INTRODUCTION

Dendritic cells (DC) are professional antigen presenting cells that originate from bone marrow progenitors and are found in peripheral organs like skin where they capture and process antigens (Austyn, 1996, 1998; Peters et al., 1996; Cella et al., 1997; Banchereau and Steinman, 1998). They then migrate to lymphoid tissues and present the processed antigens in the context of major histocompatibility complex (MHC) class I and II molecules to elicit specific T cell responses. DC are more potent in antigen presentation than other antigen presenting cells like B cells and macrophages. Most importantly, DC are unique in their ability to prime naive T cells and thus serve a key role in initiating primary immune responses. Therefore, they constitute a link between the innate and the adaptive immune system. Given their unique properties DC are a particularly attractive cell type for immunotherapy of diseases, such as cancer (Girolomoni and Ricciardi Castagnoli, 1997; Schuler and Steinman, 1997).

So far the study of molecular and biochemical properties of DC has been hampered by limitations in the cell number and purity of the cell populations obtained. During the past few years several protocols have been developed to generate DC from bone marrow, peripheral blood leukocytes or from CD34+ stem cells by in vitro differentiation in the presence of specific combinations of cytokines, like GM-CSF (granulocyte-macrophage colony stimulating factor) and TNF-α (tumour-necrosis factor-α ; Peters et al., 1996 and references therein; Cella et al., 1997; Austyn, 1998; Banchereau and Steinman, 1998). Several dendritic cell lines were also obtained, however, such cells maintain a largely immature phenotype and cannot be induced to mature in vitro (Paglia et al., 1993; Elbe et al., 1994; Xu et al., 1995; Volkmann et al., 1996). We previously described an alternative approach for generation of DC as clonal and homogenous cell populations that are most suitable for further molecular and biochemical studies. This system is based on the conditional transformation of DC progenitor cells from chicken bone marrow with a v-Rel estrogen receptor (ER) fusion protein v-RelER, where v-Rel activity is under the control of the ER hormone binding domain (Boehmelt et al., 1992, 1995). The v-rel oncogene, a retrovirus transduced version of c-rel, belongs to the NF-κB/Rel transcription factor family (Gilmore et al., 1996). Members of this family are involved in the regulation of genes that control important immune functions in different cell types (Baueerle and Baltimore, 1996; Attar et al., 1997; Siebenlist, 1997; Sha, 1998) in particular, one of them, RelB, is highly expressed in dendritic cells and has been implicated in normal DC

SUMMARY

Polarised expression pattern of focal contact proteins in highly motile antigen presenting dendritic cells

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Dendritic cells are professional antigen presenting cells that capture antigens and migrate to lymphoid tissues to elicit specific T cell responses. Here we used an in vitro differentiation system for generating highly motile dendritic cells from chicken bone marrow progenitors by employing the conditional v-Rel estrogen receptor (ER) fusion protein v-RelER. Molecular mechanisms of dendritic cell motility were investigated. Differentiation of v-relER progenitors into dendritic cells is associated with a reduction in cell-cell and cell-extracellular matrix interactions as cells acquire motility. We demonstrate that v-relER progenitors and dendritic cells express several adhesion receptors and components of adhesion complexes. Differentiation of v-relER cells was accompanied by downregulation of focal adhesion kinase (FAK), a key molecule of adhesion complexes, but ectopic FAK expression did not affect cell adhesion and motility. Interestingly, v-relER dendritic cells exhibit a polarised expression pattern of actin and vimentin, with actin being highly concentrated at the leading edge of the cells where lamellipodia are formed. FAK, paxillin and tyrosine phosphorylated proteins are found at both poles of the cell and colocalise with actin at the leading edge, while surface β1 integrin is confined to the uropod at the rear. CD34+ stem cell-derived human dendritic cells also exhibited an elongated bipolar morphology, mode of migration and a polarised pattern of actin-vimentin expression similar to v-relER dendritic cells.

Key words: Dendritic cell, Cell migration, v-Rel, Polarity, Adhesion molecule, Uropod, FAK
Interestingly, a similar polarisation was also observed in cells with a polarised expression pattern and colocalise with actin. Organised in classical focal adhesion plaques but rather exhibit focal contacts are expressed in v-relER DC that are not adhesive properties of v-relER DC. Several components of adhesions. However, these cells have equivalent structures of sites of organisation of actin stress fibres. Other more focal adhesion plaques are localised at the tips of the cell and represent sites of organisation of actin stress fibres. Other more motile cell types like macrophages lack stress fibres and focal adhesions. However, these cells have equivalent structures of similar protein composition but of smaller size and higher turnover called focal complexes (Allen et al., 1997).

In this work we investigated in more detail the motility and adhesive properties of v-relER DC. Several components of focal contacts are expressed in v-relER DC that are not organised in classical focal adhesion plaques but rather exhibit a polarised expression pattern and colocalise with actin. Interestingly, a similar polarisation was also observed in cells present in DC preparations obtained from human CD34+ hematopoietic progenitors.

