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Blasts in transient leukemia in neonates with Down syndrome differentiate into basophil/mast-cell and megakaryocyte lineages in vitro in association with down-regulation of truncated form of GATA1

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Running title:
Differentiation of transient leukemia blasts and GATA1
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Summary

Mutations of GATA1, leading to aberrant expression of a truncated form of GATA1 (called GATA1s), are present in transient leukemia (TL) in neonates with Down syndrome. Using these molecular markers of TL, we investigated the growth and differentiation potential of TL blasts in the presence of hematopoietic growth factors (HGFs). Interleukin-3, stem cell factor and granulocyte-macrophage colony-stimulating factor potently stimulated the growth of TL blast progenitors and induced differentiation towards basophil/mast cell lineages, whereas thrombopoietin induced differentiation towards megakaryocytes. GATA1s was expressed in TL blasts in all 5 patients examined but was down-regulated during differentiation induced by these HGFs, while full-length GATA1 was not expressed throughout the culture. GATA1 mutations were detected in TL blasts in 4 patients, including one patient with two distinct mutations. The cells of this patient exhibited identical and only mutated sequences both before and after culture with HGFs, confirming the leukemic cell origin of these differentiated cells. Erythroid differentiation of TL blasts was not evident with any HGFs. These data indicate that TL blasts have the potential to grow and differentiate towards particular hematopoietic lineages in the presence of specific HGFs and that the down-regulation of GATA1s might be involved in blast cell differentiation.

Key words:

Down syndrome; transient leukemia; GATA1; hematopoietic growth factor; mast cell
Introduction

Children with Down syndrome (DS) are at higher risk of developing leukemia (Hitzler & Zipursky 2005; Malinge et al, 2009). In patients with DS under the age of 3 years, acute megakaryoblastic leukemia (AMKL), a very rare subtype of leukemia in non-DS patients, is the predominant type of leukemia. In about 10% of neonates with DS, there are hematological abnormalities indistinguishable from AMKL, usually detected on routine study or incidental blood examination performed because of another unrelated illness (Hitzler & Zipursky 2005; Zipursky 2003). This disorder spontaneously disappears within several months without any therapy and has been given a variety of names, including transient myeloproliferative disorder, transient abnormal myelopoiesis and transient leukemia (TL).

Recently, mutations affecting the GATA1 gene, which encodes one of the GATA family of zinc-finger transcription factors, have been found almost exclusively in megakaryoblastic leukemias of DS, including TL and AMKL (Groet et al, 2003; Wechsler et al, 2002). Most of these mutations are located within exon 2 and all of these mutations result in the lack of 50-kDa full-length GATA1 and the generation of a 40-kDa truncated form of GATA1 (called GATA1s) that lacks the N-terminal transactivation domain (Gurbuxani et al, 2004; Hitzler & Zipursky 2005; Wechsler et al, 2002). We have hypothesized that TL may be a very special form of leukemia arising in the fetal liver, since myelofibrosis, which is one of the characteristics features of AMKL, is not present and, instead, hepatic fibrosis is often seen in TL (Miyauchi et al, 1992). The dominant action of GATA1s, leading to hyperproliferation of hematopoietic progenitor cells in the yolk sac and fetal liver, has recently been shown (Li
et al, 2005), but the role of the GATA1s protein in the pathogenesis of TL remains unknown.

Another unique feature of TL, which is unusual for acute leukemia, is that mature blood cells of a variety of myeloid lineages appear in vitro after culture of TL blasts in the presence of growth stimulants, including hematopoietic growth factors (HGFs) (Denegri et al, 1981; Suda et al, 1987). This finding suggests that TL blasts may have the capability of differentiation into mature cells, which might be involved in the spontaneous remission of TL. However, it has not been clearly shown how the growth and differentiation of blast progenitors in TL are regulated by individual HGFs and which lineage(s) of the differentiated cells in vitro are really derived from TL blasts since it is difficult to completely exclude the influence of co-existing normal hematopoietic progenitors in the samples examined due to a lack of specific markers of TL. At the present time, useful molecular markers of TL clones are available, that is, GATAI mutations and the resultant aberrant expression of GATA1s coupled with the absence of full length GATA1 protein. In this study, by utilizing these molecular markers, we demonstrated that TL blasts are capable of differentiating towards uncommon basophil/mast-cell and megakaryocytic lineages, but not towards an erythroid lineage, in vitro in the presence of specific HGFs and that differentiation is closely associated with down-regulation of aberrant GATA1s protein, a phenomenon that has not previously been described.
Materials and Methods

