Intercellular transport of lysosomal acid lipase mediates lipoprotein cholesteryl ester metabolism in a human vascular endothelial cell–fibroblast coculture system

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We present results from studies of human cell culture models to support the premise that the extracellular transport of lysosomal acid lipase has a function in lipoprotein cholesteryl ester metabolism in vascular tissue. Vascular endothelial cells secreted a higher fraction of cellular acid lipase than did smooth muscle cells and fibroblasts. Acid lipase and lysosomal β -hexosaminidase were secreted at approximately the same rate from the apical and basolateral surface of an endothelial cell monolayer. Stimulation of secretion with NH_4Cl did not affect the polarity. We tested for the ability of secreted endothelial lipase to interact with connective tissue cells and influence lipoprotein cholesterol metabolism in a coculture system in which endothelial cells on a micropore filter were suspended above a monolayer of acid lipase-deficient (Wolman disease) fibroblasts. After 5–7 d, acid lipase activity in the fibroblasts reached 10%–20% of the level in normal cells; cholesteryl esters that had accumulated from growth in serum were cleared. Addition of mannose 6-phosphate to the coculture medium blocked acid lipase uptake and cholesterol clearance, indicating that lipase released from endothelial cells was packaged into fibroblast lysosomes by a phosphomannosyl receptor-mediated pathway. Supplementation of the coculture medium with serum was not required for lipase uptake and cholesteryl ester hydrolysis by the fibroblasts, but was necessary for cholesterol clearance.

Results from our coculture model suggest that acid lipase may be transported from intact endothelium to cells in the lumen or the wall of a blood vessel. We postulate that delivery of acid hydrolases and lipoproteins to a common endocytic

compartment may occur and have an impact on cellular lipoprotein processing.

Introduction

We are studying the extracellular transport of lysosomal acid lipase in human cell culture models of vascular tissue to determine its role in lipoprotein cholesteryl ester metabolism and to learn how it relates to the abnormal lysosomal hydrolase activity and lipid accumulation that are characteristic of atherogenesis. Lysosomal acid lipase, also termed acid cholesteryl ester hydrolase, or cholesterol esterase (3.1.1.13), is critical for the hydrolysis of triglycerides and cholesteryl esters that cells acquire by receptor-mediated endocytosis of lipoproteins (Goldstein and Brown, 1977). A hereditary deficiency of acid lipase activity, in patients with Wolman disease—or the clinically less severe condition, cholesteryl ester storage disease—is characterized by accumulation of esterified cholesterol and triglyceride in several tissues (Schmitz and Assmann, 1989). Brown *et al.* (1976) showed that defective lipoprotein cholesteryl ester metabolism in fibroblasts from affected patients was corrected by culture with fibroblasts that had normal acid lipase activity or the corresponding conditioned medium. We studied acid lipase secretion by fibroblasts (Sando and Henke, 1982) and purified the extracellular form of the enzyme from large scale fibroblast microcarrier culture (Sando and Rosenbaum, 1985). When applied to cultured lipase-deficient fibroblasts, the purified lipase was internalized via a mannose 6-phosphate receptor-mediated route and hydrolyzed lysosomal cholesteryl esters.

The concept of receptor-mediated packaging of lysosomal enzymes was formulated to explain observations in cultured fibroblasts that acid hydrolases were secreted, recaptured through a specific recognition and uptake process, and compartmentalized in a functional state in lysosomes (Neufeld, 1981). The secretion-recap-

ture route for lysosomal enzyme packaging is now recognized to be quantitatively less important than one in which phosphomannosyl-mediated recognition of the nascent endogenous enzymes occurs within intracellular organelles (Creek and Sly, 1984). A general physiological role for the process of intercellular lysosomal enzyme exchange remains to be assigned. Lysosomal enzyme uptake by mammalian cells may be mediated by different processes and recognition systems, depending on the nature of the cellular interaction, the cell type, and structural properties of a given lysosomal enzyme (Olsen *et al.*, 1981; Owada and Neufeld, 1982; Shepherd and Stahl, 1984; McNamara *et al.*, 1985; von Figura and Hasilik, 1986; Dong and Sahagian, 1990).

One of our objectives has been to identify factors that regulate the secretion and uptake of lysosomal acid lipase in specific cell types, to determine how the localization and intercellular exchange of the enzyme affect its function in normal and diseased tissues. Its crucial role in lipoprotein metabolism has led to the consideration that acid lipase function is closely linked to atherogenesis (deDuve, 1974; Wolinsky *et al.*, 1979; Fowler and Brown, 1984; Schmitz and Assmann, 1989), and this directed our interest to properties of acid lipase transport in cells related to vascular tissue. We focused on endothelium because of its importance in regulating the localization and metabolism of substances in the vessel wall (Gimbrone, 1986) and because of the consequences of endothelial injury for atherogenesis (Ross and Glomset, 1976; Minick *et al.*, 1977). We discovered that human umbilical vein endothelial cells secreted substantially more acid lipase than did human vascular smooth muscle cells and fibroblasts. This finding prompted further study of the transport process and properties of the secreted endothelial lipase.

Endothelial cells exhibit structural (Muller and Gimbrone, 1986) and functional (Simons and Fuller, 1985) polarity with respect to their apical and basolateral surfaces. Thus, the vectorial transport of extracellular matrix remodeling factors (Unemori *et al.*, 1990) and bioactive lipids (Stoll and Spector, 1987, 1989) is thought to have an important role in endothelial cell metabolism and communication with neighboring cells. In investigations of other cell types that exhibit a polarized phenotype, the secretion of lysosomal enzymes by osteoclasts was highly polarized and has been related to a critical role for this process in bone metabolism (Baron *et al.*, 1988), whereas that of cathepsin D, by Ma-

din-Darby canine kidney cells, was bidirectional (Caplan *et al.*, 1987). To determine whether extracellular acid lipase was distributed on the luminal or basolateral side of the endothelium, we studied the polarity of secretion of the enzyme from endothelial cells grown on polycarbonate micropore filters in a system that allowed for sampling of medium in contact with each side of the cell monolayer.

