Collagen-bound low density lipoprotein modifies endothelial cell adhesion to type V collagen: Implications for atherosclerosis

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SUMMARY

Low density lipoprotein (LDL) is retained in the extracellular matrix of the arterial wall where it is considered to be atherogenic, but little is known about how cell adhesion to the matrix is affected by collagen-bound LDL. We tested the effect of native, oxidized and acetylated LDL reacted with adsorbed monomeric type I, III and V collagen on endothelial cell adhesion to collagen using a colorimetric adhesion assay. We found that none of the LDL species affected adhesion to type I and III collagen, but that collagen-bound native and acetylated LDL enhanced attachment to type V collagen, whereas bound oxidized LDL inhibited adhesion to this collagen. We suggest that oxidized LDL associated with type V collagen in the arterial wall would favor de-endothelialization and contribute to atherogenesis and thrombosis.

Keywords: Low density lipoprotein, cell adhesion, type V collagen, atherosclerosis, endothelium.

Abbreviations: AcLDL, acetylated LDL; DMEM, Dulbecco's modified Eagle's medium; LDL, low density lipoprotein; natLDL, native LDL; oxLDL, oxidized LDL.

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1 INTRODUCTION

Critical interactions between atherogenic lipoproteins, extracellular matrices and vascular wall cells astutely govern the progression of atherosclerotic disease. Atherogenesis is especially exacerbated when circulating low density lipoprotein (LDL) seeps into the arterial wall and is sequestered in the subendothelial matrix. Bound to the matrix, LDL is prone to oxidation by reactive oxygen species, peroxynitrite and enzymes such as lipoxygenases and myeloperoxidase [1]. Oxidized LDL (oxLDL) is particularly atherogenic, because it elicits recruitment of blood monocytes into the artery, transformation of macrophages and smooth muscle cells into foam cells, cell proliferation, inflammatory responses, and changes in cell adhesive properties [2]. Furthermore, oxLDL is thought to cause endothelial cell insult and to inhibit re-endothelialization of injured areas of the arteries [3, 4]. Consequently, accumulation of LDL in the arterial wall contributes to the development of atherosclerosis and related diseases.

Although LDL is clearly retained in the extracellular matrix both *in vivo* and *in vitro*, surprisingly little definitive information is available on which specific component LDL reacts with, including collagen itself, the major constituent of the matrix. The incorporation of LDL into the matrix and its binding to collagen is thought to be facilitated by intermediaries such as lipoprotein lipase [5-9], glycosaminoglycans [10], and decorin, a small dermatan sulfate-rich proteoglycan [11], and it is likely that additional accessory molecules will be discovered. With regard to direct interactions between LDL and collagen gels, several experimenters [12-14] viewed type I collagen as a major trapper of native LDL (natLDL) and especially of oxLDL. In contrast, Pentikainen *et al.* [11] reported that LDL does not bind appreciably to plastic-adsorbed type I collagen alone. Greilberger *et al.* [15] described limited association of natLDL with adsorbed type I collagen at low ionic strength, but strong binding of oxLDL. However, at physiological ion concentration they found that both natLDL and oxLDL associate with this collagen in equivalent amounts. These binding kinetics have been confirmed by Takei *et al.* [16] for type I collagen gels. NatLDL and oxLDL also bind to type III collagen about as well as to type I collagen, but both lipoproteins react less avidly with type V collagen [14, 15]. Quantitative experimental studies on interactions between type V collagen and various forms of LDL are lacking.

Since subendothelial matrix-bound LDL is thought to profoundly affect numerous aspects of cell behavior including adhesion, we studied the attachment of endothelial cells to plastic-adsorbed type I, III and V collagen that had been reacted with natLDL, oxLDL or acetylated LDL (acLDL) following adsorption. We reasoned that the LDL modifications might elicit specific differences in cell adhesion. We found that type V collagen and LDL together constitute a hitherto unrecognized biologically active adhesive substrate of possible relevance to atherogenesis in general.

2 METHODS

Cell culture – Endothelial cells were isolated enzymatically from bovine aortas obtained from a slaughterhouse using established procedures and were characterized by staining of von Willebrand factor. They were cultivated in DMEM with 10% FCS under 5% CO_2 at 37°C. Cells passaged less than ten times and grown to subconfluency were used.

