Stromal Inhibition of Megakaryocytic Differentiation Correlates with Blockade of Signaling by Protein Kinase C-ε and ERK/MAPK*

Received for publication, April 27, 2001
Published, JBC Papers in Press, June 6, 2001, DOI 10.1074/jbc.M103825200

Adam N. Goldfarb‡§, Loretta L. Delehanty‡, Dongyan Wang‡, Frederick K. Racke¶, and Isa M. Hussaini‡

From the ‡Department of Pathology, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908 and the ¶Department of Pathology, Johns Hopkins Medical Institutions, Baltimore, Maryland 21287

Contact with bone marrow stromal cells maintains normal and leukemic hematopoietic progenitors in an undifferentiated state. Recently, stromal contact has been shown to diminish the yield of megakaryocytes in cultures of primary human hematopoietic stem cells. This inhibition may explain the poor megakaryocytic engraftment frequently observed after bone marrow transplantation. In the current study, stromal co-culture is shown to render K562 cells refractory to megakaryocytic induction. This stromal inhibition correlated with the selective down-regulation in K562 cells of protein kinase C-ε (PKC-ε), which has recently been implicated in regulation of megakaryocytic lineage commitment. In addition, the stromal inhibition correlated with inactivation of the ERK/MAPK pathway, which has also been implicated in promoting megakaryocytic development. Forced expression of PKC-ε by retroviral transduction was insufficient to reverse the stromal blockade of ERK/MAPK signaling or of megakaryocytic induction. Thus stromal interruption of ERK/MAPK signaling occurred independently of PKC-ε levels and correlated more closely with megakaryocytic blockade. These findings provide potential mechanisms for stromal inhibition of hematopoietic differentiation and possibly for the poor megakaryocytic engraftment seen after bone marrow transplantation.

The influence of bone marrow stromal cells on hematopoiesis has been the subject of intensive investigation for many years. Dexter et al. initially showed that stromal cells could support long term hematopoiesis in vitro through a mechanism in which hematopoietic stem cells were sustained in an undifferentiated, proliferative state (1). Whitlock and Witte similarly demonstrated the importance of bone marrow stromal cells in maintenance of B cell progenitors in long term culture conditions (2). As with primary progenitor cells, leukemic cells also have demonstrated reliance on stromal cells for survival and proliferation (3). A critical aspect of stromal function in the majority of these co-culture systems appears to be the inhibition of hematopoietic differentiation.

Stromal influences on hematopoietic differentiation occur through multiple mechanisms including secretion of soluble factors, elaboration of extracellular matrix, and direct contact (4). In several cases, a direct contact mechanism has been associated with dramatic inhibition of hematopoietic differentiation. For example, differentiation blockade of 32D myeloid progenitor cells occurred through engagement of their receptor Notch1 by the stromally displayed hJagged1 ligand (5). Recently, direct stromal contact was shown dramatically to diminish the yield of megakaryocytes from primary human CD34+ stem cells by an unknown mechanism (6).

To understand how the bone marrow microenvironment might impair megakaryocytic development, we have sought to characterize the effects of stromal co-culture on megakaryocytic lineage commitment. Of particular interest are signal transduction pathways critical in megakaryopoiesis that might be perturbed by stromal contact. One such pathway is that involving ERK/MAPK,1 sustained activation of which is required for proper megakaryocytic differentiation in a number of model systems including primary human stem cell cultures (7–11). Another essential signaling element is protein kinase C (PKC), in particular the ε isozyme, which potentiates GATA-1 activation of a megakaryocytic promoter (12). Treatment of cell lines as well as primary human stem cells with the PKC-ε-selective agonist ingelase 3,20-dibenzooate promotes megakaryocytic differentation (12, 13). Furthermore, evidence also supports cross-talk between the PKC-ε and ERK/MAPK signaling pathways (14, 15).

