Fibronectin Stimulates Macrophage Uptake of Low Density Lipoprotein-Heparin-Collagen Complexes

Domenick J. Falcone and Brian G.J. Salisbury

In this study, we investigated whether fibronectin will enhance macrophage uptake of particulate complexes of low density lipoproteins (LDL) and whether fibronectin's opsonic effect could be modulated by the heparin component in these model matrices. We isolated a heparin fraction (HepFn) based on its affinity to fibronectin. HepFn appeared more charged than unfractionated heparin, as evidenced by enhanced electrophoretic mobility and ability to effect a cathodic shift in the electrophoretic migration of fibronectin. HepFn lacked the smaller molecular weight species present in unfractionated heparin. Macrophage endocytosis of LDL-heparin-collagen complexes, as evidenced by the intracellular accumulation of LDL-derived cholesterol esters and endogenously synthesized cholesteryl esters, was enhanced by fibronectin. When LDL matrix complexes were prepared with HepFn, fibronectin's opsonic properties were significantly enhanced. F(ab)2 fragments of anti-fibronectin, capable of inhibiting fibronectin's opsonization of gelatin-derivated latex particles, inhibited the fibronectin-dependent stimulation of cholesteryl ester synthesis by macrophages exposed to LDL-HepFn-collagen complexes. Thus, fibronectin stimulates macrophage endocytosis of LDL matrix complexes. The affinity of the constituent glycosaminoglycan for fibronectin is important in the regulation of this phenomenon. (Arteriosclerosis 8:263–273, May/June 1988)

Macrophage ingestion of low density lipoprotein (LDL) is dramatically enhanced when LDL is insolubilized by components of the extracellular matrix. Our previous experiments have shown that LDL incorporated into a model extracellular matrix containing heparin, fibronectin (Fn), and denatured collagen (gelatin) was readily phagocytosed by macrophages. However, in contrast to either LDL or chemically modified LDL, in which the degradation of lipoprotein paralleled the uptake, the endocytosis of insoluble LDL matrix complexes rapidly exceeded the macrophage's capacity to degrade LDL. Consequently, lipoprotein-driven, cholesterol esterification measured in these cells was limited by the slow rate of lysosomal hydrolysis of endocytosed LDL. Although it has been suggested that the interaction of Fn with glycosaminoglycans and collagen may have important biologic consequences in localizing monocyte-macrophages at sites of tissue injury and stimulating the uptake of tissue debris, it was not clear from our earlier studies if the enhanced uptake of LDL matrix complexes by macrophages was a consequence of simply insolubilizing LDL with matrix components or due to the presence of Fn in the model matrix.

Integral to the latter phenomenon would be the interaction of lipoprotein matrix complexes with Fn. It has been demonstrated that Fn binds heparin, heparan sulfate, and hyaluronic acid. In addition, Fn co-distributes and co-precipitates with heparan sulfate and chondroitin sulfate proteoglycans. Among the various interactions between Fn and glycosaminoglycans, Fn exhibits the highest affinity for heparin. Each Fn monomer contains at least three distinct binding sites capable of interacting with heparin with differential affinity and sensitivity to cations. Although it has been shown that Fn binds heparin, heparan sulfate, and hyaluronic acid, a more recent study indicates that it may interact with Fn fibril formation, which enhances Fn binding to native or denatured collagen, and enhances the binding of Fn-collagen complexes to macrophages. Indeed, it has been reported that heparin enhances Fn-mediated phagocytosis of gelatin-derivated latex particles by macrophages.

Since the binding of sulfated glycosaminoglycans to Fn can regulate both the interaction of Fn with collagen and of Fn-collagen complexes with macrophages, then the interaction of lipoprotein with glycosaminoglycans demonstrating high affinity to Fn may be important in the uptake of LDL matrix complexes. This report describes the isolation of a heparin fraction (HepFn) based on its affinity to Fn and its effect on LDL uptake by macrophages when incorporated into a complex containing heparin, collagen, and Fn. Specifically, we measured the uptake of both complex-derived apolipoprotein and cholesteryl ester and the subsequent stimulation of endogenous cholesteryl ester synthesis. These experiments demonstrate that Fn stimulates macrophage ingestion of LDL-heparin-collagen complexes. Fn's opsonization of LDL matrix complexes was enhanced...
when complexes were prepared with a heparin fraction exhibiting high affinity to Fn and was blocked by F(ab)\(_2\) fragments of anti-Fn.

