Cultured extrahepatic cells possess a specific high affinity binding site (receptor) for high density lipoprotein (HDL) that is induced by cholesterol delivery to cells. To characterize the binding recognition site(s) on HDL, the ability of HDL to interact with cultured human fibroblasts was assayed after chemical alteration of specific apoprotein amino acid residues. Reduction and alkylation, acetylation, and cyclohexanedione treatment of HDL3 had little or no effect on cellular binding. Treatment of HDL3 with tetraniitromethane (TNM), however, caused a large dose-dependent decrease in binding, with maximum inhibition at 3 mM. Amino acid analysis of the TNM-treated particles showed specific alteration of tyrosine residues, but sodium dodecyl sulfate-gel electrophoresis demonstrated apoprotein cross-linking coincident with decreased binding. These results suggest that modification of HDL tyrosine residues and/or cross-linking of HDL apoproteins alters the ligand site recognized by the HDL receptor. Gradient gel electrophoresis, molecular sievel chromatoigraphy, and electron microscopy showed only minor changes in size distribution and shape of HDL3 particles after treatment with 3 mM TNM, but at higher TNM concentrations, coalescence and aggregation of particles was evident. Treatment of HDL3 with 3 mM TNM affected neither its promotion of the low affinity (receptor-independent) cholesterol efflux from cells nor its ability to accept cholesterol from an albumin suspension, yet promotion of high affinity (receptor-dependent) cholesterol efflux from cells was abolished. The finding that TNM treatment of HDL3 decreases both its receptor binding and its promotion of cellular cholesterol efflux from cells without substantial alteration of its physical properties supports the hypothesis that the HDL receptor functions to facilitate cholesterol transport from cells.

Experimental procedures

Cells—Normal human skin fibroblasts were grown from explants of punch biopsies of skin from the inner thighs of normal volunteers in plastic tissue culture flasks containing Dulbecco’s minimum essential medium plus 10% fetal bovine serum (growth medium) at 37°C in humidified incubators equilibrated with 5% CO2, 95% air. Cells were trypsinized from stock flasks (2-14 passages), seeded in 35-mm plastic Petri dishes using 2 ml of medium containing 5 x 10^5 cells, and grown in growth medium until just before reaching confluence, usually 6-10 days after plating. Cells were then washed twice with a PBS-albumin medium (0.2 g/l of KCl, 0.2 g/l of KH2PO4, 3.0 g/l of NaCl, 2.16 g/l of Na2HP04, 7H2O, 2.0 g/l of bovine serum albumin, at pH 7.4) and incubated for 48 h with serum-free culture medium containing albumin, 2.0 g/l, plus the indicated concentration of cholesterol. Cholesterol was dissolved in 95% ethanol (10 mg/ml) before addition to the albumin medium. The final ethanol concentra-
Mass-cellular lipids were extracted by the hexane-isopropyl alcohol procedure as modified for lipoprotein (21). HDL was subjected to phenylagarose affinity chromatography to remove apo A and apo E (14).

Liposomes—Phospholipid proteoliposomes were made by cholate dialysis (22) using egg lecithin (Applied Science Laboratory) and apo A-I in an approximate molar ratio of 300:1. This method previously has been reported to produce uniform round bilamellar vesicles which, when prepared with cholesterol, are biologically active substrates for the enzyme lecithin-cholester-alicyclosome transferase (22). When indicated, vesicle phospholipids were radiolabeled by the addition of [methyl-14C]dipalmitoylphosphatidylcholine to the egg lecithin.

Chemical Modification of Lipoproteins and Liposomes—Acetylation was performed according to the method of Frerken-Conrat (23) as modified by Baud et al. (24). An HDL solution containing 2.5-5 mg of protein/ml was diluted 1:1 (v:v) with saturated sodium acetate and, while being continuously stirred for 90 min in an ice bath, acetic acid was added to an 8-8 molar ratio to a total volume equivalent to 1.5 mg/ml of HDL protein. Alkylation and reduction of HDL was carried out according to the mercaptosuccic-oioamidocete method of Weisgraber et al. (25) using a concentration of HDL similar to that used in acetylation. Cyclohexanedione (Aldrich) treatment was performed following the method of Fatty and Smith (26) as modified by Mahley et al. (27) using the same HDL concentrations as described for acetylation. Effective modification was confirmed by agarose gel electrophoresis and amino acid analysis.