In previous investigations where coculture systems were devised to model interactions between endothelium and smooth muscle cells in the vascular wall, endothelial cells influenced smooth muscle cell cholesterol and lipoprotein metabolism by modifying lipoprotein particles and by elaborating factors, such as mitogens and eicosanoids, that altered connective tissue cell growth and function (Davies *et al.*, 1985; Hajjar *et al.*, 1987; Horrigan *et al.*, 1988). To investigate the possibility that endothelial acid lipase can be transported to connective tissue cells and affect cholesteryl ester metabolism, we cocultured endothelial cells with lipase-deficient fibroblasts; this system enabled us to quantitate intercellular transfer of the lipase and its effect on cholesterol mobilization in the target cells.

Results

Acid lipase secretion from cultured endothelium and other human cells

Cultured human umbilical vein endothelial cells secreted acid lipase into the culture medium over a period of several days, during which intracellular activity was constant. Secretion of the lipase and lysosomal β -hexosaminidase (3.2.1.30) was enhanced by the addition of NH_4Cl to the culture medium (Figure 1). Only 1%–3% of the activity of the cytoplasmic enzyme, lactate dehydrogenase (1.1.1.27), was detected in the medium and this was not affected by NH_4Cl . In experiments presented here, cultured human vascular smooth muscle cells and skin fibroblasts secreted $\sim 1/6$ and $1/2$, respectively, of the lipase activity that was secreted by endothelial cells under basal conditions (Figure 2). However, the connective tissue cells were more sensitive to treatment with NH_4Cl , and fibroblasts secreted as much acid lipase as did endothelial cells when they were maximally stimulated. Although results varied among experiments, the intracellular lipase activity of endothelial cells was usually $\sim 25\%$ – 50% lower than that of smooth muscle cells and fibroblasts. Treatment of confluent cultures of

the above cell types with NH_4Cl had little effect on intracellular acid lipase activity.

Properties of secreted endothelial cell acid lipase

To obtain a sufficient quantity of acid lipase for characterization, secretions were collected from four T-75 flasks of confluent endothelial cells that were maintained for 3 d with or without 6 mM NH_4Cl in serum-free WRC 935 medium (Wolfe *et al.*, 1988). We compared the secreted endothelial lipase with the purified secreted hu-

Figure 1. Secretion of acid lipase, β -hexosaminidase, and lactate dehydrogenase by cultured endothelial cells. Human umbilical vein endothelial cells were prepared as described in Methods and grown in 12-well plates. After 5 d, cells were rinsed, then pretreated, to remove serum components, with serum-free Waymouth medium that contained 1 mg/ml fatty acid free bovine serum albumin. To minimize background ammonia levels in the culture medium that might arise from hydrolysis of glutamine, a liquid concentrate of MB 752/1 Waymouth medium, without glutamine, was purchased from Grand Island Biologicals; diluted; and supplemented with 2 mM L-glutamine, 8 $\mu\text{g}/\text{ml}$ insulin (Sigma; bovine pancreas), and antibiotics (as for standard fibroblast growth medium), just before use. After 4 h this was replaced with fresh serum-free medium that contained the indicated concentrations of NH_4Cl . The fresh media had the expected concentrations of NH_4Cl , as was determined with use of a colorimetric assay for ammonia (Sigma kit, reagent # 640-1 and 640-2), and these did not change significantly during the culture period. After 4 or 20 h, as indicated, medium and cells were harvested and samples analyzed for enzyme activity as described in Methods. Enzyme activities in cells and secretions were assayed in duplicate and normalized to cell protein. Values are given as the ratio, in percent, of extracellular to intracellular activity, and represent the average of determinations from duplicate cultures of the following: acid lipase after 4 h (●) or 20 h (○), β -hexosaminidase after 4 h (■), and lactate dehydrogenase after 4 h (◆). Intracellular acid lipase activity was 10 U/mg under basal conditions.

Figure 2. Secretion of acid lipase by cultured human skin fibroblasts and vascular smooth cells. Fibroblasts (AG 1519) and umbilical vein smooth muscle cells were cultured as was described in Methods. One week after being seeded, confluent cell monolayers in 12-well plates were treated and assayed for acid lipase activity as was described in the caption for Figure 1. Secretions and cell lysates were prepared from fibroblasts (■) and two different lines of smooth muscle cells (●, ○), which were exposed to varying concentrations of NH_4Cl for 20 h; intracellular lipase activity was 20, 21, and 9.5 U/mg, respectively, in extracts from cells maintained under basal conditions.

man fibroblast acid lipase/cholesteryl ester hydrolase (Sando and Rosenbaum, 1985). The two activities were indistinguishable with regard to catalytic behavior and pH dependence. Endothelial acid lipase was eluted from a column of decylagarose as a single peak with a profile identical to that of the major form of secreted fibroblast acid lipase. As with the fibroblast enzyme, a molecular weight of $\sim 50\,000$ was estimated by gel filtration chromatography. Like the major fibroblast acid lipase, but in contrast to a minor, fibroblast acid 4-methylumbelliferyl (4-MeUmb)¹ oleate hydrolase/phospholipase A, the endothelial lipase was stable for several hours in the presence of 0.4 M dithiothreitol. The secreted endothelial lipase was precipitated by agarose-linked monoclonal antibodies against the purified fibroblast acid lipase/cholesteryl ester hydrolase. Endothelial lipase that was secreted in the presence of NH_4Cl was indistinguishable, by the above criteria, from that secreted without stimulation.

¹ Abbreviations: BME, Basal Medium Eagle; FBS, fetal bovine serum; MEM, Minimal Essential Medium; 4-MeUmb, 4-methylumbelliferyl; MM199, modified Medium 199; NAD, nicotinamide adenine dinucleotide.