Patients and cells

TL blasts were obtained from the peripheral blood of 5 newborn patients with Down syndrome and TL after informed consent of their parents was obtained in accordance with the Declaration of Helsinki (Table 1). TL was diagnosed based on morphology of the blasts, the expression profiles of lineage-associated antigens (CD7, CD13, CD33, CD41, CD56, glycophorin A, etc.), a chromosome analysis demonstrating trisomy 21 without additional abnormalities and the clinical course of spontaneous remission. Mutations of the GATA1 gene were detected in patients 1 through 4 (Table 1) and, although GATA1 exon 2 of TL blasts in patient 5 exhibited a wild type sequence, aberrant expression of GATA1s and lack of full-length GATA1 was demonstrated by western blot analysis in all 5 patient samples as described below. Two leukemia cell lines, K562 and KU812 were purchased from the Human Science Research Resources Bank (Osaka, Japan), and CMK (derived from megakaryoblastic leukemia in DS) was a gift from Dr E. Ito of Hirosaki University, Japan. The use of human cells in experiments was approved by Tokyo Dental College Ichikawa General Hospital Institutional Ethics Committee.

HGFs

Granulocyte colony-stimulating factor (G-CSF) was provided by Chugai Pharmaceutical (Tokyo, Japan), and the other HGFs were purchased from manufacturers; stem cell factor
(SCF) (BioVision, Mountain View, CA, USA), interleukin-3 (IL-3) (Strathmann Biotech AG, Hamburg, Germany), granulocyte-macrophage CSF (GM-CSF) (Prospec-Tany, Rehovot, Israel), thrombopoietin (TPO) (PeproTech EC, London, UK) and erythropoietin (EPO) (Calbiochem, La Jolla, CA, USA). These HGFs were all human recombinant forms and, except for EPO, used at a final concentration of 50 ng/mL; EPO was used at a final concentration of 250 ng/mL.

**Blast cell enrichment**

Mononuclear cell fractions were obtained from heparinized peripheral blood of patients with TL and cryopreserved in liquid nitrogen. Prior to the experiments, these cells were cultured either in suspension in α-minimum essential medium (αMEM) supplemented with 20% fetal calf serum (FCS) overnight or, for some samples, for 2 days with additional 10% medium conditioned by human bladder carcinoma cell line 5637 (5637-CM), rather than used directly after thawing from liquid nitrogen; monocytes were removed by plastic adherence during this period and lymphocytes were depleted on the following day using the immuno-magnetic beads method (Dynal/Invitrogen, Oslo, Norway) with monoclonal antibodies against CD3 (UCHT1; Ancell, Bayport, MN, USA) and CD20 (B9E9; Sigma, Saint Louis, MO, USA). The above procedures were aimed at facilitating recovery, and minimizing loss, of TL blasts after thawing, particularly in the limited amount of samples, as well as minimizing the influences of co-existing normal cells. These methods have been published and the absence of significant interactions between growth factors used in consecutive cultures has been demonstrated.
(Miyauchi et al, 1987). In fact, using some patient samples, we confirmed that there were no significant differences in the data obtained with and without pretreatment of 5637-CM. With cytospin specimens, the yield of blast enrichment after the above procedures was morphologically confirmed to be >95% in all cell samples.

**Colony assay**

Leukemic colony formation was assessed using a clonal culture with methylcellulose as previously described (Miyauchi et al, 1987). TL blasts were plated in 96-well plates at a concentration of 5 x 10^3 to 10^4 cells per well and colonies were counted after 5 to 7 days.

**Suspension culture**

To assess morphological changes in TL blasts in the presence of HGFs, TL blasts were cultured in suspension at a concentration of 2.5 to 5 x 10^5 cells/mL in 24-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ, USA) in 0.25 to 0.3 mL of αMEM containing 20% FCS and HGFs.

**Immunocytochemistry**

Cytospin specimens were fixed in buffered formol acetone (pH 7.0) and immersed in 100% methanol containing 0.3% H_2O_2 at room temperature for 30 min to inhibit endogenous peroxidase activity. The specimens were then treated for antigen retrieval with 0.1-M citrate buffer (pH 6.0) for c-kit or with a high pH target retrieval solution (DAKO Japan, Tokyo,
Japan) for tryptase in a microwave oven at 90°C for 15 min and naturally cooled. Immunocytochemistry was performed using an automated slide preparation system (Ventana Japan, Yokohama, Japan). Primary monoclonal antibodies against human mast cell tryptase (AA1; Abcam, Cambridge, MA, USA) and c-kit (104D2; DAKO, Tokyo, Japan) were applied at a dilution of 1:200 and 1:50, respectively.

