Evidence for Distinct Intracellular Signaling Pathways in CD34+ Progenitor to Dendritic Cell Differentiation from a Human Cell Line Model


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Evidence for Distinct Intracellular Signaling Pathways in CD34⁺ Progenitor to Dendritic Cell Differentiation from a Human Cell Line Model


Intracellular signals that mediate differentiation of pluripotent hematopoietic progenitors to dendritic cells (DC) are largely undefined. We have previously shown that protein kinase C (PKC) activation (with phorbol ester (PMA) alone) specifically induces differentiation of primary human CD34⁺ hematopoietic progenitor cells (HPC) to mature DC. We now find that cytokine-driven (granulocyte-macrophage CSF and TNF-α) CD34⁺ HPC→DC differentiation is preferentially blocked by inhibitors of PKC activation. To further identify intracellular signals and downstream events important in CD34⁺ HPC→DC differentiation we have characterized a human leukemic cell line model of this process. The CD34⁺ myelomonocytic cell line KG1 differentiates into dendritic-like cells in response to granulocyte-macrophage CSF plus TNF-α, or PMA (with or without the calcium ionophore ionomycin, or TNF-α), with different stimuli mediating different aspects of the process. Phenotypic DC characteristics of KG1 dendritic-like cells include morphology (loosely adherent cells with long neurite processes), MHC I⁺/MHC IIbright/CD83⁺/CD86⁺/CD14⁺ surface Ag expression, and ReLB and DC-CK1 gene expression. Functional DC characteristics include fluid phase macromolecule uptake (FITC-dextran) and activation of resting T cells. Comparison of KG1 to the PMA-unresponsive subline KG1a reveals differences in expression of TNF receptors 1 and 2; PKC isoforms α, β, βII, and μII; and ReLB, suggesting that these components/pathways are important for DC differentiation. Together, these findings demonstrate that cytokine or phorbol ester stimulation of KG1 is a model of human CD34⁺ HPC to DC differentiation and suggest that specific intracellular signaling pathways mediate specific events in DC lineage commitment. The Journal of Immunology, 1999, 162: 3237–3248.

How multipotent hematopoietic stem cells become mature blood cells is a paradigm of cellular differentiation and lineage commitment. Complex hematopoiesis underlies the generation of each component of the immune system, including T, B, and professional APCs (such as monocyte/macrophages and dendritic cells (DC)1). Evidence now suggests that DC play the central and critical role in both initiating and controlling the primary immune response via cognate interactions with T and B cells (1). In humans, dendritic cells are a heterogeneous population shown in vitro to arise from multiple distinct hematopoietic progenitor cells (HPC) along distinct differentiation pathways. These progenitors include CD14⁺ monocytes (2–5) and CD34⁺ bone marrow cells (in humans a heterogeneous population of multipotential progenitors of which the true pluripotent stem cell is a subset). CD34⁺ CD10⁻ HPC give rise to the lymphoid DC lineage (6), whereas CD34⁺ CD86⁺ HPC are progenitors for myeloid DC and monocyte/macrophages (7).

Broadly characterized from a cellular standpoint, there are two stages of DC differentiation (8). The first stage involves differentiation of multipotential HPC to immature (or unactivated) DC, cells with high capacity for Ag uptake but relatively poor ability to activate T cells. This differentiation is induced in vitro by exogenous cytokines (GM-CSF and TNF-α (with or without other cytokines) for CD34⁺ HPC (9), and GM-CSF and IL-4 for monocytes (2–5)), CD40 receptor cross-linking (CD34⁺ HPC) (10), or calcium ionophore alone (monocytes) (11). The second stage involves maturation of immature to mature DC, cells that have decreased Ag uptake capability but are much more potent in activating T cells. An alternative viewpoint is that DC maturation is actually activation of resting DC. In vivo this second stage is probably triggered by “danger signals,” which may include live bacteria and components (LPS, DNA), viral infection, and inflammatory cytokines (reviewed in Ref. 1). In vitro, maturation/activation can be induced by a number of stimuli, including cytokines (TNF-α with or without other cytokines, reviewed in Ref. 12), monocyte-conditioned medium (13), or CD40 receptor cross-linking (10).
We have previously reported that the phorbol ester PMA alone induces differentiation of human CD34+ HPC into mature and fully functional DC, suggesting that direct activation of protein kinase C (PKC) by itself is sufficient to trigger this lineage commitment (14). PMA-driven CD34+ HPC→DC differentiation is specific for DC (no other lineages are generated) and does not involve proliferation (differentiation is complete within 7 days), although about 50% of the input number of CD34+ HPC are lost (in part via apoptosis). All these characteristics distinguish PMA from receptor-mediated differentiation signals (cytokines, CD40 receptor cross-linking). Since exogenous stimuli (including TNF-α, IL-1β, IL-4, and CD40) (15–19) used to induce CD34+ HPC→DC differentiation also activate PKC as part of their downstream signaling cascade, it is likely that PMA is activating the component of this cascade that specifically initiates DC differentiation. Consistent with this, we now report that inhibition of PKC activation suppresses generation of DC by GM-CSF and TNF-α stimulation of CD34+ HPC.

Aside from a potential role for PKC activation, relatively little is known about what intracellular signaling pathways (and the genetic programs they initiate) are involved in HPC→DC differentiation. Signaling via intracellular calcium flux alone (by calcium ionophore) induces monocyte→DC differentiation (11), although we have not observed this for CD34+ HPC (D.C.S.L and K.P.L., unpublished observations). The downstream components involved in this calcium signaling are uncharacterized. Another signaling pathway involves the lipid second messenger ceramide, which mediates down-regulation of Ag uptake during cytokine-driven maturation/activation of DC (20). Downstream of these signaling pathways, the transcription factor RelB (a member of the NFκB family) appears to play an important role in DC differentiation based on apparent function (21–24) and the phenotype of RelB knockout mice (25, 26). RelB expression is up-regulated in vitro during cytokine- or PMA-driven DC differentiation (2, 14). What genes RelB specifically regulates during DC differentiation is currently unknown.

