Differentiation of monocyte-derived dendritic cells is associated with upregulation and activation of Rac-1 small GTPase

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Abstract Critical changes occurring in Rac-1 molecule, a cytoskeleton organizing small GTPase associated with cell ruffling, have been analyzed in dendritic cells (DCs) derived from monocytes cultured with granulocyte-macrophage colony-stimulating factor and IFN-\(\alpha\) or IL-4. Although with different kinetics, both agents induced activation of Rac-1 molecule and, more importantly, an upregulation of both protein expression and mRNA transcription. These findings strengthen the role of Rac-1 molecule in the induction of DC differentiation and suggest that, besides its activation, the upregulation of Rac-1 molecule might also play a role in the acquisition of DC mature phenotype.

Keywords: Dendritic cells; Rac-1; Interleukin-4; Interferon-\(\alpha\); Cytoskeleton; Small GTPases

1. Introduction

Dendritic cells (DCs) are professional antigen presenting cells considered as essential regulators of both innate and acquired immune responses [1–3]. In addition to their phenotypic and functional features, DCs show a specific morphology that distinguishes them from others of the immune system, i.e., the dendritic-like protrusions. Accordingly, DCs need a dynamic organization of cytoskeleton, which can render them capable of a flexible activity leading to their phenotypic and migratory properties [4,5]. The actin microfilament integrity and function are pre-requisites for the occurrence of important events associated to cell polarization and movements as well as to activities such as antigen presentation or phagocytosis [6]. Actin cytoskeleton organization is controlled by proteins belonging to the Rho family [7–9]. The Rho family encompasses three different subfamilies of small GTPases, all controlling the actin cytoskeleton: (i) Rho subfamily, which induces the assembly of F-actin stress fibers, typical of adhering cells; (ii) Rac subfamily, involved in the ruffling activity, typical of spreading cells; and (iii) Cdc42 subfamily, which is of relevance in the generation of the cell filopodia [10]. Interestingly, some lines of evidence have previously indicated that the impairment of this molecules results in the hindering of both extension and contraction of dendrites in mature DCs [11,12]. Furthermore, Rac-1 molecule activation seems to be required for short-range migration and T cell priming [13]. In this context, the present work deals with the molecular changes occurring to Rac-1 molecule during monocyte-derived DC differentiation induced by granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 4 (IL-4) (IL-4-DCs), an extensively used and well known DC differentiating agent, or by GM-CSF and interferon \(\alpha\) (IFN-\(\alpha\), IFN-DCs) a cytokine recently described as a potent DC maturing agent [14].

2. Materials and methods

2.1. Cell culture and treatments

DCs were generated from peripheral blood mononuclear cells isolated by Ficoll-Hypaque (Flow Laboratories, Hornby, Ont.) as stated elsewhere (14) obtained from 14 different healthy donors. Highly enriched monocytes (95% CD14+) were cultured at 6·10\(^{5}\)/ml in RPMI 1640 medium supplemented with 15% heat-inactivated fetal calf serum, 2·10\(^{-5}\)M L-glutamine and penicillin–streptomycin and 250 ng/ml GM-CSF (PeproTech, London, England) and either IL-4 (250 U/ml, IL-4-DCs) (R&D Systems, Minneapolis, MN) or natural IFN-\(\alpha\) (Alfaferone, AlfaWasserman) at the concentration of 10000 U/ml (IFN-DCs). After 3 (for IFN-DCs) or 5 days (for IL-4-DCs) of culture, 200 ng/ml lipopolysaccharide (LPS) (Eschericia coli serotype 011:B4, Sigma, St. Louis, MO) was added to DCs for 24 h. To inactivate Rho molecules, Clostridium difficile Toxin B (CdB) (10 ng/ml, Sigma–Aldrich), a bacterial protein toxin able to impair the interaction of Rho molecules with their substrates [15], was used. The toxin was administered to DCs 4 h before treatments with IL-4 or IFN-\(\alpha\) cytokines and lefted in the medium during DC differentiation.

