Effect of Tunicamycin on the Metabolism of Low-Density Lipoproteins by Control and Low-Density-Lipoprotein-Receptor-Deficient Human Skin Fibroblasts

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Preincubation of normal human skin fibroblasts with tunicamycin, which inhibits *N*-glycosylation of glycoproteins, resulted in a dose-dependent and reversible inhibition of binding and internalization of homologous low-density lipoproteins by the cells. The degradation of the internalized lipoproteins was not affected by the drug. Comparative studies with fibroblasts deficient in low-density-lipoprotein receptors indicated that tunicamycin exerts its inhibitory effect only via the receptor-mediated high-affinity binding and uptake of lipoproteins. These results suggest that expression of low-density-lipoprotein receptors on the cell surface of human skin fibroblasts depends on intact *N*-glycosylation.

Human skin fibroblasts internalize LD lipoproteins by a high-affinity receptor-mediated process and receptor-independent bulk-phase endocytosis (Goldstein & Brown, 1974). Structural features required on the LD lipoprotein particle for binding to the receptor have been characterized in several studies (Mahley *et al.*, 1977; Weisgraber *et al.*, 1978; Filipovic *et al.*, 1979; Filipovic & Buddecke, 1979), whereas little is known about LD-lipoprotein receptor itself; it has been suggested to be a glycoprotein (Basu *et al.*, 1978; Chatterjee *et al.*, 1978; DiFerrante *et al.*, 1978).

In the present study tunicamycin was used as a probe to investigate the glycoprotein nature of the LD-lipoprotein receptor. Tunicamycin interferes specifically with the synthesis of *N*-glycosidically linked oligosaccharides, thus leading to the formation of glycoproteins deficient in asparagine-linked oligosaccharides (Tkacz & Lampen, 1975; Struck & Lennarz, 1977). The strong inhibition of receptor-mediated binding and uptake of LD lipoprotein observed in tunicamycin-treated fibroblasts suggests that *N*-glycosidically linked oligosaccharides are a constituent of the LD-lipoprotein receptor or a component required for the expression of the LD-lipoprotein receptor on the cell surface. While this study was in progress Chatterjee *et al.* (1979) came to similar conclusions.

Abbreviation used: LD lipoprotein, low-density lipoprotein.

Materials and Methods

Tunicamycin was a gift from Dr. R. L. Hamill, Lilly Research Laboratories (Indianapolis, IN, U.S.A.). [^{14}C]Glycine (sp. radioactivity 188 Ci/mol), [$2\text{-}^3\text{H}$]mannose (sp. radioactivity 4.1 Ci/mol) and ^{125}I (carrier-free) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Lipoproteins

Human LD lipoproteins (density 1.02–1.055 g/ml) were isolated and labelled with ^{125}I as described previously (Filipovic *et al.*, 1979), yielding specific radioactivities of 9×10^4 – 14×10^4 c.p.m./ μg of LD lipoprotein. Lipoprotein-deficient human serum was prepared as described by Fung *et al.* (1977).

Cell lines

Human skin fibroblasts deficient in LD-lipoprotein receptor (GM 1915) were obtained from the Institute for Medical Research, Camden, NJ, U.S.A. Control fibroblasts were cultured from skin explant of a healthy donor. Cells were grown in plastic dishes in Eagle's minimal essential medium supplemented with 10% (v/v) foetal calf serum (LS Labor Service, München, W. Germany) as described by Cantz *et al.* (1972).

Incubation experiments

All experiments were performed with cells grown to confluency in 25 cm² plastic dishes. On days 5–10 the growth medium was replaced by 3 ml of fresh medium containing 5% (v/v) lipoprotein-deficient human serum, 35 mM-dimethyl sulphoxide and up to 3.6 μ M-tunicamycin. After indicated periods of incubation 5 μ g of ¹²⁵I-labelled LD-lipoprotein protein/ml was added and the incubation was continued for 6–48 h. Cells were washed eight times with ice-cold buffered saline containing 0.2% (w/v) bovine serum albumin (Goldstein & Brown, 1974) treated with 0.1% tissue culture trypsin (LS Labor Service) or dissolved with 1.0 ml of 0.5 M-NaOH. Binding plus uptake and degradation were determined as described by Goldstein & Brown (1974). For determination of the incorporation of [¹⁴C]glycine or [2-³H]mannose, cells were incubated in the presence of tunicamycin or cycloheximide for 24 or 6 h respectively, before the addition of [¹⁴C]glycine (16 μ Ci/ml) or [2-³H]mannose (2 μ Ci/ml). After incubation for 6 h, cells were washed seven times with buffered saline, scraped off with a rubber 'policeman', collected by centrifugation at 1000 rev./min (800 g) for 3 min and suspended by eight freeze–thaw cycles in 0.5 ml of water. Cells and medium were precipitated at 4°C with trichloroacetic acid (final concn. 5%). The precipitate was dissolved in 0.5 ml of 2.5 M-NaOH and samples were used for determination of radioactivity and protein (Kaltwasser *et al.*, 1965). Neither tunicamycin nor cycloheximide had any effect on the cell viability, as monitored by the Nigrosine test (Kaltenbach *et al.*, 1958).