We report here that co-culture of K562 cells with a stromal monolayer strikingly rendered these cells completely resistant to megakaryocytic induction with highly potent stimuli such as the phorbol ester, TPA (12-O-tetradecanoylphorbol-13-ester), and TPA-conditioned medium (7, 12). For biochemical characterization, TPA-treated K562 cells grown alone or in stromal co-cultures were compared for expression of an array of PKC isozymes. While treated cells cultured alone showed marked up-regulation of PKC-ε protein levels, in stromal co-cultures treated (and untreated) cells showed complete loss of detectable PKC-ε protein and mRNA. Such effects were not observed for any PKC isozyme other than ε. Analysis of ERK/MAPK activation showed strong, sustained induction of ERK phosphorylation in treated cells cultured alone. By contrast, treated cells co-cultured with stroma showed complete loss of detectable ERK activation.

We next determined whether down-regulation of PKC-ε levels, as opposed to blockade of ERK phosphorylation, represented the primary mechanism for stromal inhibition of differentiation. Accordingly, enforced PKC-ε expression was achieved by retroviral...
transduction of cells. Despite high levels of PKC-ε in transduced cells, stromal co-culture still abrogated ERK phosphorylation secondary to TPA treatment. Furthermore, although the PKC-ε-transduced cells showed evidence of spontaneous differentiation, the cells remained completely susceptible to stromal blockade of megakaryocytic induction. We conclude, therefore, that stromal contact may potently block megakaryocytic lineage commitment through multiple parallel mechanisms including interference with sustained ERK/MAPK signaling and inactivation or down-regulation of PKC-ε.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The bone marrow stromal cell line, HESS-5, was kindly provided by Dr. Takashi Tsuji of the Pharmaceutical Frontier Research Laboratory (Japan Tobacco, Inc., Yokohama, Japan) (16). These cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. The human hematopoietic cell line K562 was obtained from the American Type Culture Collection (ATCC) and grown in RPMI 1640 media with 10% fetal bovine serum at 37 °C, 5% CO₂. All experiments using K562 employed mid-log phase cells at a density of 0.5–1.0 × 10⁶ cells/ml. TPA-conditioned media was obtained as previously described by 72 h of treatment of either K562 or HEL cells with 25 nM TPA (Sigma) followed by harvesting and dialysis of the supernatant (7, 12). For megakaryocytic differentiation induction, cells were treated directly with 25 nM TPA or were resuspended in conditioned media followed by incubation at 37 °C, 5% CO₂ for 48 h. Co-cultures were initiated by seeding K562 cells onto confluent monolayers of HESS-5 cells grown in six-well plates. Harvesting of K562 cells off of the stromal monolayers was accomplished by repeated gentle pipetting of media to dislodge the weakly adherent cells. Microscopic examination was employed to rule out stromal disruption and contamination of harvested cells.

**Flow Cytometry**—Staining of cells for surface CD41a and glycophorin A employed the direct phycoerythrin-conjugated antibodies HIP8-PE and GA-R2-PE (PharMingen, San Diego, CA) at a concentration of 10 μg/ml. Phycoerythrin-conjugated, isotype-matched antibody controls were also used at 10 μg/ml. Flow cytometric analysis was performed on a FACScaliber system utilizing CellQuest software (Becton Dickinson, San Jose, CA).

**Immunoblot and Northern Blot Assays**—For immunoblotting, equivalent numbers of cells for each specimen were washed with phosphate-buffered saline and resuspended in 100 μl 1 x SDS-polyacrylamide gel electrophoresis loading buffer/10⁶ cells. Samples were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Equivalent lane loading was verified by Ponceau staining of membranes. Probing of membranes and chemiluminescent signal detection followed previously described protocols (12, 17). Antibodies to PKC isozymes consisted of rabbit polyclonal antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and were used at dilutions of 1/1000 or 1/2000. Anti-phospho-ERK consisted of a rabbit polyclonal antibody purchased from Promega (Madison, WI) and was used at a dilution of 1/2000. For Northern blot analysis total cellular RNA was isolated from cells using the Trizol reagent (Life Technologies, Inc.). 20 μg/lane of glyoxalated total RNA underwent electrophoresis on 1.0% agarose gels followed by electrotransfer to Zeta probe nylon membranes (Bio-Rad Laboratories). Prehybridization, hybridization with randomly primed 32P-labeled probes, and washing were carried out as previously described (17). The probe for PKC-ε consisted of a 1.9-kilbase HindIII fragment from the cDNA described by Soh et al. (15). The probe for glyceraldehyde-3-phosphate dehydrogenase has been previously described (17).

