Effects of Tunicamycin on the Binding and Degradation of Low Density Lipoproteins and Glycoprotein Synthesis in Cultured Human Fibroblasts*

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Subroto Chatterjee,‡ Peter O. Kwiterovich, Jr., and Catherine S. Sencer
From the Departments of Pediatrics and Medicine, Lipid Research Clinic, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

SUMMARY
Tunicamycin, an antibiotic that prevents the glycosylation of newly synthesized protein, was found to inhibit the high affinity binding and degradation of low density lipoproteins in normal human cultured fibroblasts. This inhibition of the binding and degradation of low density lipoproteins was time-dependent and was reversible upon removal of tunicamycin from the medium. Glycoprotein synthesis was inhibited more than 74% in tunicamycin-treated cells, while protein synthesis was minimally inhibited. After tunicamycin was removed from the medium, incubation of the cells in medium supplemented with lipoprotein-deficient serum for 24 to 48 h reversed the inhibitory effect of the antibiotic on both glycoprotein synthesis and the binding and degradation of low density lipoproteins. These observations indicate that there is a relationship between glycoprotein synthesis and the binding and degradation of low density lipoproteins. Glycosylation of the receptor for low density lipoproteins may be necessary for its intracellular migration or for its normal orientation on the cell surface. Alternatively, the glycosylation of an intracellular or cell surface glycoprotein(s) may be necessary in order for the receptor for low density lipoprotein to function normally. Since tunicamycin blocks the synthesis of N-acetylglucosaminyl pyrophosphoryl polyisoprenol (Takacz, J. S., and Lampon, J. O. (1975) Biochem. Biophys. Res. Commun. 65, 248-257; Struck, D., and Lennarz, W. J. (1977) J. Biol. Chem. 252, 1007-1013) and results in the synthesis of glycoproteins deficient in asparagine-linked oligosaccharides, the data further suggest a possible role of polyisoprenyl compounds in the synthesis of glycoproteins in cultured human fibroblasts. The possible role of this pathway in a feedback regulation of the receptor by low density lipoproteins remains to be elucidated.

Plasma low density (beta) lipoproteins (LDL) are the major carriers of plasma cholesterol in man. The metabolism of LDL has been studied in vitro in a variety of cell types, including cultured fibroblasts, smooth muscle cells, leukocytes, and isolated hepatocytes (1). A pathway for the metabolism of LDL has been proposed, based primarily on the studies of Goldstein and Brown in cultured human fibroblasts (1). The pathway may be summarized as follows. LDL is first bound by a specific high affinity receptor that is located within the "coated pit" on the cell surface. The bound lipoprotein is then internalized by absorptive endocytosis and is transferred to the lysosome where the protein moiety is catabolized and cholesteryl esters are hydrolyzed. The cholesterol that is released is then utilized to satisfy cellular requirements including membrane synthesis. In addition, cholesterol suppresses the further de novo synthesis of hydroxymethylglutaryl coenzyme A reductase, the rate limiting enzyme of cholesterol biosynthesis, and also appears to inhibit the synthesis of the LDL receptor itself, thus limiting the further entry of LDL-cholesterol into the cell. The inherited inability of the cell specifically to bind LDL has been shown to result in marked alterations in the metabolism of both LDL and cholesterol in cultured fibroblasts from patients with homozygous familial hypercholesterolemia (1).

Very little is known about the structure of the LDL receptor except that it seems to be protein in nature (1). We, and others, have suggested previously that the LDL receptor may be a glycoprotein (2-5). If so, receptor function should be altered by agents that block glycoprotein synthesis.

Tunicamycin, an antibiotic from Streptomyces lysosuperficus, contains N-acetylgalactosamine and has been shown to interfere selectively with glycoprotein biosynthesis by inhibiting the formation of dolichol-bound N-acetylgalactosamine derivatives. Since this reaction is involved in the synthesis of the core sequence for N-glycosidically linked oligosaccharides, treatment with tunicamycin results in the synthesis of glycoproteins deficient in asparagine-linked oligosaccharide(s) (6, 7).

Tunicamycin has been previously used in cultured fibroblasts to study the biosynthesis of fibronectin, a major cell surface glycoprotein. Although synthesis of fibronectin does not absolutely require glycosylation via a lipid-linked pathway, the carbohydrate-deficient pool of fibronectin inside the cell is subject to enhanced proteolytic degradation. Thus, the enhanced rate of fibronectin degradation results in decreased quantities of this glycoprotein on the cell surface and the cell culture medium (8). An essential role of glycosylation of proteins by the lipid-linked pathway has been indicated by studies in which tunicamycin was found to inhibit N-acetylgalactosaminyl (P)-P dolichol synthesis and to block the development at specific stages of sea urchin embryos (9). Indirect evidence suggesting the involvement of these compounds in the replication, assembly, and maturation of virus, and in the secretion and turnover of fibronectin in chick fibroblasts has also been reported.