**Methods**

**Materials**

Fresh, human, platelet-poor plasma for Fn and lipoprotein isolations was provided by the New York Blood Center (New York, NY). Dulbecco’s modified Eagle’s medium (DMEM), Roswell Park Memorial Institute (RPMI)-1640, Dulbecco’s phosphate-buffered saline (DBPS), penicillin/streptomycin sulfate, and trypsin-EDTA were purchased from Flow Laboratories (McLean, VA). Iodo-\(^{131}\)I-acetic acid (5 to 15 mCi/mmol), \(^{1-14}\)C-oleic acid (40 to 60 mCi/mmol), \(^{9,10-3}\)H-oleic acid (2 to 10 Ci/mmol), cholesteryl-1,2,6,7-\(^{3}H\)-ilinoleate (75.3 Ci/mmol), 1,2-\(^{3}H\)-cholesterol (40 to 60 Ci/mmol), \(^{4-14}C\)-cholesterol (50 to 60 mCi/mmol), iodine-125 (17 Ci/mg, carrier-free), and Aqua sol were obtained from New England Nuclear (Boston, MA). Gelatin-Sepharose 4B, DEAE-Sephacel, heparin-Sepharose CL-6B, and Sephadex G-200 (Superfine) were obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Heparin (sodium salt) was purchased from Sigma (St. Louis, MO), and chondroitin 6-sulfate (sodium salt) was a product of Seikagaku Kogyo and purchased through Miles (Naperville, IL). Heparan sulfate (sodium salt) was an NIH-reference standard previously provided by Martin B. Mathews under NIH Contract No1-AM-5-2205. Calf-skin collagen was purchased from Cabiochem-Behring (La Jolla, CA). F(ab)\(_2\) fragments of anti-Fn (37 mg/ml) prepared from F(ab)\(_2\) fragments of anti-Fn (37 mg/ml) prepared from Cappel/Cooper Biomedical (Malvern, PA). Carboxylated Fluoresbrite microspheres (0.28μm, 2.5% solids, 2 × 10\(^{12}\) particles/ml) were purchased from Polysciences (Warrington, PA).

Additional specific products included: Durapore membranes and Swinnex filters (Millipore, Bedford, MA); Diaflo YM-2 ultrafiltration membranes (Amicon, Danvers, MA); Nunc tissue culture dishes (Nunc, Denmark); Linbro tissue culture plates (Flow); Isogel (FMC, Rockland, ME); Brilliant blue R (Aldrich, Milwaukee, WI); and Celagram-II cellulose acetate membranes (Shandon Southern, Sewickley, PA). All other chemicals or solvents were reagent grade or better and were obtained from Sigma Chemical Company.

**Isolation of Plasma Fibronectin**

Fn was isolated according to the procedures described by Ruoslahti et al.\(^{23}\) with minor modifications. Fresh, human plasma was filtered to remove particulates, and 1.0 mM phenylmethylsulfonyl fluoride (PMSF) was added as a protease inhibitor. The plasma was applied to a column of gelatin-Sepharose. Unbound proteins were washed out with 0.1 M sodium phosphate/0.01 M citrate buffer (pH 7.5) containing PMSF. Fn was eluted from the gelatin-Sepharose with 5 M urea/50 mM Tris-HCl (pH 7.5). The eluate was applied to a column of DEAE-Sephacel, and unbound material was washed out with 5 M urea/50 mM Tris-HCl. Fn was eluted with a linear NaCl gradient (0 to 0.3 M) in 5 M urea/50 mM Tris-HCl. The isolated Fn was concentrated to approximately 1 mg/ml by ultrafiltration (YM-2 filter) and was stored at -20°C. Before use, Fn was exhaustively dialyzed against 0.15 M NaCl/0.3 mM EDTA (pH 7.5). Freshly purified Fn migrated as a doublet (\(M_r = 220,000\)) in reduced SDS-polyacrylamide gels.

Fn was carboxymethylated (Cm) with iodo-\(^{131}\)I-acetic acid as described previously.\(^{1}\) Following Cm, \(^{131}\)Cm-Fn was applied to a heparin-Sepharose column equilibrated with 50 mM Tris acetate (pH 7.0) containing 1.0 mM PMSF and 0.02% sodium azide. Unbound, labeled Fn was washed from the column and discarded. Bound Fn was eluted from the heparin-Sepharose with a linear NaCl gradient (0 to 3M) in 50 mM Tris acetate (pH 7.0). The isolated Fn was concentrated and dialyzed as above.

**Fractionation of Heparin**

Purified Fn was adsorbed onto a column of gelatin-Sepharose equilibrated with 50 mM Tris acetate (pH 7.0) containing 1.0 mM PMSF and 0.02% sodium azide. The column was washed free of unbound or excess Fn and 5 mg of heparin in water was added. Fractions were collected and monitored for uronic acid and absorbance at 280 nm. Bound heparin (HepFn) was eluted from the column with 1.5 M NaCl in 50 mM Tris acetate buffer. The eluant was concentrated by ultrafiltration (YM-2 membrane) to approximately 10 to 15 ml and was dialyzed against water; heparin was isolated by precipitation with 1% CPC in the presence of sulfate ions.\(^{25}\) The heparin-CPC precipitate was dissolved with a small volume of 2 M NaCl absolute ethanol (100:15, vol/vol), and heparin was recovered by several cycles of ethanol precipitation.\(^{25}\) The recovered HepFn, pooled from several isolations, was approximately 2 mg. In an effort to isolate a heparin fraction that was unable to bind Fn, 3.6 mg of heparin was applied to the gelatin-Sepharose column in which 25 to 30 mg of Fn was adsorbed. Unbound heparin was recovered by the procedures described above and was reapplied to the regenerated affinity column. The unbound heparin fraction was recovered for a second time and fractionated again. The recovered, unbound fraction was approximately 250 μg.

The heparin fractions were analyzed by cellulose acetate electrophoresis in a cadmium acetate buffer (pH 4.1).\(^{26}\) Heparin fractions were also analyzed by chromatography on a Sephadex G-200 column equilibrated with 50 mM NaCl (pH 6.0) containing 0.005% thimerosal.