Electron microscopy

Electron microscopic observation was performed as previously described (Miyauchi et al, 1997) with 2.5% glutaraldehyde for fixation.

Western blot analysis

Twenty micrograms of total cellular protein was resolved under reducing conditions using SDS-PAGE electrophoresis (Multigel-II mini12.5%; Sekisui Medical, Tokyo, Japan). The primary antibodies and their dilutions used were as follows: anti-GTATA1 (C-terminus) goat polyclonal antibody (M20; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:200 dilution), anti-GATA2 rabbit polyclonal antibody (H-116; Santa Cruz Biotechnology; 1:200 dilution) and anti-α-tubulin mouse monoclonal antibody (Ab-1; Oncogene Research Products, Cambridge, MA, USA; 1:1000 dilution).

GATA1 gene mutation analysis

Genomic DNA was extracted using a QIAamp DNA blood midi kit (Qiagen, Valencia, CA,
USA). Genomic DNA obtained from blast-enriched samples were amplified with primers as previously described (Hitzler et al, 2003). Amplified products were gel-purified using a QIAEX II gel extraction kit (Qiagen) and cycle sequenced with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) in both directions. The sequence was analyzed on a 3130 Genetic Analyzer (Applied Biosystems). The PCR product from patient 4 was subcloned into pGEM-T Easy (Promega, Madison, WI, USA) and sequenced with T7 and SP6 primers.

**Results**

Using colony assay, we first determined which HGF stimulates the growth of TL blast progenitors, a minor population of blasts that have the potential to proliferate and give rise to large population of descendant blasts with little growth potential (Griffin & Löwenberg 1986; McCulloch 1983). Very similar patterns of colony formation in response to HGFs were noted in cells from all 5 patients examined (Figure 1A): IL-3, GM-CSF and SCF were potent stimulators of colony formation, particularly with IL-3 being the strongest in all cases. The activity of TPO was generally less potent than that of the former three, although it exceeded that of GM-CSF in one case (Pt. 5). G-CSF and EPO exhibited very weak or no activity. The growth-stimulating effects of HGFs in suspension cultures were similar to those in colony assay (Figure 1B). Cell number greatly increased after 7-day suspension cultures in the presence of IL-3, GM-CSF and SCF, and less effectively in the presence of TPO, while G-CSF and EPO exhibited very limited effects. Hence, it was shown that IL-3, GM-CSF and
SCF are potent stimulators of the growth of blast progenitors in TL.

We next examined morphological changes of TL blasts in suspension culture in the presence of each HGF. Before culture, the enriched TL cell samples from each of the 5 patients consisted of >95% blasts with frequent cytoplasmic blebs, indicating a megakaryoblast morphology (Figure 2A, E, Figure 4A). After 7 days of suspension culture in the presence of IL-3, GM-CSF or SCF, varying proportions of maturing or mature basophils with coarse basophilic granules and vacuolated granules, with the latter being probably due to water-soluble granule contents characteristic of basophils, and atypical nuclei appeared in the samples from each of the 5 patients (Figure 2B, Figure 3A). Electron microscopic observation of these cells demonstrated spherical, vacuolated cytoplasmic granules containing an electron-dense core matrix with a background of variably extracted granule contents and particulate material, consistent with the ultrastructure of basophil granules (Figure 2C, D). In addition to basophils, elongated, irregular-shaped cells, each with a single nucleus and numerous large basophilic cytoplasmic granules, also appeared in varying numbers, depending on the sample and culture conditions (Figure 2F). These cells were negative for peroxidase, except for a minor proportion of weakly positive cells (Figure 2G, arrow), contained cytoplasmic granules exhibiting metachromasia when stained with toluidine blue at pH 3.0 (Figure 2H) and some cells exhibited positive immunocytochemical staining for c-kit and mast-cell-tryptase (Figure 2I, arrow). Electron microscopic observation demonstrated that cytoplasmic granules of these cells contained substances with various types of structures (Figure 2J, K): 1) a homogeneous, electron-dense material; 2) a particulate material,
consistent with basophil granules; 3) scroll-like inclusions, identical to mast cell granules; and 4) both 2) and 3) in the same granules. These findings indicate that these are hybrid cells with characteristics of both basophils and mast cells. In long-term suspension cultures, these cells with basophil/mast cell features from patient 4 remained viable for up to 6 months in the presence of IL-3 alone. Quantification under light microscopic observation showed that these elongated cells with the features of hybrid basophil/mast cells tended to account for larger proportions in the presence of IL-3, being 50% of total cells in patient 3, >30% in patient 5 and 20% in patient 1 (Figure 3A). Although only a minor proportion of such cells were seen on culture day 7 in the case of patient 4, the proportion increased from 1% to 24%, along with an increase in basophils from 11% to 36%, by day 14 in the presence of IL-3.