For each TNM (Aldrich) modification, a fresh stock solution was prepared by dilution of TNM at room temperature in 95% ethanol to 1 mg/ml. A solution of TNM (4-72 µg/ml) was added to a 1:1 (v:v) reaction mixture containing 0.5-1 mg/ml of protein (28). After incubation for 1 h at 37°C, the mixture was allowed to stand at room temperature for 1 h, after which it was dialyzed extensively against saline/EDTA at 4°C. This procedure was based on previously described methods (29, 29) and on methods personally communicated by Dr. Y.-D. I. Chen, Stanford University.

HDL Binding—To determine cell binding of [3H]-HDL at 37°C, cells were rapidly washed twice with PBS-albumin medium (was diluted 1:1 with PBS-albumin medium containing 1.0-2.0 mg/ml albumin and 125I-HDL at a protein concentration of 1.0-2.0 mg/ml). The cells were then added to solutions containing HDL or proteoliposomes at protein concentrations of 1.5-3.0 mg/ml. Cell binding was determined by washing twice with PBS-albumin medium containing 1.0-2.0 mg/ml albumin and 125I-HDL at a protein concentration of 1.0-2.0 mg/ml. The cell-binding assay was performed following the method of Mancini et al. (35) as modified by Ahrens et al. (36). Specific binding was determined by subtracting binding in the presence of excess unlabeled HDL or proteoliposomes from total binding. Results demonstrated that modification of cysteine residues by sulfide-acetamide bond formation was irreversible under these hydrolysis conditions. For cyclohexanedione-treated samples, hydrolysis was performed in the presence of mercaptoacetic acid (2%, v/v) to stabilize the cyclohexanedione derivative of arginine (26, 27). It was estimated that cyclohexanedione treatment modified 30 to 40% of the arginine residues in HDL. To estimate the degree of acetylation of lysine residues, the indirect method of Wolf and Singer (38) as modified by Ahrens et al. (36) was used. Briefly, native and acetylated HDL were treated with dinitrofluorobenzene to convert n-acetylated lysines to acetyl-stable dinitrophenylated derivatives prior to hydrolysis. Results indicated that more than 80% of the lysine residues were acetylated by acetic anhydride treatment.

Patty Acid Analysis—Lipids were extracted from HDL in chloroform-methanol (2:1, v/v) and lipid classes were separated by thin layer chromatography as previously described methods (15). The phospholipid silica spot and the combined triglyceride plus cholesteryl ester (neutral lipid) spots were suspended in ethanolic (95%) KOH (0.1 M) and lipids were saponified for 1 h at 80°C. The liberated free fatty acids were extracted in hexane, the solvent was evaporated under N2, the residue was redissolved in 1% H2SO4 in methanol was added. After heating at 80°C for 2 h, the methyl esters were extracted in hexane and the solvent was dried under N2. Samples were resuspended in 0.1 ml of hexane and analyzed in a Hewlett-Packard 5790A gas chromatographer with flame ionization detector and HP-5991A integrator. Fatty acids were separated on a 30 m X 0.25 mm inside diameter, 0.2-µm-thick capillary column.
The area under the peaks for each fatty acid was integrated and expressed as percent of the total area for all peaks.

Other Methods—Electron microscopy of HDLb negatively stained with 1% sodium phosphotungstate at pH 7.4 was performed by previously described methods (38). Lipoprotein triglyceride and phospholipid mass were determined by previously described methods (39, 40). Protein content of lipoproteins and cells was determined by the method of Lowry et al. (41).

RESULTS

To identify the domain(s) on HDL apoproteins that are recognized by the HDL binding site, we employed methods of protein alteration specific for certain amino acid residues. Acetylation, reduction and alkylation, or cyclohexanedione treatment were performed on HDLb to alter lysine, cysteine, and arginine residues, respectively. These modifications caused little or no change in the ability of HDLb to compete for binding of 125I-HDLb to fibroblasts (Fig. 1a), suggesting no significant involvement of these amino acid residues in recognition of HDLb by its cellular binding site.

