In vitro differentiation of human monocytes to macrophages results in depletion of antioxidants and increase in *n*-3 fatty acids levels

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Abstract The lipid composition and α-tocopherol content of human monocytes were investigated before and after their differentiation to macrophages. The total lipid and protein content per number of cells increased after the differentiation of monocytes by approximately four-fold; a two-fold increase in docosahexaenoic and docosapentaenoic acids and a two-fold decrease in linoleic acid were also noted. As opposed to an initial monocytic vitamin E content of 4.75 pmol/10^6 cells, macrophagic vitamin E levels were undetectable. Changes in vitamin E and fatty acids contents in macrophages, with respect to monocytes, appear to reflect the lipid composition of fetal calf serum, that is low in vitamin E and has a proportionally higher docosahexaenoic acid content than adult human serum.

Key words: Macrophage; Monocyte; Fatty acid; Antioxidant; Atherosclerosis

1. Introduction

The first progenitor cell derived from the hematopoietic stem cells (HSCs) is a colony-forming unit (CFU), which can take the monocyte pathway and initially give rise to proliferating monoblasts; these differentiate into promonocytes and finally into mature circulating monocytes. Circulating monocytes are believed to be a replacement pool for tissue-resident macrophages, for example lung macrophages.

Cultured macrophages, derived from circulating monocytes, are widely employed in studies of biochemical processes involved in atherosclerosis. Recruited macrophages in atherosclerotic lesions are indeed crucial in the pathophysiology of atherosclerosis: macrophage uptake of oxidatively modified low-density lipoprotein (LDL) via scavenger receptors gives rise to foam cells in the atheroma [1]. In addition, macrophages resident in lesions contribute to the onset and progression of the atheroma by: releasing free radicals (thus increasing the oxidation of LDL), synthesizing bioreactive lipids and coagulation cascade components, and by secreting proteases and protease inhibitors, and cytokines and chemokines [2].

Despite the widespread use of cultured macrophages – derived from monocytes by differentiation – little is known about the potential contribution of the lipid modifications of such cells, as compared to the progenitor monocytes, induced by the common cell culture techniques. Lipids that can not be synthesized by animal cells, such as the polyunsaturated fatty acids (PUFA), and antioxidants must be provided to the cells either through the diet (in vivo) or by the medium in cell cultures. Thus, the present investigation aimed at assessing the modifications of lipid and antioxidant composition of human monocytes before and after their macrophagic differentiation.

2. Materials and methods

2.1. Cell isolation

Human monocytes were obtained as described by Colli et al. [3,4]. Briefly, blood was obtained by venipuncture from healthy human volunteers, employing sodium citrate 3.8% as anticoagulant. Mononuclear cells were obtained by Histopaque® (Sigma, St. Louis, MO, USA) gradient centrifugation at 450×*g* for 30 min.

After repeated washes with RPMI medium (Sigma, St. Louis, MO, USA) to minimize platelet contamination, the cell preparation was seeded in Petri dishes (Corning, Milan, Italy); monocytes were separated from lymphocytes by adherence and were subsequently cultured in RPMI medium supplemented with 10% fetal calf serum (Gibco, Milan, Italy), 0.5% penicillin-streptomycin-Fungizone (Bio-Whittaker), and 2 mmol/l glutamine.

The cells were allowed to differentiate to macrophages for 2 weeks, after which they were characterized by the presence of the CD68 antigen [3,4].

2.2. Evaluation of lipid and protein composition

Macrophages were rinsed with saline phosphate buffer and then were removed from the Petri dishes by means of a rubber cell lifter. Lipids were extracted with chloroform:methanol (2:1, by volume) added with BHT (5 μg/ml), according to Folch et al. [5], and quantified by microgravimetry. The phospholipid fraction of cell lipids were separated by thin-layer chromatography [4]. Fatty acids were quantified by gas-liquid chromatography after their derivatization to methylesters [6]. The same methodology was employed to assess the lipid composition of fetal calf sera and of human serum for comparative evaluation (the latter was obtained by standard procedures from healthy volunteers).

The triacylglycerol and phospholipid contents of the lipid extract were determined by employing commercially available kits (Verbena, Milan, Italy). The protein content was measured according to Lowry et al. [7].

2.3. Vitamin E determination

For the evaluation of the α-tocopherol content, cells and media were extracted with hexane after protein precipitation with ethanol. α-Tocopherol levels were measured by HPLC, employing fluorimetric detection [8].