**Retroviral Transduction**—The retroviral vector consisted of MSCV-IRES-GFP (MIG) kindly provided by the laboratory of Dr. Stephen Goff (Howard Hughes Medical Institute, Columbia University College of Physicians and Surgeons) (18). To generate MIG, the full-length cDNA of PKC-ε, released as an Xhol fragment from the pHACE-ε plasmid (kindly provided by Dr. Jae-Won Soh, Herbert Irving Comprehensive
Cancer Center, Columbia University College of Physicians and Surgeons) (15), was ligated into the XhoI site of MIG. Generation of amphotropic retrovirus using transient transfection of the Phoenix packaging line and infection of cells by spinoculation followed the protocols posted by Dr. Gary Nolan (Department of Molecular Pharmacology, Stanford University School of Medicine) (www.stanford.edu/group/nolan/protocols.html). Retrovirally transduced, GFP+ cells were purified by sorting on a Becton Dickinson FACS Vantage cell sorter (Becton Dickinson).

RESULTS

Co-culture with Bone Marrow Stromal Cells Blocks Megakaryocytic Lineage Commitment—Previous studies have shown that inclusion of bone marrow stromal cells in megakaryocytic cultures of human CD34+ stem cells diminishes the yield of megakaryocytes by ~50% (6). This inhibitory effect is mediated by direct contact of hematopoietic progenitors with stromal monolayers (6, 19). We therefore tested whether stromal co-culture influenced the initial phases of megakaryocytic differentiation, i.e. lineage commitment, in a biochemically characterized model system of megakaryocytic induction (20).

Remarkably, co-culture of K562 cells with the bone marrow stromal cell line HESS-5 completely blocked the morphologic/light scatter changes associated with megakaryocytic induction, using either TPA-conditioned medium (Fig. 1) or direct TPA treatment (not shown). As shown in Fig. 2, the stromal blockade was not limited to morphologic/light scatter changes but also included the immunophenotypic changes associated with early megakaryocytic induction. In particular, stromal co-culture blocked both the up-regulation of the megakaryocytic marker CD41a (Fig. 2A), as well as the down-regulation of the erythroid marker glycophorin A (GPA) (Fig. 2B). Down-regulation of GPA has been shown to be a very sensitive marker of early megakaryocytic induction (21). Thus, stromal co-culture rendered K562 cells completely refractory to megakaryocytic induction by two potent stimuli, TPA-conditioned media and TPA itself.

Stromal Co-culture Abrogates PKC-ε Up-regulation and ERK/MAPK Activation—The PKC family has long been known to play a role in megakaryocytic differentiation (22–24). Recently, the PKC-ε isozyme has been specifically implicated in the programming of megakaryocytic lineage commitment (12). We therefore examined by immunoblot the expression of several PKC isozymes in K562 cells induced to undergo differentiation alone or in contact with stromal cells. As shown in Fig. 3, induction of megakaryocytic differentiation with either conditioned medium or TPA was associated with clear up-regulation of PKC-ε. None of the other PKC isozymes examined (α, γ, δ, η) showed up-regulation with megakaryocytic induction. Notably, stromal co-culture blocked both the up-regulation of PKC-ε associated with megakaryocytic induction. By contrast, none of the other PKC isozymes manifested down-regulation secondary to stromal contact. PKC-δ demonstrated weak up-regulation in K562 cells co-cultured with stroma.

To better understand the mechanism for stromal blockade of PKC-ε up-regulation, we performed Northern blots on cells induced to undergo megakaryocytic differentiation either alone

![Stromal Blockade Of Megakaryocytic PKC-ε and Erk Signaling](https://example.com/stromal-blockade.png)

**Fig. 3.** Stromal co-culture blocks both the up-regulation of PKC-ε and the activation of ERK that are associated with megakaryocytic induction. A, immunoblot analysis of PKC isozymes, phospho-ERK, and total ERK expression in K562 cells treated as indicated in the absence or presence of stromal monolayers. The membrane was stripped and reprobed multiple times for the assessment of the indicated proteins. B, Northern blot analysis of PKC-ε mRNA levels in K562 cells treated as indicated in the absence or presence of stromal monolayers. To control for lane loading the membrane was stripped and reprobed for glyceraldehyde-3-phosphate dehydrogenase.