In this paper, we report the effects of tunicamycin on the binding and degradation of LDL by normal cultured human fibroblasts. The data suggest a link between glycoprotein synthesis and the high affinity binding and degradation of LDL.

MATERIALS AND METHODS
Na[14C]I (13 to 17 mCi/μg in 0.05 N NaOH), L-[U-14C]leucine (>300

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‡ To whom all inquiries pertaining to this work should be addressed.

† 1\textsuperscript{1} The abbreviation used is: LDL, low density lipoproteins.
phosphate-buffered saline containing 0.1% bovine serum albumin and five times with phosphate-buffered saline. The monolayers were solubilized in 1 N NaOH and suitable aliquots were withdrawn for measurement of protein and determination of radioactivity by gamma counting.

**RESULTS**

**Effects of Tunicamycin on the Viability and the Rates of Incorporation of [3H]Leucine and [14C]Glucosamine into Normal Human Fibroblasts**—Trypan blue dye exclusion studies indicated that all fibroblast cultures remained viable (>89%) throughout the experiments. The data in Tables I and II indicate that the rates of incorporation of [3H]leucine into normal human fibroblasts treated with tunicamycin or in the

| Time | Incorporation of L-[U-3H]Leucine | Incorporation of D-[U-14C]Glucosamine |
|------|---------------------------------|--------------------------------------|
| h    | Tunicamycin | Control | Inhibition | Tunicamycin | Control | Inhibition |
| 6    | 470.5 | 489.0 | 3.8 | 47.6 | 51.9 | 6.9 |
| 24   | 414.5 | 457.0 | 9.3 | 26.4 | 48.0 | 45.0 |
| 48   | 410.4 | 414.2 | 7.0 | 11.0 | 42.0 | 73.9 |

**Table II**

| Time | Incorporation of L-[U-3H]Leucine and D-[U-14C]Glucosamine in normal human fibroblasts treated with tunicamycin for 48 h and subsequently incubated in lipoprotein-deficient serum-supplemented medium
|---|---|---|---|---|---|
| h | cpm/μg protein | % | cpm/μg protein | % |
| 6 | 366.5 | 428.0 | 14.4 | 53.7 | 65.9 | 18.6 |
| 24 | 363.2 | 445.0 | 19.6 | 60.0 | 49.2 | nil |
| 48 | 380.5 | 380.5 | nil | 49.3 | 49.1 | nil |

**Low Density Lipoproteins and Glycoprotein Synthesis in Vitro**

mCi/mmol), and D-[U-14C]glucosamine (>200 mCi/mmol) were purchased from New England Nuclear. Fetal bovine serum, minimum essential medium, antibiotics, and other tissue culture supplies were obtained from Grand Island Biological Co. Other materials and reagents for biochemical assays were obtained from sources previously reported (10). Tunicamycin was the gift of Dr. A. A. Buchsbaum of Merck, Sharp and Dohme Co. Three normal human fibroblast lines were used in this study. The cell lines were derived from skin biopsies from the forearms of two adult males (R. W., P. O. K.) and one adult female (L. W.) and were characterized as normal using the following procedures. 

**TABLE I**

| Time | Incorporation of L-[U-3H]Leucine and D-[U-14C]Glucosamine |
|------|-------------------------------------------------------------|
| h    | Tunicamycin | Control | Inhibition | Tunicamycin | Control | Inhibition |
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**RESULTS**

Effects of Tunicamycin on the Viability and the Rates of Incorporation of [3H]Leucine and [14C]Glucosamine into Normal Human Fibroblasts—Trypan blue dye exclusion studies indicated that all fibroblast cultures remained viable (>89%) throughout the experiments. The data in Tables I and II indicate that the rates of incorporation of [3H]leucine into normal human fibroblasts treated with tunicamycin or in the

