Incorporation of Oleic Acid into Lipid by Foam Cells in Human Atherosclerotic Lesions

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ABSTRACT

The incorporation of ^{14}C-labeled oleic acid into phospholipid, cholesterol ester, and triglyceride in human arterial intima (normal and atherosclerotic) obtained from kidney transplant donors has been investigated in vitro. Most of the oleic acid was incorporated into phospholipid, in both the normal intima and the atherosclerotic lesion, but a greater proportion of label was diverted to cholesterol ester in the lesion. Representative sections of the vessels used for metabolic studies were taken for radioautography, and these demonstrated localization of ^{14}C to foam cells, but very little localization in the region of spindle-shaped cells. It was concluded that lipid synthesis in the human atherosclerotic lesion takes place predominantly in the intimal foam cells.

ADDITIONAL KEY WORDS human atheroma lipid metabolism fatty acids arterial wall metabolism intimal cells phospholipid cholesterol ester radioautography

■ The origin of lipid in the early human atherosclerotic lesion has been the subject of considerable investigation over recent years. Whereas phospholipid accumulation depends on synthesis in the intima (1), the cholesterol in the lesion appears to be derived from the plasma (2). The origin of cholesterol ester, which increases more rapidly than any other moiety, is not clear. Analysis of the plasma and intimal lipids indicates that the human fatty streak has a higher proportion of cholesterol oleate and a lower proportion of cholesterol linoleate than both the adjacent normal intima and the plasma (3-5). These findings may be explained by differential uptake or removal of cholesterol esters by the lesion, but may equally well indicate the esterification of cholesterol in the arterial wall. The latter seems likely in view of the many studies demonstrating lipid synthesis in the arterial wall (6-10). In-vitro studies in experimental animals (6, 11, 12) have demonstrated incorporation of labeled fatty acids either taken up by the intima or synthesized by it into cholesterol ester in the intima. In man, the umbilical artery has been shown to take up ^{14}C-labeled linoleic acid and incorporate it into phospholipid and triglyceride, but not to any extent into cholesterol ester (13). Little information is available, however, on the uptake and incorporation of fatty acids in atherosclerotic lesions in man.

In the present paper, the uptake of ^{14}C-labeled oleic acid by human atherosclerotic lesions and by the adjacent normal intima has been examined in vessels obtained from renal transplant donors. The localization of such uptake and metabolism by the foam cells in the lesion has been investigated by radioautography, in a similar way to that described previously (14) for lesions of cholesterol-fed rabbits.

Materials and Methods

Oleic acid 1-^{14}C, specific activity 32.5 or

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43.2 mc/mmole, (Radiochemical Centre, Amersham, England) was dissolved in 0.05N sodium hydroxide and taken up in an excess of bovine albumin (Armour). Radiochemical purity was verified by thin-layer chromatography and by gas liquid radiochromatography as described below. The oleic acid chromatographed as a single spot, and gas liquid radiochromatography indicated > 99% oleic acid.

Fresh human vessels were obtained from cadaveric renal transplant donors, within 1 hour after cessation of vital functions. Portions of thoracic aorta, iliac or renal arteries containing uncomplicated atherosclerotic lesions were set up in an incubation medium containing equal volumes of Hanks' solution (15) and normal human serum, together with a known amount (approximately 0.5 to 2.0 μc/ml incubation medium) of sodium 1-^{14}C oleate: albumin. After incubation for 4 hours at 37°C, a portion of the vessel was taken for radioautography and the remainder dissected into macroscopically normal and atherosclerotic lesions, the intima then being stripped from media and adventitia in both portions. The intima (normal and lesion) was extracted with chloroform: methanol (2:1 v/v) according to the method of Folch et al. (16). The relative amounts of normal and atherosclerotic artery varied considerably in different experiments, and in experiment 2, as most of the artery used was atherosclerotic, no separation into normal and atherosclerotic was attempted.