Even using agents that directly activate signal transduction pathways, a number of obstacles makes study of the intracellular and genetic events involved in DC differentiation difficult. The rarity of CD34+ HPC (0.1–1% of bone marrow mononuclear cells) makes sufficient isolation for larger scale studies laborious. In addition, CD34+ HPC are a heterogeneous population of progenitors already committed to different lineages (for example, see Ref. 6), which makes analysis of bulk starting populations problematic. Receptor-mediated CD34+ HPC→DC differentiation involves cell proliferation, which complicates identification of differentiation-specific processes. These stimuli also generate mixed populations of cells (DC, monocytes, neutrophils) that require additional purification of DC before definitive analysis (reviewed in Ref. 12). Finally, CD34+ HPC have traditionally been difficult to transfect/transduce (27), making genetic manipulation involving these approaches less effective.

Many of these problems could be circumvented in a cell line model of DC differentiation. Czerniecki et al. have suggested that the pro-myelocytic HL-60 cell line differentiates into DC with calcium ionophore, thus representing a potential model of monocyte→DC differentiation (11). It has recently been reported that committed CD34+ precursors for myeloid DC and macrophages are CD86+ positive (7). We have found that the CD34+ human myeloblastic cell line KG1 (28) also expresses CD86. KG1 is a cytokine-responsive human CD34+ myelomonocytic cell line derived from a patient with erythroleukemia. KG1 was originally described to differentiate to the monocyte/macrophage lineage (based on morphologic and biochemical assays) when stimulated with phorbol ester (29–31), although DC-specific markers were not available at that time. The KG1a subline of KG1 was indifferent to the effects of PMA (and cytokines) and is characterized as less differentiated than KG1 (29, 30, 32). KG1 and KG1a have qualitative differences in PKC activation and substrate phosphorylation (30, 33, 34). We now report that phorbol ester or cytokine (GM-CSF and TNF-α) induces KG1 to differentiate into dendritic-like cells (DLC) based on morphology, surface Ag phenotype, function, and gene expression. In contrast, KG1a do not differentiate. Characterization of stimuli required to induce DLC differentiation as well as comparison to KG1a provide evidence that distinct intracellular signaling pathways are involved and mediate specific components of differentiation.

Materials and Methods

Cells and culture
CD34+ HPC were isolated from organ donor bone marrow by immunomagnetic selection as previously described (35). Purified cells were typically >95% CD34+ as determined by FACS analysis with a second non-cross-blocked anti-CD34 mAb. For generation of DC, CD34+ HPC were cultured for 14 days at 5 × 106 cells/ml in complete culture medium (IMDM, Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FCS (HyClone, Logan UT), 100 μM t-glutamine, and 100 μM penicillin/streptomycin solution (Life Technologies)) with GM-CSF (200 U/ml) and TNF-α (2.5 ng/ml; all from R&D Systems, Minneapolis, MN). Where indicated, staurosporine (Calbiochem, San Diego, CA) was added at 0.1 ng/ml, a dose sufficient to block PMA-induced proliferation of human T cells. At the end of the culture period, nonadherent cells and adherent cells were resuspended (3 mM EDTA), washed, and concentrated by centrifugation.

KG1 and KG1a cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in IMDM/20% FCS/100 mM t-glutamine/100 U penicillin/streptomycin solution (Life Technologies)) with GM-CSF (200 U/ml) and TNF-α (10 ng/ml), ionomycin (100 ng/ml; Calbiochem). The four-cytokine combination of GM-CSF (2 ng/ml), IL-3 (5 ng/ml), IL-6 (5 ng/ml), stem cell factor (SCF, 120 ng/ml) has previously been found to induce robust proliferation of primary CD34+ HPC (35). Where indicated, bisindolylmaleimide I (bis; Calbiochem) was used at 5 μM.

Flow cytometric analysis and mAbs
Adherent and loosely adherent cells were harvested with 3 mM EDTA, washed twice, and resuspended in staining medium (PBS, 5% FCS, 2% BSA, and 0.1% sodium azide). Phenotypic analysis of cells (2 × 106) was performed by flow cytometry using saturating concentrations of the following mAbs: CD1a (clone SK9), CD2 (S5.2), CD4 (SK3), CD3 (L233), CD4 (MO9), CD34 (1G12), CD80 (L307.4), and CD154 (89-76; all from Becton Dickinson, San Jose, CA); CD10 (H110A), CD33 (WM53), CD40 (SC3), CD45RA (H100), CD86 (IT2.2), and CD90 (SE10; all from PharMingen, San Diego, CA); CD83 (HB15, Immunotech, Westbrook, ME); MHC class I (H58A) and MHC class II (H42A, both from Veterinary PharMingen, San Diego, CA); CD1a (clone SK9), CD2 (S5.2), CD4 (SK3), CD13 (L138), CD14 (MO9), CD34 (1G12), CD80 (L307.4), and CD154 (89-76; all from Becton Dickinson, San Jose, CA); CD10 (H110A), CD33 (WM53), CD40 (SC3), CD45RA (H100), CD86 (IT2.2), and CD90 (SE10; all from PharMingen, San Diego, CA); CD83 (HB15, Immunotech, Westbrook, ME); MHC class I (H58A) and MHC class II (H42A, both from Veterinary Medical Research Development, Pullman, WA); c-fms (3-4A4), TNF receptor 1 (N-20), and TNF receptor 2 (C-20; all from Santa Cruz Biotechnology, Santa Cruz, CA). Appropriate conjugated isotype-matched Abs were used as controls. Ten thousand cells were analyzed on a Coulter Elite flow cytometer (Coulter, Hialeah, FL) through a viable cell gate as determined by forward and right angle light scatter parameters to exclude subcellular particles.