**Isolation of Lipoproteins**

LDL (1.019 ≤ d ≤ 1.063 g/ml) was isolated from fresh human plasma by ultracentrifugation.\(^{27}\) Isolated LDL was dialyzed against 150 mM NaCl/0.3 mM EDTA (pH 7.4) at 4°C for 24 hours. Purity of the LDL preparation was determined by electrophoresis in composite agar-agarose gels.\(^{28}\) Acetylation of LDL (acyetyl LDL) was performed according to the method of Goldstein et al.\(^{29}\) as described previously.\(^{1}\) Acetyl LDL migrated to a more cathodic position than native LDL when electrophoresed in agar-agarose gels. LDL was iodinated by the method described by Bilheimer et al.\(^{30}\) Both labeled and unlabeled LDL preparations co-migrated following electrophoresis in agar-agarose gels.

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rose gels. More than 99.9% of the $^{125}$I-LDL (73 811 dpm/μg protein) was precipitable with trichloroacetic acid. In addition, LDL was also labeled by incubating LDL with $^3$H-cholesterol and cholate in dimethyl sulfoxide and dialyzed free of unincorporated label as described previously. The percentage of LDL-$^3$H-cholesterol precipitable by acid or heparin was more than 99%.

**Isolation of Mouse Peritoneal Cells**

Resident peritoneal macrophages were obtained from male BALB/c mice (Rockefeller University) or Swiss Webster mice (Charles River) by the method of Edelson and Cohn as described previously. Peritoneal cells were harvested in D-PBS, collected by centrifugation (400 g for 10 minutes), and were resuspended in DME or RPMI containing 20% heat-inactivated fetal calf serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml). Peritoneal cells were aliquoted into plastic Petri dishes (2 x 10$^6$ cells/35 x 10 mm) or 12-well Linbro plates (5.0 x 10$^5$ cells/well) and were incubated in a humidified CO$_2$ incubator at 37°C for 1 hour. Dishes were then washed vigorously with DPBS, and monolayers were incubated overnight with supplemented DME or RPMI. The experiments were performed the next day.

**Complexing Heparin and Other Glycosaminoglycans with Fibronectin**

The interaction of Fn with various heparin fractions and other glycosaminoglycans was analyzed by electrophoresis in 1% composite agarose-galactomannan gels (isosel) with 50 mM Tris acetate buffer (pH 7.0). Gels were run at 150 V/20 mA for 2 to 2.5 hours with cooling. Proteins were fixed in 10% acetic acid for 30 minutes and were washed free of buffer and acid overnight in water. After drying, gels were stained with 0.1% Brilliant blue R in water/ethanol/acetic acid (65:25:10, vol/vol/vol).

**Binding of $^{14}$C-Cm-Fibronectin to LDL Heparin or LDL Hep$^{fn}$**

LDL (355 μg in 0.15 M NaCl/0.3 mM EDTA) was added to 3 ml of 7.5 mM CaCl$_2$ containing 100 μg heparin (Hep) or Hep$^{fn}$, and was allowed to stand 1 hour at 4°C. Complexes (LDL-heparin or LDL-Hep$^{fn}$) were diluted to 10 ml with 7.5 mM CaCl$_2$ and were dispensed into 1 ml aliquots to which various concentrations of labeled Fn were added. The binding of Fn to these complexes was measured in two ways: by co-precipitation and by a filter binding assay. The co-precipitation of Fn with LDL-heparin complexes was performed as previously described. The filter binding assay was performed as follows by using syringe-loaded Swinnex filters. Samples (1 ml) containing LDL, heparin, and labeled Fn were passed through a prewashed 0.2 μm Durapore filter (GVWP; 13 mm), and the filter was washed by passing through 5 ml of 7.5 mM CaCl$_2$. The filters were removed from the holders and were placed overnight in 500 μl 0.5 M NaOH to solubilize the bound $^{14}$C-Cm-Fn. Vials received 10 ml of Aquasol and were assayed in a liquid scintillation spectrometer.

**Preparation of LDL Complexes with Heparin, Fibronectin, and Collagen**

Insoluble complexes of native LDL, $^{125}$I-LDL, heparin or Hep$^{fn}$, Fn, and denatured collagen were prepared as previously described, with the following modifications. Heparin or Hep$^{fn}$ (100 μg), Fn (1 mg), and denatured collagen (1 mg) were mixed and allowed to stand at room temperature for 15 minutes. The volume was adjusted to 2.9 ml with water, and 355 μg of LDL was added. CaCl$_2$ was added to a final concentration of 7.5 mM. Under these conditions, all of the added LDL and heparin was recovered in the insoluble complexes (data not shown). The concentrations of Fn and gelatin that co-precipitated with the LDL-heparin complexes were not determined. LDL matrix complexes prepared as described above were resuspended in DPBS before addition to cell incubation medium.

In addition to the above procedures, lipoprotein matrix complexes containing calf-skin collagen were prepared as follows: LDL was complexed to heparin or Hep$^{fn}$ in the presence of 7.5 mM CaCl$_2$. LDL-heparin complexes containing 100 μg LDL and 28 μg heparin or Hep$^{fn}$ were isolated by centrifugation and resuspended in a small volume of 0.15 M NaCl/0.3 mM EDTA (pH 7.0) containing 280 μg of calf-skin collagen and collagen and 280 μg Fn. The complexes prepared by this procedure were added directly to the cell incubation medium.

**LDL Uptake, Cell Content, and Degradation**

$^{125}$I-LDL incorporated into complexes prepared with heparin or Hep$^{fn}$ was incubated with macrophage monolayers for 1 hour. LDL uptake, cell content, and degradation were determined by the method of Goldstein and Brown as described previously. Monolayers were treated with 0.5% trypsin/0.02% EDTA for 30 minutes to remove noningested complex-derived LDL. Cells were washed twice with DPBS, and cellular protein was digested with 0.2 M NaOH. Aliquots were removed for gamma counting and protein determinations by the method of Lowry et al.. Uptake, cell content, and degradation were expressed as μg of LDL per mg of cell protein.

