Adhesion to Fibronectin Induces Megakaryocytic Differentiation of JAS-REN Cells

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Abstract. Background: Binding of integrins to the extracellular matrix elicits various responses. We have previously reported a megakaryocytic-erythroid cell line (JAS-R) that showed phenotypic changes after adhesion to plastic dishes. However, the matrix protein and the mechanism responsible for megakaryocytic differentiation still remain unknown. Materials and Methods: JAS-REN (erythroid) cells were cultured on dishes coated with various proteins. The cells were treated with RGDS, a tetrapeptide ligand to integrins, or phorbol ester (12-o-tetradecanoylphorbol-13-acetate, TPA) for 48 hours and then were harvested. Subsequently, the cell surface markers were analyzed using flow cytometry and gene expression was studied by RT-PCR. Results: The JAS-REN cells adhered to fibronectin-coated dishes, but showed poor adhesion to dishes coated with collagen, laminin or poly-D-lysine. The TPA-stimulated JAS-REN cells showed an increase in the expression of integrin αIIbβ3 complex (CD41a) and integrin β3 (CD61), while glycoporphin A (CD235a) expression was decreased. JAS-REN cells that were adherent to fibronectin-coated dishes also showed a similar pattern of phenotype to TPA-treated cells, but the changes were not so prominent. RT-PCR revealed that TPA treatment altered the gene expression profile of JAS-REN cells, making it similar to that of JAS-RAD (megakaryocytic) cells. The RGDS-treated and fibronectin adherent JAS-REN cells also showed a mostly similar expression profile to JAS-RAD cells, but these two stimuli did not alter the gene expression profile as TPA stimulation did. Transcription factors, FLI1 and GFI1, were induced by all stimuli. Conclusion: Signals triggered by adhesion to fibronectin result in the induction of FLI1 that may play a pivotal role in the lineage shift of JAS-REN cells from erythroid to megakaryocytic.

Our understanding of hematopoietic cell differentiation has advanced markedly over the last two decades (1). It is well-known that the surrounding milieu has an important role in maintaining the stemness or inducing the differentiation of hematopoietic cells (2). However, little is known about the relationship between adhesion to the extracellular matrix and differentiation of leukemic cells. Megakaryocytes and erythroblasts have been shown to originate from a common myeloid progenitor (3), and cells derived from megakaryocytic leukemia or erythroleukemia frequently show both erythroid and megakaryocytic features (4-7). These cells have been demonstrated to undergo differentiation into either the erythroid or megakaryocytic lineage in response to various differentiation inducers (4-7). However, the mechanisms that regulate the differentiation of leukemic cells into either of these lineages remain unclear.

Cell surface marker analysis has shown that leukemic cell lines express lineage-specific receptors for matrix proteins (8-10). Megakaryocytic leukemias express several integrins that allow the cells to adhere to fibrinogen, von Willebrand factor, fibronectin, collagen and vincrin (4-6). Recent studies have revealed that signals originating from integrins are involved in regulating the survival, proliferation and differentiation of hematopoietic cells through alteration of the gene expression profile and modulation of structural proteins (11-13). However, how integrin-mediated adhesion influences differentiation into megakaryocytes or erythroid cells still remains unclear.

We have previously demonstrated that a human megakaryocytic-erythroid cell line (JAS-R) develops two phenotypes depending on adhesion to the culture dish (14, 15). Adherent cells show a megakaryocytic phenotype, while cells suspended in the medium are erythroid. It requires about two months of culture for this process to occur and transcription factors (GATA DNA-binding transcription factors) are involved.
factors and the ETS family of genes) are presumed to be involved in the lineage shift. In the present study, the extracellular matrix proteins responsible for adhesion-dependent megakaryocytic differentiation of JAS-REN cells were investigated.

**Materials and Methods**

**Cell culture.** JAS-REN and JAS-RAD cells (14, 15) were cultured in Corning 430167 cell culture dishes (Corning Inc., Corning, NY, USA) that contained RPMI 1640 medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (Gibco BRL, Gaithersburg, MD, USA) at 37°C under 5% CO2 in a humidified incubator. The cells were used for experiments after entering the exponential growth phase. RGDS oligopeptide was purchased from Peptide Institute Inc. (Minoh-shi, Osaka, Japan). Coated culture dishes were obtained from BD-Bioscience (San Jose, CA, USA), and 12-o-tetradecanoylphorbol-13-acetate (TPA) was purchased from Sigma (St. Louis, MO, USA). To study the adhesion to culture dishes, the JAS-REN and JAS-RAD cells were seeded onto culture dishes coated with collagen (type I and type IV), fibronectin, laminin, or poly-D-lysine. After 48 hours, each dish was gently washed three times with culture medium to remove suspension cells, and cell adherence was assessed under a microscope. To study the megakaryocytic differentiation, the JAS-REN cells were cultured on fibronectin-coated dishes or treated with either 30 nM TPA or 1 mM RGDS for 48 hours. To remove adherent cells from the culture dish, the JAS-REN cells were first washed twice with PBS(–) and further incubated with fresh PBS(–) for 15 min. After pipetting, cells were harvested as adherent cells.

