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Antagonistic regulation of macrophage phenotype by M-CSF and GM-CSF: Implication in atherosclerosis

Isabelle Brochériou[a,b], Seraya Maouche[a], Hervé Durand[a], Vincent Braunersreuther[c], Gilles Le Naour[b], Alexei Gratchev[d,e], Fabien Koskas[a,g], François Mach[c], Julia Kzhyshkowska[d,e], Ewa Ninio[a,*]

[a] INSERM UMRS937, Université Pierre et Marie Curie UPMC-Paris 6, Faculté de Médecine Pierre et Marie Curie, 91 Boulevard de l'Hôpital, 75634 Paris, France
[b] Department of Pathology, Hôpital de la Pitié-Salpêtrière, AP-HP, Paris, France
[c] Division of Cardiology, Foundation for Medical Research, University Hospital, Geneva, Switzerland
[d] University Medical Centre Mannheim, Ruprecht-Karls University of Heidelberg, Germany
[e] Institute of General Pathology and Pathophysiology, Russian Academy of Medical Sciences, Moscow, Russia
[g] Department of Vascular Surgery, Hôpital de la Pitié-Salpêtrière, AP-HP, Paris, France

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ABSTRACT

Objectives: We characterized the transcriptional profiles of GM-CSF- (GM-MØ) and M-CSF-induced macrophages (M-MØ) and investigated in situ a subset of differentially expressed genes in human and mouse atherosclerotic lesions.
Methods and results: Using microarrays we identified a number of genes and biological processes differentially regulated in M-MØ vs GM-MØ. By varying in culture the M-CSF/GM-CSF ratio (0–10), a spectrum of macrophage phenotypes was explored by RT-QPCR. M-CSF (10 ng/ml) stimulated expression of several genes, including selenoprotein-1 (*SEPP1*), stabilin-1 (*STAB1*) and CD163 molecule-like-1 (*CD163L1*) which was inhibited by a low dose of GM-CSF (1 ng/ml); M-CSF inhibited the expression of pro-platelet basic protein (*PPBP*) induced by GM-CSF. Combining Tissue Microarrays/quantitative immunohistochemistry of human aortic lesions with RT-QPCR expression data either from human carotids vs mammary non-atherosclerotic arteries or from the apoE null mice normal and atherosclerotic aortas showed that, *STAB1*, *SEPP1* and *CD163L1* (M-CSF-sensitive genes) and *PPBP* (GM-CSF-sensitive gene) were expressed in both human arterial and apoE null mice atherosclerotic tissues.
Conclusion: A balance between M-CSF vs GM-CSF defines macrophage functional polarisation and may contribute to the progression of atherosclerosis.

1. Introduction

Monocyte-derived macrophages (MØ) are essential cells for the formation of atherosclerotic lesions as they contribute to local arterial wall inflammation and become lipid-laden foam cells. Macrophages are phenotypically diverse and their heterogeneity depends upon local environment and stimuli [1]. GM-CSF and M-CSF produced by various cells in response to injury are the major survival/mitogenic factors for the macrophage lineage with the capacity to activate and induce their differentiation [2–4]. Initially M1 and M2 macrophage phenotypes were described by analogy to Th1 and Th2 lymphocyte subsets; macrophages cultivated with either GM-CSF (denominated GM-MØ) or with M-CSF (M-MØ) were named as proinflammatory M1 and anti-inflammatory M2 phenotypes respectively [5,6]. Subsequently a class of "classically activated" macrophages was described, i.e. macrophages activated by interferon-gamma (IFNgamma) alone or in concert with either lipopolysaccharide (LPS) or tumor necrosis factor (TNFalpha) and also called M1 macrophages. Finally, the "alternatively activated" macrophages were obtained upon incubation with IL-4 or IL-10 and called M2 macrophages [7,8]. Classically activated M1 macrophages express several pro-inflammatory cytokines, such as IL-1, TNFalpha and IL-6, while their M2 counterparts are considered as anti-inflammatory as they generate IL-10 and TGFbeta.