2.2. Flow cytometry analyses

Cell staining was performed using mouse monoclonal antibodies (mAbs) FITC or PE conjugate. Cells were incubated for 30 min at 4 °C with the following mAbs: CD1a (IgG1, FITC clone H149), HLA-DR (IgG2a, FITC clone G46-6), CD80 (IgG1, FITC clone L307.4) CD83 (IgG1, PE-clone HB15e) (all from BD Biosciences, San Jose, CA). Cells were then washed with cold PBS and analyzed with a FACScan (Becton Dickinson, Mountain View, CA, USA) equipped with a 488 argon laser. At least 10000 events have been acquired. As regards quantitative analyses of intracellular Rac-1 small-GTPase pro-
tein, cells were treated with Fix & Perm permeabilization kit (Caltag Laboratories, Burlingame, CA) and stained with mAbs for the detection of Rac-1 (Upstate Biotechnology), for 1 h at 37 °C. After washing, cells were incubated for 30 min at 37 °C with FITC-labeled anti-mouse mAbs (Sigma). Cells were then analyzed with a FACScan (Becton-Dickinson). At least 20000 events have been acquired. Data were recorded and statistically analyzed by a Macintosh computer using CellQuest Software. Statistical significance of flow cytometry studies was calculated by using the parametric Kolmogorov-Smirnov (K/S) test.

2.3. Scanning electron microscopy
IFN-DCs and IL-4-DCs were collected and plated on poly-L-lysine coated slides, fixed with 2.5% glutaraldehyde and post-fixed in 1% OsO4 as previously described [14]. The samples were examined with a Cambridge 360 scanning electron microscopy.

2.4. Static cytometry analyses
To visualize intracellular distribution of Rac-1 small-GTPase protein, monocytes and DCs were collected and plated on poly-L-lysine coated slides, fixed for 30 min in 3.7% paraformaldehyde, and permeabilized with 0.5% triton X-100. Cells were then stained using Rac-1 mAb (Upstate, Biotechnology) for 1 h at 37 °C. After washing, cells were incubated for 30 min at 37 °C with FITC-labeled anti-mouse mAb (Sigma). For F-actin detection, cells were stained with fluorescein–phalloidin (Sigma) at 37 °C for 30 min. Finally, all samples were mounted with glycerol–PBS (2:1) and observed by intensified video microscopy by using a Nikon Microphot fluorescence microscope equipped with a color chilled 3CCD camera (Zeiss, Germany).

2.5. Rac-1 activation assay
IFN-DCs and IL-4-DCs were collected at different times after cytokine treatment of monocytes (1, 2, 3 and 5 days) with or without the additional incubation step with LPS (24 h). Pull down assay was performed by using small GTPase evaluation Kit for Rac-1 (Cytoskeleton, Denver, CO) as established by the manufacturer’s protocol. Samples were analyzed by SDS–PAGE and Western blot analysis. Briefly, after cell lysis, proteins were loaded with PAK-PBD beads, possessing a high affinity for Rho-GTPases [16]. The immunoblot was analyzed by densitometry and normalized as a function of the total proteins loaded in the assay as previously described [16].

2.6. RT-PCR
Total cellular RNA was extracted using the RNase Kit (Quiagen). RNA was reverse transcribed into cDNA and amplified by PCR using the Access RT-PCR System (Promega) according to the manufacturer’s instruction. GAPDH and Rac-1 set of primers were synthetized by M-Medical-GENENCO (Cornaredo). The following couples of primers were used: GAPDH: sense 5'-GTC TTC ACC ATG GAG AAG-3', antisense 5'-CTC CCA AGA AGA ATT AGG GT-3'. Rac-1: sense 5'-GTC TTC ACC ATG GAG AAG-3', antisense 5'-CTC CCA AGA AGA ATT AGG GT-3'.