Recent reports (42, 54) suggested that alteration of HDL by TNM, presumably specific for tyrosine residues, abolished its binding to hepatocyte membranes. To determine if tyrosine-containing segments of human HDL apoproteins constitute the ligand site recognized by the HDL binding site of cultured fibroblasts, the binding of TNM-treated HDLb was compared with that of control HDLb by means of competition studies. When HDLb had been treated with TNM at a concentration of 3 mM, its ability to compete for 125I-HDLb binding was almost totally lost (Fig. 18). Even at a 10-fold excess, TNM-treated HDLb competed poorly with the 125I-labeled ligand. This is in contrast to the effective competition by native HDLb seen even at low concentrations. To examine for possible dose dependency of this TNM effect, HDLb was treated with TNM at concentrations of 10^-3 mM to 30 mM and tested for its ability to compete for 125I-HDLb binding (Fig. 2). There was no significant effect on binding competition up to a TNM concentration of 0.1 mM. Above 0.1 mM TNM there was a precipitous decrease in competition and then a plateau beginning at 3 mM. At the higher concentrations of TNM, less than 15% of 125I-HDLb binding was displaced, even at a 10-fold excess of unlabeled ligand (Fig. 1b).

Previous studies demonstrated that both major HDL subclasses, HDLb and HDLz, interact with the HDL binding site on cultured human skin fibroblasts (14). On a protein basis, HDLb and HDLz compete for 125I-HDLb binding to similar extents (Fig. 3). Competitive binding of both HDLb and HDLz was nearly abolished when the lipoproteins were treated with TNM.

Treated HDLb samples across a range of TNM concentrations were subjected to agarose electrophoresis along with untreated HDLb (Fig. 4). Increased electrophoretic mobility was noticeable at a TNM concentration of 0.3 mM, corresponding to the TNM concentration at which HDLb binding competition was decreased (Fig. 2).

TNM alteration is relatively specific for tyrosine residues, especially at a pH of 8 or less (29, 43). Amino acid analysis of altered and unaltered HDLb showed that TNM treatment caused a shift of the entire tyrosine peak into the phenylalanine peak (Fig. 5), without substantial changes in the other major amino acid peaks (Table I), consistent with a specific conversion of tyrosine to 3-nitrotyrosine (28). Since the acid
Fig. 4. Agarose gel electrophoresis of native and TNM-treated HDLs. The samples as those used in the competition studies described in Fig. 2, as indicated by the concentration of TNM used in the modification. Approximately 30 μl of each sample was applied to the gel.

AMINO ACID ANALYSIS

![Amino acid analysis graph]

Fig. 5. Amino acid analysis of native (-----) and TNM-treated (---) HDLs. Only the amino acid peaks eluting near tyrosine are shown, since the other portions of the chromatogram are similar. The samples were chromatographed separately.

Table I

| Amino acid residue | Ratio of TNM-treated to native HDLs |
|--------------------|-------------------------------------|
| Aspartic acid      | 1.06                                |
| Threonine          | 1.06                                |
| Serine             | 1.07                                |
| Glutamic acid      | 1.06                                |
| Glycine            | 1.09                                |
| Valine             | 1.00                                |
| Leucine            | 1.11                                |
| Tyrosine           | 0.06                                |
| Phenylalanine      | 0.97                                |
| Lysine             | 0.97                                |
| Histidine          | 0.77                                |
| Arginine           | 0.92                                |

*Calculated for TNM-treated HDLs by subtraction of estimated value for 3-nitrotyrosine.

No

TNM 0.03 0.3 3 30 mM

albumin → apo A-I apo A-II

Fig. 6. SDS-PAGE of native and TNM-treated HDLs. The concentration of TNM used in modification was as indicated. Approximately 3 μg of HDL protein was added to each well and chromatographed as described under “Experimental Procedures.”

Fig. 7. Gradient gel electrophoresis of native and TNM-treated HDLs. HDLs was treated with the indicated concentration of TNM and chromatographed as described under “Experimental Procedures.” Molecular weight standards were as follows: thyroglobulin (669,000), apoferritin (443,000), catalase (240,000), lactate dehydrogenase (140,000), albumin (67,000).

from the gel. The oligomer formation and eventual failure of entry into the gel strongly suggested that cross-linking between HDL apoproteins was caused by TNM treatment (44).