Collagen preparation – Type I and type III collagen were isolated from calf skin by acid-extraction and limited pepsin digestion, respectively, and purified by fractional salt precipitation [17]. Type V collagen, obtained from bovine amnion by pepsin digestion, was first purified as described by Rhodes & Miller [18]. The final precipitate described by the authors was solubilized in 0.02 mol/L sodium acetate, pH 4.8, and chromatographed on a Whatman CM-52 cation exchange column (Biometra, Göttingen, Germany). The two chain form of type V collagen, $[\alpha1(V)]_2\alpha2(V)$, was eluted in a single peak following type IV collagen using a 0-0.1 mol/L NaCl gradient. Purified collagens were tested for homogeneity using polyacrylamide gel electrophoresis, lyophilized and stored at -20°C.

Isolation and modification of LDL – NatLDL in the density range of 1.02-1.06 g/ml was isolated from pooled human

plasma by sequential differential ultracentrifugation and dialyzed extensively against 0.15 mol/L NaCl with 5 mmol/L EDTA and then against 0.15 mol/L NaCl. OxLDL was made by oxidizing natLDL for 6 hours at 37°C using copper sulfate [19]. The oxidation status of oxLDL was estimated on the basis of the amount of thiobarbituric acid-reactive substances present as described by Thomas & Jackson [20]. The oxLDL used contained an equivalent of about 5 nM malondialdehyde/mg protein and thus was weakly oxidized (data not shown). AcLDL was obtained by reacting natLDL with acetic anhydride [21]. Completeness of acetylation was verified by agarose gel electrophoresis. The finished products were sterile-filtered and stored at 4°C for no longer than nine days before use.

Coating of microtiter plates – Lyophilized collagen was solubilized in 0.1% acetic acid at a concentration of 1 mg/ml and adsorbed to 96-well Polysorb microtiter plates (Nunc, Wiesbaden, Germany) overnight at 4°C at six successive three-fold dilutions from 10 to 0.04 µg/ml in volumes of 100 µl per well. Two wells were used per collagen type and concentration in each experiment. Following adsorption, the plates were washed with water, blocked with 0.5% BSA and washed again. The adsorbed collagen in the wells was incubated with 100 µl per well of the LDL preparations diluted in Dulbecco's PBS without calcium and magnesium chloride (Sigma, Deisenhofen, Germany) to a protein concentration of 100 µg/ml or with 100 µl of neat PBS overnight at 4°C. The plates were washed again and used immediately for the adhesion tests.

Cell adhesion assay – Cultivated cells were suspended by trypsinization and washed with serum-free DMEM to minimize interference by serum proteins during attachment. Approximately 40,000 cells in 100 µl of serum-free medium were added to each well and allowed to attach at 37°C for 3 hours. The plates were carefully washed twice by hand with PBS to remove non-adherent cells. The relative number of cells in each well was assayed colorimetrically using a MR 5000 plate reader (Dynatech, Krefeld, Germany) essentially as described by Kueng *et al.* [22], except that 1% toluidine blue in PBS was used for staining and that extinctions were measured at 590 nm. Measured extinctions were linearly proportional to cell numbers in the range of 2,000 to at least 30,000 cells (r = 0.9985, $p < 0.001$; data not shown).

3   RESULTS

The adhesion of endothelial cells to increasing amounts of adsorbed type I, III and V collagen reacted with natLDL, oxLDL or acLDL and to the same concentrations of adsorbed, unreacted collagens was analyzed. All solutions from which the collagens were adsorbed contained pure collagen molecules as shown by polyacrylamide gel electrophoresis under non-reducing and reducing conditions (Figure 1) in monomeric form as assessed by rotary shadowing and electron microscopy (data not shown). The adhesion experiments were repeated four times. Examination of the extinction curves for type I and III collagen with and without LDL showed that adhesion rose rapidly with the amount of collagen in the adsorption solution, reaching saturation at a collagen concentration of < 2 µg/ml. Therefore, to pool the results, all extinction data for each experiment were scaled by a factor such that the average of the extinctions for those wells containing pure type I collagen at the concentrations 3.33 and 10 µg/ml (four values) corresponded to 1. The adjusted experimental data, scaled in this way and pooled over the four experiments, are given graphically in Figure 2.
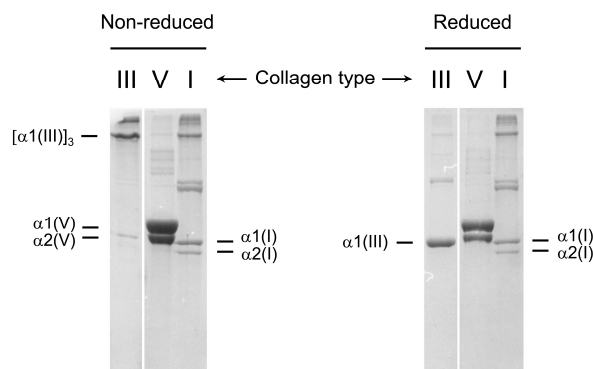