MATERIALS AND METHODS

Cell culture
v-relER transformed bone marrow cells were isolated and cultured as described (Boehmelt et al., 1995). v-relER clones #14, 22 and 25 were used. Cells were grown in standard medium containing 10^-6 M estrogen (Sigma) at 3.5x10^6 cells/ml. Standard medium was Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 8% fetal calf serum (FCS, Sebak), 5% chicken serum (ChS, Sigma), 10 mM Hepes, pH 7.3, and 100 units/ml penicillin/streptomycin (Gibco-BRL). Tested batches of FCS and ChS were used. To induce differentiation of v-relER DC, cells were seeded in standard medium (2.5x10^6 cells/ml) containing 10^-6 M estrogen antagonists ICI 164,384 or RU 58668 (kindly provided by A. Wakeling, Zeneca, UK, and D. Martini, Roussel-UCLAF, France, respectively (Van de Velde et al., 1994; Boehmelt et al., 1995); both antagonists gave identical results (data not shown). Cells were replated at day 2 and routinely recovered at day 3 of differentiation. Chicken embryo fibroblasts (CEF) and HD3 erythroblasts were grown in standard medium but containing 2% ChS (Boehmelt et al., 1995). Cell number and cell size were determined with the CASY-1 Cell Counter and Analyser System (Schräfe Systems, Reutlingen, Germany).

To generate v-relER cells ectopically expressing FAK, a recombinant retrovirus vector was constructed by cloning chicken FAK cDNA (Cobb et al., 1994; kindly provided by J. T. Parsons, Charlottesville, Virginia, USA) between XhoI and EcoRI sites of pSFCV-LE vector (Fuerstenberg et al., 1990). Virus stocks were generated in CEF as previously described (Briegel et al., 1993) and used for infection by coculture of virus releasing CEF with v-relER cells (clone 22; 2 days). Neomycin resistant cells were selected in standard growth medium containing 0.8 mg/ml G418 (Gibco-BRL) as mass cell populations and in methocel colony assays (2 mg/ml G418) for isolation of individual clones, and grown in standard growth medium.

Human DC were generated by in vitro differentiation of CD34+ stem cells. Briefly, mobilised peripheral blood mononuclear cells were collected by apheresis from patients after obtaining informed consent followed by CD34+ selection employing immunomagnetic bead purification. CD34+ cells with 85-99% purity were obtained and grown in RPMI 1640 (Gibco-BRL) supplemented with 10% FCS (Gibco-BRL) and 100 U/ml penicillin/streptomycin (Gibco-BRL) in the presence of GM-CSF, TNF-α and SCF (stem cell factor; modified from Caux et al., 1992; Strunk et al., 1996). Cells were cultured at 2x10^6 cells/ml until day 4; cell density was then reduced to 0.5x10^6 cells/ml and cells were recovered at day 8-14 of culture and used for experiments. SCF was present only until day 6. Growth factors were: recombinant human GM-CSF, 1000 U/ml, Novartis, Basel, Switzerland; recombinant human TNF-α, 500 U/ml, Bender, Vienna, Austria; recombinant human SCF, 100 ng/ml, Amgen Inc. Thousand Oaks, Calif., USA.

Analysis of cell motility
Cells were seeded on culture dishes that were coated with CEE-conditioned medium (CCE; Boehmelt et al., 1995) at 1.5x10^6 cells/ml and monitored by time-lapse video microscopy using a Axiovert microscope (Zeiss) equipped with a small custom-made incubator (37°C, 5% CO2) and CCD camera. The motility of individual cells was evaluated by tracking their movement over 5 hours; speed of migration was calculated in μm/hour.

For transmigration assays 1x10^6 v-relER progenitor cells or DC were seeded in 0.5 ml culture medium in the upper chamber of transwell cell culture inserts (3 μm pore size, high pore density, Falcon). Bottom chambers contained 0.5 ml of the respective culture medium. After different time points the number of transmigrated cells in the bottom chamber was determined and evaluated relative to the total number of cells seeded. Transwell membranes or bottom chambers were coated with CCE medium for 16 hours at 4°C or left untreated before seeding the cells.

Adhesion assays
To evaluate cell-matrix interactions, v-relER cells were grown in estrogen containing culture medium or induced to differentiate for 3 days.
in the presence of RU 58668 (see above). Single cell suspensions were seeded in tissue culture dishes that were coated with CCE (Boehmelt et al., 1995) or human plasma fibronectin (2 μg/cm²; Biomol, Hamburg, Germany), and allowed to adhere for 0.5, 3 or 6.5 hours at 37°C. Cells were mechanically dislodged by gentle shaking of the culture dish and cell numbers in the supernatant were determined. Experiments were done in triplicates and the percentage of cells remaining on the culture dish relative to the total number of cells seeded was evaluated.

Cell-cell interactions were analysed in reaggregation assays. Single cell suspensions of v-relER progenitors or v-relER DC were generated by extensive pipetting, and cells were seeded on standard tissue culture dishes at 3 × 10⁶ cells/ml in standard medium. Images were taken after 15 minutes, 1, 4 or 24 hours with Axiovert microscope (Zeiss) equipped with a ProgRes 3012 CCD camera (Kontron Elektronik, Munich, Germany).