**Cellular Accumulation of $^3$H-Cholesteryl Esters**

LDL-$^3$H-cholesterol was complexed to LDL matrix complexes prepared with either heparin or Hep$^{fn}$, collagen, and increasing concentrations of Fn were incubated with macrophage monolayers for 5 hours in a humidified CO$_2$ incubator at 37°C. Each dish also received excess unlabeled oleic acid (100 mM oleic acid/20 mM albumin) to serve as a substrate for re-esterification to $^3$H-cholesterol liberated during hydrolysis of LDL-$^3$H-cholesterol. After incubation, monolayers were exposed to 0.5% trypsin/0.02% EDTA to remove noningested complexes; these were washed twice with DPBS. Lipids were extracted in situ by the method of Hara and Randir as described previously. After visualization with rhodamine 6G, labeled lipids were scraped into scintillation vials containing 10 ml Aquasol and were assayed. The cell protein...
remaining on the culture dishes was dissolved in 0.2 N NaOH, and an aliquot was removed for protein determination. The accumulation of labeled cholesteryl ester was expressed as dpm/mg of cell protein.

**Cellular Incorporation of \(^{14}\text{C}\)- or \(^{3}\text{H}\)-Oleic Acid Into Cholesteryl Oleate**

To each dish, an aliquot of \(^{14}\text{C}\)- or \(^{3}\text{H}\)-oleic acid and albumin mixture prepared as described by Goldstein et al.\(^{36}\) was added to give a final concentration of 100 \(\mu\text{M}\) oleic acid. After a 5-hour incubation in a humidified CO\(_2\) incubator at 37°C, the cultured cells were washed twice with DPBS. Lipid was extracted and fractionated as outlined above. Esterification was expressed as the nmoles of labeled oleic acid incorporated into cholesteryl esters per mg of cell protein.

**Fluorescent Phagocytosis Assay**

Phagocytosis of gelatin-derivatized fluorescent latex particles (0.28 \(\mu\text{m}\)) was determined as previously described.\(^{37}\) On the day of the assay, macrophages were washed to remove serum and were incubated with media (RPMI containing 100 \(\mu\text{g/ml}\) heparin); the media were supplemented with Fn (50 \(\mu\text{g/ml}\)) or Fn and F(\(\text{ab}\)_2) fragments of anti-Fn (Ab; diluted 1:100, vol/vol. Each well then received 10\(^{6}\) gelatin-derivatized fluorescent latex particles. After a 1.5-hour incubation, noningested particles were removed by trypsinization (0.5% trypsin and 0.02% EDTA, 20 minutes). Cells were solubilized with 0.2 N NaOH and fluorescence was measured in a Turner filter fluorometer.

**Statistical Analysis**

When indicated by variance ratio testing, heteroscedastic data were converted by log transformation \([x' = \log(x + 1)]\) before parametric analysis.\(^{38}\) The mean cellular uptake of \(^{125}\text{I}\)-LDL matrix complexes containing either heparin or HepFn were compared by using the \(t\) test.\(^{38}\) The mean cellular incorporation of labeled oleic acid into cholesteryl esters stimulated by various LDL matrix complexes was compared by using a single-factor analysis of variance. When indicated by a significant \(F\)-statistic, pairwise comparisons were performed by multiple range testing procedures.\(^{38}\) The uptake of fluorescent latex particles by control cells and cells exposed to either Fn or Fn and anti-Fn were compared by single-factor analysis of variance. The effect of Fn on mean cellular accumulation of \(^{3}\text{H}\)-cholesteryl esters by macrophages incubated with LDL-\(^{3}\text{H}\)-cholesterol linoleate complexed with either heparin or HepFn and collagen was analyzed by using two-factor analysis of variance.

**Results**

**Properties of Heparin Fraction Isolated by Its Affinity to Fibronectin**

Commercial heparin was fractionated on a gelatin-Sepharose column to which purified Fn was first adsorbed. When analyzed by cellulose acetate electrophoresis, the unfractionated heparin was resolved into two bands: a diffuse cathodic band and an adjacent denser anodic band (Figure 1). The various heparin fractions migrated in a fashion similar to commercial heparin (two bands) with little or no difference in the diffuse band. In contrast, there were clear differences in the migration of the dense anodic band. In the case of HepFn, the trailing edge of this band was shifted cathodically (Figure 1, Lane 2). This was a characteristic and reproducible property of various HepFn isolates and was unaffected by the buffer system. In contrast to HepFn, the leading edge of the anodic band of a heparin fraction serially depleted of Fn binding activity was shifted more anodically (Figure 1, Lane 3).

The size distributions of heparin fractions were compared by chromatographing equivalent amounts of heparin and HepFn (Figure 2) on a Sephadex G-200 column. Un-
fractionated heparin eluted at an approximate $K_v$ of 0.35, slightly skewed to the right. $\text{HeP}_{\text{Fn}}$ eluted at a similar $K_v$; however, its elution profile was less polydisperse and did not contain a population of smaller species present in the unfractionated heparin. An insufficient amount of unbound heparin precluded its analysis.