Figure 1: *SDS polyacrylamide gel electrophoresis of collagens type I, II and V preparations used in the adhesion experiments.* The preparations show the expected pattern of each of the single collagen polypeptide chains as described by Rhodes & Miller [18]. Bands not labeled in this figure represent oligomers of the single collagen chains such as $[\alpha1(I)]_2\alpha2(I)$, $[\alpha1(I)]_2$, and $\alpha1(I)\alpha2(I)$.

Comparison of the adhesion curves in Figure 2.A and 2.B shows that endothelial cells adhered about as readily to type I as to type III collagen regardless of whether the collagen had been reacted with LDL or not; collagen-bound LDL had no effect on adhesion to these collagens. In comparison to type I and III collagen, the affinity of the cells to type V collagen was significantly lower for all substrates and for all collagen coating concentrations tested ($p < 0.01$) except the lowest, 0.04 µg/ml (cf. Figures 2.A and 2.B with 2.C). Saturation of adhesion for cells on type V collagen was reached more slowly and at noticeably higher collagen concentrations (>3 µg/ml) than for cells on the other collagens. Moreover, adhesion to type V collagen was strongly dependent both on the presence of collagen-bound LDL and on the composition of the LDL (Figure 2.C); compared to adhesion to the pure collagen alone, adhesion to type V collagen was elevated in the presence of natLDL and especially of acLDL, and compared to natLDL, oxLDL inhibited endothelial cell adhesion to type V collagen, whereas acLDL enhanced adhesion. To quantify these relationships, we calculated the percentages of cells adhering to type V collagen with oxLDL and acLDL relative to the number of cells that adhered to type V collagen with natLDL for the two collagen concentrations (3.33 and 10 µg/ml) at which adhesion approached maximum (Table 1). Only about two thirds as many cells adhered to type V collagen with associated oxLDL as to collagen with natLDL, whereas approximately one quarter more cells adhered to collagen with acLDL than to collagen with natLDL. These differences were significant at the levels indicated in Table 1. Approximately twice as many cells adhered to collagen with acLDL as to collagen with oxLDL ($p < 0.001$; Figure 2.C).

Table 1: *Endothelial cells adhering to adsorbed type V collagen reacted with oxidized low density lipoprotein (oxLDL) and acetylated low density lipoprotein (acLDL) relative to cells adhering to collagen reacted with native low density lipoprotein (natLDL).*

Concentration µg/ml	natLDL %	oxLDL %	acLDL %
3.33	100	70*	135*
10	100	68[‡]	120[†]

Probabilities of the observed number of adhering cells being the same as the number of cells that adhered to collagen with natLDL,* $p < 0.01$, [†] $p < 0.05$, [‡] $p < 0.001$, were estimated using Student's t-test. Concentrations given refer to the amount of collagen in the coating solutions.

4   DISCUSSION

We examined whether adhesion of vascular endothelial cells to adsorbed monomeric interstitial collagen is altered when LDL is complexed with the collagen. Adhesion to type I and III collagen is not affected by collagen-bound LDL. In contrast, adhesion to type V collagen is strongly influenced by the presence of LDL and by the composition of the LDL. Most importantly, adhesion is significantly inhibited by collagen-bound oxLDL compared to adhesion to this collagen with associated natLDL. We conclude that endothelial cell adhesion to type V collagen is modified by collagen-bound LDL.