Gradient gel electrophoresis demonstrated that marked aggregation of HDLs particles did not occur until the HDLs was treated with TNM in excess of 3 mM (Fig. 7). Although increases in particle size became evident at 3 mM TNM, nearly all of the particles had the same distribution as untreated HDLs. Similar results were observed when particles were analyzed by molecular sieve chromatography (Fig. 8). At a TNM concentration of 3 mM, the peak was shifted slightly to the left, indicating a slight increase in average Stokes radius of the particles. This suggested a minor change in particle size distribution that could have occurred with apoprotein cross-linking. In contrast, at 10 mM TNM, there was a significant shift and broadening of the peak, suggesting a spectrum of increase in particle size. Electron microscopy also revealed that treatment of HDLs with 3 mM TNM had little effect on particle morphology (Fig. 9). At 3 mM TNM, the particles were similar in size and shape to native HDLs and

TNM-altered HDLs was compared to native HDLs by 10–15% gradient SDS-PAGE (Fig. 6). At relatively low concentrations of TNM (0.03 to 0.3 mM), there was evidence of polymerization of HDL apoproteins, while at the highest TNM concentrations (3–30 mM) HDL was largely excluded

hydrolysis used for amino acid analysis destroys tryptophan, it is not known if this amino acid is also affected by TNM treatment.

Effect of TNM treatment on amino acid composition of HDLs

The area under the chromatographic peak representing each amino acid residue was measured for native and 10 mM TNM-treated HDLs in separate chromatography runs of equal amounts of protein. The values for the TNM-treated HDLs were divided by those for the native particle. Only the major peaks are shown. A similar degree of tyrosine modification was observed for HDL proteins treated with 3 mM TNM (data not shown).

Amino acid residue Ratio of TNM-treated to native HDLs

Aspartic acid 1.06
Threonine 1.06
Serine 1.07
Glutamic acid 1.06
Glycine 1.09
Valine 1.00
Leucine 1.11
Tyrosine 0.06
Phenylalanine 0.97
Lysine 0.97
Histidine 0.77
Arginine 0.92

*Calculated for TNM-treated HDLs by subtraction of estimated value for 3-nitrotyrosine.
there was no evidence of interparticle aggregation. In contrast, at TNM concentrations of 10–30 mM, the particles appeared to aggregate in clumps and chains and much larger particles were formed, suggesting that treatment of HDL₃ with the higher TNM concentrations led to coalescence of two or more HDL particles.

Measurements of the lipid mass in HDL₃ demonstrated that TNM treatment caused modest changes in the lipid to protein ratio over the TNM concentration range that induced loss of HDL binding (Fig. 10). With the exception of unesterified cholesterol, the ratio of lipids to protein in HDL₃ progressively decreased with increasing concentrations of TNM. The unesterified cholesterol to protein ratio increased to a maximum value at the lowest concentration of TNM tested. This increase in unesterified cholesterol content in association with decreases in esterified lipids suggested that TNM may have promoted ester hydrolysis. However, the relative composition of fatty acids in both phospholipids and neutral lipid esters (triglyceride plus cholesteryl ester) appeared to be unaltered by TNM treatment (Table II), indicating that, unless all ester bonds were affected equally, hydrolysis of HDL lipid esters could not account for the decrease in lipid to protein ratio. The fatty acid composition data also showed that TNM did not have a marked effect on the degree of unsaturation of acyl groups, indicating that nitration of lipid double bonds did not occur to any significant extent.

To further investigate the possibility that TNM blocked HDL binding by altering the interaction between HDL apoproteins and lipids, studies were conducted using recombinant phospholipid-apoprotein vesicles. Preliminary studies (16) demonstrated that proteoliposomes prepared with egg lecithin and apo-A-I, the major apoprotein in HDL, were as effective as native HDL₃ particles in competing with ¹²⁵I-HDL₃ binding. When apo-A-I proteoliposomes were treated with 3 mM TNM, there was a broadening of the apo-A-I band on SDS polyacrylamide gels and a slight retardation in mobility, similar to that observed with HDL₃ (compare Fig. 11, inset, to
500 Cellular Binding of Altered HDL

