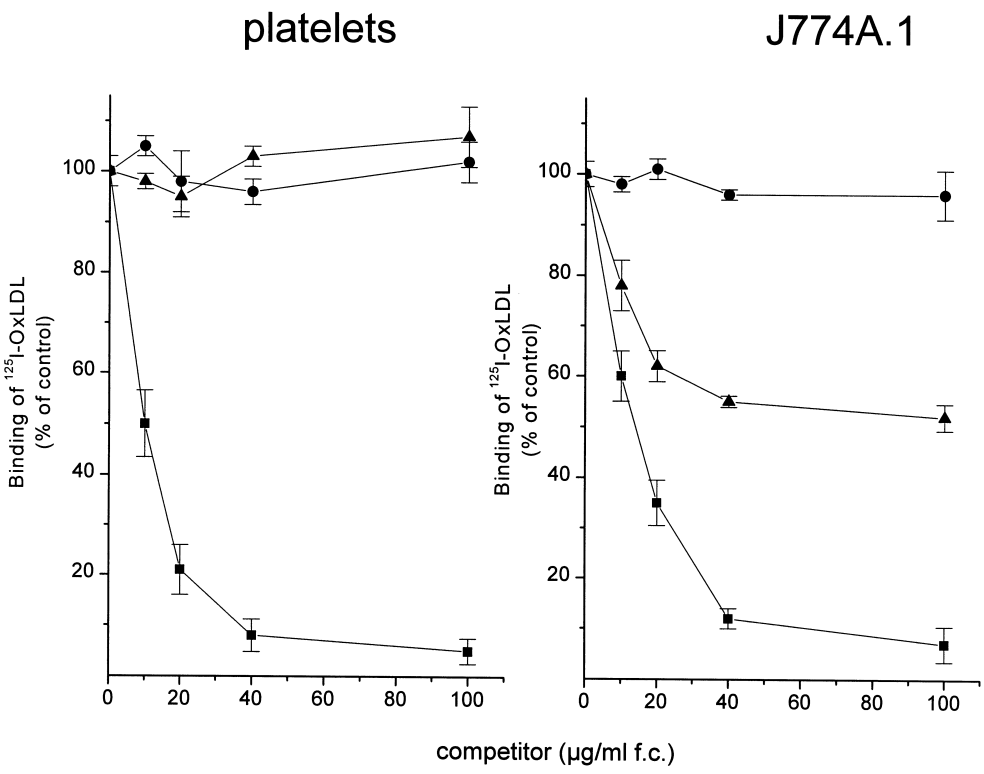


Fig. 1. Influence of native LDL (●), AcLDL (▲) and OxLDL (■) on the total binding of $^{125}$I-OxLDL (10 μg/ml) to human platelets and J774A.1 cells. Each data point represents the mean of three experiments in duplicate ± standard deviation (S.D.).

compete with platelet bound OxLDL, a finding that is corroborated by recently published data [19], as is the fact for AcLDL that can partly compete with the binding of OxLDL to the macrophages [2]. Native LDL does not appear to have any influence on the binding of OxLDL in either of the investigated cell types.

To more thoroughly investigate the binding properties of platelet OxLDL receptors, established antagonists of currently classified SRs were used. As shown in Fig. 2, maleylated albumin (an antagonist of OxLDL binding to SRs of both class A and B) is capable of effectively competing with the binding of OxLDL to both cell types, essentially reducing OxLDL binding to both platelets and J774A.1 macrophages to background levels.

In contrast, fucoidan (as polyinosinic acid, not shown), a selective inhibitor of OxLDL binding to SRs of class A, is only capable of exercising an inhibitory influence on the binding of OxLDL in J774A.1 cells (reducing binding to 36 ± 8%). In human platelets, an increase rather than a decrease in the OxLDL binding can be observed.

Both the inability of fucoidan to compete with the OxLDL binding to human platelets and the fact that platelets show no binding specificity for AcLDL provide compelling evidence for a lack of class A SRs on human platelets.

Instead, all data presented clearly argue for an involvement of class B SRs in OxLDL binding to human platelets. Interestingly, even the observed increase of modified LDL binding in the presence of fucoidan has been observed in this class of receptors [23].

SRs of class B consist of two members, namely SR-BI and CD36. While it is generally accepted that SR-BI shows specificity for AcLDL (which also competes with OxLDL for binding to this receptor), it could be shown by several groups that in the case of CD36, there would seem to be an exclusive ligand specificity for OxLDL [19].[24,25] However, there are findings that seem to contradict this [23,26,27].