Flow cytometry

For flow cytometry v-relER progenitors and v-relER DC were recovered, washed with 1% bovine serum albumin (BSA, fraction V, Sigma) in PBS and incubated with primary antibody (1 hour). The following anti-chicken monoclonal antibodies were used: anti-β1 integrin (W1B10, Sigma), anti-α2β1 (MEP17; McNagny et al., 1992), anti-ααβ2 integrin (clones 3-6 and 2B1) and anti-ααβ7 integrin (clone 13-26; kindly provided by T. Göbel, Basel, Switzerland), anti-DN-GRASP (BEN1; Corbel et al., 1996), anti-HEM-CAM antibody (Vainio et al., 1996), anti-CD44 (clone AV6; a kind gift from T. F. Davison, Compton, UK) and anti-MHC class II (2G11, Salomonsen et al., 1991). Cells were then reacted with FITC-conjugated anti-mouse IgG (1 hour, Sigma), washed and resuspended in PBS containing 1% BSA and propidium iodide (2 μg/ml; Sigma) for gating on viable cells. Cells were analysed by flow cytometry using a FACScalibur device with CELLQuest software (Becton Dickinson).

Western blotting

Cells were lysed in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40 (for analysis of paxillin, vinculin and Syk) or in 50 mM Na-phosphate, pH 7.6, 100 mM NaCl. 1% Nonidet P-40 (for analysis of FAK, Crk and v-Rel) containing protease and phosphatase inhibitors (1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM Na3VO4, 50 mM NaF). Protein concentrations were measured to normalise for equal protein loading. Samples were applied to SDS-PAGE gels (7.5% or 10%) and blotted onto nitrocellulose membranes (Schleicher and Schuell, BA85) using a semi-dry blotting system (Pharmacia).

Antibody reactions were performed as described by Bartunk et al. (1996). Briefly, membranes were blocked overnight in TBS (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 5 mM KCl, 0.6 mM Na2HPO4, 0.7 mM CaCl2, 0.5 mM MgCl2) plus 1 mM EDTA, 1 mM Na3VO4, 0.05% Tween-20 and 3% BSA followed by two washes with 100 mM Tris-HCl, pH 8.0, 200 mM NaCl, 0.2% Tween-20 (wash buffer). Samples were reacted with primary antibody in blocking buffer for 1 hour. The following antibodies were used: anti-FAK, anti-paxillin, anti-Csk, anti-Crk (all Transduction Laboratories), anti-vinculin (Sigma) and monoclonal anti-Rel antibody (Rel#6; Kabrun et al., 1991). Subsequently, blots were washed and incubated with the respective secondary antibody (ECL kit, Amersham) in TBS supplemented with 5% non-fat milk powder for 45 minutes. All incubations were done at room temperature. Blots were developed with ECL reagents and exposed to film.

Immunoprecipitation

Cells were seeded in serum free medium at 3 × 10⁶ cells/ml on CCE-coated dishes. After 2 hours Na3VO4 (1 mM) was added and cells were incubated for an additional hour. Cells were then lysed in modified RIPA buffer containing protease and phosphatase inhibitors (PMSF, leupeptin, aprotinin, 1 mM Na3VO4 and 50 mM NaF) and subjected to immunoprecipitation as described by Cobb et al. (1994). Briefly, cell lysates (0.7 mg protein) were incubated with anti-FAK monoclonal antibody (7.5 μg; clone 2A7, Upstate Biotechnology) or anti-paxillin monoclonal antibody (2 μg; Transduction Laboratories) for 2 hours at 4°C. Anti-mouse IgG agarose (Sigma) was added followed by incubation for an additional 2 hours. Immunoprecipitated complexes were recovered, washed and resolved in 7.5% PAGE. Proteins were transferred onto nitrocellulose membrane and processed for western blotting as above. Membranes were probed with an anti-phosphotyrosine specific antibody (4G10, Upstate Biotechnology). Subsequently blots were stripped and reprobed with anti-FAK or anti-paxillin monoclonal antibodies (Transduction Laboratories).

Immunofluorescence

v-relER cells were cultured in the presence of estrogen or differentiated with RU 58668 for 2 days, and allowed to adhere to CCE coated slides (6-8 hours, 37°C). Culture medium was carefully removed and cells were fixed with 3.7% paraformaldehyde in PBS (15 minutes), and washed with PBS, PBS and fixed as above. Following fixation cells were permeabilised with 0.5% NP-40 in PBS (15 minutes), incubated with 0.5% FCS in PBS (30 minutes) to block unspecific binding and reacted with primary antibody. The following mouse monoclonal antibodies were used: anti-vimentin (VIM 3B4, Boehringer Mannheim), anti-FAK and anti-paxillin (Transduction Laboratories), anti-phosphotyrosine (4G10, Upstate Biotechnology Inc.). For β1 integrin staining, live cells were incubated with anti-chicken β1 integrin antibody (W1B10, Sigma), in serum free medium for 1 hour on fibronectin (FN) coated culture dishes. Cells were fixed by adding paraformaldehyde to a final concentration of 3.7%, washed in PBS and allowed to bind to adhesion slides (10 minutes; Bio-Rad). Incubation of primary antibody was for 1 hour, followed by reaction with FITC-conjugated anti-mouse IgG (1 hour; Sigma). Samples were also stained for actin with TRITC-labelled phallolidin (Sigma). DAPI (0.5 μg/ml; Sigma) was used to stain nuclei. Samples were washed with PBS and mounted in Mowiol 4.88 (Hoechst) containing 50 mg/ml DABCO (Sigma) as anti-bleaching agent. All incubations were done at room temperature. Photographs were taken with a Axiophot II fluorescence microscope (Zeiss) equipped with a Photometrics Quantix CCD camera. Confocal microscopy was done on samples treated as above with a Leika TCS 4D-I equipment (Bensheim, Germany). Images were processed with IPLab Spectrum and Adobe Photoshop software.