THIN-LAYER CHROMATOGRAPHY

Following extraction and counting, the lipid extracts from the normal and atherosclerotic intimas were separated into phospholipid, cholesterol/diglyceride, unesterified fatty acid, triglyceride, and cholesterol ester by thin-layer chromatography on Silica Gel G (Merck) using diethyl ether:acetic acid:n-hexane (38:3:100 v/v/v) as developing solvent. The phospholipid moiety was further fractionated into individual phospholipids by thin-layer chromatography by the method of Skipski et al. (17). Identification of phospholipids was made by comparison of Rf values with standards (Applied Science Laboratories, State College, Penn., U.S.A.) as set out previously (18). Following separation of the lipid extracts into the various lipid components or the individual phospholipids, the proportion of the ^{14}C-label in each moiety was determined by scraping the plates and counting directly, using the method of Snyder (19). All counting was done using a Packard Tricarb Spectrometer. All counts/min recorded were more than twice background, and counting was performed so that even where the ^{14}C-labeled component was present in very small amounts, total counts were not less than 500. Most samples, however, were counted for a minimum of 2,000 counts. Duplicate samples and duplicate counts of the one sample were routine.

GAS LIQUID CHROMATOGRAPHY

The specific activity of the ^{14}C-labeled oleic acid present in the cholesterol ester and phospholipid fractions was determined by gas liquid radiochromatography using an F & M Model 5750 Gas Chromatograph as described previously (14). In some cases a Pye Argon Chromatograph in conjunction with the Pye Radiochromatography monitor was used (20). Specific activities were expressed as counts/min of ^{14}C/mμmole oleic acid using methyl palmitate of known specific activity (35.2 counts/min/mμmole) as a standard. The standard deviation for duplicate determinations of the specific activity of the standard methyl palmitate (total counts/mass) was 7.0%, counting between 700 and 1,000 counts at a flow rate of 12 to 15 ml/min for the F & M, and 30 ml/min for the Argon Chromatograph. At these flows methyl palmitate peaks occupied approximately 30 to 60 seconds. Standard deviations of duplicate determinations carried out on individual days were appreciably less than the above figure, presumably due to slight changes in flow rate from day to day. The latter would alter the total counts accumulated by altering the counting time but would not alter the mass of a particular peak. The background radioactivity accumulating and integrated with the Pye Argon Chromatograph was 30 to 40 counts/min and with the F & M Chromatograph 20 to 30 counts/min. Sample counts accumulated over a peak time of approximately 1 minute and ranged (unless indicated in Table 3) from 40 to 710 total counts in the series reported. Duplicate determinations were routine. It was not possible to determine the specific activity of the oleic acid in the cholesterol ester in some of the samples by this means in view of their low specific activity. The ^{14}C present in the cholesterol ester fraction was therefore determined directly by counting the methyl esters prepared from the cholesterol ester and then determining the chemical amount of oleic acid in the same sample following gas liquid chromatography, using an internal standard (cholesterol heptadecanoate) to quantitate the cholesterol oleate. Specific activities by these two methods agreed where comparison was possible.

RADIOAUTOGRAPHY

The portion of artery reserved for radioautography was fixed for 4 days in 1% calcium chloride and 4% formaldehyde in physiological saline. Sections 6μ thick were cut using an Inter-

national Cryostat Model CTI. Radioautographs were prepared with Kodak AR10 Stripping Film. After exposure the radioautographs were developed and stained through the film with Sudan IV and hematoxylin.

Results

The clinical data for the four cases used for in-vitro metabolic studies are given in Table 1. The causes of death were not primary metabolic disorders, and the lesions were either fatty-streak (W.H.O. Grade I) or fibrofatty lesions (W.H.O. Grade II). In case 2, the fibrofatty lesions were so extensive that a normal area of vessel could not be obtained. Renal and iliac arteries were obtained, in addition to aorta, for metabolic study from case 4.

The percentage distribution of ^{14}C-labeled oleic acid among phospholipid, diglyceride, fatty acid, triglyceride, and cholesterol ester is shown in Table 2. Since the relative amount of normal and atherosclerotic tissue varied considerably in the different experiments, no comparison of the amount of ^{14}C-labeled oleic acid taken up and incorporated into combined lipid by normal and atherosclerotic portions of the intimas could be made. In both the normal intima and atherosclerotic lesion, most of the ^{14}C-labeled oleic acid was incorporated into phospholipid. There is, however, a higher proportion of ^{14}C-labeled oleic acid incorporated into cholesterol ester in all of the atherosclerotic intimal lesions than for the corresponding normal intima. This is particularly evident for the fatty-streak lesion where the normal intima incorporated 1.9% of the oleic acid into cholesterol ester compared with the adjacent atherosclerotic intima