Although growth-stimulating activity of TPO was generally weaker than those of the former three HGFs, TPO potently induced a different pattern of morphological differentiation. Mature megakaryocytes with large cytoplasms, containing fine granules, and multi-lobed nuclei as well as small immature megakaryocytes, including micromegakaryocytes, with single or multi-lobed nucleus and similar cytoplasmic features appeared in large numbers after 7 days in the presence of TPO (Figure 4B), accounting for more than 50% in three patients (Pts. 3, 4 and 5) (Figure 3B). The proportion of CD41-positive cells of megakaryocytic lineage, as measured by flow cytometry, increased from 44 to 87% (Pt. 3), from 62 to 75% (Pt. 4) (Figure 4C), from 20 to 34% (Pt. 1) and from 5 to 8% (Pt. 2) during culture in the presence of TPO. Electron microscopic examination demonstrated platelet demarcation membrane system and platelet specific granules in both large multi-lobed cells
G-CSF and EPO on their own were not active in inducing morphological changes. However, in cultures treated with EPO and SCF in combination, numerous basophilic and polychromatic erythroblasts appeared in cultures of three patient samples (Pts. 1, 2 and 5) on day 14 (data not shown), although no or only a small number of erythroblasts were noted on day 7. On day 21, basophilic erythroblasts decreased in number and many orthochromatic erythroblasts appeared in addition to polychromatic erythroblasts. These cells accounted for about 80% of the overall cell population. These erythroblasts were unexpectedly shown to be derived from normal erythroid progenitors as described below.

Compared with full-length GATA1, GATA1s has a reduced transactivation potential because of the absence of the N-terminal transactivation domain (Shimizu et al, 2001; Wechsler et al, 2002), but the absence of full-length GATA1 can be compensated for by the expression of GATA1s at high levels in GATA1 germline mutant (GATA1.05) mice expressing a very low level of GATA1 (Shimizu et al, 2001). In view of these previous findings, we speculated that the expression of GATA1s might be up-regulated in TL blasts when differentiation is induced by HGFs. However, on the contrary, GATA1s expression decreased to low levels, and became undetectable under certain conditions, on day 5 through 14 when the cells were cultured in the presence of any of the following HGFs; IL-3 (Pts. 1 through 5), GM-CSF (Pts. 3 and 4) and TPO (Pts. 3 and 4) (Figure 5A, B, and data not shown), although GATA1s was expressed at high levels before culture in all 5 patients. The expression of full-length GATA1 was not detected throughout the culture either with IL-3, GM-CSF or TPO.
CMK cells used for control did not show differentiation and did not exhibit any changes in the level of GATA1s expression throughout the 14-day culture in the presence of IL-3 or GM-CSF, although cell growth was augmented by these HGFs (data not shown). Hence, down-regulation of GATA1s was shown to be closely associated with differentiation of TL blasts. The cells from two patients (Pts. 1 and 2) cultured in the presence of EPO and SCF in combination showed different results: the expression of GATA1s decreased from day 7 through day 21, but strong expression of full-length GATA1 was detected on days 14 and 21 (Figure 5C), indicating that the numerous erythroblasts seen in these cultures were derived from a small number of normal erythroid precursors that had been present in the original samples.

GATA2 is another member of the GATA family of zinc-finger transcription factors that have an overlapping but distinct expression pattern, compared with that of GATA1; GATA2 is expressed at high levels in hematopoietic stem cells and its expression decreases along the erythroid differentiation pathway, where GATA1 expression increases (Labbaye et al., 1995; Leonard et al., 1993). To elucidate whether GATA2 is involved in differentiation of TL blasts, we examined the expression of GATA2. GATA2 was expressed in TL blasts at very low levels before culture (Pts. 1 through 4) (Figure 5D, E). In the presence of IL-3, GATA2 expression transiently increased to high levels on day 3, then rapidly decreased to a very low level on day 5 in all 4 patient samples examined (Pts. 1 through 4) (Figure 5D). It increased again to a slightly higher level on day 7 (Pt. 3) or 14 (Pts. 2 and 4). However, GATA2 expression progressively decreased and finally disappeared on day 3 through day 14 in the
presence of GM-CSF (Pts. 3 and 4) or TPO (Pts. 3 and 4) (Figure 5E).