In human plasma [9] and in swine arterial wall [10] the concentrations of M-CSF are increased in the presence of coronary artery disease and those of GM-CSF are increased in aortic sinus lesions of apoE null atherosclerotic mice [11]; moreover GM-CSF mRNA is expressed in human vascular cells, including endothelial, smooth muscle cells and macrophages, as showed by in situ hybridization [12]. Recently, GM-CSF was proposed as a key regulator of intimal cell proliferation in mouse lesions [13]. Therefore the relative ratio between GM-CSF and M-CSF in situ may lead to "priming" and to "activation" of monocytes/macrophages and their phenotypic

* Corresponding author. Tel.: +33 1 40779768, fax: +33 1 40779728.
E-mail address: ewa.ninio@upmc.fr (E. Ninio).

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changes. The aim of this study was to characterize the transcriptional profiles of human cultured GM-MØ and M-MØ and to identify new markers specific of each phenotype in atherosclerotic plaques. We will not use the M1 and M2 terminology and designate the in vitro-cultured macrophages as GM-MØ and M-MØ.

Our strategy consisted on a global identification of differentially expressed genes in GM-MØ and M-MØ, followed by selection of potential candidate genes among these genes based on known biological function and differential expression analysis in new RNA preparations. Since macrophage phenotypes were proposed to be a continuum, without a clear separation between M1 and M2 types [5], we explored how the expression of a subset of the selected genes was modulated by gradually varying concentrations of GM-CSF and M-CSF in culture. Finally, the most modulated genes when confirmed by RT-quantitative PCR (RT-QPCR) were studied using tissue microarrays (TMA) and immunohistochemistry (IHC) in human aortas and by RT-QPCR in human and mouse atherosclerotic lesions. Our data suggest that balance between M-CSF and GM-CSF is essential for the macrophage polarisation in vitro and may contribute to the progression of atherosclerosis.

2. Materials and methods

2.1. Monocyte/macrophage culture and RNA isolation

Blood mononuclear cells were isolated from 6 buffy-coats of healthy individuals (Etablissement Francais du Sang, Rungis, France) using Ficoll–Paque (GE Healthcare, Piscataway, NJ, USA) and monocytes were positively selected by MACS CD14 Microbeads (Miltenyi Biotech).

$CD14^+$-monocytes were seeded (3×10^6 cells/well) in RPMI 1640 medium/gentamycin (Lonza, Verviers, Belgium) in 6-wells Primaria dishes (Becton Dickinson, Franklin Lakes, NJ, USA) for 1 h; the medium was replaced with fresh one supplemented with either 10% FCS and 10 ng/ml of granulocyte macrophage colony stimulating-factor (GM-CSF, Sigma, St Louis, MO, USA) to obtain GM-macrophages (GM-MØ), or 10 ng/ml of macrophage colony stimulating-factor (M-CSF, Sigma) to obtain M-macrophages (M-MØ) [6,14]. In selected experiments ($n = 6$) M-CSF and GM-CSF were mixed together to obtain a gradient from 0 to 10 ng/ml. Cells were cultured for 6 days to induce the differentiation. In 2 independent experiments, the macrophage cultures with either M-CSF or with GM-CSF were supplemented at day 0 with Il-10 (25 ng/ml; R&D Systems, Lille, France). Total RNA was extracted using RNeasy mini kit (Qiagen, Hilden, Germany) and subjected to Agilent Bioanalyser (RNA 6000 nano-kit; Agilent, Palo-Alto, CA, USA); only RNA with RNA Integrity Number (RIN) >8 were used.

2.2. Experimental design for microarrays

We used 18 RNG/MRC two-color oligonucleotide microarrays [15] to generate global mRNA expression profiles for GM-MØ and M-MØ. The microarray experiments were conducted at the postgenomic plateform P3S (Pitié-Salpêtrière Hospital, Paris), using hybridization of GM-MØ vs M-MØ cRNA on the same array. Amplification of mRNA, microarrays and QPCR experiments are described in online Supporting information.

