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Normolipidemic Xanthelasma Palpebrarum: Lipid Composition, Cholesterol Metabolism in Monocyte-derived Macrophages, and Plasma Lipid Peroxidation

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The lipid compositions of 8 normolipidemic xanthelasma palpebrarum (XP) lesions were analyzed using thin-layer chromatography, with the adjacent uninvolved skin used as control. The lesions were found to be composed predominantly of cholesterol, mostly cholesteryl ester, whereas in the control specimens phospholipids predominated. The degradation rates of ^{125}I-low-density lipoprotein (LDL), oxidized LDL, and acetyl LDL, and the rates of intracellular cholesterol synthesis from 1,2-^{14}C-acetate, in blood monocyte-derived macrophages (MDM) from 3 normolipidemic patients, were similar to those of MDM from 3 normal control subjects. The mean levels of lipid peroxides and conjugated dienes under basal conditions, as well as following the addition of a free radical-generating compound (2,2'-azobis-2-amidinopropane hydrochloride) to the plasma of 14 normolipidemic XP patients were significantly higher than those of 14 age- and sex-matched normal controls.

We conclude that the predominant lipid accumulated in normolipidemic XP lesions is cholesteryl ester, but there is no evidence for intrinsic cellular cholesterol metabolism derangement in blood MDM from patients which could account for this. Since macrophage cholesterol accumulation can also result from enhanced uptake of increased levels of oxidized LDL, the increased plasma lipid peroxidation (derived from oxidized LDL) might lead to accumulation of cholesterol in macrophages and formation of foam cells via this mechanism. ***Key words: atherosclerosis; low-density lipoprotein; degradation; synthesis.***

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Xanthelasmas constitute the most common form of cutaneous xanthoma, appearing more frequently in females than in males (1). Although xanthelasma is a type of xanthoma, 25–70% (on average 50%) of patients in most series have been found to be normolipidemic (defined initially as having normal cholesterol and triglyceride levels) (1). The most frequent Fredrickson hyperlipidemic phenotype in hyperlipidemic xanthelasma patients is type IIa (1). Less frequent are types IIb, III, and IV (1). Many of the hyperlipidemic phenotypes that are associated with xanthelasmas have long been associated, in numerous studies, with an increased risk of atherosclerosis (2). The reason for the appearance of xanthelasmas in normolipidemic individuals, and whether they constitute a marker of increased risk of atherosclerosis, is more obscure (1). Previous studies of lipid composition of xanthelasmas, which showed that the main lipid stored is cholesterol, predominantly in its esterified form (3, 4), included, except for one case (4), xanthelasmas from hyperlipidemic individuals only (3, 4).

There is some similarity between cholesterol-accumulating xanthomas and atheroma formation, at least experimentally (5, 6). In both, the foamy macrophage seems to play a major role (7, 8). The major pathway for delivery of exogenous cholesterol to most cells is the uptake of low-density lipoprotein (LDL). LDL receptor activity in these cells is tightly coupled to the level of cellular cholesterol, thus preventing its intracellular accumulation. Monocyte-derived macrophages (MDM), however, also have scavenger, or acetyl-LDL, receptors, which are not regulated by intracellular cholesterol levels (9, 10). It has been demonstrated in vitro that LDL modified by acetylation could induce the transformation of macrophages into foam cells via the uptake by these scavenger receptors (10). Of special interest is the oxidative modification of LDL, which is believed to occur in vivo. Lately there has been growing evidence to suggest that oxidized LDL might represent the modified LDL responsible for cholesterol loading of macrophages and for promoting atherogenesis in vivo (11, 12). Therefore, a possible mechanism by which the dermal macrophage could contribute to the formation of normolipidemic xanthelasmas is by enhanced LDL or modified LDL uptake. Since dermal macrophages are derived from blood monocytes (13), the putative enhanced uptake and degradation of LDL due to intrinsic cellular factors might also become apparent in blood MDM. In order to investigate these possibilities, we studied the lipid composition of normolipidemic XP, plasma lipid peroxidation levels, and the rates of LDL, oxidized LDL, and acetyl-LDL degradations and intracellular cholesterol synthesis in MDM derived from these patients.