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control cells were similar at 6, 24, and 48 h of incubation. The inhibition of protein synthesis by tunicamycin was less than 10% at each time interval examined (Table I). After tunicamycin had been removed from the cell culture medium, there was some decreased incorporation of leucine into the cells that had been previously treated with tunicamycin compared to the controls after 6 and 24 h of incubation. By 48 h of incubation, the incorporation of [14C]leucine into the control cells and those previously treated with tunicamycin were indistinguishable. These data indicate that there were insignificant alterations in the rates of protein synthesis in the cells treated with tunicamycin. In contrast, the rate of [14C]glucosamine incorporation, as used as an index of glycoprotein biosynthesis, was markedly decreased in the cells treated with tunicamycin after 24 and 48 h of incubation. There was no short term difference between the incorporation of [14C]glucosamine after 6 h in the tunicamycin and the control cells. At 24 and 48 h, there was a 45 and 74% inhibition of glycoprotein synthesis, respectively. However, after tunicamycin was removed from the medium, there was a sudden burst of glycoprotein synthesis by these cells, as judged by a 5-fold increase in the incorporation of [14C]glucosamine into the trichloroacetic acid-insoluble fractions (Table II). This enhanced incorporation of glucosamine by these cells into the glycoprotein fraction was maintained 24 h after tunicamycin had been removed. By 48 h, the incorporation of glucosamine into the cells previously treated with tunicamycin was very similar to that found after 6 h of incubation with tunicamycin (Tables I and II). Therefore, there was no inhibition of glycoprotein synthesis in these cells 24 and 48 h after tunicamycin had been removed from the medium.

**Effects of Tunicamycin on 125I-LDL Binding and Degradation**—Untreated cultured fibroblasts bound as much LDL as cells which were treated with tunicamycin for 6 h (Fig. 1A). The degradation of LDL by these cells was also equally unaffected after 6 h in tunicamycin (Fig. 2A), indicating that the internalization of LDL and bulk-phase endocytosis were also unaffected. However, after 24 h of incubation with tunicamycin, there was approximately a 50% inhibition of high affinity LDL binding (Fig. 1C); this decreased specific (high affinity) binding of LDL was associated with a similar decrease in LDL degradation that occurred through the “LDL pathway” by bulk-phase endocytosis (Fig. 2C). When tunicamycin was removed from the culture medium, there was a gradual increase in the high affinity binding of LDL (Fig. 1A and B), accompanied by a similar increase in LDL degradation (Fig. 2B). The binding and degradation of LDL in the nonspecific components of the curves remained parallel with those from the normal control cells. In addition, the amount of LDL bound in the untreated cells after 24 h was increased from 400 to 500 ng/ml of LDL protein in the high affinity portion of the curve (Fig. 1A and B). After 48 h of treatment with tunicamycin, inhibition of more than 75% of high affinity LDL binding was observed (Fig. 1C); this decreased specific (high affinity) binding of LDL was associated with a similar decrease in LDL degradation that occurred through the “LDL pathway” by bulk-phase endocytosis (Fig. 2C).

**FIG. 1.** Effects of tunicamycin and its subsequent removal on 125I-low density lipoprotein binding in normal human fibroblast cultures. Normal human fibroblasts were seeded (2 x 10^6 cells/dish) into 60-mm Petri dishes and incubated for 6 days in minimum essential medium containing 10% fetal bovine serum. On the 6th day, medium was removed, the monolayers were washed three times with sterile phosphate-buffered saline and incubated at 37°C in medium containing 5% lipoprotein-deficient serum with and without tunicamycin (10 μg/10 μl in 0.01 N NaOH). To the control cultures were added 10 μl of 0.01 N NaOH. At 2-, 24-, and 44-h intervals of incubation of cells with and without tunicamycin, and at similar intervals after the removal of tunicamycin from the medium (by repeated washing with sterile phosphate-buffered saline), monolayers were incubated with 0 to 100 μg of 125I-low density lipoprotein for 4 h. Subsequently, the medium was removed. The monolayers were washed thrice with phosphate-buffered saline containing 0.2% bovine serum albumin and five times with phosphate-buffered saline. Following overnight solubilization with 1.0 N NaOH, the protein content and radioactivity were measured in the cell extracts. Each point represents the average of duplicate determinations of 2 separate dishes of a typical experiment. A, B, and C reflect 125I-LDL binding in fibroblasts with and without tunicamycin treatment for 6, 24, and 48 h, respectively. D, E, and F reflect 125I-LDL binding in fibroblasts that were removed from tunicamycin treatment and corresponding control cultures which were incubated in lipoprotein-deficient serum medium for 6, 24, and 48 h, respectively. Control cultures without tunicamycin are indicated by the broken line.