Apoptotic cells were identified by annexin V staining as previously described (36). Briefly, cells were washed twice with cold PBS and then resuspended in a binding buffer (from the manufacturer) containing HEPEs with 2.5 mM CaCl2 at a concentration of 1 × 106/ml. One hundred microliters of this suspension was reacted with 10 μl of annexin V-FITC (10 μg/ml (R&D Systems)) and 10 μl of propidium iodide reagent (50 μg/ml) in PBS. The mixture was gently vortexed and then incubated for 15 min at room temperature in the dark. Following incubation, 400 μl of the binding buffer (1× concentration) was added to each tube. Analysis by FACS was conducted within 1 h of assay completion for optimal results. Controls of unreacted cells, propidium iodide-stained cells, and annexin-V-FITC-stained cells were run first to optimize settings.
FITC-dextran uptake

Analysis of micropinocytosis by FITC-dextran uptake has been previously described (37). Activated KG1 and KG1a cells were resuspended at 10^6/ml in IMDM/20% FCS. FITC-dextran (Sigma) was added at a final concentration of 1 mg/ml, and the mixture was incubated at either 4 or 37°C for 30 min. The cells were washed four times with cold PBS containing 1% FCS and analyzed by flow cytometry.

T cell activation

Human PBMC were obtained by leukapheresis from normal healthy adult donors. T cells were purified by negative selection as previously described (38). Cells were resuspended in RPMI 1640 (Life Technologies) supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 20 mM HEPEs. T cells (1 x 10^6/well) were cultured in the absence (in medium) or the presence of the indicated number of gamma-irradiated (3000 rad ^137 Cs), in vitro-generated DC or DLC. Cultures were incubated for 3 days (DC) or 5 days (DLC) at 37°C in a humidified 5% CO_2 in air atmosphere. T cell proliferation induced by freshly isolated allogeneic PBMC was used as a reference. Proliferation was assessed after the addition of 0.5 µCi/well [methyl-3H]thymidine (New England Nuclear, Boston, MA) for the final 24 h of culture. Cells were harvested using a 96-well cell harvester, and [methyl-3H]thymidine incorporation was measured using a Beta Plate scintillation counting system (Pharmacia/LKB, Gaithersburg, MD). All determinations were performed in triplicate and expressed as the mean counts per minute ± 1 SD.

RT-PCR and Northern blot analysis

Total RNA was isolated from cultured cells by the RNA STAT-60 (Tel-Test) according to manufacturer’s recommendations. Ten micrograms of purified RNA was incubated at 37°C for 15 min with 100 U of RNase-free DNase in a 50-µl reaction, followed by phenol/chloroform extraction and ethanol precipitation. Samples of 4 µg of DNAse-treated RNA were reverse transcribed to cDNA by random primer extension in the presence of 0.1 µCi of [α-32P]dATP using Superscript-II Reverse Transcriptase (Life Technologies) following the manufacturer’s instructions. The RT reaction was terminated by heating at 70°C for 5 min, and radiolabeled cDNA was then purified on Spin-20 columns (International Mould Engineering, Onderton, MD) by centrifugation at 3500 rpm for 3 min. To normalize the input in all cDNA samples, equal amounts of cDNA loads were cloned in each PCR reaction containing 0.5 µM gene-specific primer pairs. Oligonucleotide primers specific for human DC-CK1 (sense, AGT CCC ATC TGC TAT GCC CAG; antisense, TAC GAA GAG TGG AAG GGA AAG) and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH; sense, ATG GAG GAG GTG ACG GTC GGA GCA GGA; antisense, ACG GGG CAG AGA TGA TGA TGG CTC) were used. PCR was performed at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min for 30 cycles followed by a final extension time of 10 min at 72°C. The amplified products (15 µl) were then incubated at 94°C for 5 min with 1 x 10^6 cpm of purified [α-32P]dATP-labeled gene-specific probes followed by an incubation at 55°C for 10 min. Hybridized PCR products were then resolved on 6% PAGE followed by quantitation on a PhosphorImager scanner using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Northern blot analysis was performed as previously described (39). Briefly, total RNA from unstimulated and stimulated KG1 and KG1a cells were isolated at the times indicated and equalized by serial dilution and quantitated by ethidium bromide visualization. Equal amounts of RNA were separated on a 6% PAGE followed by quantitation on a PhosphorImager scanner using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Results

Inhibition of PKC activation blocks cytokine-driven CD34+ HPC→DC differentiation

We have previously shown that PMA activation of PKC induces human CD34+ HPC→DC differentiation, which could be blocked by the PKC inhibitor staurosporine. To assess whether cytokine-induced CD34+ HPC→DC differentiation also involves PKC intracellular signaling, we cultured CD34+ HPC in GM-CSF and TNF-α for 14 days with or without 0.2 nM staurosporine, a dose 3.5-fold less than the IC_{50} of PKC (0.7 nM; Calbiochem Technical Report) but at least 10- to 200-fold lower than the IC_{50} for protein kinase A and tyrosine kinases (40). Cell viability at 14 days was >90% in both culture conditions, but there was a greater expansion of cell number without staurosporine (3 x input number vs 1 x with staurosporine). As can be seen in Fig. 1A, CD34+ HPC cultured in cytokine alone developed characteristic DC morphology (loosely adherent large cells with irregular shape, prominent dendritic processes, and hair-like cytoplasmic projections). In contrast, cultures containing staurosporine lacked cells with neurite processes and had greatly reduced numbers with hair-like cytoplasmic projections.