Since HDL₃ treated with 3 mM TNM showed greatly decreased binding competition and yet had minimal particle aggregation or distortion of particle size, this preparation was selected for studies of HDL-mediated cholesterol efflux from cultured cells. To determine any direct effect of TNM on the ability of HDL to accept cholesterol, exchange of [³H]cholesterol from an aqueous albumin suspension was measured in the presence of native or TNM-treated HDL₃ (Table III). Both the treated and untreated HDL particles were isolated from the cholesterol-albumin suspension by immunoprecipitation using antisera containing anti-apo-A-I demonstrated by radial immunodiffusion to possess near-complete reactivity toward TNM (3 mM)-treated HDL₃. The ability of HDL₃ to accept cholesterol from the albumin suspension was unaltered by TNM treatment. Despite normal ability to accept cholesterol in a cell-free system, TNM-treated HDL₃ was much less effective than HDL₃ in promoting cholesterol transport from cultured fibroblasts, whether measured by the appearance of [³H]cholesterol or unesterified cholesterol mass in the medium or by the decrease in [³H]cholesterol or cholesterol mass in the cell (Table IV). When [³H]cholesterol efflux from cells was measured as a function of the concentration of native HDL₃, both a high and a low affinity component were evident (Fig. 13). TNM treatment greatly reduced the high affinity component of efflux without affecting the low affinity component.

To examine the dose dependency of the TNM effect on HDL-mediated cholesterol transport from cells, HDL₃ particles treated with different concentrations of TNM were compared for their ability to remove cholesterol from cells over short term incubations (4 h). As an assay of net cholesterol transport, the relative rate of cholesteryl ester formation by cells was measured after exposure to HDL₃. Previous studies (5, 6, 53) have demonstrated that the cholesterol esterification rate is a function of the amount of excess unesterified cholesterol in cells and thus can be used as a sensitive biochemical assay for changes in net flux of cholesterol into or out of cells. With an increasing dose of TNM, there was a reduction in the ability of HDL₃ to remove cholesterol from cells as evidenced by a diminished ability to suppress the rate of cholesteryl ester formation (Fig. 14). The TNM-mediated reduction in cholesterol transport was dose-dependent over a concentration range below 3 mM, similar to that observed for its effect on cellular binding of HDL₃. As with binding, the major

| Lipoprotein (25 µg protein/ml) | Incubation time (h at 37 °C) | Immuno precipitable cholesterol µg/mg lipoprotein protein |
|-----------------------------|---------------------------|-----------------------------------------------|
| Native HDL₃                 | 1                         | 79 ± 13                                      |
|                             | 25                        | 197 ± 10                                    |
| TNM-HDL₃                    | 1                         | 84 ± 8                                       |
|                             | 25                        | 212 ± 10                                    |

Fig. 11. Effect of TNM treatment on the ability of apo-A-I proteoliposomes to compete for ¹²⁵I-HDL₃ binding to fibroblasts. Proteoliposomes were prepared as described under “Experimental Procedures.” Untreated and 3 mM TNM-treated vesicles were used as competitors for ¹²⁵I-HDL₃ binding during a 1-h, 37 °C incubation as described for Fig. 1. The inset shows SDS-PAGE of the indicated vesicles after staining with Coomassie Blue. Results are representative of six separate experiments performed at either 0 °C or 37 °C with six different preparations of proteoliposomes.

Fig. 12. High performance gel filtration chromatography of untreated (A) and TNM-treated (B) apo-A-I proteoliposomes. Proteoliposomes were prepared with [³H]phospholipids and analyzed by high performance gel filtration chromatography as described under “Experimental Procedures.” The column was eluted at a flow rate of 1.0 ml/min and fractions of 1 ml each were collected and analyzed for [³H]radioactivity (broken line). Protein content was monitored continuously at 280 nm (solid line). Elution time indicates time after V₀. The concentration of TNM used was 3 mM. Lipoproteins and standard proteins of known molecular weight are indicated as follows: 1, LDL; 2, thyroglobulin (670,000); 3, HDL; 4, ovalbumin (45,000); and 5, myoglobin (17,000).

