CD4+, CD8+ and CD19+ Cells at Individuals with Dyslipidemia

Populações Celulares Periféricas (CD4+, CD8+ e CD19+) em Indivíduos Dislipidémicos

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ABSTRACT

Introduction: The past decade has witnessed an increasing recognition that inflammatory mechanisms play a central role in the pathogenesis of atherosclerosis and its complications. Recently, attention was focused on the potential role of plasma markers of inflammation as risk predictors among those at risk for cardiovascular events. Of these potential markers, C-reactive protein (CRP), IL6, metalloproteinases, ICAM, VCAM and other molecules, have been extensively studied. On the other hand, to our knowledge, there are only a few studies on the role of inflammatory cells, like T and B lymphocytes in the atherosclerosis.

Material and Methods: By Flow Cytrometry analysis we have determined on dyslipidemic people and on a control group, the percentage of some peripheral inflammatory cells, like CD3+, CD4+, CD8+, CD19+, CD56+, CD56CD8+, DN, CD25+, CD26+, CD25CD3+, CD26CD3+, CD25CD26CD3+, CCR5+, CCR5CD3+, CCR5CD4+, HLADR+, HLADRCD4+, HLADRCD8h+, HLADRCD8low+, HLADRCD8+, CD95+, CD95CD95L+, CD3CD95+, CD3CD95L+, CD62L+, CD3CD62L+, CD69+, CD69CD3+ e CD69CD4+.

Results: In the present study we have particularly studied the percentage of CD4+, CD8+ and CD19+ cells. The CD4+ cells have been significantly reduced in the people with dyslipidemia.

Discussion: We do not know the peripheral numbers of the subtype Th1 and Th2, neither the percentage of CD4+CD25+ cells (regulatory T cells). We have not find any differences on the percentage from the CD8+ and CD19+ cells.

Conclusions: In spite of the identified limitations resulting from the small-sized samples, it was possible to show a reduction of some molecules after application of acetylsalicylic acid.

Keywords: Biological Markers; Blood Cells; Dyslipidemias; Flow Cytometry.

RESUMO

Introdução: Os mecanismos imunológicos e inflamatórios têm um papel crucial no desenvolvimento da aterosclerose e na sua tradução clínica. São inúmeros os estudos que procuraram relacionar os mais diversos marcadores inflamatórios – leucócitos, proteína C reactiva, interleucinas, quimiocinas, moléculas de adesão, metaloproteinases, etc – com os factores de risco clássicos da aterosclerose, a formação da placa e os fenómenos clínicos. Não são tantos, que tenhamos conhecimento, os trabalhos que analisaram o comportamento das diversas células mononucleares na fisiopatologia da aterosclerose. Sendo os monócitos/macrófagos e os linfócitos células fundamentais no desencadear e posterior evolução desta doença vascular, procurámos determinar as percentagens das diversas populações celulares periféricas em indivíduos dislipidémicos e em normolipidémicos.

Material e Métodos: Por citometria de fluxo, determinámos em indivíduos com dislipidemia e num grupo controlo, as concentrações no sangue periférico dos CD3+, CD4+, CD8+, CD19+, CD56+, CD56CD8+, DN, CD25+, CD26+, CD25CD3+, CD26CD3+, CD25CD26CD3+, CCR5+, CCR5CD3+, CCR5CD4+, HLADR+, HLADRCD4+, HLADRCD8h+, HLADRCD8low+, HLADRCD8+, CD95+, CD95CD95L+, CD3CD95+, CD3CD95L+, CD62L+, CD3CD62L+, CD69+, CD69CD3+ e CD69CD4+.

Resultados: Embora na sua grande maioria não tenham sido encontradas diferenças significativas entre os dois grupos de participantes, verificaram-se em algumas populações celulares, resultados que nos mereceram alguns comentários. Neste artigo debruçámo-nos apenas sobre as populações positivas para os CD4, CD8 e CD19.