Table 1. Cultured endothelial barrier to passage of macromolecules^a

Sample applied	Time (hours)	Transfer across membrane (percent of applied)	
		– cells	+ cells
To upper chamber			
Acid lipase	0.5	19.9 ± 3.2 ^b	<0.1 ^c
	1.0	24.8 ± 1.2	<0.1
	2.0	35.7 ± 1.8	3.8 ^d
Albumin	1.0	31.9 ± 5.5	1.7 ± 0.7
To lower chamber			
Acid lipase	0.5	2.4 ± 0.2	2.1 ± 0.8
	1.0	8.0 ± 0.4	3.6 ± 0.4
	2.0	21.0 ± 4.2	3.5 ± 0.5
Albumin	1.0	5.5 ± 0.4	0.4 ± 0.1

^a Primary cultures of human umbilical vein endothelial cells were grown on 4.5 cm² Transwell polycarbonate filters and maintained in culture as described in Methods. After 4 d, medium was removed from the confluent monolayers and the filter insert and lower well were rinsed and pretreated for 1 h with serum-free Waymouth medium (see Figure 1 legend) to remove serum components. Fresh medium that contained 10 mg/ml fatty acid free bovine serum albumin (Sigma) was added to the upper or lower chamber of the culture system; fibronectin-coated filters that had not been inoculated with cells served as controls. After 1 h, medium in both chambers was sampled for protein. Medium was replaced with serum-free Waymouth medium containing 1 mg/ml of albumin; and purified human fibroblast acid lipase (20 U/ml), prepared as described by Sando and Rosenbaum (1985), was added to the upper or lower chamber. Aliquots of medium removed from both chambers at the indicated times were assayed for acid lipase activity as described in Methods. The data shown were obtained from 2 different experiments (and sets of cultures) in which the transport from the upper to lower chamber, or the reverse, was studied.

^b The percentage of total lipase or albumin that was recovered in the recipient chamber is given as the mean ± SE of determinations on 3 cultures.

^c Activity in the lower chamber was below the detection limit of the assay.

^d Average of determinations on 2 wells.

Polarity of lipase transport from endothelial cells

Each experiment was preceded by a test for the impermeability of endothelial monolayers to bovine serum albumin. Results from a control experiment, in which impermeability of the monolayers to acid lipase was also tested, are shown in Table 1. Only a small fraction of the albumin or lipase applied to either chamber crossed the cell monolayer, compared with that which passed through a fibronectin-coated membrane without cells.

At early time points secretion of acid lipase to the lower chamber was only slightly favored

(Figure 3). Stimulation of secretion by NH₄Cl did not change the distribution. Similar results were obtained for lysosomal β -hexosaminidase (data not shown). Results during the first few hours were considered to be more representative of the dynamic in vivo state than after longer culture times, when the accumulation of secreted products may have disturbed the integrity of the endothelial barrier. The increased fractional distribution in the lower compartment after several hours was consistent with lipase diffusion across the monolayer, as affected by a concentration gradient, which favored the chamber with the larger volume.

These findings indicated that the secreted acid lipase would be available for interaction with cells on either side of an intact endothelial monolayer. We tested for the transport of endothelial acid lipase to cells of connective tissue origin by coculturing endothelial cells with acid lipase-deficient (Wolman disease) fibroblasts in an apparatus identical to that used for polarity studies, which prevented cell contact while al-

Figure 3. Polarity of acid lipase secretion by endothelial cells grown on microporous membranes. Endothelial cells were grown on microporous membranes in the Transwell system as was described in Methods. After 5 d, cells were rinsed and maintained in serum-free Waymouth medium for 4 h, then for 1 h with medium that contained 10 mg/ml of bovine serum albumin in the upper chamber, to monitor the integrity of the monolayer. Less than 1% of albumin/h was transferred from the upper to the lower chamber. Both chambers were sampled for secreted enzyme activity at designated time points by the removal of 50 μ l of medium from triplicate cultures. Values represent the mean $\pm$ standard error of secreted acid lipase activity in the upper (filled symbols) and lower chamber (open symbols) from unstimulated cells (circles) and from cells stimulated by the addition of 6 mM NH₄Cl to the culture medium (diamonds).

lowing for exchange of macromolecules between the coculture partners.

Acid lipase activity and accumulation of cholesteryl ester in Wolman disease fibroblasts

Because they have a low basal level of acid lipase activity and express a specific pathway for lysosomal enzyme uptake, Wolman disease fibroblasts served as good target cells for lipase transport studies (Sando and Henke, 1982). At acidic pH values (4.2), Wolman disease fibroblasts had ~15% of the 4-MeUmb oleate hydrolase activity of normal cells, whereas neutral 4-MeUmb oleate hydrolase activity was not affected. When assayed with a cholesteryl oleate or triolein dispersion, the acid lipase activity in Wolman fibroblasts was <5% of that in normal fibroblasts (Sando and Henke, 1982). These findings are in agreement with those by other investigators (Cortner *et al.*, 1976; Burton *et al.*, 1980; Hoeg *et al.*, 1982), although one group reported that neutral lipase activity was depressed in Wolman disease fibroblasts (Messiah *et al.*, 1983). A large fraction of the 4-MeUmb oleate hydrolase activity in Wolman disease fibroblast extracts is attributable to an acid phospholipase A, which is separable from the acid lipase/cholesteryl ester hydrolase by hydrophobic chromatography (Sando and Rosenbaum, 1985), is not reactive with anti-lipase antibodies, and is inactivated by treatment with high thiol concentrations (Sando and Schlom, unpublished). Our ability to control for this background activity enabled us to use the sensitive and convenient fluorogenic assay to accurately measure the activity of the acid lipase/cholesteryl ester hydrolase (see Tables 3 and 5).

Wolman disease fibroblasts have high levels of cholesteryl ester compared with barely detectable amounts in normal fibroblasts. We followed the accumulation of cholesteryl ester by Wolman disease fibroblasts as a function of serum lipids in the medium (Table 2). Cell cholesteryl ester mass was comprised of material retained from the original transplant as well as that which accumulated during new growth in complete serum. Fibroblasts in serum-free chemically defined media accumulated little additional esterified cholesterol, but grew poorly. The lipid-poor, CPSR-1 supplement supported growth almost as well as did complete serum, while not promoting cholesteryl ester accumulation. Fibroblasts maintained in serum accumulated cholesterol rapidly in the first few

Table 2. Cholesteryl ester accumulation in acid lipase-deficient fibroblasts—dependence on serum in the growth medium

Serum or substitute	Time (days)	Cell protein (μg/well)	Cholesterol (nmol/mg protein)	
			Free	Esterified
Experiment 1 ^a				
10% FBS	12	165 ± 8 ^b	119 ± 6	161 ± 10
10% CPSR-1	12	130 ± 11	81 ± 2	44 ± 1
ITS	12	44 ± 5	91 ± 12	56 ± 6
HL-1	12	39 ± 1	89 ± 7	63 ± 5
Experiment 2 ^c				
10% FBS	1	69 ± 17	111 ± 6	186 ± 18
	6	172 ± 10	86 ± 4	122 ± 3
	14	102 ± 10	139 ± 9	237 ± 13
	1	68 ± 3	57 ± 3	106 ± 3
	6	127 ± 12	59 ± 6	62 ± 7
	14	142 ± 9	107 ± 5	67 ± 7