Fig. 6). TNM treatment of the proteoliposome almost completely abolished its ability to compete for ¹²⁵I-HDL₃ binding (Fig. 11). When proteoliposomes were prepared with [³H]phosphatidylcholine and characterized by high performance liquid chromatography, the apo-A-I and radiolabeled phospholipid eluted together as a single peak with an apparent size similar to HDL (Fig. 12A). Except for a slightly broader peak, the elution profile for TNM-treated proteoliposomes was the same as that of the untreated vesicle, with both protein and phospholipid eluting together in a single population of particles (Fig. 12B). These results indicate that TNM treatment blocks competitive binding of apo-A-I proteoliposomes without causing marked alteration in the size distribution and lipid composition of particles.
TABLE IV

Effect of TNM treatment on ability of HDL₃ to transport cholesterol from fibroblasts

Fibroblasts were incubated for 48 h with 50 μg/ml [¹⁴C]cholesterol (1 μCi/ml), washed five times with PBS-albumin, and then incubated with serum-free medium containing albumin plus the indicated lipopro-tein (20 μg/ml). After 48 h, the cells were chilled to 0 °C, the medium was collected and assayed for [¹⁴C]cholesterol and cholesterol mass, and the washed cells were extracted with hexane/isopropyl alcohol for unesterified cholesterol mass and radioactivity determinations. Net change in medium cholesterol mass was calculated as the difference in unesterified cholesterol in medium from dishes with and without cells. Cell-free values for medium containing no HDL₃ or TNM-HDL₃ were 0.12, 0.52, and 0.59 μg of cholesterol/ml, respectively. Net change in cellular cholesterol mass and radioactivity was calculated from the difference between zero time (145 μg of cholesterol/mg of cell protein) and 48-h values. The [¹⁴C]cholesterol content of the cells and medium was converted to mass equivalents using the mean value for the specific activity of unester-ified cholesterol/ml, respectively. Net change in cellular cholesterol mass and

| Net change in unesterified cholesterol | Lipoprotein added | Lipoprotein added |
|----------------------------------------|-------------------|-------------------|
|                                       | None              | TNM-HDL₃         |
|                                       | ¹⁴C cholesterol-48 h⁻¹, mg cell protein⁻³ |
| Medium                                |                   |                   |
| ³H                                    | +17 ± 2⁺⁺⁺       | +38 ± 3          |
| Mass                                  | +11 ± 1⁺⁺⁺       | +35 ± 1⁺⁺⁺      |
| Cellular                              |                   |                   |
| ³H                                    | -19 ± 4⁻⁻⁻       | -40 ± 2⁻⁻⁻      |
| Mass                                  | -13 ± 3⁻⁻⁻       | -31 ± 6⁻⁻⁻      |

⁺ Different from value for native HDL₃, p < 0.05.

FIG. 13. Effect of TNM-treatment on ability of HDL₃ to promote [¹⁴C]cholesterol efflux from fibroblasts. Cultured human fibroblasts were preincubated with medium containing 50 μg/ml [¹⁴C]cholesterol, thoroughly washed, and then incubated with medium containing the indicated concentrations of native (●) or TNM-treated (○) HDL₃. The appearance of [¹⁴C]radioactivity in the medium was measured after 4 h at 37 °C. Values are the mean of triplicate determination ± S.E.

reduction in cholesterol transport occurred at TNM concentra-tions below which there were marked changes in the size and shape of HDL particles. These results show a close association between the inhibitory effect of TNM on HDL₃ binding and HDL₃-mediated cholesterol transport from cells.

DISCUSSION

Previous work in our laboratory has provided evidence for the existence of an HDL receptor on cultured human fibro-blasts (15), human smooth muscle cells (15), and bovine aortic endothelial cells (45). To characterize the binding domains on HDL involved in ligand recognition by that receptor, we altered HDL₃ by methods specific for certain amino acid residues. Acetylation (24) and reduction and alkylation (25) failed to alter competitive binding of HDL to its binding site on cultured fibroblasts. This implies a lack of involvement of lysine and cysteine residues in recognition of HDL by its binding site. Cyclohexanedione treatment caused only a slight decrease in competitive binding of HDL₃, suggesting that arginine residues are also not involved in the interaction of HDL with cells. However, since this treatment modified less than half the arginine residues in HDL₃, the possibility of arginine involvement cannot be completely eliminated. The degree of inhibition of LDL binding to its receptor by cyclohexanedione treatment was found to be a function of the number of arginine residues modified (27).