Discussão: A menor concentração das células CD4+ na nossa população de dislipidémicos foi aparentemente inesperada devido ao relacionamento existente entre este tipo celular, os factores de risco e a aterosclerose. Não foram determinados os subtipos Th1 e Th2, nem a população de células reguladoras CD4+CD25+, que poderiam explicar a menor percentagem de células CD4+ nos nossos dislipidémicos. Relativamente às células CD8+ e CD19+, apresentaram percentagens sobreponíveis nos dois grupos de participantes.

Conclusões: Apesar das limitações evidenciadas em resultado da reduzida dimensão das populações, foi possível demonstrar uma redução em algumas moléculas após aplicação de ácido acetilsalicílico.

Palavras-chave: Células Sanguíneas; Citometria de Fluxo; Dislipidemia; Marcadores Biológicos.

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INTRODUCTION

Originally considered primarily as a degenerative disease affecting large and medium-sized arteries, atherosclerosis is regarded today as a chronic inflammatory disease leading to an acute ischaemic syndrome, due to the plaque rupture.

The presence of T lymphocytes and macrophages in atherosclerotic plaques suggests that immunological mechanisms are involved in its pathogenesis.[1] It is presently recognized that T and B cells, monocyte, dendritic cells (DC) and natural killer cells (NK) are crucial for atherosclerosis development and progression.[2-3]

Importantly, lipoprotein (namely LDL) transfer and retention in the artery wall is a critical determinant for triggering atherosclerosis lesions. LDLs are subjected to the action of several highly reactive species with oxidative capacity, synthesized by endothelial, smooth muscle cells and macrophage,[4-5] becoming more immunogenic and OxLDL-specific immunological responses have already been identified.[6] One of the main actions of these OxLDL is the stimulation of interleukins, chemokines, adhesion molecules and metalloproteinase production by endothelial cells, smooth muscle cells and macrophages, in turn attracting circulating mononuclear cells, inducing their adhesion to the endothelium and their migration to the sub-endothelial space were they will trigger and perpetuate inflammatory mechanisms leading to the plaque formation and subsequent rupture.[7-8]

Most studies have addressed the role of macrophage in inflammatory and immunological events underlying atherosclerosis pathophysiology.

Several animal and human studies have also drawn attention to the presence of T and B lymphocytes in atherosclerotic lesions, enhancing the role of these cells in their pathophysiology.[9] Studies carried out in apolipoprotein E-deficient mice (Apo $E^{-/-}$) as well as in other LDL receptor deficient mice ($LDLR^{-/-}$) demonstrated that CD4 Th1 lymphocytes producing interferon-gamma and interleukin 12 play a prominent role in the induction of atherosclerosis, while CD4 Th2 cells, producing interleukin 10, may have a primarily protective role.[10-11]

In what concerns CD4 cells, we would like to mention a specific cellular subtype characterized by CD4 and CD25 expression, producing anti-inflammatory cytokines IL10 and TGF-β, known as CD4+CD25+ T cells. These cells, also designated as regulatory T cells (Treg), suppress effector CD4 lymphocytes, with supposedly anti-atherosclerotic properties.[12,13] Significant reductions of atherosclerosis lesions have been observed in Apo $E^{-/-}$ mice recipients of CD4+CD25+ T cells.[14] Patients with acute coronary syndromes display a reduction in the frequency of CD4+CD25+ T cells, as well as a reduction of their suppressivefunction.[15]

In what concerns B lymphocytes, there is a scarcer volume of research regarding their potential action in atherosclerotic vascular disease. These cells produce anti-OxLDL antibodies and *anti-heat shock protein* (HSP), both admittedly involved in atherosclerosis pathophysiology.[16,17] These antibodies have been detected in atherosclerosis animal models and in patients with coronary heart disease.[18] Some studies are in line with a protective role of these antibodies regarding atherosclerosis. Immunization of rabbits and mice with OxLDL induced an increase in anti-OxLDL antibody production and a reduction in atherosclerosis lesions.[28,29] Furthermore, a marked reduction in anti-OxLDL levels and a significant increase in aortic atherosclerosis lesions in mice both negative for LDL receptor and with less than 1% of B cells, fed with a western diet, has been observed.[30]

OBJECTIVES

We sought to compare peripheral cell populations between patients with dyslipidemia and sub-clinical vascular disease and normolipidemic individuals. Sub-clinical valvular heart disease was assessed through measuring carotid intima-media thickness by Doppler ultrasound imaging. This study focuses on CD4, CD8 and CD19 expression.

