β1-Integrins determine the dendritic morphology which enhances DC-SIGN-mediated particle capture by dendritic cells

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Abstract

The morphology of antigen-presenting dendritic cells (DCs) is characterized by the presence of numerous long dendrites. The formation of these processes is shown to require the interaction between the β1-integrin (CD29) on the surface of the DCs and fibronectin in the extracellular matrix. This interaction occurs at focal contacts formed at the tips of dendrites, which contain high concentrations of the β1-integrins, actin and the cytoskeletal proteins vinculin, paxillin and talin. Dendrites contain an extensive microtubule (MT) network, and are retracted in the presence of the MT inhibitor colchicine, suggesting that MTs are essential for dendrite stability. The dendritic morphology is shown to contribute directly to an enhanced ability to capture dendritic cell specific ICAM-3 grabbing nonintegrin (DC-SIGN)-coated beads. Time-lapse photography demonstrates that dendrites are highly dynamic structures, with cells extending and retracting multiple dendrites in different directions over a 3-h period. This motility increases the area scanned by an individual DC by over 2-fold. The unusual combination of a dendritic morphology and high motility is likely to play a major role in the efficient function of DCs as sentinels of the immune system.

Introduction

The presence of long cytoplasmic processes, often many times the length of the cell body, is characteristic of several cell types, including both neurons and antigen-presenting dendritic cells (DCs). Indeed, because of this shared morphology, skin DCs were, in fact, originally classified as nerve cells (1). Dendritic morphology may increase the efficiency of antigen contact and uptake, but there are little data in support of this hypothesis. Furthermore, the molecular events which determine dendritic morphology remain incompletely understood. Although it is possible to obtain cells with many of the properties of tissue DCs by culture of monocytes in granulocyte macrophage colony-stimulating factor (GM-CSF)/IL-4 (2), these cells cultured on plastic show considerable membrane ruffling and motility (3) but do not form prominent dendrites such as those described in vivo. In a previous study (4), we developed a model which showed that culturing DCs on fibronectin (FN), a major and ubiquitous constituent of the extracellular matrix (ECM) [reviewed in (5)], induces them to form long, slender dendrites. These dendrites can extend several times the length of the cell body, and are reminiscent of the typical in vivo morphology. The formation of these processes was shown to reflect major re-organizations of the actin cytoskeleton, and required the co-ordinated activity of RhoA, Rac1 and Cdc42, members of the Rho family of GTPases implicated in diverse cellular events, including cell motility, phagocytosis and cell division (6).

In the present study, we investigate this in vitro model further. Dendrite formation is shown to be characterized by the formation of focal contacts containing β1-integrin and associated with localized actin polymerization. Once formed, dendrite integrity is maintained by the formation of microtubular structures reminiscent of neuronal cells. However, this dendrite
formation is transient, and DCs rapidly retract and extend multiple dendritic processes in different directions. Demonstrating the link between this mobile dendritic morphology and the efficiency of antigen capture by DCs required the development of a novel assay of particle capture, in conjunction with computer-assisted image analysis of time-lapse movies.

**Methods**

**DC culture**

Human PBMC-derived DCs were obtained from PBMCs of healthy volunteers (4). The cells were >85% DCs as judged by phenotypic analysis.

**mAbs**

mAbs used were as follows: CD1a (NA1/34) IgG2a supernatant (a gift from A. McMichael, Oxford, UK); CD11a (ICRF38), CD11b (ICRF44), CD11c (ICRF319) CD18 (B1/18), β1/CD29 (PS52) α4/CD49d (7.2R) all IgG1 purified (a gift from N. Hogg, Cancer Research UK, London, UK); CD14 (HB246) IgG2b supernatant (American Type Tissue Collection); CD86 (BU63) IgG1 supernatant (a gift from D. Hardie, University of Birmingham, Birmingham, UK); HLA-DR (L243) IgG2a supernatant (a gift of P. Beverley, Jenner Institute, Compton, UK); vinculin (HVIN-1), talin (8D4), tubulin (2-28-33) IgG1 purified (all from Sigma, Poole, Dorset, UK); paxillin (349) IgG1 purified (BD Transduction Laboratories, Lexington, KY, USA), LSP1 (a gift from K. Pulford, Oxford University, Oxford, UK).