Chen and colleagues originally reported (42) that TNM modification of rat HDL altered its interaction with isolated hepatocyte membranes and perfused rat liver and they sug-gested that tyrosine was present in the HDL binding recognition site. To test for possible tyrosine involvement in the active ligand site of human HDL for its receptor on extrapatic cells, native and TNM-modified HDL were compared in their ability to compete with HDL for its binding to human fibroblasts. In agreement with the earlier report (42), TNM treatment of HDL profoundly decreased its competitive bind-ing to fibroblasts. Amino acid analysis of TNM-HDL in the present study showed that tyrosine was specifically altered, suggesting the presence of tyrosine at the active ligand site. Furthermore, the increased mobility of TNM-HDL on agarose gel electrophoresis was consistent with the concept that formation of 3-nitrotyrosine had occurred, resulting in an increased negative charge of the particle. However, it is impos-sible to draw firm conclusion regarding tyrosine involvement at the active ligand site in light of the results of SDS-PAGE analysis which clearly showed protein cross-linking due to TNM treatment. Significantly, the TNM dose-response curve for binding competition paralleled the degree of cross-linking evident by SDS-PAGE. Cross-linking as a side reaction to TNM was first described in 1968 by Doyle and colleagues (45) in the case of collagen, γ-globulin, and carboproteinase A. TNM has been reported to cross-link certain other proteins.

FIG. 14. The effects of TNM treatment on the ability of HDL₃ to modulate cholesteryl ester formation in fibroblasts. Fibroblasts were incubated with serum-free medium containing albumin plus 50 μg/ml cholesterol. After 36 h, cells were incubated with the same medium without cholesterol to allow for equilibration of intracellular pools of cholesterol. After 12 h, cells were washed five times with PBS-albumin and incubated with medium containing 1 mg/ml albumin alone (broken horizontal line) or plus 10 μg of protein/ml of HDL₃ treated with the indicated concentration of TNM. After 4 h, the rate of cholesteryl ester formation was measured by 1-h pulse incubations with [¹³C]oleic acid as described under “Experimental Procedures.” Each point represents the mean ± S.E. of four incuba-tions. Samples of HDL₃ are the same as those shown in Fig. 7.
(47, 48), although many additional proteins do not appear to undergo this side reaction (46, 49, 50). Even though TNM-mediated cross-linking is believed to specifically involve tyrosine residues (47, 51), the cross-linking reaction itself alters the secondary structure of an affected protein. Thus, TNM cross-linking is likely to affect accessibility of many amino acid residues other than tyrosine at some distance from the cross-linking site.

Although involvement of specific amino acids was not demonstrated conclusively, the current study provides additional evidence that binding of HDL₃ to cholesterol-treated cells is mediated by HDL apoproteins rather than lipid. SDS-PAGE analysis revealed dose-dependent TNM cross-linking of HDL apoproteins which closely paralleled the dose-dependent effect of TNM on binding. As evidenced by gradient gel filtration, electrophoresis, and electron microscopy, the size and shape of HDL₃ particles was not altered substantially by treatment with 3 mM TNM, a concentration that led to maximum suppression of HDL₃ binding to cells. At concentrations below 3 mM, TNM had only a modest effect on the lipid to protein ratio of HDL₃. On an HDL₃ protein basis, TNM treatment led to the same degree of loss (~25% at 3 mM TNM) of all the major lipid classes except unesterified cholesterol. Analysis of HDL lipids by gas chromatography indicated that the loss in phospholipid, cholesteryl ester, and triglyceride was not the result of extensive hydrolysis of ester bonds or nitration of acyl group double bonds. It is possible that TNM treatment led to a modification of the physical properties of a proportion of HDL₃ lipids so that they were incompletely recovered during extraction. This conclusion is supported by results from a recent study by Chacko (54), who demonstrated that TNM treatment caused an increase in the proportion of phospholipid and cholesteryl esters that remained associated with protein during lipid extraction, suggesting that the apparent lipid loss was due to cross-linking of some of the HDL lipids to apoprotein. Regardless of the mechanism involved, it is unlikely that the relatively minor changes in lipid composition alone could account for the striking suppression of competitive binding that was observed when HDL₃ was treated with 3 mM TNM.