MATERIAL AND METHODS

Characterization of the populations studied

Control group (C), comprising 12 healthy normolipidemic individuals of both genders (six male), with ages 30 to 70 (mean age – 51.33 years).

Dyslipidemia group (D), comprising 38 patients selected from a group of patients, comparable in terms of gender (22 male) and age (mean age – 55.76) to the controls, but with higher total cholesterol (> 200mg/ dl) or LDL cholesterol (> 130mg/ dl) and / or triglyceride levels (> 200mg/ dl) in association with sub-clinical carotid atherosclerotic lesions. These patients were not following any lipid-lowering therapy for at least one month, having obviously maintained any antihypertensive, antiplatelet or any other essential therapy.

All participants gave informed consent and the present study was approved by the Ethics Committee of *Hospitais da Universidade de Coimbra*.

A clinical assessment of all participants was carried out, including body weight, height, body mass index, abdominal / hip circumference and blood pressure determination and any clinical sign of atherosclerosis (heart murmurs, peripheral pulses) or dyslipidemia signs (corneal arcus, xanthelasma / xanthoma, hepatosplenomegaly) was assessed.

Family history, as well as food habits, tobacco use and drinking patterns, the use of medication and other

lifestyle characteristics were considered.

Lipid profile (total cholesterol, HDL, LDL and triglyceride), Apo A1, Apo B100, Apo(a), Apo E genotyping, glucose, uric acid, CK, alkaline phosphatase, transaminases, gamma-GT, bilirubin, urea, creatinine, thyroid tests and urine sediment analysis were obtained, as well as ceruloplasmin and transferrin serum levels. All the tests were obtained after a 12-14h fast.

An ECG was performed, as well as an assessment of possible atherosclerosis lesions, with carotid intima-media thickness obtained by Doppler imaging.

Ankle-brachial index (ABI) and, whenever deemed necessary, accompanied by Doppler ultrasound of lower limb arteries.

Characterization of membrane markers

The following monoclonal antibodies were used:

- CD8 FITC, CD19 FITC, CD3 PE, CD56 PE and CD4 PECy5 -Lymphogram "Cytognos"
- CCR5 PE - RD Systems
- CD8 PE, CD3 Cy5, CD4Cy5 – Dako
- CD95 FITC, CD26 PE, CD62L, HLA DR FITC - Immunotech
- CD4 FITC, CD25 FITC, CD69 PE - CLB
- CD95L PE – Caltag

A flow cytometer ('FacsCalibur'-*Becton Dickinson*) and triple immunofluorescence was used for acquisition of samples:

- FL1 – FITC (fluorescein)
- FL2 – PE (phycoerithrin)
- FL3 – PE Cy5 phycoerithrin-cyanine 5)

Acquired data was stored and subsequently analysed using "*Paint-a–gate*" software, allowing for identification of the different cellular populations.

Statistical analysis

A 95% confidence interval was calculated for longitudinal data and variance analysis for comparison of the means (ANOVA), both between groups and in the same group, in successive moments. We adopted a significance level of 95% ($p < 0.05$).

SPSS v.15 software was used.

The results are presented as absolute or relative numbers and include the mean as well as standard deviation.