**FIG. 2.** Effects of tunicamycin and its subsequent removal on 125I-low density lipoprotein degradation in normal human fibroblast cultures. The protocol of this experiment was identical with that described in the legend to Fig. 1. Following incubation for 4 h, medium from tunicamycin-treated, tunicamycin-treated and washed cultures as well as control cultures were removed. Medium was precipitated with ice cold 50% trichloroacetic acid. The trichloroacetic acid-soluble fractions were processed further for the measurement of LDL degradation as described elsewhere (11). A, B, and C reflect 125I-LDL degradation in fibroblasts with and without tunicamycin treatment for 6, 24, and 48 h, respectively. D, E, and F represent 125I-LDL degradation in fibroblast cultures that were removed from tunicamycin treatment and corresponding control cultures which were incubated in medium containing 5% lipoprotein-deficient serum for 6, 24, and 48 h, respectively.

**DISCUSSION**

The present study evaluates the relation of glycoprotein synthesis to LDL binding and degradation in cultured normal human fibroblasts. The most important finding is that a marked inhibition of glycosylation of certain newly synthe-
sized proteins completely prevented the binding and degradation of LDL via the high affinity receptor mechanism. One possible explanation of these observations is that the amount or rate of export, or both, of newly synthesized LDL receptors to the cell surface are severely inhibited, presumably due to underglycosylation; consequently, the total number of newly synthesized LDL receptors on the cell surface is substantially reduced. The available data do not exclude several other possible explanations. For example, the exteriorization of the internal pool of LDL receptors may conceivably not involve prior glycosylation. In this case, the transport and incorporation of the LDL receptor to the cell surface may be dependent upon some other cellular glycoprotein. Alternatively, the decrease in glycoprotein synthesis may be associated with an altered organization of other membrane proteins or glycoproteins on the cell surface of the fibroblasts that affect the accessibility of the LDL receptors for the specific binding of LDL.

Since tunicamycin blocks the synthesis of N-acetylgalactosaminyl pyrophosphoryl oligosaccharide (6, 7) and results in the synthesis of glycoproteins deficient in asparagine-linked oligosaccharide in other systems, the inhibition of the high affinity binding and degradation of LDL and the reversibility of these phenomena upon removal of tunicamycin from the medium raise the possibility that the first step in the glycosylation of the newly synthesized LDL receptor in cultured human fibroblasts may involve an asparagine-glucosamine linkage. If glycosylation is indeed necessary for LDL receptor synthesis or secretion, or both, then decreased glycosylation due to tunicamycin treatment may have several additional effects. (a) It may alter the migration and localization of the LDL receptors within the coated pits and the subsequent migration towards the cell surface; or (b) it may increase proteolytic degradation of newly synthesized LDL receptors. For example, in a recent study, Leavitt et al. (16) found that in tunicamycin-treated vesicular stomatitis virus- and simian virus-infected baby hamster kidney cells, all of the viral proteins were synthesized, whereas the glycoproteins were devoid of carbohydrates. Moreover, the nonglycosylated glycoproteins were not detected on the cell surface when lactoperoxidase-mediated iodination or indirect immunofluorescence staining or chymotrypsin treatment was employed. In contrast, the glycosylated glycoproteins were readily detectable by these methods (16). Subsequently, these investigators found that the nonglycosylated glycoproteins had altered solubility properties relative to their glycosylated counterparts and, hence, altered intracellular migration and exteriorization on the cell surface. In another study, tunicamycin was reported to have decreased the cellular pool of fibronectin in chick embryo fibroblasts (8) which, in turn, was reflected in decreased quantities of this glycoprotein on the cell surface and in the culture medium. Brown and Goldstein (17) have shown that prior incubation of normal human fibroblasts with cholesterol, 25a-hydroxycholesterol, or LDL progressively reduced the ability of these cells to bind 125I-LDL at the high affinity site. Thus, decrease in binding can be interpreted as due to the proportional decrease in total number of receptors rather than altered binding affinity or cellular LDL metabolism. Using this criterion and adding cycloheximide to the culture medium, the half-life of the LDL receptor was reported to be approximately 25 h. Recently, 25a-hydroxycholesterol has been found to inhibit the incorporation of radioactive acetate into cholesterol and dolichol in cultured smooth muscle cells by 82 and 91%, respectively (18). At the same time, 25a-hydroxycholesterol diminished the synthesis of phosphoryl oligosaccharides and dolichol-dependent glycoproteins (18). Considered together, these observations indicate that glycosylation of newly synthesized LDL receptor protein may be necessary for proper orientation of LDL receptors and to function on the fibroblast cell surface.

The possible involvement of the glycoproteins and dolichol pathway in LDL receptor function and regulation suggests a number of metabolic steps that may be involved in the pathophysiology of familial hypercholesterolemia. Primary aberrations in this pathway may be due to one or more genetic mutations. 

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