RNase protection

Total RNA was prepared as described by Boehmelt et al. (1992). For RNase protection analysis the following riboprobes were generated: FAK (tyrosine kinase region, position 1695 to 1860; Schaller et al., 1983) in pSP64 (Promega). Plasmids were linearised with PvuII (actin) and anti-sense probes were prepared following RNaseA/RNaseT1 digestion samples were resolved in 6% polyacrylamide, 8 M urea denaturing gels and detected by autoradiography.

RESULTS

v-relER cell differentiation is accompanied by reduction in cell-ECM interactions and loss of cell-cell contacts

One of the most striking features of v-relER DC is their motility in culture which is associated with an elongated
bipolar cell morphology (Fig. 1; Boehmelt et al., 1995). v-relER DC are at the leading edge devoid of organelles and present lamellipodia-like cytoplasmic protrusions. Organelles, vacuoles and other intracellular compartments are confined to the opposite side of the cell body (Boehmelt et al., 1995). Interestingly, many v-relER DC have at the rear of the cell a globular structure, termed uropod, which is not observed in v-relER progenitors (Fig. 1). The uropod has been implicated in recruitment of leukocytes through specific cell-cell interactions (del Pozo et al., 1997). Finally, v-relER DC are also smaller than v-relER progenitor cells (8.7±0.4 μm and 9.6±0.5 μm, respectively).

To analyse the motility of v-relER DC quantitatively, v-relER progenitor cells were differentiated by estrogen antagonist treatment for 3 days and seeded on tissue culture surfaces that were coated with chicken embryo fibroblast (CEF)-conditioned medium (CCE) as a source of ECM components (Boehmelt et al., 1995). As expected, v-relER DC maintained their elongated bipolar morphology on CCE coated culture surfaces. v-relER progenitors also kept their spherical appearance while some cells with spiny processes were also seen (Fig. 1). Cell migration was then analysed by time-lapse video microscopy. As shown in Fig. 2A and B v-relER DC exhibited an extensive motility, migrating an average of 88 μm/hour. The movement was, however, not directed, at least under the culture conditions employed so far. v-relER progenitor cells did not migrate and were sessile.

V-relER DC were also active in transmigration assays and clearly more effective than undifferentiated cells (Fig. 2C). Interestingly, coating of transwell surfaces with CCE medium efficiently enhanced v-relER DC transmigration. CCE treatment of the lower chamber left DC transmigration unaffected, indicating that CCE does not contain any potent chemotactic activity.

Next we wanted to assess how the high motility observed for v-relER DC relates to the strength of binding to ECM. To this end v-relER progenitors and DC were seeded onto CCE coated culture dishes and following incubation for 0.5, 3 and 6 hours cells were mechanically dislodged and the proportion of adherent cells was determined. While v-relER progenitor cells effectively adhered to substrate (60-70% after 3 hours, Fig. 3A) v-relER DC were consistently found to adhere more weakly, and only 20-25% of the cells seeded remained bound to CCE coated culture dishes. In this assay the percentage of cells bound to substrate reflects the strength of adhesion, rather than the presence of two cell populations (adherent and non-adherent) in the cultures. Since fibronectin (FN) represents a major ECM component produced by fibroblasts, v-relER cell binding to FN was analysed. Again v-relER progenitor cells adhered more efficiently than v-relER DC (Fig. 3A). Furthermore, both v-relER progenitors and v-relER DC bound more tightly to CCE coated dishes than to FN, indicating that CCE medium contains additional components that mediate adhesion. Consequently, a number of other commercially available ECM compounds were tested, like collagen type I, laminin and thrombospondin, and only collagen was found to effectively cause adhesion of v-relER cells. Adhesion of v-relER progenitor cells to CCE or FN coated culture dishes also caused a reduction in cell proliferation by about 20% as determined by [3H]thymidine incorporation, while v-relER progenitors grown on untreated dishes showed normal growth rates. Differentiated v-relER DC did not proliferate and accordingly, there was no effect on [3H]thymidine incorporation with these cells, irrespective of
whether they were cultured on CCE coated, FN coated or uncoated dishes. In addition to differences in cell-ECM contacts, changes in cell-cell interactions were also observed when v-relER cells differentiated. When grown in suspension v-relER progenitors clustered in large cell aggregates indicating that cell-cell contacts were formed. Following induction of differentiation these interactions were lost and v-relER DC generated single cell cultures (data not shown). To further analyse this observation, v-relER cells were grown in estrogen containing medium or differentiated by antagonist treatment (3 days), and single cell suspension cultures were generated by disrupting cell aggregates mechanically by extensive pipetting. The capacity of v-relER progenitor cells and v-relER DC to reaggregate after various periods of time was then monitored microscopically (Fig. 3B). While v-relER progenitors started to reaggregate after about 1 hour and formed large cell clusters with more than 100 cells/aggregate within 24 hours, v-relER DC remained as single cell cultures (Fig. 3B).