2.3. Mouse studies

ApoE null C57BL/6J mice (males) were fed a high-cholesterol diet (1.25% cholesterol, 0% cholate; product #D12108; Research Diets, New Brunswick, NJ) in conventional housing as in [16]. Littermate mice (10 weeks old) were fed high-cholesterol diet for 0, 6 and 12 weeks ($n = 8$/group), euthanized, and analyzed for vascular lesions within the aorta. 19 Aortas (from the beginning of the aortic arch, just after the aortic roots, to the iliac bifurcation) were snap-frozen in liquid nitrogen (LN2) and were used for total mRNA extraction with Tri-reagent (MRC Inc, Cincinnati, OH) [16].

2.4. Human arterial samples

Fragments of fresh human full-thickness resections of carotids ($n = 20$) obtained from the Department of Vascular Surgery at Pitié-Salpêtrière Hospital and left internal mammary arteries ($n = 6$) obtained during coronary artery bypass graft surgery, from the Department of Cardiology were used. Patients approved to use the post surgery waste material. Briefly, carotid fatty streaks ($n = 9$) and plaques ($n = 17$) were dissected and immediately extracted with Trizol (Invitrogen) for total RNA which was purified as described above. The non-diseased mammary arteries were processed in a similar way. The classification of the lesions was performed according to Stary et al. [17].

The Tissue MicroArrays (TMA) included 51 aortic samples from different individuals: 19 fatty streaks, 20 atherosclerotic plaques, 7 complicated plaques and 5 normal arteries. For each lesion three representative areas were selected from hematoxylin- and eosin-stained sections of a donor block. Core cylinders (diameter: 0.6 mm) were punched and deposited into a recipient paraffin block using a specific arraying device (Beecher Instruments, Alphelys ring, MD) [18]. Five-μm TMA sections were used for IHC analyses described in online supporting information as well as the tissue microarray's histomorphometry analysis.

3. Experimental results

3.1. Transcriptional difference between the M-CSF and GM-CSF macrophages

This study was designed to compare the patterns of gene expression in GM-MØ and M-MØ. The morphology of the 2 macrophage populations was distinct after 6 days of culture; GM-MØ were round-shaped, whereas the M-MØ were elongated in shape (Supporting Figure 1A) as described in [6]. Additionally, both GM-MØ and M-MØ became lipid laden upon incubation with phospholipolyzed LDL (Supporting Figure 1B).

The analysis of differential gene expression between different macrophage phenotypes was performed using 12 arrays composed each of 21,668 probes corresponding to 19,724 distinct genes (data available in NCBI GEO: GSE18275). At this stage of selection we used a non stringent threshold for considering a gene for further study (1.5 FC and $p < 0.05$ non-adjusted). Using this criterion, the comparison of GM-MØ vs M-MØ identified 57 differentially expressed genes, thirty one genes were upregulated in GM-MØ compared to M-MØ and 26 genes were upregulated in M-MØ. Hierarchical clustering (Fig. 1) illustrates differential expression of genes in GM-MØ vs M-MØ.

Gene set enrichment analysis based on the literature and previously published data [14] was conducted by constructing a collection of 52 gene sets representing GO biological processes associated with monocyte-to macrophages differentiation. An over-representation of genes involved in immunity and defense, macrophage-mediated immunity, and cytokines and chemokines-mediated signaling pathways was observed in GM-MØ as compared to M-MØ, (Supporting Table 2).

3.2. Selection of candidate genes and validation by RT-QPCR

A subset of genes was extracted from those differentially expressed in GM-MØ vs M-MØ (Fig. 1) according to their differential expression and possible role in atherosclerosis for validation by RT-QPCR using new MØ preparations (Table 1 and data not