TABLE 1

Clinical Data Related to Arteries Obtained for In-Vitro Studies

Case no.	Cause of death	Sex	Age	Artery obtained	Lesion type
1	Head injury	Male	18	Thoracic aorta	Fatty streak
2	Cerebral tumor	Male	48	Thoracic aorta	Extensive confluent fibrofatty
3	Subarachnoid hemorrhage	Male	42	Thoracic aorta	Fibrofatty plaques
4	Head and chest injuries	Male	32	Thoracic aorta Renal artery Iliac artery	Fibrofatty plaques

TABLE 2

Percentage Distribution of ^{14}C-Labeled Oleic Acid Among Intimal Lipid Fractions after Incubation In Vitro

Case no.	Lesion type	Incubation medium counts/min × 10^6/ml	% Uptake	Phospholipid	Diglyceride	Fatty acid	Triglyceride	Cholesterol ester
				Aorta				
1	Normal	3.7	0.260	48.7	5.8	32.1	11.1	1.9
	Fatty streak	3.7	0.080	37.5	3.6	30.3	12.7	15.6
2	Fibrofatty	0.77	0.540	72.1	4.2	6.1	11.0	6.2
3	Normal	2.61	0.580	40.4	4.6	38.8	11.8	4.4
	Fibrofatty	2.61	0.071	26.0	4.1	50.7	10.1	9.1
4	Normal	1.45	0.171	34.4	6.3	33.4	18.0	8.1
	Fibrofatty	1.45	0.359	29.7	7.1	26.4	22.1	14.8
				Renal Artery				
4	Normal	1.45	0.163	40.6	6.4	32.2	15.3	5.6
	Fibrofatty	1.45	0.138	39.2	7.0	19.0	21.7	13.2
				Iliac Artery				
4	Normal	1.45	0.260	35.5	6.4	35.4	18.3	4.8
	Fibrofatty	1.45	0.320	33.0	5.3	16.4	26.3	19.1

TABLE 3

Specific Activities of Phospholipid, Triglyceride, and Cholesterol Ester (counts/min/mμmole oleic acid)

Case no.	Lesion type	Phospholipid	Triglyceride	Cholesterol ester
		Aorta		
1	Normal	154	54	93*
	Fatty streak	169	34	18
3	Normal	213	194	30
	Fibrofatty	338	116	42*
4	Normal	232	225	<36†
	Fibrofatty	163	103	32
		Renal Artery		
4	Normal	141	47	<35†
	Fibrofatty	204	50	20
		Iliac Artery		
4	Normal	110	18	<75†
	Fibrofatty	299	51	27

*Determined by direct counting and separate assay by gas liquid chromatography. †Counts less than twice background, precluding accurate determination.

TABLE 4

Percentage Distribution of ^{14}C-Labeled Oleic Acid Among Individual Intimal Phospholipids

Case no.	Lesion type	Origin	Lysolecithin	Sphingomyelin	Lecithin	Phosphatidyl inositol	Phosphatidyl ethanolamine	Solvent front
				Aorta				
1	Normal	1.0	0.6	2.0	60.9	15.5	6.1	14.0
	Fatty streak	3.9	1.0	2.6	55.1	11.2	7.2	18.9
2	Fibrofatty	1.5	1.3	1.7	68.3	9.3	11.3	6.8
3	Normal	0.2	0.3	0.5	69.0	16.9	8.8	4.4
	Fibrofatty	0.3	0.4	0.9	69.3	11.1	12.2	5.8
4	Normal	0.5	0.8	0.4	72.7	9.7	8.4	7.5
	Fibrofatty	0.2	0.4	0.9	72.5	9.7	9.5	7.0
				Renal Artery				
4	Normal	0.0	0.0	0.0	76.7	13.0	5.7	4.5
	Fibrofatty	0.0	0.0	0.0	78.1	7.0	8.3	6.5
				Iliac Artery				
4	Normal	0.5	0.9	0.5	72.4	11.2	8.3	5.0
	Fibrofatty	0.3	0.4	0.2	74.2	8.8	9.1	7.0

in which 15.6% of the labeled oleic acid was diverted to cholesterol ester. The specific activity of the oleic acid present in the phospholipid, triglyceride and cholesterol ester is given in Table 3. In most of the experiments the specific activity for the phospholipid, triglyceride, and cholesterol ester of the atherosclerotic lesion is of the same order as that for the corresponding normal intima. For the fatty-streak lesion (case 1), the specific activities of both triglyceride and cholesterol ester are lower for the lesion than for the normal intima. It can be seen also from Table 3 that the specific activity of the phospholipid oleic acid is several times higher than that for the cholesterol ester oleic acid in the equivalent portion of artery whether normal or atherosclerotic.