In summary, differentiation of v-relER progenitors into DC was associated with specific changes in morphology, reduction in cell-ECM interactions and loss of cell-cell contacts as cells acquired the ability to migrate.

Expression of cell adhesion molecules in v-relER cells
Cell-ECM interactions and cell-cell contacts are mediated by

![Fig. 2. v-relER DC are highly motile in vitro. (A) v-relER progenitors or v-relER DC were seeded on CCE coated culture dishes and analysed by time-lapse video microscopy. Cell motility was evaluated by tracking the movement of individual cells for 5 hours. Representative migration patterns of 15 v-relER progenitors and 8 v-relER DC are shown. v-relER DC moved long distances, however, their migration was not directed, while v-relER progenitors were largely sessile. (B) Values obtained from the quantification of trajectories were normalised for 1 hour and the velocity of migration (\(\mu m\) per hour) for 25 representative cells plotted with decreasing migration rates. (C) Transmigration assay of v-relER DC. Motility of v-relER progenitor cells and DC was assessed in transwell assays. The number of cells that migrated through 3 \(\mu m\) pores within 24 hours is shown as a percentage of the total number of cells seeded. Bottom chamber coated with CCE (lanes 1, 2); transwell surface coated with CCE (lanes 3, 4); untreated control (lanes 5, 6).](image)

![Fig. 3. v-relER cell differentiation causes reduction in cell-ECM interactions and loss of cell-cell contacts. (A) v-relER progenitors and v-relER DC were seeded as single cell suspensions on CCE or fibronectin coated, or untreated culture dishes (CCE, FN and control, respectively) and allowed to adhere for 0.5, 3 and 6.5 hours. Non-adherent and loosely adherent cells were mechanically dislodged and the proportion of adherent cells relative to the number of cells seeded was determined. The means of triplicate values with standard deviations are shown. (B) Cell-cell interactions were evaluated in reaggregation assays. Single cell suspension cultures of v-relER progenitors and v-relER DC were seeded in standard medium plus estrogen or estrogen antagonist RU 58668, respectively, and their capacity to reaggregate after 15 min, 1, 4 and 24 hours was monitored microscopically. v-relER progenitor cells start to form cell aggregates after 1 hour and after 24 hours cultures resemble normal suspension cultures, while v-relER DC remain as single cell cultures.](image)
Specific surface adhesion molecules. Therefore it was of particular interest to investigate whether specific changes in the expression of cell adhesion receptors might account for the reduction in cell-ECM interactions and the loss of cell-cell contacts that occurred during v-relER cell differentiation. To this end v-relER progenitors and DC were analysed for $\beta_1$ integrin expression by flow cytometry, since $\beta_1$ integrins comprise the largest subfamily of ECM receptors (like those for FN and collagen) and might be involved in v-relER cell binding to ECM. Molecules that mediate cell-cell interactions like DM-GRASP and HEM-CAM were also studied.

It was found that v-relER progenitors expressed high levels of $\beta_1$ integrin which were further upregulated when cells differentiated (Fig. 4). Cells were negative for integrins like $\alpha_2\beta_1$, $\alpha_\alpha\beta_2$ and $\alpha_4\beta_7$ (data not shown). We note, however, that the number of antibodies available for chicken integrins is rather limited which has so far prevented a more extensive analysis. v-relER cells also expressed high levels of DM-GRASP (BEN/SC1; Corbel et al., 1996), an adhesion molecule of the immunoglobulin superfamily thought to generate cell-cell contacts through homophilic interactions. Following v-relER cell differentiation only a minor reduction in DM-GRASP expression was seen (Fig. 4) indicating that homophilic binding via DM-GRASP is unlikely to be involved in the loss of cell-cell interactions observed when cells differentiate. Both v-relER progenitors and DC showed high levels of CD44 but neither of them expressed HEM-CAM, a member of the immunoglobulin superfamily found on early hematopoietic progenitor cells (Vainio et al., 1996; Fig. 4 and data not shown). As expected, v-relER cells exhibited high levels of MHC class II that were upregulated when cells differentiated into v-relER DC.

**v-relER cells express components of focal adhesion complexes**

Since v-relER cells abundantly expressed $\beta_1$ integrin and adhered to components of the ECM, we sought to determine whether proteins found in focal contacts were present in v-relER cells. Therefore v-relER progenitors and v-relER DC were prepared and analysed by western blotting using a panel of specific antibodies. Two independent v-relER clones were investigated and CEF served as experimental control (Fig. 5A). FAK is one of the key molecules of focal contacts and directly binds the $\beta_1$ integrin cytoplasmic domain (Clark and Brugge, 1995; Schwartz et al., 1995; Parsons, 1996; Yamada and Geiger, 1997), therefore it was important to examine its expression in v-relER cells. Interestingly, while v-relER progenitors effectively expressed FAK its expression in v-relER DC was much lower (Fig. 5A and B). Other components of focal contacts, like the structural proteins paxillin and vinculin, the tyrosine kinase Csk and the adapter molecule Crk, were also present, but their levels remained unchanged upon differentiation. Syk tyrosine kinase was barely detectable in v-relER cells and tensin was only seen in CEF control (Fig. 5A and data not shown). We also observed that the anti-paxillin antibody stained an additional band of 55 kDa which might represent the recently described paxillin related Hic-5 protein (Matsuya et al., 1998).