RESULTS

As regards lipid profile, patients in group D presented significant differences, as would be expected, in total cholesterol levels (307.74 *versus* 189.67 mg/dl; $p = 0.000$) and LDL-cholesterol (172.18 versus 112.45 mg/dl; $p = 0.000$). Triglyceride levels were also higher in patients with dyslipidemia (285.47 *versus* 142.42 mg/ dl; $p = 0.104$), although without statistical significance. HDL-cholesterol levels (49.79 *versus* 48.33 mg/dl; $p = 0.720$) overlapped in both populations. TC/HDL-C ratio, which has a strong prognostic relevance and should be ideally less than 5, was significantly different in both populations, with values in group D related to a higher cardiovascular risk than controls (6.55 *versus* 4.05; $p = 0.001$).

Percentage results regarding peripheral cellular populations are presented in table 1.

Although we have not observed significant differences regarding cell populations between the patients in both groups, the results obtained require some reflection. As above-mentioned, in the present study, we will only discuss the results concerning CD4+, CD8+ and CD19+ expression.

The frequency of CD4+ cells was higher in controls than in patients with dyslipidemia (46.233 *versus* 40.300; $p = 0.026$).

The frequency of CD8+ cells did not show any significant differences in both groups ($p = 0.783$).

CD19+ cells, presented similar frequencies in both groups ($p = 0.945$).

CD25 expression was similar in both populations ($p = 0.108$), tending to be less frequent in patients with dyslipidemia (11.076 *versus* 12.700).

DISCUSSION

We are aware that serum cell populations may not exactly represent what happens in the vascular wall and within atherosclerosis lesion. For instance, CD4+ cells reactive to human HSP have been detected in the atherosclerosis plaque but not in peripheral blood.[31] Availability of tissue samples would be ideal, but out of reach of our present work.

CD4 antigen is present in T cells and behaves like a co-receptor of major histocompatibility complex class II molecules set to activate these cells. It is a marker of thymic differentiation and is also present in some thymocytes, monocytes/macrophages and granulocytes.

CD4+ cells were significantly reduced in group D patients. Several studies addressed the relationship between CD4+ cells, risk factors and clinical or subclinical vascular disease. In one study of Japanese men with metabolic syndrome, CD4+, CD3+ and memory cells (CD4+CD45RO+) presented a positive correlation with BMI, plasma levels of glucose and triglyceride and were inversely correlated with HDL-C level.[32] Another study showed once again a positive correlation between CD4+, CD3+, CD8+ and CD8+CD28+ with diabetes.[19] In addition, another study showed that the CD4+Th_1/CD4+CD25+ ratio was positively associated with the occurrence of acute coronary syndrome.[34] A positive correlation has also been found between CD4+CD28- lymphocytes and the risk of an ischaemic stroke.[20] Different results have been obtained in one study carried out in patients submitted to renal transplant, where an inverse correlation was found between CD4+ levels