**Immunostaining and confocal microscopy**

For localization of F-actin, fixed cells were permeabilized with 0.2% Triton X-100 for 5 min, and incubated with 0.1 μg ml⁻¹ tetramethyl rhodamine-5-isothiocyanate- or FITC-conjugated phalloidin (Sigma) for 45 min. For the localization of other markers, cells were permeabilized with 0.2% Triton X-100 for 5 min, and then incubated with the appropriate mouse anti-human antibody (supernatants or 5 μg ml⁻¹ when concentration was known) for 45 min, followed by a 45-min incubation in the dark with a 1:20 dilution of FITC-conjugated rabbit anti-mouse IgG (Dako, Glostrup, Denmark). All antibodies were diluted in 0.5% BSA (Sigma) in PBS. Finally, the cover slips were mounted by inverting them onto 5 μl mowiol mountant (Calbiochem, San Diego, CA, USA) containing p-phenylenediamine (1 mg ml⁻¹) as an anti-bleach agent. The same procedure as above was carried out for immunofluorescence of membrane proteins but the permeabilization step was omitted. The cover slips were examined on a Zeiss confocal microscope using Zeiss 40 × 1.3 oil immersion objectives.

**Flow cytometry**

Cells were harvested, washed twice in HBSS and resuspended at a concentration of 1 × 10⁶ to 5 × 10⁶ cells ml⁻¹ in blocking buffer [HBSS containing 10% rabbit serum (GIBCO BRL, Paisley, UK) and 0.1% sodium azide] for 15 min at 4°C. A total of 50 μl of cells was added to each well of a 96-well round-bottomed plate (Falcon) as required. Cells were then incubated for 30 min on ice with 50 μl of primary antibody (supernatants or 5 μg ml⁻¹ when concentration was known). After washing three times with blocking buffer, cells were incubated for a further 30 min on ice with a 1:20 dilution of FITC-conjugated rabbit anti-mouse IgG (Dako) in blocking buffer. Cells were subsequently washed three times in HBSS containing 0.1% sodium azide and fixed in 100 μl of 3.7% formaldehyde diluted in HBSS. Finally, 50 μl of HBSS containing 0.1% sodium azide was added to each well. Stained cells were stored in the dark at 4°C and analysed on a FACScan (Beckton-Dickinson, Mountain View, CA, USA) using WinMDI software within 3 days of staining. For each sample, 10,000 events were recorded. The data were examined relative to a negative control sample with no primary antibody or the relevant isotype control. All antibodies were titrated and used at saturating concentrations.

**Quantitative assays of adherence and process formation**

A total of 10^⁵ PBMC-derived DCs or macrophages were seeded onto autoclaved 13-mm glass cover slips (BDH, Poole, Dorset, UK) coated overnight at 4°C with 10 μg ml⁻¹ bovine plasma FN (Sigma) in HBSS or 1% BSA (Sigma) in PBS or HBSS alone, as indicated, in 15-mm four-well plastic dishes (Nunc). Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ for varying time points. Cells were washed in HBSS to remove non-adherent cells and fixed in 3.7% formaldehyde in HBSS for 15 min and either processed for immunofluorescence (see above) or analysed directly under a light microscope. The number of cells adhering to a substrate was determined by counting the number of adherent cells in 10 random fields, at a magnification of ×200, under each different test condition. Ten such fields were counted from three independent experiments and a mean value was calculated from the 30 fields that had been quantified. The presence or absence of dendrites (defined as a process longer than the cell body) was recorded for each cell. The percentage of DCs forming dendrites was determined using the equation

\[
\text{Percentage of DCs with dendrites} = (\text{no. DCs with} > 1 \text{ dendrites/no. of DCs adhered}) \times 100.
\]

A minimum of 100 cells was counted for each experimental group.