The absence of full-length GATA1 expression in cells after culture with IL-3, GM-CSF and TPO indicates that the differentiated basophil/mast cells and megakaryocytes are probably of leukemic origin. To confirm this, the mutational state of the GATA1 gene was analyzed in cells before and after 7-day suspension culture in the presence of IL-3 (Pts. 2, 3), GM-CSF (Pts. 2, 3, 4) or TPO (Pts. 3, 4). All samples after culture demonstrated mutations of GATA1 exon 2 that were identical to those detected before the culture (Table 1). Although wild type sequence was also detected after PCR amplification of the samples of patients 2 and 3 both before and after culture, possibly due to coexisting cells with normal GATA1, the sample from patient 4 both before and after culture exhibited only mutated sequences. Mutational analysis is based upon PCR and is not a quantitative method, but the absence of amplification of the wild type sequence by PCR indicates that the vast majority of cells had GATA1 mutations and, therefore, confirms that the differentiated basophil/mast cells and megakaryocytes were of leukemic cell origin at least in the case of this patient. Interestingly, two different types of GATA1 mutations were detected in the cells of patient 4, demonstrating that TL blasts of this patient were comprised of biclonal leukemic cell populations (Figure 6).

Discussion

In this study, we showed that IL-3, GM-CSF and SCF are potent stimulators of the growth and differentiation of blast progenitors in TL. Toki et al (2009) recently reported that
SCF alone could support the growth of TL blasts but, in their study, GM-CSF was not tested and IL-3 was tested at a concentration below that used in our study. This appears to be the reason for somewhat different results compared to the present data. These HGFs were active in inducing differentiation of TL blasts towards basophil/mast cells. Although differentiation of TL blasts into basophils has previously been described (Suda et al, 1987), the mast cell nature of differentiated TL blasts was demonstrated for the first time in this study: positive immunostaining for tryptase and the presence of a scroll-like ultrastructure in the cytoplasmic granules are highly specific for mast cells. The elongated cell shape, which is commonly seen in tissue mast cells, and the relatively long life span of these cells in suspension culture, as has been reported in the case of murine mast cells (Majewski et al, 2006), are consistent with the characteristics of mast cells. However, these cells were morphologically not typical mast cells and simultaneously possessed the ultrastructural characteristics of basophils. Such hybrid cells, indicating a common precursor origin of the two cell types, have been observed in states of disturbed myelopoiesis (Parkin et al, 1980; Zucker-Franklin 1980). It is known that GATA1 plays a key role for the development of normal mast cells (Kitamura et al, 2006). Defective differentiation of mast cells has been documented in unusual trilineage colonies consisting of megakaryocytes, erythroid and mast cells formed by cells from mice genetically engineered to express a low level of GATA1 (Migliaccio et al, 2003), and mast cell differentiation could be induced from GATA1-null murine proerythroblasts by stimulation with IL-3 (Kitajima et al, 2006). Based on these data, it appears feasible that GATA1 mutations, resulting in the lack of full-length GATA1 and aberrant expression of GATA1s, play a role in abnormal
differentiation of TL blasts into hybrid basophil/mast cells.

In contrast to TL blasts, CMK cell line, derived from DS-AMKL, did not differentiate in the presence of any HGF. Additional mutations in CMK cells could be involved in this, but it has been shown by microarray analysis that PRAME, which is a dominant repressor of retinoic acid receptor signaling and known to play a tumorigenic role in a variety of cancers (Epping et al, 2005), is expressed in DS-AMKL but not in TL (McElwaine et al, 2004). A potential explanation for the difference between CMK cells and TL blasts could be that retinoic acid signaling pathway is disturbed in CMK by the expression of PRAME.

Expression of GATA1s was down-regulated to a low level during the differentiation process in the presence of IL-3, GM-CSF and TPO. Since GATA1s is able to bind DNA and Friend of GATA1 (FOG-1), an important cofactor for GATA1, GATA1s may interfere with the normal differentiation process in TL by acting in a dominant negative manner against proteins that participate in hematopoietic cell differentiation, and down-regulation of GATA1s may trigger activation of other transcription factors that can induce differentiation of TL blasts. GATA-2 has been shown to participate in mast cell differentiation (Tsai & Orkin 1997) and we observed a transient increase in the expression of GATA2 on day 3 in cells cultured with IL-3 in all samples tested. Other transcription factors that may be upregulated and involved in differentiation of TL blasts need to be determined.