Table 1 - Cell populations

		N	Mean	Standard Deviation	Standard Error	95% Confidence Interval		*P*
CD3	P	37	73.508	1.2361	1.2361	71.001	76.015	0.240
	C	12	6.3816	1.8422	1.8422	72.329	80.438	
CD4	P	37	40.300	7.6530	1.2582	37.748	42.852	0.026
	C	12	46.233	8.1492	2.3525	41.056	51.411	
CD8	P	37	23.900	6.2737	1.0314	21.808	25.992	0.783
	C	12	24.500	7.2770	2.1007	19.876	29.124	
CD19	P	37	12.62	4.177	0.687	11.23	14.01	0.945
	C	12	12.72	3.879	1.120	10.25	15.18	
CD56	P	37	13.589	6.6275	1.0896	11.379	15.799	0.196
	C	12	10.842	5.1078	1.4745	7.596	14.087	
CD56CD8	P	37	6.035	3.2411	0.5328	4.955	7.116	0.244
	C	12	4.833	2.4103	0.6958	3.302	6.365	
DN	P	37	3.089	2.1216	0.3488	2.382	3.797	0.275
	C	12	3.883	2.2910	0.6614	2.428	5.339	
CD25	P	37	11.076	3.0142	0.4955	10.071	12.081	0.108
	C	12	12.700	2.8832	0.8323	10.868	14.532	
CD26	P	37	39.41	11.317	1.861	35.64	43.18	0.050
	C	12	46.99	11.392	3.289	39.75	54.23	
CD25CD3	P	37	8.408	2.0973	0.3448	7.709	9.107	0.016
	C	12	10.200	2.3460	0.6772	8.709	11.691	
CD26CD3	P	37	36.922	11.2472	1.8490	33.172	40.672	0.038
	C	12	44.908	11.4119	3.2943	37.658	52.159	
CD25CD26CD3	P	37	5.157	1.7321	0.2848	4.579	5.734	0.000
	C	12	7.642	1.8676	0.5391	6.455	8.828	
CCR5	P	35	2.234	1.2943	0.2188	1.790	2.679	0.199
	C	12	2.942	2.3666	0.6832	1.438	4.445	
CCR5CD3	P	35	1.603	1.0537	0.1781	1.241	1.965	0.279
	C	12	2.017	1.3381	0.3863	1.166	2.867	
CCR5CD4	P	35	1.069	0.7688	0.1299	0.804	1.333	0.867
	C	12	1.025	0.7944	0.2293	0.520	1.530	
HLARD	P	37	26.489	7.7643	1.2764	23.900	29.078	0.014
	C	12	20.308	5.2219	1.5074	16.991	23.626	
HLADRCD4	P	37	7.722	3.8419	0.6316	6.441	9.003	0.015
	C	12	4.508	3.7833	1.0922	2.105	6.912	
HLADRCD8h	P	37	4.486	1.9518	0.3209	3.836	5.137	0.001
	C	12	2.325	1.6526	0.4771	1.275	3.375	
HLADRCD8low	P	37	1.557	0.9648	0.1586	1.235	1.878	0.027
	C	12	0.843	0.8695	0.2510	0.291	1.396	
HLADRCD8	P	37	6.068	2.5047	0.4118	5.232	6.903	0.001
	C	12	3.167	2.3689	0.6838	1.662	4.672	
CD95	P	37	21.076	11.2630	1.8516	17.320	24.831	0.487
	C	12	18.650	7.0150	2.0251	14.193	23.107	
CD95CD95L	P	37	0.470	0.4169	0.0685	0.331	0.609	0.064
	C	12	0.235	0.1699	0.0490	0.127	0.343	
CD3CD95	P	37	17.511	10.3625	1.7036	14.056	20.966	0.966
	C	12	12.375	6.8094	1.9657	13.049	21.701	
CD3CD95L	P	37	1.219	1.2778	0.2101	0.793	1.645	0.400
	C	12	0.892	0.6302	0.1819	0.491	1.292	
CD62L	P	37	60.046	14.3597	2.3607	55.258	64.834	0.087
	C	12	67.933	10.5455	3.0442	61.233	74.634	
CD3CD62L	P	37	43.792	12.8940	2.1198	39.493	48.091	0.066
	C	12	51.700	11.8714	3.4270	44.157	59.243	
CD69	P	37	11.705	7.0168	1.1536	9.366	14.045	0.024
	C	12	16.750	4.5900	1.3250	13.834	19.666	
CD69CD3	P	37	5.468	4.2780	0.7033	4.041	6.894	0.003
	C	12	9.758	3.7922	1.0947	7.349	12.168	
CD69CD4	P	37	3.511	2.8682	0.4715	2.555	4.467	0.008
	C	12	6.125	2.6840	0.7748	4.420	7.830	

and the occurrence of cardiovascular events. A CD4+ concentration in the higher quartile corresponded to a risk ten times lower as the one associated to the concentration in the lower quartile.[21] In a study with HIV+ patients, in which, as in ours, carotid disease was assessed by Doppler imaging, a higher concentration of CD4+ was associated with a lower progression of the carotid disease (p =0.01).[22] On the other hand, in healthy individuals aged between 60-70, memory CD4+CD45RO+ T cells and CD19+CD80+ activated B cells correlated positively and significantly with carotid intima-media thickness.[23]

CD8 is present in T cells as a co-receptor for activation of these cells, recognizing the antigens connected to MHC class I molecules. It is also present in some thymocytes and in some dendritic cells.