Fig. 1. Cytokine-induced phenotypic differentiation and surface ruffling. Treatments with IL-4 or IFN-α cytokines lead to increased expression of DC differentiation markers (CD1a, CD80, CD83 and HLA-DR). Note different kinetics between the two cytokines. LPS (arrows) administration resulted in bolstering marker expression in both experimental conditions. Also note that LPS administration was performed at different time points (5 days with IL-4 and 3 days with IFN-α) due to the different kinetics of DC maturation with cytokines (first and third rows). In the same vein, scanning electron microscopy analyses (second and fourth rows) clearly show that cell surface protrusions and ruffling were early detectable in both experimental conditions (1 day) but they were observable earlier in IFN-α treated cells (fourth row).
GTC-3'; antisense 5'-CAT GCC AGT GAG CTT CCC GTT CA-3';
Rac-1 sense: 5'-TCT TCT CCT TCA GTT TCT CGA TCG-3';
antisense 5'-CAA GAA GAT TAT GAC AGA TTA CGC CC-3'.
To avoid contamination, in all experiments a control RT-PCR without RNA was conducted. For densitometric analysis, the reaction products were electrophoresed through a 1.8% agarose gel (Bio-Rad). Ethidium bromide-stained gels were scanned with a densitometer (Bio-Rad Multi Analyst) and Rac-1 mRNA levels were normalized to GAPDH mRNA levels.

3. Results and discussion

3.1. Cytokine-induced DC differentiation

In the evaluation of the stage of differentiation/activation of DCs, a great importance is generally given to the morphological analyses [17]. Thus, we first carried out a scanning electron microscopy (SEM) analysis for comparing IL-4-DCs with IFN-DCs at early times after cytokine treatment of monocytes. Parallel analyses were also conducted evaluating the typical differentiation markers of DCs. With respect to undifferentiated monocytes (not shown), the exposure to IL-4 (Fig. 1, second row) or to IFN-α (Fig. 1, fourth row) resulted in a remodeling of the cell with the occurrence of a series of cell surface modifications, e.g., surface ruffling, that are typical of cell spreading and can be easily detected by SEM observations. However, significant differences were detectable between cells exposed to IL-4 and those exposed to IFN-α treatment. In particular, a pronounced cell ruffling and spreading was rapidly induced by IFN-α (after 2 days) whilst these changes were detected after 5 days with IL-4 (compare SEM pictures in Fig. 1). In the same vein, parallel analyses aimed at evaluating surface expression of typical DC differentiation/activation markers (CD1a, HLA-DR, CD80, CD83), also indicated a more rapid response of IFN-DCs with respect to IL-4-DCs (Fig. 1, first and third rows, results obtained from a representative healthy donor). Interestingly, IFN-α induced the expression of CD83, a maturation antigen, after 1 day of treatment. As expected, LPS treatment further increased cell surface ruffling and induced an upregulation of surface marker expression typical of mature DCs (Fig. 1, arrows indicate LPS administration).

3.2. Cytoskeleton rearrangement during DC differentiation

Literature data suggest that morphological changes detected on the surface of DCs (i.e., surface ruffles) are specifically associated with the activation of a small GTPase of the Rho family: the Rac-1 molecule [18]. Hence, we analyzed the actin network and Rac-1 arrangement in both IL4-DCs and IFN-DCs. With respect to monocytes, we found: (i) a redistribution of Rac-1 molecule in both experimental conditions (Fig. 2, panel A, upper rows) and (ii) a rearrangement of actin filaments (Fig. 2, panel A, bottom rows). However, according to literature [14] these changes were detected at shorter time points in IFN-DCs (3 days) with respect to IL-4-DCs (5 days). Furthermore, after LPS treatment these changes were even more...
pronounced (Fig. 2, panel A). These cytoskeletal modifications actually parallel SEM observations depicted above (see Fig. 1) and show for the first time a specific rearrangement and redistribution of Rac-1 molecule during DC differentiation. Interestingly, treatment with a bacterial protein toxin able to impair Rho-GTPase function, the *C. difficile* toxin B (CdB), before cytokine administration, clearly hindered DC actin cytoskeleton remodeling. CdB treated cells failed to show any sign of cell ruffling after both IL-4 (not shown) or IFN-α treatments, as detected by SEM analysis (Fig. 2, panel B, middle micrograph). In fact, the cytokine-induced Rac-1 and microfilament redistribution was impaired by CdB (Fig. 2, panel B, upper and bottom micrographs, respectively).