**Inhibitors and blocking antibodies**

A total of 1 μM cytochalasin (Sigma), 1 μM colchicine (Sigma) or the appropriate mouse mAb (5 μg ml⁻¹ or supernatant) diluted in complete medium of RPMI-1640 (GIBCO BRL) supplemented with 50 μM 2-β-mercaptoethanol (GIBCO BRL), 100 U ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin, 2 mM L-glutamine (all from Ciare Hall Laboratories, Cancer Research UK) and 10% heat inactivated (56°C for 30 min) FCS (GIBCO BRL) was added to 10^⁵ DCs 30–60 min prior to adherence or following the adherence of DCs to FN, as indicated. The number of cells adhering to the substrate and the percentage of cells forming dendrites were determined as above.
Time-lapse photomicroscopy

A total of $5 \times 10^5$ DCs were adhered for 30 min onto FN-coated 32-mm glass cover slips at 37°C in perfusion open-closed chambers (H. Saur, Reutlingen, Germany). Cells were left for 15 min post-treatment before initiating the time-lapse recordings. Time-lapse images were recorded using Kinetic Imaging Ltd (Wirral, UK) software. Images were recorded every 10–15 min for 3 h. During filming, DCs were maintained at 37°C and in an atmosphere of 5% CO2. Time-lapse images were analysed using ImageJ software (Wayne Rasband, National Institutes of Health, USA, http://rsb.info.nih.gov/ij/) using the macros ‘Find edges’ and ‘Area Calculator’ provided with the software.

Bead-capture assay

A total of $3 \times 10^4$ DCs were adhered for 60 min on FN-coated glass cover slips at 37°C as above. Cells were washed once and the cover slips were then inclined at an angle of 45° to the horizontal. A total of $5 \times 10^5$ immunomagnetic beads (Dynabeads sheep anti-rabbit IgG, Dynal, Liverpool, UK) pre-loaded with PE-conjugated antibody to DC-SIGN (eBioscience, eB-h209, rat IgG2a) were added to the top of the cover slip and allowed to run down the surface to which the DCs were attached. Cover slips were then transferred to 3.7% formaldehyde (BDH) and stored in the dark at 4°C. Beads were visualized by confocal microscopy using BioRad Lasersharp software. Within each field, the numbers of total and cell-associated beads were counted and the percentage of cell-bound beads was calculated. The average percent over three fields was then calculated. The experiment was repeated on three different DC preparations, and the results shown are the mean ± standard error of the mean for the three independent experiments.

Results

Dendrite formation requires FN

Human PBMCs were cultured in GM-CSF and IL-4 for 7 days as described previously (4). These cells are CD1a positive, CD86 low, CD14 negative or low and MHC class-II intermediate, consistent with an immature DC phenotype (see below Fig 2). DCs rapidly adhered and formed long, thin dendritic processes when seeded onto FN-coated cover slips, but not on uncoated cover slips, or on cover slips coated with type I or type II collagen (data not shown). The number of cells forming processes was dependent on the concentration of FN, with optimum results obtained by coating with 10 μg ml⁻¹ FN or above (Fig. 1). The DC cultures retained their characteristic morphology for at least 24 h in culture on FN, although individual cells cycled between rounded and extended forms.

The distribution of integrins and integrin-associated proteins on the surface of DCs

Integrins are known to be expressed on DCs. Previous studies have shown that β2-integrins (CD18/CD11c) are important in DC–T cell interaction (7), and that β1-integrins CD29, CD49d and CD49e, which function as important receptors for FN on many cell types, are also expressed (8). As CD29 and CD18 are both expressed on >75% of DCs (Fig. 2), and at much higher levels than their respective α chains, the distribution of these integrins together with HLA-DR was examined on the surface of the DC in relation to the F-actin within (Fig. 3). Both CD29 and CD18 are distributed along the whole cell body, just above a thin cortical layer of F-actin. However, concentrations of both integrins were also found associated with concentrations of F-actin at the ends of the dendrites (see inset, arrows) where extension of processes primarily takes place (4). For comparison, the distribution of HLA-DR (Fig. 3) was more uniform, and there was little evidence of concentrations at the points of dendrite attachment or extension. Isotype control antibodies showed no staining (data not shown).