**Interaction of Heparin and Fibronectin**

Since heparin is polyanionic, its binding to Fn should alter Fn migration in an electric field. We tested for heparin-induced shifts in Fn migration by use of uncharged agarose-galactomannan gels. As seen in Figure 3A, the pre-incubation of Fn with unfractonated or fractionated heparins leads to an increased cathodic migration. $\text{HeP}_{\text{Fn}}$, isolated by its affinity to adsorbed Fn, was most effective in this regard (Figure 3A, Lane 2). Surprisingly, a heparin species that did not bind to Fn adsorbed on gelatin-Sepharose bound to soluble Fn under identical ionic conditions. This was evidenced by the relatively small shift in the migration of Fn observed when depleted heparin was mixed with Fn (Figure 3A, Lane 4). For comparison, two other polyanions were also examined (Figure 3B). Chondroitin sulfate had no effect on Fn migration (Lane 3), whereas the addition of heparan sulfate led to a small, but discernible, increase in Fn mobility (Lane 2).

The cathodic shift in Fn migration observed in the presence of heparin was dependent on heparin concentration (Figure 3C). As little as 1 μg of heparin per 100 μg Fn resulted in increased mobility. This was further accentuated with 10 μg of heparin; however, no further displacement was observed with 100 μg of heparin. Additionally, heparin induces Fn aggregation.\(^{19}\) This is evidenced in Figures 3A and 3C by the darkly stained material remaining at the origin. Furthermore, under similar conditions, $\text{HeP}_{\text{Fn}}$ induced greater aggregation than unfractionated heparin (Figure 3A, Lane 2 vs. Lanes 3 or 4).

In addition to multiple heparin binding domains, each Fn molecule contains two domains that bind collagen and gelatin.\(^{30, 40}\) We tested whether the migration of Fn could
be modified by its interaction with another ligand to which it strongly interacts. As seen in Figure 3D, gelatin migrates anodically (Lane 1); Fn migrates cathodically, and its cathodic migration is enhanced by heparin (Lanes 2 to 4). The addition of gelatin to Fn led to band dispersion, which was accentuated in the presence of heparin or HepFn (Lanes 5 to 7).

We have previously demonstrated the incorporation of Fn into LDL-heparin complexes. Since HepFn was isolated by its ability to bind adsorbed Fn, LDL-heparin complexes prepared with HepFn should bind more Fn. We examined this possibility by measuring the incorporation of $^{14}$C-Cm-Fn into LDL-heparin complexes. When compared to native Fn, Cm generated a large fraction of Fn that failed to bind to heparin-Sepharose (Figures 4A and 4B). Only Cm-Fn, which did bind the heparin-Sepharose and eluted at a similar ionic strength as native Fn, was used in the subsequent binding studies. As indicated in Figure 5, in both assay systems, more labeled Fn bound to LDL-HepFn than to LDL-heparin complexes.

**Modulation of Macrophage Catabolism of LDL Matrix by Interaction of Heparin with Fibronectin**

We investigated whether the incorporation of a fraction of heparin with high affinity to Fn into LDL-heparin-Fn-denatured collagen complexes would modulate their uptake by macrophages. For these purposes, we used $^{125}$I-LDL as a tracer for endocytosis of apolipoproteins. As seen in Table 1, macrophages internalized significantly more LDL when insolubilized in a matrix containing HepFn ($p<0.05$). Furthermore, more than 75% of endocytosed LDL remained undegraded. These results confirm a previous report, and demonstrate that the macrophage's hydrolytic apparatus is rapidly overwhelmed by the uptake of these insoluble LDL matrix complexes. Similar accumulation of undegraded LDL was observed when macrophages were exposed to insoluble LDL proteoglycan and LDL-dextran sulfate complexes. However, once internalized, macrophages can slowly degrade LDL matrix and liberate hydrolytic products, including free cholesterol. Since endogenous cholesteryl ester synthesis is dependent on the availability of lipoprotein-derived cholesterol, the differential uptake of these complexes should be reflected in differential rates of endogenous cholesteryl ester synthesis. Therefore, we measured the incorporation of $^{14}$C-oleic acid into cholesteryl esters by macrophages exposed to LDL matrix complexes (Table 2). Our results demonstrate that esterification increased threefold in cells exposed to LDL matrix complexes prepared with unfractinated heparin as compared to LDL alone ($p<0.005$).
Likewise, a fivefold increase in the incorporation of $^{14}$C-oleic acid was found in cells receiving complexes prepared with Hep$_{pl}$, ($p < 0.001$). Neither complex was as stimulatory as soluble acetyl LDL, which is rapidly internalized and degraded.$^{1,29}$ These results (the stimulatory effect of Hep$_{pl}$ on the uptake of LDL sequestered in a model matrix containing heparin, Fn, and gelatin) provide indirect evidence that the interaction of Fn with components of the LDL matrix complex is important for stimulating uptake by scavenger cells.