Since the activity of key components of focal contacts, like FAK and paxillin, is modulated by phosphorylation we examined the phosphotyrosine content of both proteins. V-relER cells prior to and after differentiation were subjected to immunoprecipitation with specific antibodies and immunoprecipitates were analysed by western blotting with an anti-phosphotyrosine antibody. As shown in Fig. 5B, the phosphotyrosine specific signal of FAK was higher in v-relER progenitors than in v-relER DC and paralleled FAK protein levels. Paxillin was abundantly phosphorylated to virtually the same extent in both undifferentiated and differentiated cells while the paxillin related Hic-5 protein was apparently not tyrosine phosphorylated (Fig. 5B). However, major changes in the overall tyrosine phosphorylation pattern were clearly observed when v-relER cells differentiated, like the prominent increase in phosphorylation of a 65 kDa and 100 kDa protein in v-relER DC (see Fig. 7A, lanes 5 and 6). The identity of these proteins remains to be determined.

Downregulation of FAK during v-relER cell differentiation is interesting since it might be an inherent property of the ongoing DC differentiation program or alternatively reflect a regulation of FAK expression by v-Rel protein.
To investigate this possibility, total RNA from v-relER progenitor cells and v-relER DC was prepared and subjected to RNase protection analysis. A FAK specific band was readily detected in v-relER progenitors while it was dramatically reduced in v-relER DC (Fig. 6). Protected fragments for controls (S17 ribosomal protein and β-actin mRNA) remained constant. This result suggests that downregulation of FAK expression is transcriptionally controlled presumably through the v-relER protein.

Additionally, to determine whether FAK downregulation is involved in the changes of cell adhesion and/or motility observed during v-relER cell differentiation, FAK was ectopically expressed in v-relER cells via a recombinant retrovirus vector. Fig. 7A shows that such cells abundantly expressed FAK, and high levels of phosphorylated FAK protein were maintained when cells were induced to differentiate. It was observed that ectopic FAK expression left the changes in cell adhesion and motility, induced upon v-relER cell differentiation, unaffected (Fig. 7B and data not shown), indicating that downregulation of FAK is apparently not involved in mechanisms that determine motility of v-relER cells. Additionally, the overall pattern of tyrosine phosphorylated proteins was the same (with the exception of the ectopic FAK) as in parental v-relER cells (Fig. 7A). Moreover overexpression of FAK allowed us to examine the subcellular localisation of FAK protein in v-relER DC, since endogenous expression levels of FAK did not permit an efficient detection of the protein by immunofluorescence using several anti-FAK antibodies. Interestingly, FAK was expressed in a polarised fashion in v-relER DC, with a high concentration at the leading edge, colocalising with actin, and at the uropod (Fig. 7C).

In summary, v-relER progenitors and DC expressed several components of focal contacts like FAK, paxillin, vinculin, Csk and Crk. FAK levels, although reduced during v-relER cell differentiation, appear not to be a major determinant for adhesive properties of these cells, since ectopically and overexpressed FAK left differentiation, cell adhesion and...
motility of v-relER cells unaffected. Interestingly, in v-relER DC FAK showed a polarised expression pattern and colocalised with actin at the leading edge.

**Localisation of focal contact components in v-relER DC**

Having identified various proteins occurring in focal adhesions, we wanted to determine where they were located in v-relER DC and whether this was related to the polarised expression pattern observed for v-relER cells ectopically overexpressing FAK. To this end v-relER progenitors and v-relER DC were seeded on CCE coated surfaces, fixed and stained for paxillin, phosphotyrosine and for the cytoskeletal proteins actin and vimentin, and analysed by indirect immunofluorescence and confocal microscopy (Figs 8, 9). To detect surface β1 integrin, live cells were reacted with specific antibody in serum free medium, fixed, allowed to adhere to slides and analysed. CEFS were used as a control and stained accordingly.

v-relER DC showed a high concentration of filamentous actin (F-actin) at the leading edge of the cell where cytoplasmic protrusions are formed, while for some cells an actin staining at the uropod was also observed (Figs 7C, 8A). Actin stress fibres like those present in CEF control were not detected in v-relER DC, but some actin bundles were observed by confocal microscopy at the protrusions of the leading edge (Fig. 9). Vimentin expression was confined to the main cell body close to the nucleus and to the rear of the cell where all the organelles and intracellular compartments are located (Figs 8A, 9A; Boehmelt et al., 1995). While it is clear that depending on the stage of movement the organisation of cytoskeletal proteins undergoes extensive changes, the polarised actin-vimentin expression pattern was consistently observed for elongated bipolar v-relER DC and not seen for the majority of undifferentiated v-relER progenitor cells (Fig. 8A). This polarisation was independent of the substrate, since it was also observed for cells seeded on FN or in suspension (data not shown). The present state of analysis still leaves open the question whether the redistribution of the cytoskeletal proteins studied here causes or is a consequence of the bipolar morphology.