3. Results

The differentiation of monocytes to macrophages led to a four-fold increase in both cellular protein and lipid contents, due to the increase in cell size. In particular, the triglyceride content increased from 39 ± 1.12 to 70.2 ± 1.76 pmol/mg protein but decreased by two-fold when they where referred to the cell number (Table 1), possibly due to the increase in cell size. Also, the proportion of triglycerides over total lipids

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Table 1
Protein, total lipids, triglyceride, phospholipid, and α-tocopherol contents of human monocytes before and after their differentiation to macrophages

	Monocytes	Macrophages
Protein (μg/10^6 cells)	8.75 ± 1.23	34.91 ± 2.31*
Total lipids (μg/10^6 cells)	1.61 ± 0.53	6.12 ± 1.12*
Total lipids (mg/mg Protein)	0.184 ± 0.02	0.175 ± 0.04
Triglycerides (nmol/10^6 cells)	4.45 ± 0.3	2.01 ± 0.1*
Triglycerides (pmol/mg Protein)	39 ± 1.12	70.2 ± 1.76*
Phospholipids (nmol/10^6 cells)	16.1 ± 1.23	2.95 ± 0.8*
Phospholipids (pmol/mg Protein)	140.8 ± 4.53	103.2 ± 1.98*
α-Tocopherol (nmol/mg Protein)	0.54 ± 0.02	n.d.
α-Tocopherol (pmol/10^6 cells)	4.75 ± 0.87	n.d.

Data are means ± S.D. of four determinations; n.d. = not detectable (≤5 ng/10^6 cells).
*$P < 0.05$ following Student's *t*-test.

increased from ∼15.7 to 29.7%, whereas that of phospholipids decreased from ∼56.7 to 43.7%.

The vitamin E content of monocytes isolated from human blood was 4.75 ± 0.87 pmol/10^6 cells or 2.52 ± 0.21 pmol/μg total lipids (Table 1). After the differentiation of monocytes to macrophages, vitamin E levels were below the detection limit of the HPLC system, i.e. ≤5 ng/10^6 cells.

The fatty acid composition (total lipids) of both monocytes and macrophages is reported in Table 2. The percentage of docosapentaenoic acid (DPA, 22:5*n*-3) and docosahexaenoic acid (DHA, 22:6*n*-3) increased from 1.47 ± 0.23% to 2.93 ± 0.32% ($P < 0.05$) and from 2.48 ± 1.02 to 5.96 ± 1.21% ($P < 0.05$), respectively, whereas that of linoleic acid decreased from 7.91 ± 1.54 to 3.24 ± 1.23% ($P < 0.05$).

The fatty acid profile of the phospholipid fraction is given in Table 3. In macrophages, a marked increase in the long-chain PUFAs (LC-PUFAs) of the *n*-3 series 20:5*n*-3, 22:5*n*-3, and 22:6*n*-3 and a concomitant decrease in 20:4*n*-6, with respect to the corresponding values in monocytes, was observed.

Table 4 reports the composition in fatty acids of total lipids and the α-tocopherol content of human and fetal calf sera. It is noteworthy that the latter contains proportionally higher concentrations of *n*-3 fatty acids – especially of 22:5*n*-3 – and markedly lower amounts of linoleic acid. The *n*-3/*n*-6 ratios found in human macrophages and fetal calf serum were very similar (0.42 and 0.45, respectively).

4. Discussion

The data reported in this paper indicate that human monocytes, during their differentiation to macrophages, undergo a series of major transformations that led to a loss in antioxidant vitamins and to a proportional enrichment in LC-PUFAs of the *n*-3 series. In particular, vitamin E, the principal endogenous lipophilic antioxidant was undetectable in macrophages and previous reports demonstrated a rapid (∼3 h) loss of vitamin C [9]. Marked changes also occurred in the proportion of PUFA of the *n*-3 and *n*-6 series: the slight reduction in total *n*-6 fatty acids levels, associated with a marked increase in *n*-3 fatty acids levels, resulted in an almost three-fold increase of the *n*-3/*n*-6 ratio. It is noteworthy that the *n*-3/*n*-6 ratios in macrophages were increased, with respect to monocytes, more in phospholipids than in the total lipid fatty acids. Finally, linoleic acid levels were markedly reduced,

Table 2
Fatty acid composition of human monocytes before and after macrophagic differentiation

Fatty acid	Monocytes (% of total lipids)	Macrophages (% of total lipids)
16:0	19.71 ± 1.83	19.54 ± 1.75
18:0	18.37 ± 1.11	18.71 ± 1.24
16:1	0.25 ± 0.12	0.83 ± 0.43
18:1	19.71 ± 1.78	15.69 ± 1.53
18:2	7.91 ± 1.54	3.24 ± 1.23*
18:3	n.d.	n.d.
20:4	14.34 ± 1.12	16.59 ± 1.12
20:5	1.36 ± 0.76	1.94 ± 0.72
24:1	3.80 ± 0.35	2.88 ± 0.76
22:5*n*-6	1.20 ± 0.65	1.08 ± 0.54
22:5*n*-3	1.47 ± 0.23	2.93 ± 0.32*
22:6	2.48 ± 1.02	5.96 ± 1.21*
SFA	38.08 ± 1.73	38.25 ± 1.68
MUFA	23.78 ± 1.12	16.52 ± 1.21
PUFA	28.76 ± 0.98	31.74 ± 1.23
n-6	23.45 ± 1.12	20.91 ± 1.11
n-3	3.95 ± 0.64	8.89 ± 0.78
n-3/*n*-6	0.168	0.425
DHA/AA	0.173	0.359

Data are means ± S.D., $n = 4$. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; DHA, docosahexaenoic acid; AA, arachidonic acid.
*$P < 0.05$ following Student's *t*-test.