To test directly whether the binding of Fn to insoluble LDL matrix complexes modulated their uptake by macrophages, it was necessary to prepare a matrix containing calf-skin collagen instead of gelatin. Acid-soluble collagen forms fibrils at neutral pH and will co-precipitate with heparin, whereas gelatin is not easily precipitated by heparin except in the presence of Fn.$^{12}$ The effect of Fn on scavenger cell uptake of insoluble LDL matrix complexes was assessed by measuring both the obligatory increase in cholesteryl ester synthesis after endocytosis and hydrolysis of LDL, and the accumulation of undegraded lipoprotein-derived cholesteryl esters. Incubating macrophages with LDL-Hep$_{pl}$ complexes did not stimulate cholesteryl ester synthesis as compared to that observed in cells receiving LDL (Table 3) or in media controls (data not shown). Esterification observed in macrophages incubated with particulate LDL-heparin-collagen complexes increased 2.5-fold over controls ($p < 0.01$) and approximately fivefold when Fn was present (LDL-heparin-collagen vs. LDL-heparin-collagen-Fn; $p < 0.05$). Complexes prepared with LDL-Hep$_{pl}$-collagen dramatically stimulated cholesterol esterification as compared to controls and similar complexes prepared with unfraccionated heparin ($p < 0.001$). As expected, the incorporation of oleic acid into cholesteryl esters stimulated by the ingestion and hydrolysis of LDL-Hep$_{pl}$-collagen complexes was further enhanced by Fn ($p < 0.001$). LDL matrix complexes prepared with chondroitin sulfate, dermatan sulfate, or heparan sulfate failed to insolubilize LDL or to stimulate endogenous cholesteryl ester synthesis at concentrations of glycosaminoglycan equivalent to that used for heparin in these studies (data not shown).

We next determined if the Fn-dependent stimulation of endogenous cholesteryl ester synthesis observed in macrophages exposed to LDL-Hep$_{pl}$-collagen complexes could be inhibited in the presence of F(ab)$_2$ fragments of anti-Fn. The ability of F(ab)$_2$ anti-Fn to block the opsonic activity of Fn was first tested by monitoring cellular uptake of gelatin-derivatized fluorescent latex particles. Macrophage phagocytosis of gelatin-derivatized fluorescent latex particles increased from $3.0 \pm 0.2$ to $7.9 \pm 2.8 \times 10^6$ particles/µg cell protein (mean ± SD, $n = 4$) in the presence of Fn ($p < 0.001$). When cells were incubated with media containing gelatin-derivatized particles, Fn and F(ab)$_2$ anti-Fn (1:100, vol/vol), uptake was reduced to $0.8 \pm 0.2 \times 10^6$ particles/µg cell protein. Likewise, the Fn-dependent stimulation of cholesteryl ester synthesis in macrophages exposed to particulate LDL-Hep$_{pl}$-collagen complexes ($p < 0.01$) was blocked by the addition of F(ab)$_2$ fragments of anti-Fn (Table 4).

As shown in Table 1, cells exposed to LDL insolubilized in a matrix rapidly accumulate undegraded lipoprotein, which would not contribute to the stimulation in the incorporation of oleic acid into cholesteryl esters observed in Table 3. We therefore used LDL-$^{3}$H-cholesteryl linolate as a tracer for the accumulation of undegraded lipoprotein-derived cholesteryl esters (Figure 6). However, since LDL

### Table 2. Heparin Fraction Modulates Cholesteryl Ester Synthesis in Macrophages Exposed to Low Density Lipoprotein-Heparin-Gelatin-Fibronectin Complexes

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Cholesteryl $^{14}$C-oleate nmol/mg cell protein</th>
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<tbody>
<tr>
<td>LDL</td>
<td>0.25 ± 0.09</td>
</tr>
<tr>
<td>LDL-Hep-Gel-Fn</td>
<td>0.77 ± 0.04</td>
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<tr>
<td>LDL-Hep$_{pl}$-Gel-Fn</td>
<td>1.15 ± 0.17</td>
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<tr>
<td>Acetyl-LDL</td>
<td>4.39 ± 0.81</td>
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The values are nmol/mg cell protein and are the means ± SD of three separate cultures.

Macrophages were cultured in DME containing 100 nmoles of $^{14}$C-oleic acid (12,673 dpm/nmol) and 25 µg of LDL or complex-derived LDL. After incubation for 5 hours at 37°C, cells were washed, the lipids were extracted in situ, and cellular cholesteryl $^{14}$C-oleate was isolated by TLC.

Abbreviations are explained in the legend to Table 1.

### Table 3. Fibronectin Stimulates Cholesteryl Ester Synthesis in Macrophages Exposed to Low Density Lipoprotein-Heparin Fraction-Collagen Complexes

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Cholesteryl $^{3}$H-oleate nmol/mg cell protein</th>
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<tbody>
<tr>
<td>LDL</td>
<td>0.94 ± 0.03</td>
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<tr>
<td>LDL-Hep$_{pl}$</td>
<td>0.89 ± 0.06</td>
</tr>
<tr>
<td>LDL-Hep$_{pl}$-collagen</td>
<td>10.30 ± 1.57</td>
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<tr>
<td>LDL-Hep$_{pl}$-collagen-Fn</td>
<td>22.20 ± 4.89</td>
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<tr>
<td>LDL-Hep-collagen</td>
<td>2.44 ± 0.13</td>
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<tr>
<td>LDL-Hep-collagen-Fn</td>
<td>4.30 ± 2.50</td>
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The values are nmol/mg cell protein and are the means ± SD of five separate cultures.

Macrophages were cultured in RPMI containing 100 nmoles of $^{3}$H-oleic acid (21,112 dpm/nmol) and 25 µg of LDL or complex-derived LDL. The incorporation of labeled oleic acid into cholesteryl oleate was determined as described in the legend to Table 2. Abbreviations are explained in the legend to Table 1.