MATERIALS AND METHODS

Xanthelasma lipid composition

The tissue specimens for the lipid analysis were obtained from 8 healthy patients (one male and 7 females, aged 41–50 years; mean 46 years), with fasting plasma cholesterol and triglyceride levels within the normal ranges (cholesterol $\leq$6.48 mmol/l and triglycerides $\leq$2.02 mmol/l), undergoing cosmetic surgical removal of their xanthelasmas. In each case, the surgical ellipse contained normal-looking adjacent skin at the two poles. The surgically removed specimen was divided into two sections, one approximately one third and the other two thirds. The smaller piece, which contained part of the xanthelasma tissue along with adjacent normal-looking skin, was fixed in 10% formalin solution and processed for routine histologic examination. The remaining larger piece was further divided into two parts: one containing the xanthelasma tissue and the other the normal-looking adjacent skin. Both of these pieces were frozen at -70°C until lipid analysis.

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After thawing, the specimens were washed 3 times with cold phosphate-buffered saline (PBS) and homogenized in a Teflon/glass homogenizer (50 strokes). The lipids were extracted from the homogenized specimens with chloroform/methanol (2:1, v/v) and were dried under nitrogen and resolubilized in chloroform. The lipids were separated by thin-layer chromatography on silica gel plates using hexane/diethyl ether/acetic acid solution (130:30:1.5, v/v/v). The appropriate spots of lipids were visualized with iodine vapors, scraped into glass tubes, and the contents of esterified and unesterified cholesterol (14), triglycerides (15), and phospholipids (16) were analyzed.

Cells

Human MDM were isolated from the blood of the fasting subjects by density-gradient centrifugation using the method of Boyum (17). Twenty milliliters of blood anticoagulated with sodium heparin (final concentration 10 U/ml) were layered over 15 ml Ficoll-Paque. After centrifugation at 500 × g for 30 min at 23°C, the mixed mononuclear cell band was removed by aspiration and the cells were washed twice at 4°C in Dulbecco's modified Eagle's medium supplemented with L-glutamine (final concentration 2 mM), 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells were plated at 3×10^5 monocytes per 16-mm dish (Primaria Brand, Falcon Labware) in the same medium (0.5 ml) containing 20% autologous serum. After 2 h incubation at 37°C in 5% CO_2–95% air, non-adherent cells were removed by 3 washes with serum-free medium. The cells were plated in fresh medium containing 20% autologous serum, fed twice weekly, and used for experiments after 7 days in culture.

Lipoproteins

LDL was prepared from human plasma derived from fasted normolipidemic volunteers. LDL was prepared by discontinuous-density gradient ultracentrifugation, as described previously (18). The LDL was washed at d = 1.063 g/ml and dialyzed against 150 mM NaCl and 1 mM EDTA, pH 7.4, under nitrogen in the dark, at 4°C. LDL was then sterilized by filtration (0.22 nm) and used within 2 weeks. LDL was acetylated by repeated additions of acetic anhydride (19) to 4 mg protein/ml of LDL and diluted 1:1 (v/v) with saturated ammonium acetate at 4°C. Acetic anhydride was added at 40-fold molar excess with regard to total amino acid lysine residues in LDL, and the modification was confirmed by electrophoresis on cellulose acetate at pH 8.6 in barbital buffer (20). Lipoprotein oxidation was performed by incubation of LDL (1 mg protein/ml in EDTA-free PBS) with copper sulfate (10 µM) for 24 h at 37°C. Lipoprotein oxidation was confirmed by analysis of thiobarbituric acid-reactive substances, which measures malondialdehyde equivalents (21), by the lipid peroxidation test (22), and also by analysis of the conjugated dienes content of the lipoprotein (23).