The distribution of vinculin, paxillin and talin, three proteins that associate with β1-integrins (see below) and play a part in linking these membrane receptors to the actin cytoskeleton, is shown in Fig. 4. Distinct concentrations of all three molecules were observed at the tip of the dendrites (see arrows), usually co-localizing with concentrations of actin. Some diffuse staining was also seen along the dendrite, perhaps due to intracellular transport. Isotype control antibodies showed no staining in any part of the cell (data not shown).

Interaction between β1-integrins and FN is required for dendrite formation

In order to probe the function of the CD29 and CD18 molecules in dendrite extension, blocking antibodies to these
molecules were added to the cells prior to dendrite extension, and the number of cells adhering and forming processes was recorded (Fig. 5). CD29 antibody P5D2 partially inhibited the adhesion of DCs to the FN substratum. Those cells that did adhere were unable to form processes and adopted a rounded morphology different from any seen normally in the absence of the antibody (Fig. 5c). In contrast, antibodies to CD18 failed to block either adhesion or dendrite extension, although the same antibody blocked the adhesion of DCs to T cells completely (7). As a control, antibodies to CD1a (found at high levels on the DC surface (see Fig. 2)) did not affect the DC adhesion, dendrite formation or morphology (Fig. 5a–c). None of the antibodies tested affected cell viability over the time course of the experiment.

An intact actin and microtubule cytoskeleton is required for dendrite formation

The distributions of polymerized tubulin (microtubules (MTs)) and F-actin were quite distinct (Fig. 6a). MTs were present along the whole length of the extended dendrite, but were largely absent from the extending tips of the dendrites, where F-actin was concentrated. The roles of F-actin and tubulin, both major components of the cytoskeleton, in dendrite formation were investigated using the selective inhibitors cytochalasin B and colchicine. Neither cytochalasin nor colchicine blocked the adhesion of DCs to FN, although adhesion was slightly delayed in the presence of cytochalasin (Fig. 6b). However, both inhibitors totally blocked dendrite formation (Fig. 6b, lower). Treated cells instead adopted a rounded shape (Fig. 6c). Neither inhibitor affected cell viability over the time course of the experiment.

Dendritic morphology enhances DC-SIGN-mediated particle capture

A major function of DCs in non-lymphoid tissues is to bind and take up antigen. Receptor-mediated endocytosis of dextran by DCs has been studied extensively as a model of uptake of soluble antigen. However, the rapid diffusion of dextran in solution means that dextran capture is unlikely to be sensitive to changes in cell morphology. In order to directly correlate the presence of dendrites with increased capacity to trap antigen, a novel assay was therefore developed. Magnetic beads (~1- to 2-μm diameter) were coated with antibody against DC-SIGN, a lectin receptor facilitating binding of DCs to a number of micro-organisms (9). The antibody-coated beads were allowed to flow slowly past adherent DCs, which were then fixed and examined by microscopy (see Methods). Beads coated with isotype control did not bind to DCs significantly (data not shown). In contrast, a proportion of anti-DC-SIGN-coated beads bound to the surface of the DCs (Fig. 7a, arrows). The ability of control DCs to capture beads was compared with that of DCs pre-treated for 1 h with cytochalasin or colchicine, which had greatly reduced or no processes (Fig. 7b). As shown in Fig. 7c, the number of beads captured by the DCs was much reduced in the presence of either inhibitor. The inhibitors did not block binding of beads to DCs directly, since when adherent DCs and beads were incubated horizontally in culture dishes over an hour (allowing time for random movement of beads and hence random contacts
between beads and cells), no significant differences between groups were seen. The inhibitors did not affect levels of DC-SIGN, at least within the time frame of the experiment (Fig. 7d).