**Flow cytometry analysis.** The analysis of surface markers was performed by flow cytometry with a FACScalibar (Becton Dickinson, Franklin Lakes, NJ, USA) using FITC-conjugated monoclonal antibodies for CD61 and glycoporphin A (CD235a) (Dako Cytomation, Glostrup, Denmark). CD41a and control mouse IgG were obtained from BD PharMingen (San Diego, CA, USA).

**Reverse transcription-polymerase chain reaction (RT-PCR).** The genes which disclose differences in JAS-REN and JAS-RAD cells were selected in previous studies (14, 15). The mRNA expression was examined by RT-PCR, as previously reported (15). Briefly, the single stranded cDNA equivalent to 0.15 μg total RNA was employed for each PCR, which was set to amplify the target message for 25 cycles. The primer sequences and PCR conditions are available upon request.

**Results**

**Adhesion to culture dishes.** As shown in Figure 1, the JAS-REN cells seldom adhered to the culture dishes except for those coated with fibronectin, but their adhesion was always weak. In contrast, the JAS-RAD cells adhered to all of the coated dishes, but the extent of their adhesion was variable showing strong adhesion to fibronectin- and type I collagen-coated culture dishes, while adhesion to dishes coated with laminin or poly-D-lysine was markedly reduced compared with that to the fibronectin-coated dishes.

**Megakaryocytic differentiation of JAS-REN cells by TPA, RGDS or fibronectin-coated dishes.** When the JAS-REN cells were treated with 30 nM TPA for 48 hours, most of the cells became adherent to the culture dish (data not shown). In preparation for phenotypic investigation the adherent cells were removed from the culture dish as shown in materials and methods. The flow cytometry profiles of the TPA-treated cells are shown in Figure 2. The control JAS-REN cells were more committed to the erythroid lineage, but showed weak expression of CD41a and CD61, as demonstrated in Figure 2A. The intensity of CD41a and CD61 expression was increased by TPA treatment, while CD235a expression was decreased (Figure 2B). These findings suggested that TPA stimulation induced the megakaryocytic differentiation of the JAS-REN cells.
RGDS prevented the adhesion of JAS-RAD cells to culture dishes (data not shown). When the JAS-REN cells were treated with 1 mM RGDS for 48 hours, the treated cells remained in suspension. Flow cytometric analysis showed no obvious changes in the expression of CD41a, CD61, and CD235a (Figure 2C) compared to the controls. Subsequently the JAS-REN cells were cultured on fibronectin-coated dishes for 48 hours, after which the adherent and suspended cells were harvested separately and studied using flow cytometry. As demonstrated in Figure 2D the adherent cells showed increased expression of CD41a and CD61 and decreased expression of CD235a. In contrast, the non-adherent cells (Figure 2E) had a phenotype that was indistinguishable from the control JAS-REN cells.

Changes of gene expression stimulated by TPA, RGDS, or fibronectin-coated culture dishes. The TPA- and RGDS-treated cells and cells adherent to the fibronectin-coated dishes were harvested at 24 and 48 hours for comparison with control JAS-REN and JAS-RAD cells. TPA stimulation rapidly elicited the strong expression of megakaryocyte-related genes (Figure 3A and B) compared to the other two stimuli. In particular the genes with products located in the α-granules were strongly induced by TPA up to the levels seen in JAS-RAD cells (Figure 3A). RGDS and adhesion to fibronectin also induced the expression of megakaryocyte-related genes, but the induction of expression was weaker than with TPA. Moreover, PF4 and GPIX were not induced by 48 hours. In contrast to the induction of megakaryocytic genes, the changes of erythroid-related genes (Figure 3C) were not marked. Both HBB and ADD2 showed no changes, except for decreased expression of ADD2 in the fibronectin-adherent cells. Interestingly, the expression of erythropoietin was not induced by exposure to TPA, CD9, CD34, and MDR1 were expressed by the JAS-RAD cells, but not by the RGDS-treated or fibronectin-adherent JAS-REN cells. Only TPA stimulation induced all of these genes in the JAS-REN cells.

Exposure to TPA or RGDS and adhesion to fibronectin induced the expression of the transcription factors GFI1, FLI1 and RUNX1 except for RUNX1 induction with fibronectin, while PU.1, GATA1, FOG1 and NFE2 were unchanged (Figure 4A), GATA2 was also induced, though its expression was low. The genes related to integrin signal transduction are shown in Figure 4B. A signal inhibitor, CIB1, was not changed by any stimulation, while TPA induced Talin (TLN1), ARHGAP6, VAV3 and Zyxin, all of which are considered as signal transducers of the integrin system. Among these genes, RGDS induced ARHGAP6 and VAV3, but adhesion to fibronectin only induced ARHGAP6 after 48 hours.