Staurosporine treatment also resulted in the loss of cells expressing characteristic DC surface markers, CD83 (DC lineage specific), MHC class II (bright), and the costimulatory molecules CD80 and CD86 (Fig. 1B). Of particular significance were the loss of CD83- and CD80-positive cells, as these Ags are not expressed on monocytes (resting, in the case of CD80) (41, 42). In contrast to CD83 and CD80, there was only a modest decrease in CD14- (monocyte marker) cells and no loss of CD33 + (myeloid marker) cells. Cells from both culture conditions were CD34 negative and CD13 bright, demonstrating that staurosporine did not nonspecifically block all differentiation. These results suggest that staurosporine preferentially inhibited generation of CD83+ DC compared with CD14+ monocytes.

Given that DC are the most effective APC in activating T cells, the decreased numbers of DC in the staurosporine-treated cultures should be reflected in a similar decrease in functional potency. CD34+ HPC cultured in GM-CSF and TNF-α with staurosporine were significantly less effective in inducing allogeneic T cell proliferation than those cultured in cytokine alone, particularly at a low APC/T cell ratio (Fig. 1C). However, both were still superior to an unselected population of PBMC.

Together these data suggest that inhibition of PKC activation preferentially suppressed generation of DC from CD34+ HPC by GM-CSF and TNF-α, findings consistent with the differentiating effects of phorbol ester. However, the scarcity, heterogeneity, and difficulty of manipulating primary CD34+ HPC made further analysis of the relevant signaling and genetic events difficult. To overcome these obstacles we sought to establish a human cell line model of CD34+ HPC→DC differentiation. Although a heterogeneous lineage, DC share a constellation of defining characteristics, including morphology, lineage markers, function, and gene expression, that can be used to identify potential DLC.

KG1 develop characteristic DC phenotype (morphology, surface Ag expression) in response to cytokines or PMA

We first examined the PMA-responsive CD34+ myeloblast cell line KG1. KG1 cells in medium alone were typically round nonadherent cells (Fig. 2). When cultured in GM-CSF and TNF-α (with or without IL-4), PMA or PMA plus TNF-α (and PMA plus ionomycin; not shown), KG1 became loosely adherent and a sub-set developed long neurite processes and hair-like cytoplasmic projections (best seen by Wright staining; not shown), morphology
reported to be characteristic of DC (12). Morphologic changes began within 30 min of stimulation, were fully manifested within 48 h, and were stable over at least 2 wk in culture. Development of the dendrite/neurite morphology was progressively more prevalent and pronounced with GM-CSF plus TNF-\(\alpha\) plus PMA plus TNF-\(\alpha\) (and PMA plus ionomycin). In contrast to these stimuli, there was no effect of individual cytokines (GM-CSF, IL-4, or TNF-\(\alpha\)), a cytokine combination that does not induce DC differentiation (GM-CSF, IL-3, IL-6, and SCF), ionomycin alone, or ionomycin plus TNF-\(\alpha\) (data not shown). While KG1a also became adherent when similarly stimulated, they did not change morphology.

Mature DC are typically characterized by expression of MHC class I, MHC class II (high), the costimulatory molecules CD80 (B7-1) and CD86 (B7-2), and the DC lineage marker CD83 (reviewed in Ref. 12). Unstimulated KG1 cells expressed MHC I, MHC II (bright), the myeloid markers CD13 and CD33, CD34, CD45RA, and CD86 (dim) but not CD40, CD80, the DC marker CD83, or the monocyte marker CD14 (Table I). KG1a had lower expression of MHC class II and no CD86 staining. We also found that KG1 and KG1a differed markedly in expression of TNF-\(\alpha\) receptors 1 and 2 (Fig. 3), which may in part underlie the differences in TNF-\(\alpha\) responsiveness between the two cell lines.

KG1 stimulated with GM-CSF plus TNF-\(\alpha\), PMA, PMA plus TNF-\(\alpha\), or PMA plus ionomycin up-regulated CD83 expression with the same temporal kinetics as the morphology changes (Fig. 4). The level of CD83 expression has been correlated with the degree of DC maturation (43), and CD83 up-regulation in KG1...
was typically highest with PMA and TNF-\(\alpha\) or PMA and ionomycin. PMA also up-regulated CD40 expression (not shown) and modestly down-regulated MHC II, which was reversed by addition of TNF-\(\alpha\) or ionomycin. Up-regulation of CD86 was most pronounced with addition of ionomycin, and ionomycin alone could up-regulate CD86 without inducing CD83 expression (data not shown). PMA plus ionomycin also induced expression of CD80, although typically at low levels (not shown). The combination of PMA, ionomycin, and TNF-\(\alpha\) was no different from PMA plus TNF-\(\alpha\) or PMA plus ionomycin alone, suggesting that TNF-\(\alpha\) and ionomycin deliver the same signal. Individual cytokines (GM-CSF, IL-4, TNF-\(\alpha\)) or the cytokine combination GM-CSF, IL-3, IL-6, and SCF did not up-regulate CD83 or CD86 expression on KG1. None of the stimuli tested induced expression of CD1a or the monocyte marker CD14. In contrast to KG1, KG1a did not respond to GM-CSF plus TNF-\(\alpha\) or PMA with or without ionomycin.