CD8+ cells behave as cytotoxic cells, removing virus-infected cells, tumor cells and graft cells and do not present significant percentage differences between the groups.

Fewer studies assessed a possible relation between CD8+ cells in peripheral blood and vascular disease.[33] In patients with sleep disorder, in whom vascular disease has a higher prevalence than in the general population, a significantly higher cytotoxicity level than controls was observed in CD8+ lymphocyte, marked by the presence of CD56 and CD16.[24] Another study obtained similar results.[25]

In our study, a significantly higher percentage of CD4+ cells (0.026) in controls and similar percentage of CD8+ in both groups (p = 0.783) may suggest a greater weight of humoral immunity (CD4 TH_2) and / or cell-mediated hypersensitivity (CD4 TH_1) in patients without dyslipidemia, as well as a cellular immunity (CD8+) predominance in patients with dyslipidemia..

We assume that patients with dyslipidemia likely present with more serious and/or more unstable lesions, despite the lack of carotid artery assessment in our controls. Therefore, we are tempted to agree with some authors that refer to cellular immunity as playing a major role in atherosclerosis, assigning to humoral immunity, particularly to anti-OxLDL antibodies a protective role.[26] This concept is far from being consensual, as some studies demonstrate conflicting results.[27]

We would like to draw our attention to a remarkable aspect. As the percentage of doubly positive CD4 and CD25 cells (CD4+CD25+ cells with regulatory functions) has not been analysed, we observed that the percentage of CD4 and CD25 markers was reduced in our patients with dyslipidemia (the latter with no statistical significance). Given the importance of CD4+CD25+ cells as inhibitors of atherosclerosis pathophysiology,[23,28,29] we speculate that similar lower CD4 and CD25 values in our group D patients could represent a reduced percentage of regulatory CD4+CD25+ T-cells in patients with dyslipidemia.

In what concerns B cells, identified by CD19, it seems they mainly have a protective role regarding atherosclerosis development and were found in similar percentages in both groups. This protective action was demonstrated in several animal models where its concentration in the vascular wall was gradually reduced as the area occupied by plaques was increasing;[3] CD19+ lymphocyte congenital deficiency was associated with a higher development of atherosclerosis lesions.[30]

In summary, the results regarding the frequency of peripheral cell populations allow us to make a few comments:

The lower CD4+ cell concentration in our patients with dyslipidemia, apparently unexpected, due to the existing relation between this cell type, the risk factors and atherosclerosis, deserves further characterization and sub-phenotyping. As previously mentioned, similarly lower percentages of CD4 and CD25 (these with no statistical significance) in our patients with dyslipidemia, allow us to speculate that CD4+CD25+ cells (regulatory and with atherosclerosis supressive functions) are probably reduced in these patients.[23] The phenotype presented by CD4+ cells, mainly Th1, with gamma interferon production, or Th2, with anti-inflammatory cytokine production, as IL10, may explain the relationship between these cells, risk factors and atherosclerosis development.[17-20] A similar CD8+ cell concentration in both groups, accompanied by a significant reduction of CD4+ in patients with dyslipidemia suggests cellular immunity plays a predominant role in these patients.

CONCLUSION

Our study has some limitations, due to the small size of the population studied, particularly in the control group, as well as the fact that some patients with dyslipidemia have been following a lipid-lowering therapy until one month before characterization, which might have interfered with cellular frequencies. Sixteen patients with dyslipidemia were treated with acetylsalicylic acid in a dose intended for an anti-platelet effect. Despite the low daily dose used, between 100 and 150 mg, this may have exerted significant anti-inflammatory effects, as some studies have been able to demonstrate a significant reduction in molecules such as C-reactive protein, when acetylsalicylic acid is used in a daily dose of 75 to 300 mg.[31,32]

CONFLICTS OF INTEREST

The authors declare there are no conflicts of interest in writing this manuscript.

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