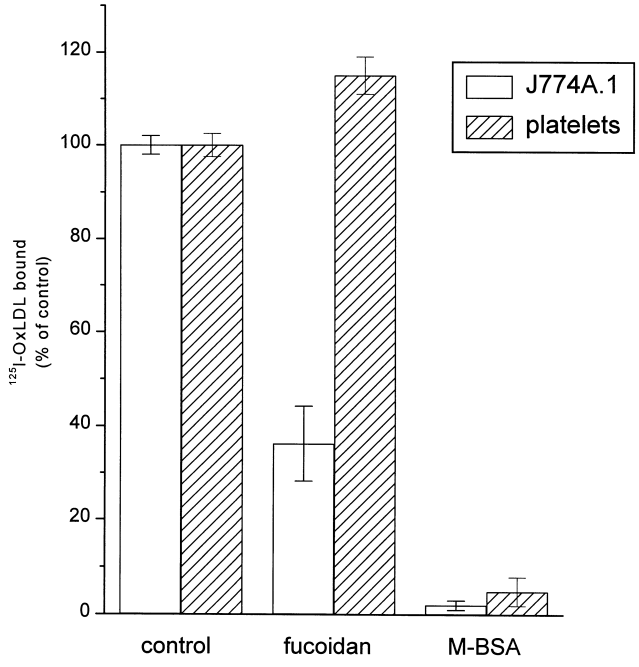


Fig. 2. Specific binding of $^{125}$I-OxLDL (10 μg/ml) to human platelets and J774A.1 cells in the absence and presence of the SR antagonists fucoidan and M-BSA (50 μg/ml each). Data represent the mean of four experiments in duplicate ± S.D.

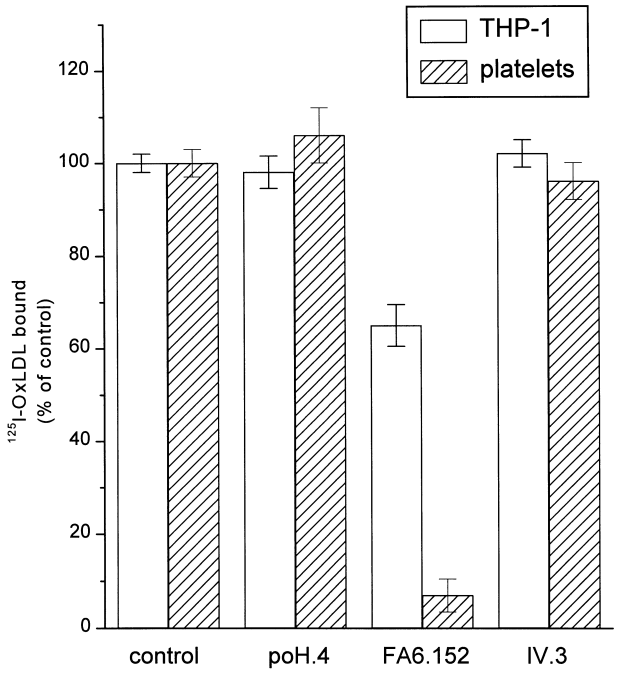


Fig. 3. Specific binding of $^{125}$I-OxLDL (10 μg/ml) to human platelets and differentiated THP-1 cells in the presence of mAbs FA6.152, IV.3 and the polyclonal antibody poH.4 (anti-CD36, CD32 and platelet GPIIb/IIIa, respectively, f.c.: 4 μg/ml each). Data represent the mean of four experiments in duplicate ± S.D.

CD36 is identical with GPIV [28] and was located as such in the platelet membrane quite some time ago where it functions as a thrombospondin and collagen receptor. The fact that CD36 has been proven to be present in the platelet membrane and in view of the above-mentioned results, CD36 would seem to be a prime candidate for the platelet OxLDL receptor.

Therefore, the influence of the mAb FA6.152 on the OxLDL binding to human platelets was of special interest, as this antibody is directed against the OxLDL binding domain of CD36 (155–183). As the binding region of CD36 for OxLDL differs in human and murine membrane proteins [29] and as respective antibodies are only able to compete with OxLDL when binding to human, but not to murine CD36 [19,29], the human monocyte cell line THP-1, that had been differentiated with PMA, was used for subsequent comparative experiments instead of murine J774A.1 cells.

As shown in Fig. 3, FA6.152 is able to reduce the specific OxLDL binding to human platelets to 7 ± 3.5%, which seems to suggest an essential (if not exclusive) role for CD36 as the platelet OxLDL receptor. In THP-1 cells, FA6.152 hinders about 35% of the OxLDL binding, which is in accordance with the reported contribution of CD36 to the overall binding of OxLDL to this cell type [19,24,25].