Signals that induce KG1→DLC differentiation also suppress proliferation and trigger programmed cell death

In primary CD34\(^+\) HPC we found the two hallmarks of PMA-driven DC differentiation are the complete inhibition of proliferation (even to exogenous cytokines) and induction of apoptosis (14). Proliferation is also absent in monocyte→DC differentiation driven by GM-CSF, IL-4, and TNF-\(\alpha\) (4) or calcium ionophore (11). As shown in Fig. 5, KG1 proliferation was suppressed by factors that induce DC/DLC differentiation (PMA (consistent with the findings presented in Ref. 44), TNF-\(\alpha\), and most significantly by PMA plus TNF-\(\alpha\), but not by a cytokine combination (GM-CSF, IL-3, IL-6, and SCF) that does not generate DC. Baseline proliferation of KG1a was 2.6-fold higher than that of KG1 and was unaffected by these stimuli (data not shown). Because the day 5 viability of KG1 was decreased in PMA (75% viable), PMA plus TNF-\(\alpha\) (70%), and PMA plus ionomycin (67%), we examined the induction of apoptosis by these stimuli (Table II). In KG1, apoptosis was triggered within 4 h by PMA plus ion and within 24 h by PMA plus TNF-\(\alpha\). Smaller increases in apoptotic cell numbers were seen at 24 h in cultures treated with PMA or TNF-\(\alpha\) alone,

| Table I. Surface Ag expression of unstimulated KG1 and KG1a\(^a\) |
|-----------------------------|---------------------|
| MHC class I | ++++ | ++++
| MHC class II | ++++ | ±
| CD1a | – | –
| CD2 | – | –
| CD10 | – | –
| CD13 | ++ | +++
| CD14 | – | –
| CD28 | – | –
| CD33 | ++ | +++
| CD34 | ++++ | ++++
| CD40 | – | –
| CD45RA | + | +
| CD80 (B7–1) | – | –
| CD83 | – | –
| CD86 (B7–2) | – | –
| CD90 (Thy-1) | – | –
| CD152 (CTLA4) | – | –
| CD154 (CD40L) | – | –
| c-fms | ± | –

\(^a\) Each + indicates an increase of 0.5 logs in mean fluorescent intensity vs. isotype matched control.

FIGURE 2. Morphology of KG1 stimulated with cytokines and/or PMA. Photomicrographs of day 7 cultures of KG1 in medium alone, GM-CSF (200 U/ml) plus TNF-\(\alpha\) (2.5 ng/ml), PMA (10 ng/ml), or PMA plus TNF-\(\alpha\) (10 ng/ml). Arrows point to neurite processes. KG1 in medium alone were nonadherent; in all other conditions they were adherent.

FIGURE 3. KG1 and KG1a expression of TNF-\(\alpha\) receptors 1 and 2. Unstimulated KG1 and KG1a were analyzed by FACS for TNF receptor 1 and 2 expression as described in Materials and Methods. The isotype-matched control is the dotted line, and Ag-specific Ab the solid line.
Correlating with a lesser inhibition of cell proliferation by these single factors. Also consistent with the proliferation data was the failure of any stimuli to induce apoptosis in KG1a (the greater baseline percentage of apoptotic cells was probably due to the higher proliferative rate).

Fluid phase macromolecule uptake by KG1 and KG1-DLC

Ag uptake (via receptor-mediated endocytosis (Fcγ, mannose receptors) or macropinocytosis of fluid phase molecules) is a functional characteristic of both DC and CD34+CD86+ DC precursors (7, 37). Ag capture (as measured by FITC-dextran uptake) changes from a high capacity in immature/unactivated DC to a lower capacity in mature/activated DC (37). Unstimulated KG1 took up FITC-dextran, and PMA did not affect uptake even though it induced other characteristic DC changes (Fig. 6). However, TNF-α alone or in combination with PMA down-modulated macromolecule uptake, suggesting that TNF-α (but not PKC activation) delivers a distinct signal that mediates the decreased Ag uptake capability associated with DC maturation.

KG1-DLC induce allogeneic T cell proliferation

The most distinctive functional characteristic of DC is the potent ability to activate T cells (45). We found that PMA-generated KG1

Figure 4. Surface Ag phenotypes of KG1 and KG1a in response to cytokine and/or PMA stimulation. KG1 cells were cultured in medium alone, GM-CSF (200 U/ml) plus TNF-α (2.5 ng/ml), PMA (10 ng/ml), PMA plus TNF-α (10 ng/ml), or PMA plus ionomycin (200 ng/ml). KG1a were cultured in PMA (10 ng/ml). Cells were harvested and analyzed by FACS. Ag-specific staining is indicated by the solid line, and the isotype-matched control is shown by the dotted line. The time of maximal surface expression varied depending on the stimulus, shown here are GM-CSF plus TNF-α and PMA plus ionomycin (day 3 in culture), and medium, PMA, and PMA plus TNF-α (day 7 in culture).

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KG1-DLC induce allogeneic T cell proliferation

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Figure 5. Inhibition of KG1 proliferation. KG1 were cultured at the cell numbers and in the conditions noted. After 54 h of culture, cells were pulsed with 0.5 μCi/well [methyl-3H]thymidine before being harvested at 72 h. [3H]TdR incorporation was measured using a Beta Plate scintillation counting system.

Figure 6. Fluid phase macromolecule uptake by KG1 and KG1-DLC. KG1 were unstimulated (medium) or cultured in PMA, TNF-α, or PMA plus TNF-α for 5 days. FITC-dextran uptake was then assayed as described in Materials and Methods and analyzed by flow cytometry. FITC-dextran uptake at 4°C by unstimulated KG1 served as the negative control.

Table II. Induction of apoptosis in KG1 and KG1a

<table>
<thead>
<tr>
<th>Condition</th>
<th>KG1 4 h</th>
<th>KG1 24 h</th>
<th>KG1a 4 h</th>
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*Cells were stimulated as indicated and assessed for induction of apoptosis (by annexin V binding) at the times indicated.
DLC were capable of stimulating allogeneic T cell proliferation (Fig. 7) and to a comparatively greater extent than unselected PBMC (as a reference population of APC). KG1 cultured in PMA and PMA plus ionomycin were significantly more potent at inducing T cell proliferation than KG1 cultured in medium, KG1α cultured in PMA with or without ionomycin, or unselected PBMC. Consistent with the morphology and surface phenotype findings above, ionomycin appeared to drive further maturation/activation over PMA alone as measured by allogenoproduction. Lack of significant T cell proliferation with PMA-treated KG1α cells demonstrates the lack of PMA carryover from the original KG1/KG1α differentiation cultures.