![Stromal Co-culture Blocks Megakaryocytic PKC-ε and Erk Signaling](https://example.com/stromal-co-culture.png)

**Fig. 4.** Retroviral transduction of K562 cells with control vector (MIG) and with PKC-ε expression vector (MeIG). A, diagram of constructs employed. B, GFP expression in transduced cells pre- and post-flow cytometric sorting. In the post-sort populations the vast majority (~99%) of the cells are GFP+. Cells were analyzed for GFP expression by flow cytometry.
or in contact with stromal cells (Fig. 3B). TPA induction of megakaryocytic differentiation was associated with a significant increase in mRNA levels of PKC-$\epsilon$, with peak levels observed at -24 h of induction. Strikingly, stromal co-culture was associated with complete loss of detectable PKC-$\epsilon$ mRNA, both in uninduced and in TPA-treated cells. Thus stromal co-culture potently down-regulated PKC-$\epsilon$ expression at the mRNA level.

Another critical determinant of megakaryocytic differentiation is the ERK/MAPK signal transduction pathway. Several groups have demonstrated a major role for ERK/MAPK signaling in promoting both megakaryocytic lineage commitment and maturation (7–11). As we have previously described (7), induction of megakaryocytic differentiation was associated with strong activation of ERK phosphorylation as detected by immunoblotting with phospho-ERK-specific antibodies (Fig. 3A). No changes in the level of total ERK protein were noted. Importantly, stromal co-culture completely blocked activation of ERK phosphorylation by either conditioned medium or TPA but had no effect on the levels of total ERK protein (Fig. 3A). Thus stromal co-culture abrogates the sustained ERK/MAPK activation characteristically associated with megakaryocytic differentiation induction.

**Forced PKC-$\epsilon$ Expression Does Not Override Stromal Inhibition of ERK Phosphorylation**—To address the significance of the PKC-$\epsilon$ down-regulation caused by stromal co-culture, retroviral transduction was used to enforce PKC-$\epsilon$ expression in K562 cells. As shown in Fig. 4A, the expression vector MIG contained the MSCV LTR upstream of the cloning site, permitting robust expression in hematopoietic cells, and the downstream IRES-GFP elements permitted selection of expressing cells on the basis of GFP positivity. Bulk populations of retrovirally transduced, GFP$^+$ cells were rapidly isolated with a high degree of purity using fluorescence-activated cell sorting (Fig. 4B). Immunoblotting demonstrated high constitutive PKC-$\epsilon$ levels in cells transduced with the expression vector for PKC-$\epsilon$, MIG, and low PKC-$\epsilon$ levels in cells transduced with the control parent vector, MIG (Fig. 5A). As expected K562-MIG control cells displayed up-regulation of PKC-$\epsilon$ in response to TPA induction and blockade of PKC-$\epsilon$ up-regulation by stromal co-culture (Fig. 5A). The MIG-transduced cells displayed further increase in PKC-$\epsilon$ levels in response to megakaryocytic induction but also maintained high levels of PKC-$\epsilon$ expression in the presence of stromal co-culture (Fig. 5A). These results confirm that the down-regulation of PKC-$\epsilon$ by stromal co-culture does not occur through proteolysis.

Anti-phospho-ERK immunoblots were performed to determine the relationship between the stromal effects on PKC-$\epsilon$ levels and the stromal effects on ERK activation. As shown in Fig. 5B, the K562-MIG control cells showed TPA activation of ERK phosphorylation, which was completely abrogated by stromal co-culture. Notably, K562-MIG cells, despite retaining expression of PKC-$\epsilon$ on stromal cells, also manifested stromal abrogation of ERK activation to a similar degree as the control cells (Fig. 5B). These results indicate that stromal abrogation of ERK activation occurs independently of stromal down-regulation of PKC-$\epsilon$ levels.