The incorporation of ^{14}C-labeled oleic acid into individual phospholipids is given in Ta-

ble 4. Most of the oleic acid has been incorporated into lecithin in both the normal and atherosclerotic portions in all of the cases studied. Further, the pattern of incorporation is similar for the two portions.

RADIOAUTOGRAPHY

Localization of ^{14}C-labeled lipid to sudanophilic mononuclear cells was evident in all lesions studied. This is illustrated in Figures 1 to 3. Figure 1 is a radioautograph of a lesion from case 1, where there was visible fatty streaking in the thoracic aorta. This lesion was essentially cellular though less so at the intimomedial junction. Most of the ^{14}C present is localized in the sudanophilic mononuclear cells. A cluster of nonsudanophilic mononuclear cells present in upper right-hand corner shows little localization.

In Figure 2, a more fibrous lesion from case 4, spindle-shaped cells can be seen which have few related silver grains, in contrast to the sudanophilic mononuclear cells with which more label is associated. On the luminal side of the lesion shown in Figure 3, large foam cells have localized ^{14}C-labeled oleic acid and its metabolic derivatives; some localization by cells with pyknotic nuclei and fragmented cytoplasm deep in the lesion is present.

FIGURE 1

Radioautograph of an aortic fatty streak, incubated with ^{14}C-labeled oleic acid, from an 18-year-old male (case 1). The luminal side of the vessel is uppermost. Localization of the ^{14}C-labeled fatty acid by intimal foam cells (F) *is apparent. There is little label associated with a cluster of nonsudanophilic mononuclear cells* (M). *The region of the intimomedial junction is denoted* IM. *Hematoxylin and Sudan IV. Exposure time, 10 days.*

FIGURE 2

Radioautograph of an aortic fibrofatty lesion from a 32-year-old male (case 4). The luminal side is uppermost. Less localization by spindle-shaped cells (S) *than by sudanophilic mononuclears* (F) *is apparent. The intimomedial junction is denoted* IM. *Hematoxylin and Sudan IV. Exposure time, 8 days.*

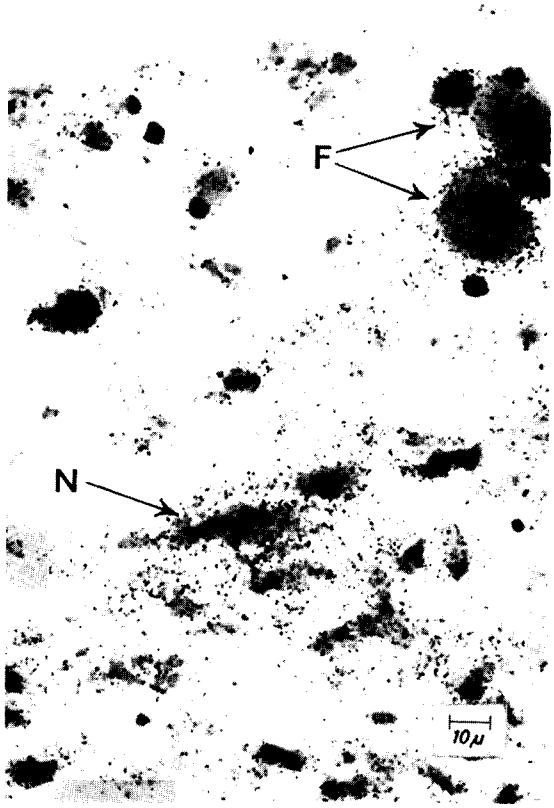

FIGURE 3

Radioautograph of a lesion from the same vessel as Figure 2. Localization of ^{14}C to large foam cells (F) *on the luminal side of the lesion (upper aspect of figure) is marked. Somewhat necrotic cells* (N) *are seen deeper in the lesion. Hematoxylin and Sudan IV. Exposure time, 8 days.*

Grain counts carried out in representative sections from all the cases studied are shown in Table 5. This data confirms quantitatively the findings illustrated in Figures 1 to 3.

Discussion

Most of the investigations in which human arterial wall has been incubated in vitro have used arteries obtained either post mortem or by surgery. Post-mortem material is usually not available immediately after death, and post-mortem changes make interpretation of metabolic data uncertain. Surgically removed arteries commonly contain advanced and complicated lesions, so that use of this material to study metabolic changes in the early atherosclerotic lesion in man is limited. The availability of vessels obtained from renal transplant donors provided an opportunity to study the lipid metabolism of early atherosclerotic lesions in vitro within 1 to 2 hours of clinical death, so that many of the advantages possible for experimental lesions are present, and the metabolic studies made in experimental animals could be extended with few of the normal limitations.