Table 3
Fatty acid composition of phospholipids (PL) of human monocytes before and after macrophagic differentiation

Fatty acid	Monocytes (% of total PL)	Macrophages (% of total PL)
16:0	22.81 ± 1.73	23.54 ± 1.75
18:0	20.37 ± 1.02	22.71 ± 1.24
16:1	0.85 ± 0.12	0.83 ± 0.43
18:1	19.71 ± 1.78	19.69 ± 1.53
18:2	10.71 ± 1.14	3.97 ± 1.13*
18:3	n.d.	n.d.
20:4	18.04 ± 1.02	10.35 ± 1.02*
20:5	0.36 ± 0.66	0.87 ± 0.62
24:1	2.91 ± 0.45	2.88 ± 0.76
22:5*n*-6	0.26 ± 0.45	0.78 ± 0.34
22:5*n*-3	1.45 ± 0.13	2.93 ± 0.32*
22:6	1.98 ± 1.01	5.42 ± 1.21*
SFA	43.18 ± 2.36	46.25 ± 1.98
MUFA	23.47 ± 1.76	23.40 ± 1.63
PUFA	32.80 ± 2.31	24.24 ± 1.99
n-6	29.01 ± 1.78	15.02 ± 0.98
n-3	3.79 ± 0.72	9.22 ± 1.28
n-3/*n*-6	0.131	0.614
DHA/AA	0.109	0.524

Data are means ± S.D., $n = 4$. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; DHA, docosahexaenoic acid; AA, arachidonic acid.
*$P < 0.05$ following Student's *t*-test.

Table 4
Fatty acid composition and α-tocopherol content of human and fetal calf sera

Fatty acid	Human serum	Fetal calf serum
16:0	25.9 ± 1.78	30.1 ± 1.87
18:0	6.5 ± 0.98	24.4 ± 2.1
16:1	1.6 ± 0.77	2.5 ± 0.76
18:1	21.6 ± 1.24	19.4 ± 1.23
24:1	0.7 ± 0.3	1.9 ± 0.5
20:3*n*-9	n.d.	0.3 ± 0.2
18:2	33.5 ± 2.3	5.8 ± 1.11
20:3*n*-6	1.5 ± 0.76	2.1 ± 1.21
20:4	6.1 ± 1.21	6.4 ± 1.11
22:4	0.2 ± 0.2	0.2 ± 0.15
22:5*n*-6	0.1 ± 0.2	0.1 ± 0.12
18:3	n.d.	0.4 ± 0.3
20:5	0.8 ± 0.4	0.9 ± 0.7
22:5*n*-3	0.2 ± 0.1	2.3 ± 0.93
22:6	1.3 ± 0.7	3.0 ± 0.91
SFA	32.4 ± 1.38	54.5 ± 1.98
MFA	23.9 ± 0.77	23.8 ± 0.76
PUFA	46.7 ± 0.65	11.7 ± 0.34
U.I.	133.7 ± 1.4	104.7 ± 1.23
n-6	41.4 ± 1.45	14.6 ± 1.23
n-3	2.3 ± 0.42	6.6 ± 1.11
n-3/*n*-6	0.07	0.45
DHA/AA	0.21	0.47
α-Tocopherol	21.67 ± 3.78	3.89 ± 1.23

Data are means ± S.D. of four determinations and are expressed as percent of total lipids, except for α-tocopherol values, that are expressed as absolute concentrations (μM). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; DHA, docosahexaenoic acid; AA, arachidonic acid; U.I., unsaturation index.

whereas both DPA and DHA levels were increased. These marked differences are not dependent on changes in the proportion of the various cellular lipid classes (triglycerides versus phospholipids), as even more pronounced modifications took place in the phospholipid class (Table 3).

It is noteworthy that fetal calf serum, as compared with human serum, has a markedly higher proportion of DPA (approximately 10-fold), whereas that of DHA is only two-fold. This may be a consequence of a relatively inefficient in vivo conversion of DPA to DHA, a process that takes place in the peroxisomes. Conversely, the macrophagic increase, as compared to monocytes, of both DPA and DHA is very similar. Hence, the loss of vitamin E, the decline of linoleic acid levels, and the accumulation of *n*-3 fatty acids appear to closely reflect contents of these compounds in the fetal calf serum (Table 4).

It is conceivable that the reduced antioxidant pool may only partially protect the unsaturated fatty acids – whose levels increased after differentiation – from oxidation; therefore the intracellular redox status may be altered, possibly interfering with the activity of certain enzymes that depend on the intracellular peroxide tone, e.g. cycloxygenases [10].

Cell cultures of macrophages are widely employed to investigate the initial steps of atherosclerosis, in which the involvement of lipid peroxidation, of LDL in particular, likely plays an important role [1]. The data reported here and elsewhere that indicate how the macrophagic fatty acid profile depends of the kind of serum adopted in culture [4] suggest that the use of human macrophages derived by differentiation from monocytes may introduce an artifactual variable linked to the altered antioxidant/fatty acids proportion; it appears appropriate to include an analysis of the fatty acid and antioxidant content of human macrophages when these are to be employed in studies of cellular systems that depend on the intracellular redox status.

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