Results and Discussion

Treatment with tunicamycin for 24 h inhibited the binding plus uptake of ¹²⁵I-labelled LD lipoprotein by control human skin fibroblasts in a dose-dependent manner. In LD-lipoprotein-receptor-deficient cells the binding plus uptake was only slightly affected by the drug (Fig. 1). Tunicamycin did not affect degradation of internalized LD lipoprotein (Fig. 1). Inhibition of binding plus uptake was observed after a lag phase of 6–12 h and was maximal after preincubation for 24 h. The tunicamycin-mediated inhibition of the binding plus uptake was completely reversible within 3 days of removal of the drug. Measurement of the LD lipoprotein bound to the cell-surface receptors of tunicamycin-treated cells provided further evidence for the assumption that the observed inhibition of binding plus uptake was caused by inhibition of binding to the cell-surface receptors. The receptor-bound fraction of the cell-surface-associated ¹²⁵I-labelled LD lipoproteins of fibroblasts treated with 1.2 μ M-tunicamycin was 50% of that of controls (Table 1).

Fig. 1. Effect of tunicamycin on the binding plus uptake (open symbols) and degradation (closed symbols) of internalized ¹²⁵I-labelled LD lipoprotein by control (O, ●) and LD-lipoprotein-receptor-deficient (Δ, ▲) human skin fibroblasts

Cells were incubated in the presence of 35 mM-dimethyl sulphoxide and the indicated concentrations of tunicamycin. After 24 h, 5 μ g of ¹²⁵I-labelled LD-lipoprotein protein/ml was added and the incubation was continued for an additional 6 h. Binding plus uptake and degradation were determined as described in the Materials and Methods section. Data are mean values ($\pm$ S.D.) for four experiments carried out in duplicate.

The inhibitory effect of tunicamycin on uptake of LD lipoproteins seems to be specific and not due to a general inhibition of adsorptive endocytosis. Thus the uptake of ³⁵S-labelled proteoglycans and binding of these molecules to the cell surface was not or only slightly inhibited by up to 3.6 μ M-tunicamycin (von Figura *et al.*, 1979).

At high concentrations, tunicamycin inhibits protein synthesis (Duksin & Bornstein, 1977; Hart & Lennarz, 1978; Olden *et al.*, 1978). Inhibition of protein synthesis by cycloheximide is known to result in an inhibition of the binding and internalization of LD lipoprotein (Brown & Goldstein, 1975). At 100 μ M-cycloheximide, which inhibited binding and uptake of ¹²⁵I-labelled LD lipoprotein to a similar extent to 1.2 μ M-tunicamycin, incorporation of [¹⁴C]glycine into trichloroacetic acid-precipitable material was less than 15% of that of controls. By contrast, 1.2 μ M-tunicamycin exerted only a slight inhibition (Table 1). It inhibited the secretion of ¹⁴C-labelled trichloroacetic acid-precipitable material rather than its formation. Under identical conditions, tunicamycin decreased incorporation of [2-³H]mannose into cellular and extra-cellular trichloroacetic acid-precipitable material to 10 and 18% of that of controls respectively. These

Table 1. *Effect of tunicamycin and cycloheximide on the binding and uptake of ^{125}I -labelled LD lipoprotein and protein synthesis by control fibroblasts*

Cells were incubated in the presence of tunicamycin or cycloheximide for 24 or 6 h respectively; $5\text{ }\mu\text{g}$ of ^{125}I -labelled LD-lipoprotein protein/ml was added, either in the absence or presence of $350\text{ }\mu\text{g}$ of unlabelled LD lipoprotein/ml, and the incubation was continued for a further 6 h. At the end of the incubation cells were washed eight times with ice-cold buffered saline containing 0.2% bovine serum albumin and then treated with trypsin. Radioactivity was determined in the trypsin supernatant, cell layer and in the trichloroacetic acid-soluble fraction of the medium (Goldstein & Brown, 1974). High-affinity binding and internalization of LD lipoprotein was calculated as the difference between ^{125}I -labelled LD lipoprotein bound and internalized in the absence and presence of unlabelled LD lipoprotein. High-affinity binding and uptake accounted for 80–85% of total binding and uptake. Incorporation of $[\text{U-}^{14}\text{C}]$ glycine was determined as described in the Materials and Methods section. Data are mean values ($\pm$ s.d.) for four experiments. The inhibitory effect of cycloheximide on uptake of LD lipoprotein is only slightly increased by preincubation for 24 h. The residual uptake under these conditions was 605 ng of LD lipoprotein/mg of cell protein.

Addition	Concentration in medium (μM)	^{125}I -labelled LD lipoprotein (ng of LD-lipoprotein protein/mg of cell protein)		$10^{-3} \times$ Trichloroacetic acid-precipitable ^{14}C -labelled material (c.p.m./mg of cell protein)	
		Surface-bound	Internalized	Cell layer	Medium
None		150 ± 18	1428 ± 162	167 ± 16	100 ± 8
Tunicamycin	1.2	76 ± 10	770 ± 67	181 ± 19	46 ± 3
Cycloheximide	100.0	72 ± 12	698 ± 71	10 ± 1	8 ± 0.6

findings indicate that defective *N*-glycosylation, rather than inhibition of protein synthesis, is responsible for the tunicamycin-mediated inhibition of LD-lipoprotein binding to the cell surface.

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