### Table 4. F(ab)$_2$ Fragments of Anti-Fn Block Fibronectin Stimulation of Cholesteryl Ester Synthesis in Macrophages Exposed to Low Density Lipoprotein-Heparin Fraction-Collagen Complexes

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Cholesteryl $^{3}$H-oleate nmol/mg cell protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL</td>
<td>0.31 ± 0.05</td>
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<td>LDL-Hep$_{pl}$-collagen</td>
<td>3.37 ± 0.60</td>
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<td>LDL-Hep$_{pl}$-collagen-Fn</td>
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<td>LDL-Hep$_{pl}$-collagen-Fn+Ab</td>
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<tr>
<td>Acetyl-LDL</td>
<td>6.04 ± 0.44</td>
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The values are nmol/mg cell protein and are the means ± SD of three separate cultures.

Macrophages were cultured in RPMI containing 100 nmoles of $^{3}$H-oleic acid (21,112 dpm/nmol) and 25 µg of LDL or complex-derived LDL. F(ab)$_2$ fragments of anti-Fn were added to the incubation media at a final concentration of 1:100 (vol/vol). The incorporation of labeled oleic acid into cholesteryl oleate was determined as described in the legend to Table 2. Abbreviations are explained in the legend to Table 1.
cholesteryl linoleate is labeled in the cholesterol moiety, a fraction of $^3$H-cholesterol liberated as a consequence of limited hydrolysis may contribute to endogenously synthesized cholesteryl esters. As expected, exposure of macrophages to LDL cholesteryl $^3$H-linoleate insolubilized with heparin (or HepFn) and collagen led to the intracellular accumulation of $^3$H-cholesterol esters. When either LDL-Hep-collagen or LDL-HepFn-collagen complexes were prepared with increasing concentrations of Fn, macrophage accumulation of $^3$H-cholesterol esters increased significantly ($p<0.001$). Furthermore, as seen in Figure 6, Fn effected a greater dose-dependent increase in $^3$H-cholesterol ester accumulation when complexes were prepared with HepFn ($p<0.05$).

Discussion

In experiments reported here, we investigated whether the interaction of Fn with LDL matrix complexes can modulate their uptake by scavenger cells. For these purposes, we used two complementary approaches. First, we isolated and partially characterized a heparin fraction, based on its affinity to Fn. If the interaction of Fn with heparin and gelatin is important to scavenger cell uptake of LDL matrix, then complexes prepared with HepFn should be internalized more efficiently. Second, using insoluble complexes of heparin, fibrillar collagen, and LDL, we determined directly if Fn can modulate the uptake of LDL matrix complexes.

When compared to unfractionated heparin, HepFn displayed a small cathodic shift in its migration in cellulose acetate, reflecting its greater charge or sulfation. In contrast, heparin serially depleted of Fn-binding species was less charged by the same criteria (Figure 1). These results are supported by the observation that heparan sulfate fractions, successively isolated from heparan sulfate-CPC complexes by dissolution with increasing NaCl concentrations, migrated more cathodically in cellulose acetate. When heparan sulfate was subsequently fractionated on Fn-Sepharose, low sulfated heparan sulfate did not bind to adsorbed Fn, whereas the fraction with the greatest charge demonstrated the highest affinity to Fn. Likewise, when heparin was fractionated on Fn-Sepharose by elution with stepwise increases in NaCl, fractions that eluted at higher ionic strength contained more sulfate.

We observed that the size distribution of HepFn was more homogeneous and more charged than unfractionated heparin. HepFn did not contain a population of smaller heparin molecules (Figure 2). These results are consistent with the observations of others who have demonstrated a correlation between larger heparin molecules or longer heparin chains and a higher affinity to Fn. In our studies, a shift in $K_w$ was not apparent; however, we did not fractionate Fn-binding heparin into the low or into the several high affinity fractions that reportedly display a considerable range in $K_w$ values with Sephadex G-100.

Fn contains a series of heparin binding domains. When isolated by proteolytic cleavage and affinity chromatography on heparin-Sepharose, these domains possessed a basic pI. The binding of polyanions to positively-charged amino acids in these domains should result in a cathodic shift in the migration of Fn in an uncharged gel medium. This was evident in Figure 3. Furthermore, this shift in Fn migration was dependent on the nature of the polyanion. For example, the HepFn fraction of heparin caused the largest cathodic shift, whereas chondroitin sulfate had no effect. This observation is consistent with the finding that Fn has little affinity with free chondroitin sulfate.

Even heparin that was serially depleted of Fn binding fractions by affinity chromatography to adsorbed Fn was able to bind to soluble Fn, resulting in a small cathodic shift. These results strongly suggest that the reported failure of a putative non-binding heparin fraction to adsorb Fn may have resulted from the masking of heparin binding sites normally available when Fn is soluble.

The substitution of HepFn for unfractionated heparin in particulate complexes of LDL, heparin, denatured collagen, and Fn resulted in the enhanced uptake of complex-derived LDL and the subsequent cholesteryl ester synthesis by scavenger cells (Tables 1 and 2). Since complexes prepared with HepFn bind more Fn, these data suggest that the cellular uptake of particulate LDL matrix complexes may be regulated by their interaction with Fn. To directly test the role of Fn in macrophage ingestion of LDL matrix complexes, insoluble Fn-free complexes were prepared with LDL, heparin, and acid-soluble calf-skin collagen.
Whereas gelatin is not easily insolubilized by heparin except in the presence of Fn, acid-soluble collagen forms fibrils at neutral pH and co-precipitates with heparin. Cellular uptake of complex-derived LDL was monitored by quantitating cholesteryl ester synthesis and the accumulation of degraded LDL-derived cholesteryl esters over an equivalent incubation period. Since internalized LDL-derived cholesteryl ester is either degraded and re-esterified or remains undegraded, then monitoring both processes provides an index of uptake.