^a Fibroblasts (GM 1606) from a patient with Wolman disease were split 1:4 and grown for 18 h in a 12-well plate (4.5-cm² wells) in MEM that contained 10% FBS, then maintained, with refeeding every 4 d, in the following: MEM + 10% FBS; MEM + 10% CPSR-1 (Sigma), a bovine plasma preparation with a reduced lipid content; ITS (Collaborative Research) in MEM; or HL-1 (Ventrex Laboratories). Serum replacements were used according to the manufacturer's recommendations. After 12 d, cell monolayers were rinsed several times with saline and harvested by scraping in 0.5 ml of saline. Cells were disrupted by sonication and extracts analyzed for cholesterol and protein as is described in Methods.

^b Cells were seeded and grown for 18 h as in Experiment 1, then maintained for the indicated times in MEM that contained either 10% FBS or 10% CPSR-1. Cultures were plated at different times so that all samples could be prepared and assayed on the same day.

^c Values for protein and cholesterol are the mean $\pm$ SE of determinations from 3 individual cultures.

days of culture, then more slowly thereafter. Thus the cholesteryl ester in Wolman disease cells is derived, for the most part, during rapid growth, from uptake of serum lipids; the period of maximum cholesteryl ester accumulation coincided with the growth phase during which uptake of low density lipoproteins by fibroblasts had previously been shown to be the greatest (Goldstein and Brown, 1974; Chait *et al.*, 1979).

Clearance of cholesteryl ester from lipase-deficient fibroblasts by coculture with endothelial cells

When confluent cultures of Wolman disease fibroblasts and endothelial cells were combined for several days, acid lipase activity in the fibroblasts was increased over background by an amount that corresponded to ~10% of the activity in normal fibroblasts (Table 3). Cell extracts

Table 3. Correction of cholesterol metabolism in Wolman disease fibroblasts cocultured with endothelial cells^a

Cell type	Coculture	Acid lipase (U/mg) ^b		Cholesterol (nmol/mg)	
		-DTT	+DTT	Free	Esterified
Fibroblast	-	1.53 ± 0.15	0.19 ± 0.07	78 ± 19	154 ± 52
	+	2.56 ± 0.32	1.18 ± 0.40	66 ± 3	11 ± 1
Endothelial	-	9.60 ± 0.42	7.74 ± 0.35	44 ± 3	0
	+	10.2 ± 1.4	7.87 ± 1.03	45 ± 1	0

^a Wolman disease fibroblasts (GM 1606) were grown to confluence for 7 d in 6-well plates (9-cm² wells). Freshly prepared endothelial cells were plated on Transwell filters, and, after incubation for 4 h, the adherent monolayers were rinsed and combined in coculture with the fibroblasts where indicated. Cocultures were maintained in MM199 medium with 20% FBS for 7 d with one medium change after 4 d. Cells were rinsed with saline, harvested, and analyzed for protein and cholesterol as is described in Methods. Values are the mean ± SE of determinations from 3 separate cultures.

^b Before assay for acid lipase activity, an aliquot of each cell extract was treated with 0.1 volume of 4 M 1,4-dithiothreitol (DTT) or water for 1 h at room temperature. The thiol was added to selectively inactivate nonspecific acid 4-MeUmb oleate hydrolase activity and lower the background of acid lipase activity in controls.

in which pretreatment with high thiol reduced the background activity almost 90% showed the same increase. Coculture did not significantly affect intracellular acid lipase activity in endothelial cells, as was borne out in numerous other experiments. Lipase activity in endothelial cell extracts was reduced ~20% by treatment with high thiol, indicating that, as with normal fibroblasts (data not shown), most of the intracellular lipase in those cells corresponded to the acid lipase/cholesteryl ester hydrolase. The cholesteryl ester content of Wolman disease fibroblasts was reduced >90% after coculture with the endothelial cells for 1 wk (Tables 3, 6, and 7).

Role for phosphomannosyl recognition system in acid lipase uptake and cholesteryl ester clearance

Mannose 6-phosphate competed with purified fibroblast acid lipase for uptake by fibroblasts (Sando and Henke, 1982). When it was added to the coculture medium, both the transport of endothelial acid lipase to fibroblasts and cholesteryl ester clearance were inhibited (Table 4). The different line of Wolman disease fibroblasts (GM 6144) that was used for this experiment had the same properties in coculture studies as did the line (GM 1606) that was used in most of the other experiments in this report, and effects of mannose 6-phosphate on lipase transport and cholesteryl ester clearance in GM 1606 fibroblasts were similar to those described for the GM 6144 line. When results from a number of studies were considered, mannose 6-phosphate did not significantly affect the level of intracellular endothelial acid lipase activity.

Role of serum in acid lipase transport and cholesterol clearance

Serum was not required for transport of endothelial lipase or for cholesteryl ester hydrolysis in recipient fibroblasts (Table 5). Our measurements very likely underestimated the extent of lysosomal hydrolysis, because in this work we did not assess the contribution of extralysosomal reesterification to the total cholesteryl ester mass. Serum was essential for cholesterol clearance from the fibroblasts. Even though coculture in serum-free medium resulted in substantial cholesteryl ester hydrolysis, total intracellular cholesterol content was unchanged from that in untreated controls, whereas both hydrolysis and clearance were promoted by coculture in serum-supplemented medium. The dependency on serum for cholesterol efflux is in agreement with results from studies of other cultured cell systems, which demonstrated that the high-density lipoprotein fraction was the active serum component (Stein *et al.*, 1975; Ho *et al.*, 1980; Oram *et al.*, 1981; Reichl and Miller, 1986).