Dendritic mobility enhances the surface area covered by dendritic processes

A striking feature of DC dendrites is their mobility which was apparent from the time-lapse photography of DC cultures (see Supplementary Figure 1S, available at International Immunology Online). There is relatively little vectorial cell movement under these conditions, but individual dendrites extended and retracted rapidly, and then re-extended in different directions. Image analysis of individual cells (Fig. 8a) was used to determine the envelope of the surface area covered by a cell over a period of 150 min (Fig. 8b) compared with the average area covered at each individual time frame. The average area of the cell shown in each of the panels of Fig. 8a was 1338 ± 120 pixels, while the overall cumulative area covered by the cell envelope throughout the recording period (Fig. 8b) was 3787 pixels, an increase of 280%. A similar analysis was carried out to determine the average area covered by all DCs within the field of a time-lapse recording (Supplementary Figure 1S, available at International Immunology Online). The average area covered by cells at each time point in one such 180-min sequence was 24 ± 2% of the surface, while the total area covered over the time of the sequence was 60%, an increase of 250%. In a second sequence (data not shown), where the density of DCs was somewhat higher, the proportion increased from 45 ± 3% to 79%. Dendrite motility therefore greatly increases the effective surface area of the DCs.

Discussion

The starting point for this study was that most previous studies of immature DCs show cells which lack significant processes.
Although DCs will adhere to a variety of surfaces, including glass and plastic, here similar to others (10, 11), we found enhanced adherence of DCs on FN, and furthermore, FN was found to be essential for dendrite formation in culture (Fig. 1).

The concentration of FN (10 µg ml⁻¹) which gives maximal dendrite formation also promotes maximal cell protrusion and polarization in CHO-K1 cells (12). In contrast to DCs, macrophages adhere rapidly and strongly to FN but assume an entirely different morphology with no dendrites (4). Interestingly, dendrite formation and neurite outgrowth is also promoted by FN in some neuronal cell types (13, 14) but not others (15).

The β1-integrins are important receptors for FNs which are expressed on the DC membrane (Fig. 2) (10, 16–18). Consistent with the requirement for FN, dendrite formation was shown also to require CD29. In the presence of an anti-CD29
blocking antibody, DCs which bound to the substratum failed to extend dendrites, and instead assumed a rounded, contracted appearance. Nevertheless, since CD29 is expressed widely, dendrite formation is not itself due to CD29 expression alone, i.e. CD29 is necessary but not sufficient. The role of the integrin $\alpha$ chains, which are expressed at much lower levels, on dendrite extension remains an important question for further study.

CD29 antibodies caused the rapid disappearance of dendrites even when it was added after adhesion had been allowed to take place (data not shown). This observation is consistent with the highly dynamic nature of dendrites in culture, although we cannot exclude that the CD29 antibody is also inducing some signalling pathway within the DCs. Concentrations of both $\beta_1$- and $\beta_2$-integrins were observed in the motile and actin-rich tips of the dendritic processes, where dynamic interactions between the DCs and ECM substratum were frequently observed. Three other cytoskeleton

Fig. 6. The effect of pre-adherence treatment with colchicine or cytochalasin on adherence to FN and dendrite formation. Panel (a): DCs were seeded on FN-coated cover slips and allowed to adhere for 120 min prior to fixation. The cells were stained with antibody to $\beta$-tubulin and subsequently a second layer of FITC-conjugated anti-mouse IgG antibody. F-actin distribution was visualized using tetramethyl rhodamine-5-isothiocyanate-conjugated phalloidin. Cells were analysed on a confocal microscope. Representative image of three independent experiments. Scale bar represents 10 $\mu$m. Panel (b): The number of adherent DCs (upper) and the percentage of DCs forming processes (lower) in 30 randomly chosen fields from three independent experiments were calculated. Mean values $\pm$ standard error of the mean for three experiments is shown in panel (c). The expression of DC-SIGN after treatment with either colchicine or cytochalasin was measured by flow cytometry (panel d).