The LDL, acetyl-LDL, and oxidized LDL were iodinated by McFarlane's method as modified for lipoproteins (23).

Cholesterol metabolism in MDM

Intracellular cholesterol synthesis and LDL, acetyl-LDL, and oxidized LDL degradation rates were studied in MDM of the 3 patients with normolipidemic xanthelasmas. A normal control was included on the same day of study of each patient. None of the subjects were taking any medication, and all were on a normal diet at the time of blood collection.

LDL degradation by macrophages

The degradation of ^{125}I-labeled LDL by cells was measured as previously described (24). Cells were washed with PBS, and 0.5 ml of serum-free medium containing 25 µg protein/ml of ^{125}I-labeled LDL (150–300 cpm/ng) was added to 16-mm dishes of cells. After 5 h incubation, the media were removed from the cells, and trichloroacetic-acid-soluble, free-iodide radioactivity was determined. Degradation rates were corrected for cell-free incubations that were carried out in parallel. Results were expressed as µg of ^{125}I-LDL degraded/mg cell protein. Protein was measured by the method of Lowry et al. (25).

Measurement of cellular cholesterol synthesis

The human MDM were incubated for 18 h in Dulbecco's modified Eagle's medium containing autologous serum. Cells were then washed and further incubated in serum-free medium for 24 h with 1,2-^{14}C-sodium acetate (1.25 mM, 10 µCi/ml), as previously described (26). At the end of the incubation, the incorporation of radioactivity into cellular non-esterified cholesterol and into the sterol moiety of cellular cholesteryl ester was determined by thin-layer chromatography, after cell lipid extraction with hexane/isopropanol (3:1, v/v) (26).

Plasma lipid peroxidation

Fasting plasmas were obtained from 14 patients, 5 males and 9 females, aged 36–75 years (mean 51 years), with normolipidemic xanthelasmas, and from 14 healthy volunteers, 5 males and 9 females, aged 33–70 years (mean 49.7 years). Specifically, the patients' triglyceride and cholesterol levels were 0.52–1.90 mmol/l (mean ± SD 1.23 ± 0.49 mmol/l) and 3.34–6.45 mmol/l (mean ± SD 5.30 ± 1.10 mmol/l), respectively, and the controls' triglyceride and cholesterol levels were 0.54–2.0 mmol/l (mean ± SD 1.19 ± 0.49 mmol/l) and 3.11–6.17 mmol/l (mean ± SD 4.8 ± 0.86 mmol/l), respectively. None of the subjects were taking any medications, and all were on a normal diet at the time of blood collection.

Blood was drawn into 1 mM Na_2-EDTA and centrifuged at 200 × g for 10 min at room temperature. Four milliliters of plasma were dialyzed against PBS overnight at 4°C. Then 2 ml of plasma were incubated for 2 h at 37°C without (control) or with 100 mM 2,2'-azobis,2-amidinopropane hydrochloride (AAPH) (Polysciences, Warrington, PA), which is a water-soluble azo compound that thermally decomposes and thus generates water-soluble peroxy radicals at a constant rate (27). Oxidation was terminated by refrigeration and the addition of 0.1 mM EDTA. Plasma lipid peroxidation was then determined by the lipid peroxidation test (22) and also by analysis of the conjugated dienes content of the plasma (23).

Statistical analysis

Each separate experiment was performed in triplicate. Student's *t*-test was used to analyze the significance of the data. The results are the means ± SD.

RESULTS

Histology

Histologic examination of the smaller piece, which contained xanthelasma tissue and adjacent normal skin, demonstrated "polarity" in each case; i.e. the bulk of the xanthelasma tissue was situated at the xanthelasma site, whereas no foam cells, or only a few, were present in the adjacent normal-looking skin. Subcutaneous fat cells were not detected in any of the biopsies, and sebaceous glands were usually not found.