Our studies reveal effects of collagen-bound LDL on adhesion, but they do not clarify whether endothelial cell attachment takes place to collagen, LDL, or both, or indirectly via other molecules. Although the cells were allowed to adhere for only a short time (3 hours) and in a serum-deficient environment, they may have been able to mobilize sufficient quantities of accessory molecules that might also play a role in attachment. Nevertheless, endothelial cell adhesion to the type I and III collagen substrates evidently does not depend on LDL and its receptors [23,24], because the kinetics of attachment to bare type I and III collagen and to these collagens with associated LDL are the same. A common receptor for both collagen and LDL, such as CD36 [25], might be involved. It is also possible that insufficient amounts of LDL bind to type I and type III collagen to influence adhesion noticeably. On the other hand, LDL is reported to have substantially higher affinity for type I collagen than for type V collagen [14, 15], and as we have shown, adhesion to type V collagen is clearly influenced by collagen-bound LDL. The observed differences in adhesion to type V collagen with LDL might be due to variations in the amounts of collagen-bound LDL and consequent differential masking of collagen-cell binding sites by the LDL, but we consider this explanation unlikely, because adhesion was enhanced by natLDL and acLDL compared to adhesion to the collagen alone. We conclude that the modalities of adhesion to type I and III and to type V collagen with collagen-bound LDL are quite different.
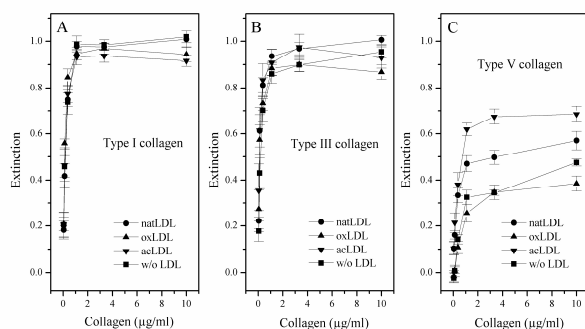

Figure 2: Kinetics of adhesion of endothelial cells to collagen with and without (w/o) collagen-bound low density lipoprotein (LDL). Cells were allowed to attach to microtiter plates with adsorbed collagen that had been reacted with LDL. Relative numbers of adhering cells were estimated colorimetrically. Extinction is in arbitrary units. The x-axis gives the collagen concentration in the coating solutions (means ± SEM; n = 8 for each concentration). (A, B) Lack of effect of any kind of LDL on adhesion to type I and III collagen. (C) Enhancement of adhesion to type V collagen by collagen-bound native LDL (natLDL) and acetylated LDL (acLDL), and inhibition of adhesion by bound oxidized LDL (oxLDL).

Viewed in the context of the artery, modification of adhesion to type V collagen by LDL could obviously have a bearing on the adhesiveness of endothelial cells to the subendothelium.

Type V collagen has traditionally been regarded as a regulator of collagen fibrillogenesis [26, 27]. However, this collagen also influences cell adhesion strongly, because endothelial cells, platelets, monocytes and smooth muscle cells adhere less to it than to the other fibril-forming collagens *in vitro* [28-31], and coatings of type V collagen inhibit endothelialization of prothetic surfaces [32]. Accordingly, type V collagen is considered antithrombogenic. Our own adhesion tests on human and porcine smooth muscle cells revealed just baseline attachment to this collagen with or without LDL, and in marked contrast to endothelial cells, no modification of adhesion whatsoever with natLDL or acLDL (data not shown). Type V collagen generally comprises only a few percent of the interstitial collagen in the normal artery, but it is enhanced under pathologic conditions [33]. It represents a major expressed collagen in cultured endothelial cells which secrete it predominantly into the cell layer and not into the medium, as is the case for type III collagen [34]. Using immunohistochemistry and an anti-type V collagen antibody (clone V-3C9; ICN, Aurora, Ohio), we have seen strong expression of type V collagen in and near the human arterial endothelium (data not shown). Hence, the basal surfaces of endothelial cells in the artery would be expected to be extensively exposed to this collagen. OxLDL accumulated in the subendothelium would also be expected to interact basolaterally with endothelial cells [16]. Along with oxLDL and other collagens, type V collagen is present in substantial quantities in atherosclerotic plaques [35, 36]. As we have shown, endothelial cell adhesion to type V collagen is inhibited by collagen-bound oxLDL. Thus, acting in concert, type V collagen with bound oxLDL would tend to impair endothelial cell adhesion in the endothelium. We think that the known toxic effect of oxLDL may be related to inhibition of adhesion of endothelial cells to the subendothelial matrix as a result of the association of oxLDL with type V collagen.

We speculate that reduction in endothelial cell adhesion to type V collagen due to collagen-bound oxLDL might be one factor promoting de-endothelialization in the hyperlipidemic artery. This may be an initial and crucial event in arterial injury. Conversely, the enhanced attachment to type V collagen with bound natLDL seen by us may represent the basal adhesion level to this collagen in the *in vivo* situation where natLDL and type V collagen complexed with type I collagen would always be present. To summarize, type V collagen with bound oxLDL would be atherogenic. The de-endothelializing tendency of oxLDL bound to type V collagen would contribute exquisitely and ultimately to plaque and thrombus formation.

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