Time dependence of cholesterol mobilization in the coculture system

To examine the influence of coculture on cholesteryl ester accumulation in rapidly growing fibroblasts, which were actively internalizing serum lipids, we initiated coculture shortly after both cell types were plated (Table 6). Fibroblast acid lipase did not increase over background until after 2 d of coculture, reflecting either minimal expression of the fibroblast uptake system or a low level of endothelial lipase secretion at short times after plating. As acid lipase activity

Table 4. Mannose 6-phosphate in the medium inhibits acid lipase uptake and cholesteryl ester clearance in Wolman disease fibroblasts cocultured with endothelial cells^a

Cell type	Coculture	Man 6P	Acid lipase (U/mg)	Cholesterol (nmol/mg)	
				Free	Esterified
Fibroblast	—	—	2.62 ± 0.14 ^b	44 ± 2	107 ± 3
	—	+	2.87 ± 0.15	50 ± 2	114 ± 3
	+	—	4.25 ± 0.31	58 ± 1	59 ± 4
	+	+	2.92 ± 0.20	62 ± 3	120 ± 6
Endothelial	+	—	22.4 ± 3.7	38 ± 2	0
	+	+	30.4 ± 1.9	44 ± 5	0

^a Wolman disease fibroblasts (GM 6144) were grown to confluence in 6-well plates, then placed in coculture or not with freshly plated endothelial cells in MM199 medium + 20% FBS for 1 d. The medium was replaced with MEM + 10% FBS. Where indicated, 2 mM mannose 6-phosphate (Sigma) was added to the culture medium, which was changed daily over a 4-d period. Cells were rinsed, harvested, and assayed for acid lipase activity, cholesterol, and protein.

^b Values are the mean ± SE of determinations from 3 individual cultures.

increased in the fibroblasts, after 2 d, intracellular cholesteryl ester decreased. Once cholesteryl ester was cleared from Wolman disease fibroblasts in coculture with endothelial cells for 1 wk, little further accumulation occurred when fibroblasts were maintained for a second week, without coculture, in the presence or absence of serum (Table 7). The fact that the cells were not undergoing rapid growth and at the same time were exposed to a high concentration of serum probably contributed to a low rate of internalization of serum lipoproteins. Acid lipase activity retained by fibroblasts after endothelial cells were removed would be expected to protect against cholesteryl ester accumulation for a brief period, but after 1 wk, activity above

baseline levels was not detectable (Table 7). This was predicted by our previous estimates of a short intracellular half-life (7–20 h) of lipase activity after uptake of the purified fibroblast enzyme.

Cholesteryl ester clearance from lipase-deficient fibroblasts mediated by coculture with other cell types

When endothelial cells were replaced with vascular smooth muscle cells in the coculture system, cholesteryl ester was cleared from the Wolman disease fibroblasts (Table 8). Acid lipase uptake by the fibroblasts was less than that which we usually observed after coculture

Table 5. Dependence on serum in the medium for clearance of cholesterol from Wolman disease fibroblasts in coculture with endothelial cells^a

Coculture	Serum (%)	Acid lipase ^b (U/mg)	Cell protein (μg/well)	Cholesterol (nmol/mg)	
				Free	Esterified
—	0	0.65 ± 0.09	156 ± 4	72 ± 11	91 ± 25
+	0	2.84 ± 0.41	147 ± 10	120 ± 3	39 ± 3
—	10	1.14 ± 0.15	321 ± 35	100 ± 8	104 ± 11
+	10	3.40 ± 0.06	260 ± 14	118 ± 4	31 ± 5

^a Wolman disease fibroblasts (GM 1606) were grown for 6 d in MEM + 10% FBS, then for 1 d in MM199 + 20% FBS, in coculture where indicated, with freshly seeded endothelial cells. The culture medium was replaced, as indicated, with either MEM + 10% FBS or the chemically defined serum-free formulation, WRC-935, which was supplemented with 5 mg/ml of bovine serum albumin and 50 μM 2-mercaptoethanol. Cells were refed with fresh medium after 3 d and, 3 d later, were harvested and assayed for acid lipase activity, protein, and cholesterol. Values are the mean ± SE of determinations from 3 individual cultures.

^b Acid lipase activity was assayed in cell extracts that were pretreated with 1,4-dithiothreitol, as in Table 3, to lower nonspecific background activity.

Table 6. Time course of cholesteryl ester clearance from Wolman disease fibroblasts placed in coculture with endothelial cells immediately after fresh seeding of both cell types^a

Time in coculture (days)	Acid lipase (U/mg)	Cholesterol (nmol/mg)	
		Free	Esterified
0	3.24 ^b	74	111
1	3.58	74	107
2	3.25	74	115
4	4.98	63	25
5	6.74	72	16
6	5.47	62	8

^a Wolman disease fibroblasts (GM 1606) and endothelial cells were cocultured in MM199 + 20% FBS for the indicated times. The experiment was designed so that all cultures were harvested and assayed at the same time. The cells were plated in 2 groups, for the 1–2 d and the 4–6 d range. Fibroblasts were combined with endothelial cells in coculture at the following times after both cell types were plated: for the 2- and 6-d points, 4 h; for the 1- and 5-d points, 24 h; and for the 4-d point, 48 h. The 0-d point was obtained from fibroblasts that were grown for 48 h after plating without exposure to endothelial cells. Fibroblasts were harvested and assayed as in experiments described in previous tables.

^b Values are the average determinations from 2 individual cultures per time point. Fibroblast protein content ranged from 222 to 249, and 376 to 448 μ g/well in the 0–2 d and 4–6 d groups, respectively.

with endothelial cells. This is in accord with our findings of a lower rate of lipase secretion from smooth muscle cells compared with that from endothelial cells (Figures 1 and 2), but it could

also indicate that smooth muscle cells secreted a form of the lipase that was less efficiently internalized by fibroblasts. We also found that cells of a human leukemic monocyte-macrophage line were capable of promoting cholesteryl ester clearance when cocultured with acid lipase-deficient fibroblasts (Sando, Dahle, and Zhou, unpublished).

Discussion

This work showed that 1) cultured endothelial cells produced a large quantity of extracellular lysosomal acid lipase in comparison with that by other human cell types; 2) the acid lipase was secreted from both the luminal and the basolateral surface of endothelial cells; and 3) when endothelial cells were cocultured with acid lipase-deficient fibroblasts, the secreted endothelial lipase was taken up by the fibroblasts and promoted lysosomal cholesteryl ester hydrolysis. These findings lend support to the hypothesis that intercellular transport of the lysosomal acid lipase is an important aspect of the enzyme's role in lipid metabolism. Our results bring to light a new mechanism whereby endothelial cells may interact with connective tissue cells to influence lipoprotein metabolism in the vascular wall.