Interestingly, in v-relER DC we did not observe the typical punctuated pattern of paxillin staining seen for focal adhesion plaques in CEF (Fig. 8B). A staining pattern indicative of smaller focal complexes, like those described for macrophages (Allen et al., 1997), was also not found. However, in v-relER DC paxillin staining was more pronounced in areas where lamellipodia are formed and at the uropod, but also found in the main cell body (Figs 8B, 9C). These areas were additionally stained with a phosphotyrosine specific antibody (Fig. 8B). β1 integrin mainly localised to the membrane at the rear of the cell, being most pronounced at the uropod (Fig. 9E).

In summary, v-relER DC do not contain classical focal adhesion plaques or focal complexes that are characterised by a punctuated pattern of paxillin and tyrosine phosphorylated proteins but rather exhibit a polarised expression of such proteins which colocalise with actin. Vimentin is found close to the nucleus and to the rear of the cell, and membrane β1 integrin is more concentrated at the uropod.

**Human DC preparations contain cells with morphology, motility and polarisation similar to v-relER DC**

Next we wanted to determine whether the properties described above for v-relER DC were also observed for human DC. Therefore human CD34+ peripheral blood stem cells were obtained and induced to differentiate into DC in vitro by treatment with GM-CSF and TNF-α. At day 8 of culture all cells expressed high levels of MHC class II and a majority of them exhibited an elongated bipolar morphology very similar to that seen for v-relER DC (Fig. 10A). Such human DC preparations were, however, more heterogeneous in cell morphology than v-relER DC and cells were also larger (mean diameter 11 μm). The elongated cells had cytoplasmic protrusions at the leading edge and uropods were also observed. Additionally, cells exhibited a high motility in culture very similar to v-relER DC. Most interestingly, they also showed the typical polarisation of...
Motility of v-relER dendritic cells

actin and vimentin as revealed by immunofluorescence, while paxillin expression was more uniform (Fig. 10B, and data not shown). Upon longer culture the proportion of elongated bipolar cells decreased as cells progressed further in their differentiation program; at day 14 the majority of cells acquired the typical morphology of ‘veiled’ DC. A more detailed characterisation of the phenotype and function of the elongated bipolar cells in human DC preparations is presently being performed and will be published elsewhere.

DISCUSSION

Cell migration is an essential property required for DC function. DC have to migrate to peripheral organs (like skin) and, following antigen uptake, home to lymphoid tissues to elicit primary immune responses. So far there are only few data on the molecules involved in DC migration (Roake et al., 1995; Austyn, 1996; Winzler et al., 1997). Such studies are difficult to perform, mainly due to the heterogeneity of the DC preparations available. Additionally, the DC cell lines generated so far only partially recapitulate DC function in vitro and are not motile (Paglia et al., 1993; Elbe et al., 1994; Volkmann et al., 1996). The v-relER differentiation system for DC employed in this paper is particularly well suited for studying DC motility on a molecular level since homogenous cell populations of highly motile DC are readily obtained (Boehmelt et al., 1995).

The migration of cells is a complex process that requires the constant formation and disassembly of cell-cell and cell-ECM interactions. Actin and vimentin are key players in this process, as demonstrated by our findings. The polarised expression of actin, vimentin, and paxillin in v-relER DC, as well as the punctuated expression of paxillin and phosphotyrosine in focal adhesions, suggest a dynamic and regulated migration process. Further studies are needed to elucidate the underlying molecular mechanisms and to correlate these with functional outcomes in vivo.
interactions (Huttenlocher et al., 1995; Lauffenburger and Horwitz, 1996). Furthermore, both theoretical estimations and experimental evidence suggest that maximal migration rates are achieved by an intermediate strength of attachment to ECM (Huttenlocher et al., 1995). Accordingly, in v-relER DC the acquisition of migratory activity is associated with profound changes in adhesive properties; v-relER DC lose cell-cell contacts and show reduced binding to ECM components. Similar changes were also observed for long-term growth factor-dependent DC cultures from mouse spleen (Winzler et al., 1997); following maturation such cells acquire a veiled morphology and become less adhesive and highly motile.