Our data indicate that in the early atherosclerotic lesion, fatty acid is actively taken up and incorporated into phospholipid, triglyceride, and cholesterol ester. The percentage of fatty acid diverted to cholesterol ester was higher in the atherosclerotic lesion than in the adjacent normal intima, and this was more

TABLE 5

Radioautograph Grain Counts (no./100 μ^2)

Case no.	Lesion type	Intima: Foam cells	Intima: Nonsudanophilic round mononuclear cells	Intima: Spindle-shaped cells	Intima: Extracellular	Media
			Aorta			
1	Fatty streak	15.6	5.0		2.9	1.1
2	Fibrofatty	15.3	4.9	4.1	2.5	1.1
3	Fibrofatty	17.7	4.0	2.6	2.2	1.2
4	Fibrofatty	18.7	5.8	4.7	4.1	4.4
			Renal Artery			
4	Fibrofatty	15.2	3.6	4.5	2.4	3.6
			Iliac Artery			
4	Fibrofatty	12.6	2.7	1.8	1.1	1.1

marked in the fatty-streak lesion than in the fibrofatty lesion. The specific activity of the oleic acid in the cholesterol ester was not appreciably greater in the lesion than in the normal intima. Such data are available for only one time interval in each experiment, however, so that an adequate comparison of the fractional turnover of cholesterol ester in the lesion and normal intima cannot be made. However, as far as they go, the specific activity data provide no indication of increased fractional turnover time of cholesterol ester in the lesion as compared with the normal. Presumably the amount of ^{14}C-labeled oleic acid incorporated into cholesterol ester in vitro per unit weight of intima was increased in the lesion.

The data presented is inadequate to assess the route of incorporation of fatty acid into either the phospholipid or cholesterol ester. The specific activity of the phospholipid was much higher, however, than that of the cholesterol ester at all time intervals, so that lecithin acyl transferase activity cannot be excluded as a source of cholesterol ester fatty acid.

The findings reported in the present paper are essentially similar to those reported previously for lesions of cholesterol-fed rabbits (14), in which oleic acid was taken up and diverted to cholesterol ester formation in the atherosclerotic intima. The type of phospholipid into which the oleic acid was incorporated in the present study also closely parallels that demonstrated for the rabbit lesion.

Our previous in-vitro study of atherosclerotic aortas from cholesterol-fed rabbits has indicated that most of the uptake and incorporation into lipid occurs in the foam cells present. This observation has been confirmed in the human lesion for foam cells present in both the fatty-streak and fibrofatty lesions. From the chemical data presented, it is apparent that most of the ^{14}C present in the lesion after incubation is present as phospholipid, but varying amounts of the other moieties synthesized, triglyceride and cholesterol ester, are also present, together with a significant amount of unchanged ^{14}C-labeled oleic acid. The radioautographic localization therefore cannot provide information relative to the incorporation into a single lipid moiety, but only to the total process involving uptake of fatty acid and its incorporation into phospholipid, triglyceride, and cholesterol ester. The conclusion that the foam cells take up oleic acid and are responsible for incorporating it into phospholipid and cholesterol ester in the human lesion does, however, make certain assumptions, namely, that the ^{14}C localized by radioautography is present, not only as lipid, but as combined lipid in the foam cell areas. It must be further assumed that, if the oleic acid is present predominantly as combined lipid in the foam cells, this did not arise by transfer of complex lipids synthesized elsewhere. These assumptions are reasonable as most of the ^{14}C-label is in fact present over foam cell areas, but there is no direct evidence in support of this in the present experiments. These possibilities have been considered and excluded in the case of rabbit atherosclerotic intima in the previous paper (14) and are discussed more fully in that context.

It appears likely, therefore, that the localization of ^{14}C-labeled oleic acid over foam cells in the present work indicates that the uptake of fatty acid and such incorporation into phospholipid, triglyceride, and cholesterol ester as occurs in the lesion is carried out in the main by the foam cells present. Further evidence is necessary, however, before one can conclude that the bulk of fatty acid synthesis and its subsequent incorporation into combined lipid in the human atherosclerotic lesion occurs predominantly in the foam cells present.

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