Our observations of the high extracellular levels of acid lipase and β -hexosaminidase in endothelial cultures, compared with those in connective tissue cell cultures, are in agreement with results obtained for β -hexosaminidase by Hasilik *et al.* (1981), who showed, in addition,

Table 7. Reaccumulation of cholesteryl ester in Wolman disease fibroblasts after pretreatment with endothelial cell coculture^a

Treatment of fibroblasts		Acid lipase (U/mg)	Cell protein (μ g/well)	Cholesterol (nmol/mg)	
Week 1, $\pm$ coculture	Week 2, $-$ coculture			Free	Esterified
–	–	0.99 $\pm$ 0.08 ^b	170 $\pm$ 3	77 $\pm$ 3	143 $\pm$ 21
+	–	3.63 $\pm$ 0.20	241 $\pm$ 35	72 $\pm$ 5	11 $\pm$ 1
–	0% serum	1.50 $\pm$ 0.11	190 $\pm$ 25	69 $\pm$ 3	88 $\pm$ 5
+	0% serum	1.53 $\pm$ 0.06	140 $\pm$ 11	53 $\pm$ 3	6 $\pm$ 1
–	20% serum	1.22 $\pm$ 0.14	430 $\pm$ 58	54 $\pm$ 3	100 $\pm$ 6
+	20% serum	1.03 $\pm$ 0.10	351 $\pm$ 20	53 $\pm$ 2	11 $\pm$ 1

^a Wolman disease fibroblasts (GM 1606) were grown for 7 d in MM199 + 20% FBS with or without exposure to endothelial cells in coculture, as indicated. Both cell types were freshly seeded at the beginning of the experiment. After 1 wk, endothelial cells were removed from the coculture group. A portion of the fibroblast cultures from each group ($\pm$ coculture) were harvested and analyzed for lipase, protein, and cholesterol. The remaining fibroblast cultures were maintained for a second week in MM199 medium that contained either 5 mg/ml bovine serum albumin (0% serum) or 20% FBS. Cells were then harvested and assayed as above.

^b Values are the mean $\pm$ SE of determinations from 3 individual cultures.

Table 8. Correction of cholesterol metabolism in Wolman disease fibroblasts cocultured with vascular smooth muscle cells^a

Cell type	Coculture	Acid lipase (U/mg)	Cholesterol (nmol/mg)	
			Free	Esterified
Fibroblast	—	3.60 ± 0.13 ^b	104 ± 3	114 ± 6
	+	4.37 ± 0.61	90 ± 4	28 ± 6
Smooth muscle	—	17.3 ± 1.1	56 ± 2	3 ± 2
	+	14.2 ± 1.7	76 ± 10	1 ± 1

^a Human venous umbilical smooth muscle cells, passage 10, were grown as described in Methods for 2 d on Transwell filter inserts and then combined, where indicated with Wolman disease fibroblasts (GM 1606), which were freshly seeded in the lower chamber of the 6-well coculture apparatus. Culture in MM199 ± 20% FBS was continued for 7 d (medium renewed after 4 d) and then cells were rinsed, harvested, and assayed for cholesterol, protein, and lipase activity. Fibroblast and smooth muscle cell cultures had 263 ± 19 and 672 ± 55 µg protein/chamber, respectively.

^b Values are the mean ± SE of determinations from 3 individual cultures.

that fibroblasts and vascular smooth muscle cells (but not umbilical vein endothelial cells) internalized high uptake forms of lysosomal enzymes. In results not reported here, we had found that acid lipase purified from fibroblast secretions was taken up by fibroblasts, but not by endothelial cells; vascular smooth muscle cells, like fibroblasts, bound the lipase by a saturable process that was sensitive to mannose 6-phosphate (Sando and Tranby, unpublished). Endothelial cells may not express surface receptors for acid hydrolase uptake. Because endothelial cells have intracellular lysosomal enzyme levels that are somewhat reduced from those of fibroblasts, an intracellular route for lysosomal enzyme packaging does exist in these cells, but may be less effective because of a receptor deficiency or some other structural anomaly along the packaging route. Cornicelli *et al.* (1983) reported that endothelial cells released ammonia into the culture medium at a level sufficient to inhibit lysosomal lipoprotein catabolism. By decreasing the acidity of endosomal compartments, lysosomotropic amines also interrupt recycling of phosphomannosyl receptors, thus diverting acid hydrolases from the normal lysosomal packaging route to extracellular secretion (Gonzalez-Noriega *et al.*, 1980). The high basal rate of hydrolase secretion by endothelial cells in our studies was not caused by excess ammonia in the culture medium; we carefully monitored ammonia levels and took precautions to minimize the contribution from glutamine hydrolysis. Endogenous factors peculiar to endothelial cells may alter endosomal acidification, thereby promoting the

extracellular localization of acid hydrolases, in analogy with a number of mutant cell lines that exhibit acidification defects (Mellman *et al.*, 1986).

We chose Wolman disease fibroblasts as a connective tissue model in this primary work because lipase transport and cholesteryl ester clearance can be rigorously quantitated in this system. Uptake of the endothelial lipase by Wolman disease fibroblasts was mediated by a phosphomannosyl recognition process. From our earlier discussion, vascular smooth muscle cells apparently recognize extracellular acid hydrolases by the same mechanism as do fibroblasts, indicating that the lipase transport properties demonstrated in our coculture system may apply to the situation in the vascular wall, in which endothelial-smooth muscle interactions are of greater significance. Macrophages can acquire acid hydrolases through a receptor-mediated process that involves recognition of mannosyl-, N-acetylglucosaminyl-, and L-fucosyl residues of glycoproteins (Shepherd and Stahl, 1984); the expression of this receptor is regulated by glucocorticoid hormones. Acid hydrolases may also be transported from macrophages (McNamara *et al.*, 1985; Dean and Martin, 1988) and lymphocytes (Olsen *et al.*, 1981; Abraham *et al.*, 1985) by direct contact between donor and recipient cells. The prevalence of leukocytes in atherosclerotic lesions points up the need to consider all of these possible mechanisms for intercellular hydrolase exchange among cells of the vascular wall. Damage to the endothelium, pathological conditions which may arise in dis-

eased tissue (such as lowered pH), and increased cellular metabolites such as ammonia would be expected to alter lipase transport and action. Further studies with coculture models should help to elucidate requirements for lipase transport and its role in lipoprotein metabolism by cells that are present in vascular tissue.