3.3. Rac-1 activation and upregulation during DC differentiation

Since small GTPases [11,19,20] are able to cycle between two conformational states, an “active” state, bound to GTP (Rac-1 GTP) and an “inactive” state, bound to GDP (Rac-1 GDP), the activation of Rac-1 molecule was first evaluated by pull-down experiments at different culture times. After short time treatments (4 h), no significant difference was detected in the activation of Rac-1 molecule between IFN-DCs and IL-4-DCs as compared to undifferentiated monocytes. By contrast, longer exposure times (1–3 days) clearly showed Rac-1 activation state in the DC cultures (Fig. 3, panel A). Notably, at earlier time points (1 day), the activation of Rac-1 molecule was less evident in IL-4-DCs with respect to IFN-DCs (Fig. 3, panel A). After LPS treatment, the activation of Rac-1 was well evident in both experimental conditions (Fig. 3, panel A, results obtained after 3 days for IFN-DCs and after 5 days for IL-4-DCs are shown). The differences in terms of differentiation pattern between IL-4-DCs and IFN-DCs were previously investigated elsewhere [14]. However, we show here that Rac-1 GTPase activation, previously hypothesized to be of importance in DC migration and homing [13], seems to reflect the differentiation pathways induced by the two cytokines, faster in IFN-DCs.

Furthermore, quantitative analyses of the expression of total Rac-1 molecule were also conducted. An increased expression of this molecule was detected by Western blot (Fig. 3A)
and flow cytometry analysis (Fig. 3B, IL-4-DCs and 3C, IFN-DCs). These results (mean values ± SD obtained from DCs from 14 healthy donors) clearly indicated that a time dependent upregulation of Rac-1 molecule in cytokine treated cells with respect to untreated monocytes (day 0). A further increase of Rac-1 molecule was also detected in IL-4-DCs and IFN-DCs after LPS treatment, reaching a peak level after 5 and 3 days treatments, respectively (Fig. 3B and C, grey histograms). Hence, in view of this protein upregulation, we decided to evaluate Rac-1 mRNA level in our DC differentiation model. Notably, a gradual upregulation of Rac-1 mRNA level was detected in IL-4-DCs (Fig. 3D) as well as in IFN-DCs (Fig. 3E). Of notice, a further increase of Rac-1 mRNA was also detected after LPS administration, but in IL-4-DCs only. (Fig. 3D).

4. Conclusions

Administration of cytokines was known to bolster differentiation of DCs in terms of phenotype, morphology, migration capability and immune priming activities [14,18]. We have shown here that Rac-1 molecule, a small GTPase involved in actin cytoskeleton network remodeling, although with different kinetics, was potently activated by both IL-4 and IFN-α administration as well as by the subsequent treatments with LPS. Intriguingly, we also found that an upregulation of Rac-1 protein, in terms of both protein expression and mRNA level, can be detected in differentiating DCs. Rac-1 GTPase, in its activated form, i.e., the GTP bound form, has been associated with a series of subcellular changes leading to increased cell polarity and to an increased spreading pattern [10]. In fact, Rac-1 small GTPase belongs to the family of Rho proteins, i.e., Rho Rac and Cdc42, that, once activated, instruct actin network remodeling associated with cell adhesion dynamics, cell ruffling and the formation of cell protrusions [7–10]. Altogether these findings, allow to identify Rac-1 upregulation and activation, as important steps in IFN and IL4-induced differentiation of monocytes into DCs allow in terms of migration ability and immunological synapse formation in response to danger signals [6].

References