Discussion

Various small molecules have been demonstrated to induce leukemic cell differentiation. One of the most potent differentiation inducers is TPA, and it induces the megakaryocytic differentiation of erythroid and megakaryocytic-erythroid leukemia cells (16, 17). The JAS-REN cells in the present study also underwent megakaryocytic differentiation in response to TPA (Figure 2). Our previous study had shown that adhesion of JAS-RAD cells to the substratum of culture dishes shifted the erythroid phenotype to a megakaryocytic one (15). In the present study both JAS-REN and JAS-RAD cells were preferentially adherent to fibronectin (Figure 1) which supports the growth of hematopoietic stem cells (18). The adhesion was dependent on a bivalent cation, integrins were considered to play a major role in the cell-fibronectin interaction.

We have previously demonstrated that the transcription factors GFI1, FLI1, and RUNX1 were highly expressed in JAS-RAD cells, while PU.1, GATA1, FOG1 and NFE2 showed similar expression in JAS-RAD and JAS-REN cells.
and other studies have also suggested that FLI1 and RUNX1 are associated with megakaryocytic differentiation (15, 19-26). In particular, FLI1 seems to have a role in megakaryocytic differentiation. In the present study RT-PCR showed that the TPA-induced alterations of these transcription factors in the JAS-REN cells produced a very similar expression pattern to that of the JAS-RAD cells. RGDS treatment and adhesion to fibronectin also altered these genes in a mostly similar manner to TPA treatment (Figure 4A). Interestingly, the expression of PU.1, GATA1, and NFE2 was unchanged by any treatment. These data suggest that the latter group of transcription factors is essential for the growth and differentiation of megakaryocytic and erythroid common progenitor cells, while induction of the former group of genes is necessary for megakaryocytic differentiation to occur. GFI1 that was increased by all stimuli has been demonstrated to play an important function in maintaining stemness of hematopoietic cells (27), but its role for megakaryopoiesis still remains unclear.

In the present study signals originating from the fibronectin-integrin interaction produced a gene expression profile in the JAS-REN cells which was similar to that of the JAS-RAD and TPA-treated JAS-REN cells. It is known that various integrins (integrins α1β3, α2β1, α3β1, α6β1, and α1β3) bind to fibronectin (28). Among them, ITGB5 was absent in JAS-R cells according to our previous microarray study (data not shown), while the expression of ITGAV and ITGA4 was similar in both JAS-RAD and JAS-REN cells, although

Figure 3. RT-PCR for genes altered by lineage shift. A: Major genes in α-granules; B: integrins and platelet-related proteins; C: erythropoietin and erythroid-related genes; D: miscellaneous genes expressed specifically in JAS-RAD cells.
ITGAV was increased by TPA stimulation (Figure 3B). In contrast, ITGA5, ITGA2B, and ITGB3 all showed considerably stronger expression in JAS-RAD cells and were induced by TPA or fibronectin in JAS-REN cells. Integrins α5β1 and αIIbβ3 bind to the RGD motif of fibronectin, and their binding to fibronectin is caused to the efficient fibronectin matrix formation (29, 30). Although integrin α4β1 has been reported to enhance thrombopoietin-induced normal megakaryocytic differentiation (31), it binds to fibronectin in an RGD-independent manner (32). Resting normal megakaryocytes bind to fibronectin through integrin α5β1 not through integrin α4β1 (28) and we consider that initial adhesion may occur mainly through integrins α5β1 and αIIbβ3. The mechanism that leads to lineage switching through these integrins is still unclear. But, we are currently focusing on the possible involvement of mitogen-activated protein kinase systems.

Approximately eight weeks of culture were required to segregate JAS-RAD cells from the original JAS-R cells (15). Using fibronectin-coated dishes, however, the lineage shift from erythroid to megakaryocytic was largely complete after one week, except for reduced expression of CD235a (data not shown). This suggests that the acquisition of the megakaryocytic phenotype is actively induced by adhesion to fibronectin, while loss of the erythroid phenotype is subordinate to megakaryocytic differentiation. After adhesion to the dish, integrin signals may have a multiplier effect that reinforces the megakaryocytic characteristics of these cells, while floating cells may rapidly lose their integrin-mediated features. Such behavior may be an intrinsic feature of megakaryocytic-erythroid cells.

In conclusion, JAS-REN cells adhere to fibronectin and without differentiation inducers their adhesion accelerates the lineage shift from erythroid to megakaryocytic with an increase of FLI1.

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Figure 4. RT-PCR for megakaryocytic-erythroid cell-related transcription factors (A) and integrin signal-related genes (B). Asterisk of TLN1 denotes 23 cycles of PCR.
References