Most of our experiments that dealt with the function of the endocytosed lipase provided direct information only with regard to the hydrolysis of cholesteryl esters that had previously accumulated in mature lysosomes. Nevertheless, our overall results suggest other mechanisms whereby transport of the extracellular lipase may have a role in lipoprotein cholesterol metabolism. The pathway for phosphomannosyl receptor-mediated internalization of lysosomal enzymes has many features in common with that for low-density lipoproteins (Brown *et al.*, 1983; Brown and Goldstein, 1986; Griffiths *et al.*, 1988). Both ligands are internalized in association with clathrin-coated membranes, transported in endocytic vesicles in a receptor-bound form, and dissociated from their receptors in an acidic prelysosomal compartment before delivery to lysosomes. Simultaneous transport of endocytosed acid lipase and its lipoprotein-bound substrates to lysosomes would be expected to enhance the efficiency of lipoprotein lipid metabolism, even though the intracellular exchange of lysosomal contents and membrane proteins may prove to be a relatively rapid process (Ferris *et al.*, 1987; Deng and Storrie, 1988). We found that, after uptake by acid lipase-deficient fibroblasts, the half-life for intracellular lipase activity was short (7–20 h) in comparison with half-lives of several days to weeks for other lysosomal enzymes (Skudlarek *et al.*, 1984), suggesting that the lipase may not accumulate in mature lysosomes. Assuming a half-life of 7 h, zero-order kinetics for enzyme synthesis, and first-order kinetics for decay of enzyme activity, ~10% of the intracellular lipase activity needs to be replaced per hour for maintenance of the steady-state level in confluent cultures of normal fibroblasts. Newly biosynthesized acid hydrolases in transit to lysosomes are believed to pass through an acidic prelysosomal compartment that is shared with the endocytic pathway (Griffiths *et al.*, 1988; Griffiths, 1989). The relatively low level of lipase activity that our results and those of Brown *et al.* (1976) indicated was sufficient for maintenance of lipoprotein cholesteryl ester metabolism may be accounted for by endogenous nascent acid lipase or endocytosed enzyme in a prelysosomal compartment. Recent

evidence supports the involvement of endosomal processing in a number of metabolic pathways. Acid protease activity that mediated prelysosomal proteolysis was demonstrated in macrophage endosomes (Diment and Stahl, 1985; Diment *et al.*, 1988). Proteolysis in a prelysosomal compartment is thought to have a role in insulin degradation (Doherty *et al.*, 1990), parathyroid hormone processing (Diment *et al.*, 1989), and antigen presentation (McCoy and Schwartz, 1988; Werdelin *et al.*, 1988). The occurrence of lipoprotein cholesteryl ester hydrolysis in endosomal fractions of rat liver was recently reported (Hornick *et al.*, 1989), and endosomal processing of lipoproteins may be important in monocyte-macrophages (Van Lenten and Fogelman, 1989). Endosomal processing may mediate the remodeling and release of lipoproteins in an altered form from a cell, as in the retroendocytosis of low-density lipoproteins by fibroblasts (Greenspan and St. Clair, 1984).

Results from our endothelial coculture studies point up a practical method for preparing cultures of lipase-deficient fibroblasts devoid of lysosomal lipid esters. Although the stored lipids could in principle be cleared by culturing for several cycles in serum-free medium, fibroblasts did not grow optimally under these conditions. Our attempts to completely remove cholesteryl esters from Wolman disease fibroblasts by adding purified acid lipase were not practical because the short lifetime of intracellular lipase activity mandated frequent supplementation with a large quantity of enzyme. The precleared lipase-deficient cell model will enable basic kinetic studies of intracellular lipoprotein cholesteryl ester and triglyceride metabolism as a function of added purified lipase, effectors of the enzyme, and highly characterized lipoproteins without complications from variable levels of endogenous lipid substrates. The coculture pretreatment procedure should also facilitate investigations into the relationship between the clinical phenotypes and biochemical abnormalities in Wolman disease and cholesteryl ester storage disease through comparative studies of lipid metabolism in patient fibroblasts. Although previous studies of this nature provided valuable qualitative information as to the consequences of acid lipase deficiency in cell cholesterol metabolism (Brown *et al.*, 1976; Burton *et al.*, 1984; Slotte, 1987), preclearing of abnormal lipid accumulations would enable a more valid quantitative comparison among mutant lines of lipid hydrolysis rates and of the disposition and regulatory effects of cholesterol and other reaction products. A similar approach should be bene-

ficial to studies of other lysosomal storage disorders.

Methods

Cell culture

Normal human skin fibroblasts (AG 1519) and cells derived from patients with Wolman disease (GM 1606, 6144) were obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository, Coriell Institute for Medical Research (Camden, NJ). Cultures were maintained in 75 or 150 cm² plastic flasks at 37°C in Eagle's Minimal Essential Medium (MEM) with Earle's salts supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT), 0.2 mM MEM nonessential amino acids, 1% Basal Medium Eagle (BME) vitamins, 2 mM L-glutamine, 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 8 U/ml of mycostatin. The medium was buffered at pH 7.4 with 0.015 M NaHCO₃ and 5% CO₂ in air. Cells were subcultured at a ratio of 1:4 with 0.5% trypsin in 0.9% NaCl.

Endothelial cells were obtained from human umbilical veins (Czervionke *et al.*, 1978), and primary cultures were prepared according to a slight modification of the method of Jaffe *et al.* (1973). The cells were suspended in modified Medium 199 (MM199) that contained 20% heat-inactivated FBS. MM199 consisted of Medium 199 that was supplemented with 1× BME vitamins, 1× BME amino acids solution, 1 g/L glucose, 0.1 g/L neomycin sulfate, and 2.2 g/L NaHCO₃, adjusted to pH 7.4. For most experiments, 1.5 ml of a suspension that contained 1 × 10⁶ cells/ml of medium was applied to 4.5 cm² polycarbonate micropore filter (0.4-µm pore) coculture inserts (Transwell, Costar, Cambridge, MA), and 2.6 ml of medium was added to the lower, 9 cm², chamber. For experiments in which larger quantities of endothelial secretions were required for characterization of properties of the acid lipase, T-75 flasks were seeded with 20 ml of a cell suspension, as above, that contained 5 × 10⁵ cells/ml. Culture surfaces had been pretreated for 1 h at room temperature with 0.33 ml/cm² of a solution of human fibronectin (Collaborative Research, Bedford, MA; 10 µg/ml in bicarbonate-free Medium 199, buffered with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], pH 7.4). Cells were incubated at 37°C in 95% air-5% CO₂ for 4 h and rinsed, and fresh medium was added. Cultures were used 3–4 d after plating unless otherwise indicated.