Fig. 7. A dendritic morphology enhances the ability of DCs to capture antibody-coated beads. DCs were seeded on FN-coated cover slips and allowed to adhere overnight in the absence (a and c) or presence of colchicine (b and c) or cytochalasin (c). The cover slips were inclined at an angle of 45° and DC-SIGN-coated beads were gently allowed to flow past the cells as described in Methods. The cells were then fixed in 3.7% formaldehyde, and examined by confocal microscopy. The proportion of beads associated with cells [as in (a)] was calculated. The mean $\pm$ standard error of the mean for three experiments is shown in panel (c). The expression of DC-SIGN after treatment with either colchicine or cytochalasin was measured by flow cytometry (panel d).
proteins vinculin, paxillin and talin were also concentrated within the dendrite tips. The association of $\beta_1$-integrins, vinculin, paxillin and talin is characteristic of focal adhesions (FAs) formed by adherent cells such as fibroblasts and epithelial cells [reviewed in (19)]. Actin stress fibres, which are linked to the ECM via FAs, were, however, never observed in DCs. DCs, like macrophages and other motile cell types (20), instead appear to interact with the ECM via small dynamic focal contacts, which form and reform, allowing rapid changes in cell shape. Since focal contacts and FAs are known to regulate the cytoskeleton via the activity of Rho GTPases (6), this model is consistent with the recent demonstration that the Rho GTPases Rho, Rac and Cdc42 also regulate DC dendrite formation (4).

MTs are also an important constituent of the cytoskeleton (21), and play a key role in stabilizing neuronal dendrites and axons (22, 23). The distribution of MTs along the length of the dendrites and the sensitivity of dendrites to colchicine suggest that they may play a similar stabilizing role in DCs. MTs also serve a second function, mediating intracellular vesicular transport in many cell types (24), but this causal interaction was not pursued in this study because colchicine, the inhibitor used here to destroy the MT formations, also destroyed the dendrites themselves.

The highly characteristic morphology of DCs both in and outside lymphoid tissue may promote enhanced interaction between DCs and microbes or other cells of the immune system. Little experimental data testing this hypothesis have been published. Uptake of fluorescent-labelled dextran, although a useful model for studying the cell biology of antigen uptake, is unsuitable for this purpose, since its rapid diffusion allows it to reach all parts of the cell surface rapidly. Instead, we studied the ability of DCs to capture antibody-coated beads as they floated past the DC surface, a model which is more analogous to the capture of microbes by DCs in vivo. Under these conditions, cells with a dendritic morphology were more than twice as efficient at antigen capture than rounded cells. This increased efficiency was not simply due to an increased surface area, since calculation of the surface area of rounded or branched DCs suggests that their surface area is rather similar (data not shown). Interestingly, further studies using this assay (25) have shown that uptake, as opposed to binding, occurs most efficiently when dendrites are retracted, suggesting that the dynamic conversion from extended to rounded morphology which is characteristic of these cells may be an important factor in maximizing the efficiency of uptake.

The morphology of DCs is unique in that the dendrites are short-lived and highly dynamic structures [Fig. 8 and (4)]. DCs in vitro therefore appear highly motile, while not showing much directional migration. It was not possible to test the importance of this factor in the bead-capture assay directly, since we could not interfere with cell motility while leaving the dendritic morphology intact. However, computer-assisted image analysis of a series of images of the DCs taken over 3 h demonstrated that dendrite motility contributed an increase of over 2-fold in the area which would be sampled by the cell, compared with the area which could be covered at any one time point. In conjunction with our earlier study (4), we propose that the unusual dynamic morphology of the DC is driven by interactions between $\beta_1$-containing focal contacts and FN, which in turn signal the rearrangement of the local actin cytoskeleton via Rho GTPases. Extending dendrites are then stabilized by the assembly of an MT network. Dendrite formation thus requires a combination of binding to the substratum (which was mediated directly via integrin/FN binding, and hence inhibited by CD29 antibodies but not cytochalasin or colchicine) and active migration and process extension, which was blocked by both CD29 antibodies and the cytoskeletal...
inhibitors. The ability to form dendrites contributes directly to an increase in DC capture efficiency, and this is further enhanced by the ability of the DCs to extend and retract dendrites in many different directions over a relatively short time period. The individual molecular components of this model will need to be tested further both in vitro and in vivo. This model suggests that DC morphology, as much as surface phenotype and cytokine secretion, may determine their remarkable efficiency in antigen processing and presentation.

Supplementary data
Supplementary data are available at International Immunology Online.

Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>DC</td>
<td>dendritic cell</td>
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<tr>
<td>DC-SIGN</td>
<td>dendritic cell specific ICAM-3 grabbing nonintegrin</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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References