Xanthelasma lipid composition

The mean cholesteryl ester and unesterified cholesterol concentrations in the xanthelasma tissue were 23 and 4.6 times higher, respectively, than those found in the adjacent normal-looking skin ($p < 0.01$) (Fig. 1). In contrast, the mean phospholipid and triglyceride concentrations in the normal-looking adjacent skin were only 2 and 1.4 times higher, respectively, than those found in the xanthelasma tissue ($p < 0.01$ and 0.05, respectively).

Altogether, cholesterol composed, on average, 88% of the xanthelasma lipids, but accounted for only 30% of the lipids in the adjacent normal-looking skin. In the xanthelasma tissue, the mean cholesteryl ester concentration was 2.4 times higher than unesterified cholesterol, whereas in the adjacent normal-

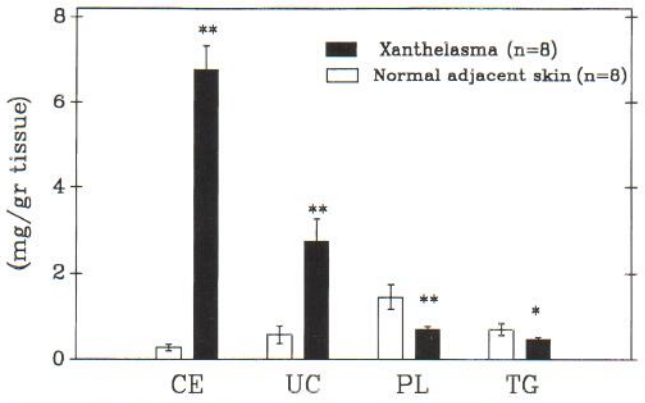

Fig. 1. Lipid composition of normolipidemic xanthelasmas versus adjacent normal-looking skin. The mean cholesteryl ester (*CE*), unesterified cholesterol (*UC*), phospholipid (*PL*) and triglyceride (*TG*) concentrations are shown. $*p<0.05$, $**p<0.01$ versus normal controls.

looking skin the mean concentration of unesterified cholesterol was twice that of cholesteryl ester.

Cholesterol metabolism in MDM

The mean values of intracellular cholesterol synthesis rates and LDL, acetyl-LDL, and oxidized LDL degradation rates did not differ significantly between patients and controls (Table I).

Plasma lipid peroxidation

The mean concentrations of conjugated dienes in the plasma derived from the patients and the controls prior to AAPH-induced lipid peroxidation were 91 ± 31 nmol/l (95% confidence interval (CI), range 75–108 nmol/ml) and 69 ± 14 nmol/ml (95% CI, range 62–76 nmol/l), respectively ($p<0.05$), and after lipid peroxidation 400 ± 62 nmol/ml (95% CI, range 367–433 nmol/ml) and 308 ± 42 nmol/ml (95% CI, range 286–330 nmol/ml), respectively ($p<0.01$) (Fig. 2). The mean plasma concentrations of lipid peroxides in the patients and controls prior to oxidation were 19 ± 14 nmol/l (95% CI, range 11–26 nmol/l) and 6.0 ± 2.6 nmol/l (95% CI, range 4.6–7.4 nmol/l), respectively ($p<0.01$), and after lipid peroxidation 554 ± 108 nmol/l (95% CI, range 497–611 nmol/l) and 429 ± 121 nmol/l (95% CI, range 364–494 nmol/l), respectively ($p<0.01$) (Fig. 2).

Table I. *Cholesterol synthesis and lipoprotein degradation in human monocyte-derived macrophages*

Results are expressed as mean ± SD.