**KG1-DLC express characteristic DC genes**

Characterization of DC has classically relied on morphology, surface Ag expression, and function. Recent molecular studies have identified genes that are expressed predominantly or exclusively by DC. These include the RelB transcription factor (see below) and the chemokine DC-CK1. The DC-CK1 chemokine is expressed only by DC and not other APC, and appears to function as a chemottractant for naive T cells (46). Unstimulated KG1 expressed low levels of DC-CK1 that were substantially up-regulated by PMA (Fig. 8A). Unexpectedly, the addition of TNF-α inhibited DC-CK1 up-regulation in KG1. The converse response was seen in KG1α (Fig. 8B), where PMA has no effect on DC-CK1 expression but PMA plus TNF-α results in up-regulation, arguing against a direct inhibitory effect of TNF-α. Since PMA plus TNF-α induces early DLC differentiation in KG1α (based on CD83 expression), these findings suggest that DC-CK1 expression is maximal during early/intermediate stages of DC maturation/activation (induced by PMA in KG1, PMA plus TNF-α in KG1α) with much less expression in uncommitted progenitors (KG1α) or fully mature/activated DC (PMA- plus TNF-α-treated KG1).

Together, these findings demonstrate that in response to specific stimuli (GM-CSF plus TNF-α, PMA with or without TNF-α, or ionomycin) KG1 undergo differentiation to cells that have morphology, surface Ag phenotype, function, and gene expression characteristic of DC. In addition, specific aspects of this differentiation appear to be mediated by specific signals.

**Role of PKC in PMA-driven KG1→DLC differentiation**

Although PMA is a stable 2,3-diacylglycerol analogue and specific activator of the classical and new isoforms of PKC (47), it is formally possible that PMA’s effects in KG1 are due to activation of a non-PKC phorbol ester receptor (such as Unc-11 or n-chimaerin) (48–50). To more rigorously prove specific PKC activation, KG1 were cultured in PMA with or without TNF-α with the highly selective PKC inhibitor bis. Bis inhibited adhesion and morphologic changes in KG1 cultured in PMA without affecting cell viability (Fig. 9A). By FACS, bis inhibited up-regulation of CD83 and CD86 in response to both PMA and PMA plus TNF-α (Fig. 9B). Bis also prevented cell death induced by PMA plus TNF-α (91% viable with PMA, TNF-α, and bis on day 5 vs 37% viable with PMA plus TNF-α alone). Finally, the addition of bis inhibited the development of allostimulatory capacity in KG1 cultured in PMA or PMA plus ionomycin (Fig. 9C). The fact that bis inhibition is considerably less effective with the addition of ionomycin is probably due to a lowered PKC activation threshold caused by increased intracellular calcium.

To further determine the nature of PKC-mediated signal transduction KG1→DLC differentiation, we asked whether the differing PMA responsiveness of KG1 vs KG1α was due to differing PKC isoform expression. There are at least 11 different isoforms of PKC (47), with individual isoforms appearing to mediate specific biological function (51, 52). As seen in Fig. 10, unstimulated KG1 expressed substantially more PKCα than KG1α. Unstimulated KG1α also did not express PKCβ1, -βII, and -μ, in contrast to KG1. Both KG1 and KG1α expressed PKCε, and neither expressed PKCγ (data not shown).

**Induction of RelB expression during PMA-driven KG1→DLC differentiation**

RelB is a member of the NFκB transcription factor family that is highly expressed in DC but not monocytes (2) and may play an important role in DC differentiation and function. Because up-regulation/activation of RelB by PMA may represent the pathway by which PKC-mediated signaling gains access to the nucleus, we
examined the kinetics of RelB expression following PMA activation of KG1 and KG1a. As can be seen in Fig. 11A, RelB gene expression in KG1 was up-regulated within 24 h, plateaued through 72 h, and then declined. Unexpectedly, unstimulated KG1a constitutively expressed RelB message, which also declined after 72 h in culture with PMA. Protein expression paralleled the inducible KG1 or constitutive KG1a gene expression of RelB (Fig. 11B). Of note is the rapidity with which RelB protein was induced in KG1, appearing within 30 min of PMA stimulation. The persistence of RelB protein after gene down-regulation suggests a relatively slow turnover rate. By electromobility shift assays the levels of free (i.e., not bound to IκB) RelB heterodimers capable of binding to NFκB sites were also up-regulated by PMA in KG1, while levels in KG1a were comparatively stable (Fig. 11C, upper panels). Examination of other NFκB family members in KG1 and KG1a revealed that nearly all protein capable of binding NFκB sites contained p50 (NFκB1) as one subunit (Fig. 11C, lower panels) as evidenced by the nearly complete supershifting by anti-p50 Abs. This suggests that in KG1/KG1a RelB exists as a RelB/p50 heterodimer.