Smooth muscle cells were isolated from human umbilical veins and characterized as was described (Fry *et al.*, 1980). Cultures in passage 6–10 were grown on fibronectin-coated surfaces in MM199 that contained 20% heat-inactivated FBS. Cells were subcultured at a ratio of 1:1–1:2 after treatment with 0.25% trypsin-0.05% EDTA.

Medium WRC-935 was purchased from Amicon (Beverly, MA). Other liquid tissue culture media were obtained from the University of Iowa Cancer Center Tissue Culture Facility. Serum replacement ITS was purchased from Collaborative Research (Bedford, MA) and HL-1 from Ventrex Laboratories (Portland, ME). Other tissue culture medium additives and reagents were purchased from Grand Island Biological Company (Grand Island, NY) or from Sigma Chemical (St. Louis, MO) when another source was not indicated.

Preparation of cell extracts

After experimental treatments in culture, media were removed from cells and treated with a buffer solution to give as a final concentration of additives in the sample: 0.01 M Na phosphate, pH 7.0; 1 mM EDTA; 10 mM 2-mercaptoethanol; and 0.02% Na azide. Cell monolayers on polycarbonate filters or in tissue culture wells were rinsed five times

with 0.9% NaCl, scraped with a rubber policeman into 0.5 ml of saline, and disrupted by treatment for 10 s at 0°C with the use of a sonifier (Heat Systems–Ultrasonics, Farmingdale, NY; 350-W instrument equipped with cup horn, used at 80% of maximum power).

Enzyme assays

Samples of cell sonicates (0.2–1 µg protein) and secretions (5–10 µl) were assayed for acid lipase, β-hexosaminidase, and lactate dehydrogenase activity with the use of a fluorometric computer-aided assay system (Sando and Sando, 1986). Assays for acid lipase and lactate dehydrogenase were performed within 4 h after samples were harvested, and β-hexosaminidase and cell protein were measured in cell extracts and secretions that had been frozen for 1–3 d. Acid lipase activity was measured in a continuous rate assay system by following the release of 4-MeUmb from 4-MeUmb oleate using a modification (Sando and Henke, 1982) of the procedure of Cortner *et al.* (1976). The reaction was initiated by addition of sample to a reaction mixture, at 32°C, which contained, in a volume of 0.4 ml, 50 µM 4-MeUmb oleate, 300 µM Na taurodeoxycholate, 20 µg/ml ovalbumin (all purchased from Sigma), 80 µM dioleoyl phosphatidylcholine (Avanti Biochemicals, Birmingham, AL), and 0.05 M Na acetate, pH 4.1. β-Hexosaminidase activity was assayed with use of a modification of the method described by Suzuki (1978). The reaction mixture, at 37°C in a volume of 0.1 ml, contained sample, 10 mM 4-MeUmb β-N-acetylglucosaminide (Sigma), and 100 µg/ml ovalbumin in citrate-phosphate buffer (0.05 M citric acid adjusted to pH 4.5 with 0.1 M Na₂HPO₄). After 5–30 min the reaction was stopped by addition of 0.5 M NaOH-glycine buffer, pH 10, and the fluorescence of 4-MeUmb measured against authentic standard. Lactate dehydrogenase activity was assayed by monitoring the rate of reduction of nicotinamide adenine dinucleotide (NAD) (Amador *et al.*, 1963) in a continuous rate assay system. The reaction mixture at 32°C contained, in a volume of 0.5 ml, sample; 0.1 M glycine, pH 10; 20 mM lactic acid (Sigma, crystalline), adjusted to pH 10 with NaOH; 1 mM NAD (Chemical Dynamics, South Plainfield, NJ); and 0.1 mg ovalbumin. Production of reduced NAD was measured fluorometrically and calibrated against authentic standard (Sigma, grade III, disodium salt).

A unit of enzyme activity is defined as 1 nmole of product formed per minute. Activity was normalized to cell protein (Lowry *et al.*, 1951).

Measurement of cholesterol

Aliquots of cell sonicates that contained 25–50 µg of protein were extracted with chloroform-methanol (Folch *et al.*, 1957) immediately after cell disruption. The lipid extract was dried under N₂, dissolved in isopropyl alcohol (Heider and Boyett, 1978), and analyzed for total and free cholesterol with use of a slight modification (Sando and Henke, 1982) of a fluorometric coupled enzyme procedure (Gamble *et al.*, 1978). Cholesterol oxidase (EC 1.1.3.6) was obtained from Sigma (*Streptomyces* sp., 25–50 U/mg protein). Cholesteryl esterase (EC 3.1.1.13) used in the determination of total cholesterol was a combination of 0.01 U/assay of enzyme from microorganisms (Boehringer-Mannheim Biochemicals, Indianapolis, IN, ~20 U/mg) and 0.01 U/assay of enzyme from bovine pancreas (Sigma, 2000–4000 U/mg). Horseradish peroxidase (EC 1.11.1.7) was purchased from Worthington Biochemical (Freehold, NJ; RZ = 3, 1070 U/mg). p-Hydroxyphenylacetic acid (Aldrich Chemical, Milwaukee, WI) was purified as described by Heider and Boyett (1978). Cholesterol and cholesteryl oleate, used as standards, were obtained from Applied Science (State College, PA).

Acknowledgments

We are indebted to Constance Schroeder for invaluable contributions to the development of the vascular cell co-culture systems. This work was supported by the Arteriosclerosis Specialized Center of Research Grant HL 14230 from the National Heart, Lung, and Blood Institute, National Institutes of Health.

Received: April 13, 1990.

Revised and accepted: June 27, 1990.

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