Additional evidence that protein is involved in the interaction of HDL with its cell-surface binding site was provided by studies comparing competitive binding of native and recombinant HDL particles that have different protein and lipid compositions. On a protein basis, HDL₃ and HDL₃ compete for 3H-HDL₃ to the same degree, even though HDL₃ is a larger particle containing substantially more lipid. Treatment with 3 mM TNM suppressed competitive binding of both HDL subclasses to the same extent. While protein-free egg-lecithin liposomes are poor competitors, proteoliposomes containing apo-A-I are as effective as HDL₃ in their ability to compete for HDL binding (17). Treatment of apo-A-I vesicles with 3 mM TNM markedly suppressed their competitive binding with only minor changes in size distribution of the particles. This suppression in binding occurred even though there was no decrease in the phospholipid to apoprotein ratio of these particles, providing additional support for the conclusion that the inhibitory effect of TNM was not due to changes in particle lipid composition.

In a recent report (19), Tabas and Tall concluded that association of HDL₃ with cultured cells involves the interaction of surface lipids of HDL₃ with the cell membranes. Their conclusions were based in part on results showing that tryptic digestion of HDL₃ proteins did not eliminate the ability of HDL₃ to interact with cells. It is possible, however, that the binding domains on HDL₃ are located within the hydrophobic regions of the apoprotein and may be protected against trypsin digestion. Since TNM is also hydrophobic, these regions may be particularly sensitive to cross-linking by TNM. When purified lipid-depleted apo-A-I was treated with 3 mM TNM in aqueous solution, very little cross-linking was evident and tyrosine residues were unmodified (data not shown), suggesting that the lipid environment was an important factor in the modification process. That the receptor binding domains on apoproteins can be located in areas protected against proteolysis is supported by results showing that treatment of LDL with trypsin had little effect on its ability to interact with its receptor (52).

Several recent studies have demonstrated that TNM treatment of HDL abolishes its interaction with high affinity binding sites on human liver (55), rat liver (54), and rat ovarian (56) membranes. Although high affinity binding was reduced, TNM-treated HDL was still capable of stimulating steroidogenesis by rat ovarian cells (56), suggesting that delivery of HDL sterol to this cell type is not mediated by binding of HDL to its high affinity site. In apparent contrast to delivery of HDL cholesterol to ovarian cells, our previous studies (14, 15) suggested that removal of cholesterol from cultured fibroblasts is facilitated by binding of HDL to high affinity sites on the cell surface. Since treatment of HDL₃ with 3 mM TNM suppressed its ability to interact with its high affinity binding site without causing major changes in lipid composition or particle morphology, we tested the feasibility of using TNM-treated HDL₃ as a negative control for studies evaluating the role of HDL binding in removal of cholesterol from cells.

The current and previous studies (14, 15) demonstrated that, as with cellular binding of HDL, a typical saturation curve for promotion of cholesterol efflux from fibroblasts contains both a high affinity, saturable component and a low affinity, nonsaturable component. We postulated that the high affinity component may represent transport of cholesterol from cells that is mediated by HDL binding to its high affinity site (14, 15, 53). This concept is supported by recent results showing that treatment of HDL₃ with TNM decreased its ability to promote cholesterol transport from fibroblasts by the high affinity process. As with HDL binding, the reduction in HDL-mediated cholesterol transport by TNM treatment was dose-dependent over a TNM concentration range of 1–3 mM. The ability of HDL₃ to promote cholesterol efflux by the low affinity process appeared to be unaffected by TNM treatment, suggesting that the capacity of the particle to accept cellular cholesterol was not abolished by TNM treatment. Moreover, TNM treatment failed to alter the ability of HDL₃ to sequester cholesterol when HDL₃ was added to a cholesterol-albumin suspension. Although cellular binding of HDL₃ does not appear to be essential for transport of cholesterol from extracellular cells to HDL₃ (7), results from the current study support the hypothesis that HDL binding may facilitate removal of cholesterol from cells when they become acutely overloaded with cholesterol. This facilitative process may be mediated by a specific cell-surface receptor for HDL that is induced in response to cholesterol loading (15).

Acknowledgments—We thank Dr. Marian Cheung for radial immunodiffusion, Dr. Teresa H. Aulinskas for molecular sieve chromatography, Dr. Tom Wight for electron microscopy, Dr. William Osborne for amino acid analysis, and Dr. Marian Childs for fatty acid analysis. We thank Vilma Fernandez, Carole Brewer, Tom Johnson, Carolyn Johnson, Sue Pengo, Audrey Griffin, Welling King, Maria Culala, Daniela Hairabedian, and Elaine Loomis for excellent technical assistance, and Ann Ferguson for skillful manuscript preparation.
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