Discussion

Why hemopoietic stem cells differentiate (or do not) into a specific mature lineage remains one of the enduring biological riddles. Seminal work by a number of groups (53–68) have identified the critical receptor-mediated stimuli (cytokines, CD40) that induce lineage commitment of human multipotential CD34+ HPC to DC in vitro. We have sought to further characterize the intracellular signaling pathways that connect these receptor/membrane events to the nucleus. We have previously shown that the PKC agonist PMA by itself directly triggers differentiation of human CD34+ HPC to DC, suggesting a critical and specific role of this pathway in lineage commitment to DC (14). This is supported by observations that the receptor-mediated stimuli (GM-CSF, IL-4, TNF-α, and CD40 cross-linking) that induce DC differentiation all can activate PKC as part of their intracellular signaling pathways (15–19, 69). We now find that the PKC inhibitor staurosporine blocks cytokine-driven CD34+ HPC to DC differentiation, indicating that PKC is part of the signaling cascade initiated by GM-CSF and TNF-α. Although staurosporine can also block tyrosine kinase activity, this is typically at a substantially higher dose than that used in these experiments (40). We have also found that more specific PKC inhibitors (e.g., bis) similarly inhibit CD34+ HPC to DC differentiation (manuscript in preparation). Staurosporine did not globally block differentiation as all cells lost the CD34 marker, and the generation of CD14+ cells (most likely monocytes) was comparatively less affected. Rather, the generation of CD83+ DC was preferentially inhibited. When considered with our previous findings that PMA treatment of CD34+ HPC only generates DC (and
Materials and Methods

scribed in RelB mRNA expression. Cells were harvested and mRNA isolated as de-

A

Expression of RelB in PMA-stimulated KG1 and KG1a.

Total RNA

RelB

Actin

B

KG1

KG1a

0.5 hr 1 hr 6 hr 24 hr 72 hr

T = 0 hr

C

KG1

KG1a

anti-RelB

anti-p50

0.5 hr 1 hr 6 hr 24 hr 72 hr

T = 0 hr

FIGURE 11. Expression of RelB in PMA-stimulated KG1 and KG1a. KG1 and KG1a were cultured in 10 ng/ml PMA for the times indicated. A, RelB mRNA expression. Cells were harvested and mRNA isolated as described in Materials and Methods. Total mRNA was equalized between samples by ethidium bromide visualization and serial dilution. Samples were separated on formaldehyde agarose gels, transferred to nylon, and probed with 32P-labeled RelB cDNA probe. The same blot was then re-probed for actin expression to verify mRNA equalization and integrity. B, RelB protein expression. Cell lysates from the indicated times points were made, and equal amounts of protein were loaded onto a SDS-PAGE gel and analyzed by Western blotting with the anti-RelB Ab C-19. C, RelB and p50 electromobility shift assay. Cell lysates were made at the times indicated. Equal amounts of protein were incubated with a labeled primer containing the NFkB binding site with or without anti-RelB or anti-p50 mAb. Samples were separated on 4% polyacrylamide gel and exposed to film. Complexes that specifically contain the labeled primer, Rel B (or p50), and anti-RelB (or p50) Ab are indicated by the supershift arrow.

no other myeloid lineages), these data indicate that the intracellular pathway that specifically initiates DC lineage commitment goes through PKC. This “wiring” may be unique to CD34+ HPC, as CD34 negative progenitors and monocytes have different responses to PMA (K. P. Lee and K. Schlienger, unpublished observations).

To further study the intracellular events involved in DC differentiation and to circumvent the problems of studying this in primary CD34+ HPC, we established a human leukemic cell line model of CD34+ HPC→DC differentiation. Work demonstrating that primary chronic myelogenous leukemia cells differentiated in vitro to functional DC suggested that this was feasible (70). Examination of the PMA responsiveness of a panel of CD34+ leukemic cell lines revealed that KG1 differentiated into cells with DC characteristics. KG1 was isolated from a patient with erythroleukemia (FAB M7) undergoing myeloblastic relapse (28). Serial passage of KG1 gave rise to the KG1a subline (71), which by a number of criteria (surface phenotype, gene expression, and growth factor unresponsiveness) is thought to be arrested at a less differentiated stage than KG1 (32, 72, 73). In response to PMA KG1 was initially characterized to differentiate into macrophages (74), although these original morphologic (adherent cells with long pseudopodia), histochemical (nonspecific esterase-positive, myeloperoxidase-negative), surface Ag (MHC II- and Fc receptor-positive), and functional (phagocytosis) findings have since been found to also be characteristic of DC (reviewed in Ref. 12). PMA does not induce differentiation in KG1a, and although KG1 and KG1a have similar numbers of phorbol ester binding sites, they differ in PKC activity, translocation, and substrate phosphorylation (30, 33, 34, 74).

We believe that recharacterization of the KG1 response to PMA reveals it to actually recapitulate differentiation of the committed CD34+ myeloid DC/macrophage progenitor to DC. Like the committed DC/macrophage progenitor (7), KG1 express MHC II, CD34, and CD86, whereas KG1a are MHC II-CD34+CD86-. Unstimulated KG1 express little or no CD40, but up-regulate expression following culture in PMA. Stimuli shown to induce CD34+ HPC→DC differentiation (GM-CSF plus TNF-α, PMA) also induce differentiation of KG1 to DLC (KG1-DLC), while non-DC cytokine(s) (GM-CSF or TNF-α alone or GM-CSF, IL-3, IL-6, and SCF) do not. Unlike monocytes, ionomycin alone does not induce DC differentiation in KG1 (or primary CD34+ HPC), and likewise, PMA (as a single agent) does not drive DC differentiation in monocytes. Suppression of KG1 cell proliferation and induction of apoptosis by PMA are also characteristics we reported for primary CD34+ HPC→DC differentiation driven by PMA.