As also shown in Fig. 3, the polyclonal antibody poH.4 directed against GPIIb/IIIa does not interfere with the binding of OxLDL. This is of special interest as GPIIb/IIIa is the platelet receptor not only for adhesive proteins such as fibrinogen and fibronectin, but also for native LDL [30]. As poH.4 has recently been shown to block the binding of native LDL to human platelets [13], this, although consistent with the results shown in Fig. 1, represents an important finding, as it clearly disputes the suggested role of GPIIb/IIIa as a receptor for both native and OxLDL [22].

Additional studies were performed with Fab fragments of IV.3, a mAb directed against human CD32 (Fc gamma RIIA) able to block the binding of monomeric IgG and IgG immune complexes to this receptor. The finding that IV.3 does not interfere with the OxLDL binding to both investigated cell types rules out the possibility that the observed inhibition of OxLDL binding to human platelets in the presence of FA6.152 might (also) be caused by blocking the binding of OxLDL to CD32 through the Fc portion of the anti-CD36 antibody.

This is of particular interest as the murine counterpart of this receptor (namely Fc gamma RIIB) has been reported to represent an OxLDL receptor [21], although this finding could only be substantiated in transfected but not in natural cells. Of course, on the basis of the available data, it cannot be precluded that platelet CD32 binds OxLDL through epitopes not related to Fc and IV.3 binding. However, findings exist that human CD32 is not involved in the xLDL binding [31] and from the data presented in this study, the contribution of CD32 to the OxLDL binding can be assumed to be minimal, at best.

The results presented here allow for the first time the identification of a natural cell type capable of exclusively binding OxLDL without showing specificity for AcLDL. Evidence of exclusive OxLDL binding is particularly interesting as AcLDL and OxLDL are sometimes considered to be equivalent ligands in relation to the SR system. Consequently, AcLDL (having the advantage of not being toxic) is often used to assess the contribution of SRs to different (patho)physiological processes.

Recently published findings obtained with CD36-transfected cells have produced conflicting statements on the question of whether CD36 is able to bind both OxLDL and AcLDL or whether it acts as an exclusive OxLDL receptor. The reasons for these controversial findings remain unclear. One possibility put forward suggests the involvement of as yet unidentified co-factors [25], although possible variations within CD36 itself should perhaps be first more closely examined.

CD36 is a heavily glycosylated protein and it is known that the *Mr* values for CD36 strongly differ between various cell types within the same species as a consequence of a different degree of cell type specific glycosylation.

It therefore cannot be discounted that the degree of glycosylation might interfere with the binding of some ligands to CD36, either directly or due to changes of the quarternary structure of the receptor protein. This gives rise to the intriguing possibility that the controversial nature of the findings might be the result of post-translational events in different cell types. Taking this into account, it could well be expected that the binding specificity of CD36 expressed not only in transfected cells but possibly also in different tissues may differ from the results shown here for human platelets.

Interestingly, there also seems to be a difference with regard to post-binding events triggered by platelet CD36 and CD36 expressed by transfected cell lines. Following the binding of OxLDL to this receptor, transfected cells have been reported to internalise and degrade OxLDL [19,24]. Although human platelets are capable of endocytosis in principle, we do not find any trichloroacetic acid-soluble degradation products following incubation with $^{125}$I-OxLDL for so long as the full

functionality of the isolated platelets is preserved (approximately 2 h). Nevertheless, this period of time was sufficient for J774A.1 macrophages to degrade 460 ± 72 ng of OxLDL/mg of cell protein when incubated with 5 μg/ml OxLDL. This finding, together with the fact that the same amount of platelet bound $^{125}$I-OxLDL can be displaced by the subsequent addition of unlabelled OxLDL at both 4°C and 37°C (not shown), is a strong argument for an inability of platelet CD36 to internalise (and degrade) OxLDL.

However, apart from the above suggested explanations suggesting CD36 heterogeneity, the possibility remains that the observed AcLDL binding by CD36-transfected cells might not solely be a consequence of differences within the receptor protein, but (also) regarding the ligand, i.e. AcLDL.

It could be shown that an even comparatively modest oxidation of LDL is adequate to effect the binding specificity for CD36 [19]. However, as even lightly oxidized AcLDL shows specificity for CD36 (unpublished results), the possibility remains that some AcLDL preparations do acquire binding specificity through inadvertent oxidation (in the course of isolation, acetylation or labelling) rather than by acetylation.

Further studies will be necessary to establish whether the binding specificity of platelet CD36 described here is also true for CD36 expressed by other natural cells. In any case, the identification of an exclusive OxLDL receptor demands a critical reassessment of some of the findings previously obtained in functional studies using AcLDL.

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