	Controls ($n=3$)	Patients ($n=3$)
Cholesterol synthesis (cpm/mg cell protein)	306 ± 32	342 ± 27
Lipoprotein degradation (ng protein/mg cell protein)		
LDL	108 ± 10	110 ± 6
Oxidized LDL	251 ± 15	234 ± 18
Acetyl-LDL	366 ± 17	356 ± 20

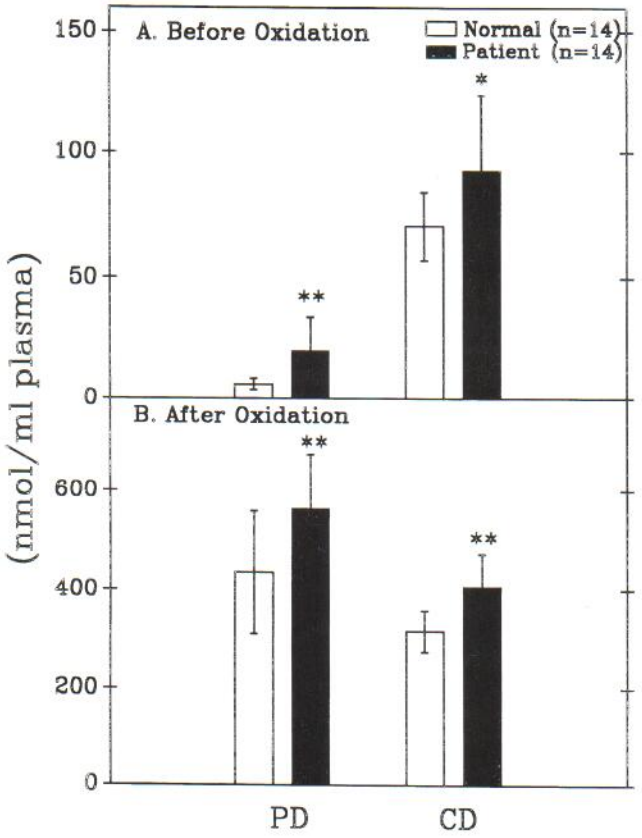

Fig. 2. Plasma lipid peroxidation (*PD*) and conjugated dienes (*CD*) before and after oxidation. $*p<0.05$, $**p<0.01$ versus normal controls.

DISCUSSION

Our findings provide evidence that the lipid composition of normolipidemic xanthelasmas is similar to that previously found in hyperlipidemic xanthelasmas (3, 4). The predominance of cholesteryl ester over unesterified cholesterol might have been due to increased esterification activity in the xanthelasma cells.

The MDM of normolipidemic xanthelasma patients did not demonstrate increased capabilities to degrade native LDL and LDL modified by acetylation or oxidation or to synthesize cholesterol de novo intracellularly. Hence, intrinsic cholesterol metabolism derangement in MDM, which could explain the formation of xanthelasmas in normolipidemic patients, was not demonstrated. It should be noted that the isolated mononuclear cells were incubated in a serum-free medium completely devoid of lipoproteins. The experimental procedures were identical for both patient and control cells, and therefore the induction of LDL receptor activity was probably similar. The monocyte/macrophages in dermal tissue, however, are exposed to lipoprotein levels which are much lower than in the serum. This may lead to enhanced LDL receptor activity in these cells, in comparison to blood monocytes, and might contribute to the formation of xanthelasmas in normolipidemic individuals. Other possible contributory factors include small changes in apolipoprotein levels in individuals with normal plasma triglyceride and cholesterol levels, which might lead to their increased uptake by dermal macrophages.

The increased levels of oxidizable LDL may be associated

with an increased risk of atherosclerosis (11, 12). Recent evidence has shown that oxidized LDL might represent the modified LDL responsible for cholesterol loading of macrophages promoting atherogenesis in vivo (12). Thus, increased mean levels of lipid peroxidation and susceptibility of the plasma of our patients to lipid peroxidation, which is by and large derived from oxidized LDL, could have a causal relationship to the formation of normolipidemic xanthelasmas and an increased risk of atherosclerosis.

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