Stimulation of KG1 with GM-CSF and TNF-α or ionomycin generates cells with a constellation of DC characteristics. Morphologically, KG1-DLC are adherent with long neurite processes in culture and have hair-like processes/veils on cytospin. By surface Ag phenotype, KG1-DLC have characteristic DC expression of MHC I, MHC II (high), CD13, CD33, the DC-specific lineage marker CD83, and the costimulatory ligand CD86 (reviewed in Ref. 12). They do not express CD1a or the DC-specific marker CD14. Expression of CD80 is present but low, suggesting that KG1-DLC are not fully mature or activated (12). Functionally, unstimulated KG1 can take up fluid phase Ag as measured by FITC-dextran uptake. TNF-α appears to deliver a signal that down-regulates Ag uptake, a hallmark of DC maturation/activation (37) consistent with the other effects (morphology, surface Ag phenotype) that TNF-α has on PMA-driven KG1→DLC differentiation. Most importantly, KG1-DLC can function to activate T cells as evidenced by proliferation in an
The inability to induce DLC differentiation in KG1a allows for comparison with KG1 of potential signaling pathways. Blockade of DC differentiation in both KG1 and primary CD34+ HPC by PKC inhibitors establishes a central role for PKC. PKC activation most likely plays a direct role and does not simply induce autocrine secretion of cytokines; for example, we find no evidence (by PCR) for induction of TNF-α expression in KG1. However, the difference between KG1 and KG1a cannot simply be the absence of PKC signaling in KG1a given that both cell lines have biochemically measurable PKC activity (75). Likewise, we have found that although both CD34+ HPC and CD34+ HPC respond to PMA, only CD34+ HPC differentiate to DC while CD34+ HPC become macrophages. This suggests that there is a qualitative component of PKC activation. Consistent with this is the differing expression of specific PKC isoforms in the cell lines. KG1a express less PKCα and no PKCβ1, -βII, or -μ compared with KG1. Given that specific PKC isoforms have been associated with specific biological events in other systems (e.g., PKCδ in T cell activation (51) and PKCβ in macrophage differentiation of HL-60 (52)), these findings suggest that the same may be true for DC differentiation. Examination of the roles of specific PKC isoforms is currently underway.

KG1a also differ from KG1 in the response to TNF-α; this is most evident in the induction of apoptosis. This difference may be due to comparatively lower expression of both TNF receptors 1 and 2 on KG1a, and this membrane “defect” is consistent with previous reports demonstrating that apoptotic responses in KG1a can be restored by directly manipulating the sphingomyelin-ceramide pathway triggered by TNF-α (76, 77). The roles of ceramide (and intracellular calcium flux) in both apoptosis and DC differentiation are under study.

In addition to the differences between KG1 and KG1a, we found that distinct signals appeared to mediate distinct, nonoverlapping biological phenomena during KG1→DLC differentiation. PKC activation by PMA appears to be a requisite inductive signal, initiating morphologic changes, surface Ag expression, and allostimulatory capacity for T cells. However, the addition of a TNF-α or calcium flux signal is necessary for full maturation/activation (as assessed by morphology, surface Ag expression, and T cell proliferation) and inhibition of cell proliferation/induction of apoptosis. Down-regulation of soluble macromolecule uptake appears to be mediated entirely by TNF-α and to be independent of PKC activation. The possibility that TNF-α and ionomycin are signaling through the same pathway is suggested by the lack of an additive effect when the two are combined (PMA, TNF-α, and ionomycin) and is being examined. Together, these data suggest that there are discrete signaling pathways involved in specific aspects of DC differentiation; PKC activation is required to initiate differentiation, and TNF-α (which initiates several intracellular pathways) and/or intracellular calcium flux is required for terminal maturation/activation and apoptosis. However, this does not explain why some single signals (PMA or CD40 cross-linking in primary CD34+ HPC, calcium ionophore in monocytes) are capable of driving complete DC differentiation/maturation from progenitors.

One possibility is that those particular pathways in those particular progenitors are directly linked to the genetic DC differentiation program. Another possibility is that the primary signal in these specific progenitors triggers autocrine secretion of additional cytokines/factors that drive terminal maturation. We are currently examining whether PMA induces TNF-α expression or calcium flux in primary CD34+ HPC.

Regardless of the specific pathway, differentiation signals have to cross into the nucleus to initiate the requisite genetic programs. Such bridges are very often preformed transcription factors, of which the NFκB family is one of the best characterized (reviewed in Ref. 78). Critical involvement of NFκB-responsive genes in DC differentiation has been found in chicken bone marrow cell lines (79). Involvement of rellNFκB family transcription factors in DC differentiation is further suggested by the observations that RelB (80, 81) is expressed at high levels in murine DC (82) and that RelB knockout mice have significant reductions in mature DC number and APC function (83, 84). In human DC nuclear localization of RelB is associated with enhanced APC function (85).

Both PMA and TNF-α up-regulate the activity of NFκB family members (including RelB by PMA) (86), and RelB up-regulation is seen during CD34+ HPC→DC (14) and monocyte→DC (2) differentiation. This is also true for KG1→DLC differentiation. However, constitutive expression of RelB in KG1a suggests that DC differentiation may not simply be due to activation of RelB. It is also possible that tonic expression of RelB somehow blocks induction of NFκB-responsive genes as has been suggested for conditional v-rel expression (79). It is unclear why RelB expression is not regulated in KG1a, but this suggests that some negative feedback signal has been lost. The rapid up-regulation of RelB expression induced by PMA in KG1a may indicate the involvement of a more immediate transcription factor, although NFκB can mediate its own transcription (78).

Together, the data presented in this report corroborate an important role for PKC activation in DC differentiation from CD34+ HPC. Characterization of the KG1 cell line model of CD34+ HPC→DC differentiation allows for a more detailed study of the signal transduction pathways involved. Our initial studies indicate that the PKC, TNF-α, and/or intracellular calcium signaling pathways mediate specific phenomenon during this differentiation. A potential connection of these pathways to the RelB transcription factor may provide a doorway into uncovering the genes that control DC differentiation and underlie the unique function of this lineage.

References