The dramatic effect of insolubilizing LDL with matrix components on the stimulation of endogenous cholesteryl ester synthesis by scavenger cells is demonstrated in Table 3. Particulate complexes of LDL, heparin or HepFn, and fibrillar collagen stimulate cholesteryl ester synthesis, whereas soluble LDL or LDL-heparin complexes did not. Furthermore, the rate of cholesteryl ester synthesis in macrophages exposed to complexes prepared with HepFn was fourfold greater than that observed in cells exposed to complexes prepared with unfractionated heparin. There are two possible explanations for these observations. Since the stimulation in cholesteryl ester synthesis is dependent on LDL uptake and hydrolysis, the data suggest that either: 1) more of the complexes prepared with HepFn are internalized, or 2) complexes prepared with unfractionated heparin are not degraded. Since the intracellular accumulation of degraded LDL-derived cholesteryl esters was similar in cells exposed to complexes prepared with heparin or HepFn, the first possibility is more likely in the absence of Fn (Figure 6). The enhanced uptake of LDL-HepFn-collagen complexes may reflect the increased anionic charge of HepFn. In these experiments, equivalent amounts of heparin and HepFn completely insolubilized LDL under conditions of low ionic strength. When complexes were suspended in physiologic saline containing collagen fibrils, more of the LDL co-precipitated with the collagen in the presence of HepFn (data not shown). In the absence of heparin or HepFn, LDL did not co-precipitate with fibrillar collagen. In the absence of collagen, LDL-heparin (or HepFn) complexes were soluble and did not stimulate macrophage cholesteryl ester synthesis (Table 3). Therefore, HepFn stimulates macrophage uptake of LDL by its more effective binding to collagen.

It would appear from these studies that the observed stimulation in ingestion of matrix-derived LDL was largely a consequence of its insolubilization. Indeed, macrophages readily ingest LDL insolubilized through complexation with other large polyanions. However, the results of these experiments showed that Fn can enhance scavenger cell ingestion of LDL matrix complexes. This conclusion is based on the observation that Fn stimulated both cholesteryl ester synthesis and the accumulation of degraded lipoprotein-derived cholesteryl esters in cells exposed to LDL matrix complexes (Table 3, Figure 6). By using the same criteria, the opsonic effect of Fn was further enhanced when complexes were prepared with a heparin fraction exhibiting high affinity to Fn. This shows for the first time that Fn can indeed opsonize extracellular matrix components and thereby stimulate uptake of sequestered ligands. However, it should be emphasized that enhanced phagocytosis is not an obligatory response by macrophages to Fn-coated targets. Thus, the interaction of Fn, collagen, and heparin may be specific.

In previous and present studies, heparin was used as a convenient and standardized reagent with which to model insoluble complexes of LDL and extracellular matrix components that may be present in the arterial wall. Heparin's high anionic charge may mimic the high charge density exhibited by arterial proteoglycans. For example, although aortic chondroitin sulfate-dermatan sulfate proteoglycan is as potent and selective as heparin in precipitating LDL, the ability of its constitutive glycosaminoglycan chains to insolubilize LDL is significantly less. In a similar manner, the ionic interaction of Fn and polyanions is dependent on charge density. Chondroitin sulfate proteoglycan can effectively bind Fn, whereas free chondroitin sulfate cannot.

In addition to exhibiting a high charge density, a general property of proteoglycans, heparin may be considered as a surrogate for heparin-like molecules in the arterial wall (i.e., heparan sulfate proteoglycans). Heparan sulfate is heterogeneous due to differences in hexuronic acid composition and sulfation. It has been reported that the physiochemical characteristics of heparan sulfate synthesized by endothelial and smooth muscle cells vary depending on their growth state. These structural differences are related to functional properties, such as the ability to inhibit smooth muscle cell proliferation. In addition, there may be local differences in the nature of the heparan sulfates in the arterial wall, such as the increased charge of heparan sulfate found in an area of pigeon aorta predisposed to lesion development. Highly charged heparan sulfate proteoglycan may play a role similar to the HepFn used in this study.

In the blood vessel, as elsewhere, glycosaminoglycan chains are linked to a protein core of a parent proteoglycan molecule. Free glycosaminoglycans or portions thereof could be generated in atherosclerotic lesions by the action of platelet and macrophage endoglycosidases and macrophage proteases. Released glycosaminoglycan chains or oligosaccharides of sufficient length possess LDL binding capacity; however, it is unlikely that free glycosaminoglycan chains (unless very highly charged) would insolubilize LDL at physiologic ionic strength. In the experiments reported here, LDL matrix complexes prepared with chondroitin sulfate, dermatan sulfate, and heparan sulfate failed to insolubilize LDL or to stimulate cholesteryl ester synthesis in macrophages. Consequently, heparin and the higher charged HepFn are analogous to intact proteoglycans, which insolubilize LDL and to stimulate cholesteryl ester synthesis in macrophages. Microscopically, heparin and the higher charged HepFn are analogous to intact proteoglycans, which insolubilize LDL and to stimulate cholesteryl ester synthesis in macrophages. Consequently, heparin and the higher charged HepFn are analogous to intact proteoglycans, which insolubilize LDL and to stimulate cholesteryl ester synthesis in macrophages.
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