Integrin receptors are important for both cell adhesion and migration as evidenced for example by knockout studies of $\beta_1$ integrin (Stephens et al., 1993; Brakebusch et al., 1997). Interestingly, v-relER progenitors express high levels of $\beta_1$ integrin that are even further upregulated when cells differentiate and acquire the ability to migrate. Such an upregulation of $\beta_1$ integrin in v-relER DC is surprising, since v-relER DC are in fact less adherent to ECM (CCE or FN) than v-relER progenitors. However, a possible explanation of these results is provided by our observation that upon differentiation $\beta_1$ integrin is redistributed to the uropod which is not bound to substrate but rather projects itself into the medium (del Pozo et al., 1997; Sanchez-Madrid and del Pozo, 1999, and data not shown). It also suggests that $\beta_1$ integrin might not be a major player in adhesion of v-relER DC to ECM.

v-relER DC express several proteins of cell adhesion complexes. Interestingly, expression of FAK, a key molecule of adhesion complexes, is downregulated when v-relER cells differentiate and acquire the ability to migrate. Other components, like paxillin, vinculin, Csk and Crk, show similar levels in both v-relER progenitors and v-relER DC. Since FAK provides a linkage between the ECM-integrin receptor complexes and multiple intracellular proteins involved in cytoskeletal organisation and signalling pathways (reviewed by Parsons, 1996; Clark and Hynes, 1997; Yamada and Geiger, 1997), it was conceivable that regulation of FAK modulated the motility of v-relER DC. However, ectopic expression of FAK in v-relER cells, that maintained high FAK protein levels independently of differentiation, left adhesive properties and motility unaffected in both v-relER progenitors and DC. Thus, FAK levels appear not to be a major determinant for the adhesive properties of these cells. Whether other FAK-related proteins like FakB, PYK2 or a FAK-related non-kinase (FRNK, comprising only the C-terminal FAK domain; Richardson and Parsons, 1996, and references therein) play a role in v-relER cell motility remains an open question. Initial data indicate that PYK2 is expressed in both v-relER progenitor and DC to similar levels (J. Madruga and M. Zenke, unpublished).

v-relER DC lack classical focal contacts and actin stress fibres, which is not surprising since such structures are most frequently found in strongly adherent and less motile cell types like fibroblasts. This observation might also be related to the reduced expression levels of the cytoskeletal proteins paxillin, vinculin and tensin in v-relER cells as compared to CEF.

**Fig. 9.** Confocal microscopy of vimentin, paxillin, $\beta_1$ integrin and actin expression in v-relER cells. v-relER DC (day 2 of differentiation) were analysed by indirect immunofluorescence and confocal microscopy (see Materials and Methods). Vimentin (A), paxillin (C), $\beta_1$ integrin (E), F-actin detected by TRITC-Phalloidin (B, D, and F), corresponding to the cells shown in A, C and E, respectively.

**Fig. 10.** Highly motile cells in human DC preparations from CD34+ stem cells exhibit a polarised expression pattern of actin and vimentin. Elongated bipolar cells present in DC preparations derived by in vitro differentiation of CD34+ cells with GM-CSF and TNF-α were analysed at day 8. (A) Phase contrast. (B) Vimentin and actin expression detected by immunofluorescence and TRITC-Phalloidin staining, respectively. Bar, 10 μm.
However, in v-relER DC both paullin and tyrosine phosphorylated proteins exhibit a polarised expression pattern and colocalise with actin most prominently at the leading edge of the cell where vesicles are formed and at the rear. This is also consistent with the pattern observed for FAK. Thus, it is very well possible that in v-relER DC the focal contact proteins are assembled in some type of adhesion complexes which are smaller in size and more transient than the focal complexes described for macrophages (Allen et al., 1997). Furthermore, the prominent vimentin expression in v-relER DC confined to the main cell body close to the nucleus and to the rear, is in accord with its function to maintain cell shape and polarity, being particularly high in cells that are subject to mechanical stress like very motile cells.

The highly polarised phenotype of v-relER DC is also associated with the presence of uropods at the rear of the cells. Uropods have been reported for various types of leukocytes when cells adhere to endothelium or ECM components and are stimulated by e.g. chemokinones or chemotactractive cytokines (del Pozo et al., 1997; Sanchez-Madrid and del Pozo, 1999). Interestingly, this structure is already observed for v-relER DC in suspension culture, even in the absence of contact with ECM substrate or of additional stimuli. To our knowledge this is the first report on the presence of uropods in DC. The fact that v-relER DC in some instances remain in contact by uropods (data not shown) indicates that a redistribution of cell adhesion molecules to this structure occurs during v-relER cell differentiation. Such changes have also been reported for other leukocytes (del Pozo et al., 1997; Sanchez-Madrid and del Pozo, 1999). In v-relER DC β1 integrin is concentrated at the uropod while other molecules that undergo a redistribution to the uropod remain elusive. Initial experiments also showed that v-relER progenitors and DC express radixin, a protein of the ERM family (Tsukita et al., 1997; data not shown), which localises to uropods in lymphocytes (Serrador et al., 1997).

In summary, the molecules involved in adhesion of v-relER cells to substrate undergo specific changes in distribution and composition during differentiation when v-relER DC acquire migratory activity. Obviously, the induction and direction of migration is controlled by the presence of specific cell surface receptors on DC that are activated by specific ligands like motility factors and chemokines. The identity of such receptors and their cognate ligands that affect migration of v-relER DC still remains to be determined. Finally, as judged by morphology, adhesive properties and mode of migration v-relER DC resemble a population of human DC obtained from CD34+ peripheral blood stem cells. Such cells also exhibited the typical polarised pattern of actin-vimentin expression that is seen for v-relER DC. It will now be interesting to analyse whether in these cells changes in gene expression and organisation of adhesion complexes are similar to those described in this paper for the v-relER cells.

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