**Forced PKC-$\epsilon$ Expression Does Not Override Stromal Inhibition of Megakaryocytic Lineage Commitment**—To explore further the significance of stromal down-regulation of PKC-$\epsilon$, K562-MIG and K562-MeIG cells were analyzed for megakaryocytic inducibility with or without stromal co-culture. We employed GPA down-regulation as an early, sensitive marker of megakaryocytic lineage commitment (21). The MeIG-transduced cells, which overexpress PKC-$\epsilon$, when grown alone showed partial spontaneous GPA down-regulation as compared with the K562-MIG control cells (Fig. 6, -Stroma). TPA induction caused complete GPA down-regulation in both K562-MeIG and K562-MIG cells cultured alone (Fig. 6, -Stroma). Most significantly, stromal co-culture completely blocked TPA induction of GPA down-regulation in both cell populations, suggesting that stromal co-culture acts to down-regulate PKC-$\epsilon$ expression, which in turn abrogates TPA induction of GPA.
megakaryocytic differentiation for both K562-MeIG and K562-MIG cells (Fig. 6, +Stroma). Thus, restoration of PKC-ε protein expression is not sufficient to override the stromal blockade of megakaryocytic lineage commitment.

**DISCUSSION**

Previous experiments with primary human hematopoietic stem cells showed that co-culture with bone marrow stromal cells significantly diminished the yield of megakaryocytes at day 5–6 of culture (6). However, the cellular mechanism responsible for the stromal inhibition of megakaryopoesis was not determined and could have involved interference with survival, proliferation, or differentiation. Our current findings indicate that stromal exposure potently inhibits megakaryocytic lineage commitment and maintains cells locked in an undifferentiated, unresponsive state. Our findings suggest that marrow stromal cells could interfere in vivo with differentiation induction of leukemic cells as well as with engraftment of megakaryocytes from transplanted stem cells.

The stromal blockade of megakaryocytic differentiation induction shown in Figs. 1 and 2 recalls earlier findings with the 32D myeloid cell line (5). Contact with bone marrow stromal cells rendered the 32D cells unresponsive to granulocyte colony-stimulating factor induction of neutrophil differentiation (5). In that system, the stromal ligand bHaggled1 engaged the Notch1 receptor on the 32D cells and blocked granulocytic differentiation by activating the Notch signaling pathway (5). This mechanism most likely does not play a role in stromal inhibition of megakaryocytic differentiation because forced expression of constitutively active Notch1 in K562 cells has been shown to block erythroid but not megakaryocytic differentiation (25). The ligands involved in stromal blockade of megakaryocytic differentiation remain unknown. In preliminary experiments, we have ruled out involvement of macrophage colony-stimulating factor, stem cell factor, and vascular cell adhesion molecule 1 (data not shown).

Stromal co-culture was associated with a marked down-regulation of PKC-ε expression at both the protein and mRNA levels (Fig. 3). This finding corroborates recently published data implicating PKC-ε in programming megakaryocytic lineage commitment (12). In the published data, PKC-ε, but not other PKC isozymes, potentiated megakaryocytic promoter activation by the transcription factor GATA-1, and the PKC-ε-selective agonist ingenol 3,20-dibenzoate induced megakaryocytic differentiation of HEL and K562 cells (12). More recently, ingenol 3,20-dibenzoate has been shown to potentiate megakaryocytic differentiation of primary human hematopoietic stem cells (13). In the current study, however, forced expression of PKC-ε by retroviral transduction was insufficient to override the stromal blockade of megakaryocytic differentiation (Fig. 6). Thus stromal blockade does not occur solely through the down-regulation of PKC-ε levels. One possibility is that stromal co-culture may also functionally inactivate PKC-ε through the modification of its phosphorylation status, recently shown to be critical in the acquisition of competence to undergo activation (26). Alternatively, stromal exposure may inhibit multiple independent pathways required for megakaryocytic lineage commitment, such that restoration of any single pathway is insufficient to override stromal inhibition. In addition, the weak up-regulation of PKC-δ associated with stromal co-culture (Fig. 3A) might contribute to megakaryocytic inhibition.

Stromal co-culture in this study was also associated with marked suppression of ERK activation (Fig. 3A). We and several other groups have previously shown that activation of the ERK/MEK pathway plays a critical role in programming megakaryocytic differentiation (7–9, 11). In particular, sustained activation of ERK for at least 24 h is required for megakaryocytic lineage commitment in a variety of model systems (7, 9, 10). Thus the abrogation of sustained ERK activation caused by stromal exposure undoubtedly contributes to the inhibition of megakaryocytic differentiation. Our data in fact indicate that abrogation of ERK activation correlates more closely with differentiation inhibition than does down-regulation of PKC-ε levels. Future studies will address the mechanisms of ERK inactivation by stromal signals and will hopefully lead to strategies for enhancement of megakaryocyte development within the native stromal milieu of the bone marrow microenvironment.

**Acknowledgments**—We thank Drs. Takashi Tsuji and Gary Nolan for kindly providing valuable cell lines. We also thank Drs. Stephen Goff and Jae-Won Soh for generous sharing of plasmids.

**References**

1. Dexter, T. M., Allen, T. D., and Lajtha, L. G. (1977) *J. Cell. Physiol.* 91, 335–344.
2. Whitlock, C. A., and Witte, O. N. (1982) *Proc. Natl. Acad. Sci. U.S.A.* 79, 3608–3612.
3. Mudrey, R. E., Portney, J. E., York, T., Hall, B. M., and Gibson, L. F. (2000) *Blood* 96, 1928–1932.
4. Torok-Storb, B., Iwata, M., Graf, L, Gianotti, J., Horton, H, and Byrne, M. C. (1999) in *Hematopoietic Stem Cells: Biology and Transplantation* (Orlic, D., Bock, T. A., and Kanz, L. eds) Vol. 872, pp. 164–175, The New York Academy of Sciences, NY.
5. Li, L., Milner, I. A., Deng, Y., Iwata, M, Banta, A., Graf, L, Marcovina, S., Friedman, C., Trask, B. J., Hood, L, and Torok-Storb, B. (1998) *Immunity* 8, 43–55.
6. Zweegman, S., Veenhof, M. A., Dehili, N., Schuurhuizen, G. J., Huigens, P. C., and Drager, A. M. (1999) *Leukemia* 13, 935–943.
7. Racke, F. R., Lewandowska, K., Goueli, S., and Goldfarb, A. N. (1997) *J. Biol. Chem.* 272, 23366–23370.
8. Whalen, A. M., Galasinski, S. C., Shapiro, P. S., Stines Nalvreini, T., and Ahn, N. G. (1997) *Mol. Cell. Biol.* 17, 1847–1858.
9. Rouyer, M.-C., Boucheron, C., Gisselbrecht, S., Dusant-Pourt, I., and Porteu, F. (1997) *Mol. Cell. Biol.* 17, 4991–5000.
10. Matsunuma, I., Nakajima, K., Waskar, H., Hattori, S., Hashimoto, K., Sugahara, H., Kato, T., Miyazaki, H., Hirano, T., and Kanakura, Y. (1998) *Mol. Cell. Biol.* 18, 4282–4290.
11. Fischelov, S., Freyssinet, J., Pichard, F., Fontenay-Roupie, M., Guesnou, M., Cheriay, M., Gisselbrecht, S., and Porteu, F. (1999) *Blood* 94, 1601–1613.
12. Racke, F. K., Wang, D., Zaidi, Z., Kelley, J., Visvader, J., Soh, J.-W., and Sord, R., and Visvader, J., Soh, J.-W., and Drager, A. M. (1999) *Leukemia* 13, 935–943.
13. Racke, F. R., Lewandowska, K., Goueli, S., and Goldfarb, A. N. (1997) *J. Biol. Chem.* 272, 23366–23370.
14. Zweegman, S., Veenhof, M. A., Dehili, N., Schuurhuizen, G. J., Huigens, P. C., and Drager, A. M. (1999) *Leukemia* 13, 935–943.
Stromal Inhibition of Megakaryocytic Differentiation Correlates with Blockade of Signaling by Protein Kinase C-ε and ERK/MAPK
Adam N. Goldfarb, Loretta L. Delehanty, Dongyan Wang, Frederick K. Racke and Isa M. Hussaini

*J. Biol. Chem.* 2001, 276:29526-29530.  
doi: 10.1074/jbc.M103825200 originally published online June 6, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M103825200

Alerts:  
- When this article is cited  
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 26 references, 17 of which can be accessed free at  
http://www.jbc.org/content/276/31/29526.full.html#ref-list-1