Mature erythroblasts that appeared on days 14 and 21 in cultures with EPO and SCF in combination expressed full-length GATA1. This result indicates that the mature erythroblasts observed in these cultures were derived from co-existing non-leukemic erythroid
progenitors. It also indicates that, although TL blasts usually acquire a partial erythroid component in their differentiation in vivo and express erythroid lineage antigens (McElwaine et al, 2004), including γ-globin and δ-aminolevulinate synthetase (Ito et al, 1995; McElwaine et al, 2004), GATA1s cannot support terminal erythroid differentiation of TL blasts in vitro. TL occurs not only in DS patients but also in phenotypically normal children whose leukemic cells harbor trisomy 21 (Zubizarreta et al, 1995), indicating that chromosome 21 contains gene(s) that play a crucial role in the pathogenesis of TL. RUNX-1 and ERG are likely candidate genes, located on chromosome 21 (Elagib et al, 2003; Hitzler & Zipursky 2005; Rainis et al, 2005); forced expression of these genes in K562 cells induces megakaryocytic differentiation in the presence or absence of TPO (Elagib et al, 2003; Rainis et al, 2005). Based upon these data, it seems possible that over-expression of Runx-1 and/or ERG as a result of trisomy 21, together with the absence of full-length GATA1, causes a shift in the balance between erythroid and megakaryocytic differentiation of TL blasts towards the latter, thereby suppressing erythroid differentiation and promoting megakaryocytic differentiation in the presence of TPO.

Mutation analyses demonstrated that the DNA samples of patient 4 had only mutated GATA1 sequences both before and after the culture with HGFs. This result indicates that differentiated cells that appeared after culture with HGFs are derived from leukemic clone and confirms that TL blasts have the potential to differentiate into basophil/mast cells and megakaryocytes in the presence of specific HGFs. It is of particular note that leukemic cells in patient 4 showed a unique biclonal nature. The presence of multiple GATA1 mutations
in the same case has been reported in some patients with DS-AMKL and peripheral blood samples of neonates with DS (Ahmed et al, 2004) and a patient with TL that arose in utero (Groet et al, 2005). The latter paper demonstrated the presence of several clonal expansions at different stages of megakaryocytic differentiation, suggesting that GATA1 mutations occur at various time points in hematopoietic ontogeny and that TL blasts are not blocked from continuous differentiation by the presence of GATA1s. Since AMKL in DS is typically clonal, it is suggested that selection of a dominant clone with acquisition of additional mutations would cause transition from TL to AMKL, which has recently been proposed as a multistep theory for DS-associated myeloid leukemias (Ahmed et al, 2004; Hitzler & Zipursky 2005).

However, GATA1 mutations were not detected in the sample from patient 5 despite the fact that TL blasts expressed aberrant GATA1s, but not full-length GATA1. This patient might carry a complete or partial deletion of exon 2 involving one or both of the primer regions. In this case, PCR-sequencing analysis results in the amplification of the remaining wild type exon 2, which is erroneously interpreted as having no mutation of GATA1 despite the translation of GATA1s.

Based on the present data, we conclude: 1) TL blasts are dependent on specific HGFs for their growth and differentiation in vitro; 2) these cells are capable of differentiating into basophils, abnormal hybrid basophil/mast cells and megakaryocytes but terminal erythroid differentiation is blocked at least in vitro; and 3) down-regulation of aberrant GATA1s is closely associated with the differentiation process. Constitutive cytogenetic abnormalities and resultant abnormal proteins are thought to be deeply involved in the
pathogenesis of cancers and could be useful molecular markers of cancer clones. The present data, however, indicate that, at least in special cases such as self-limiting TL, expression of abnormal cancer-associated proteins caused by mutations could be influenced by environmental conditions. The mechanism of this phenomenon needs to be clarified.

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Authorship

J.M. designed and performed research, analyzed data, and wrote the manuscript; Y.I., K.T, H.T., K.I. and K.S. diagnosed the disorder and contributed patient samples and clinical data; T.M. performed mutational analysis and revised the manuscript.

References


**Titles and legends to figures**

**Fig 1.** Effects of HGFs on the growth of TL blast progenitors. (A) Effects of each HGF on colony formation in methylcellulose culture, represented as colony number per cells plated in each well (5,000 cells for patients 1, 2, 4, 5 and 10^4 cells for patient 3). Data are presented as
mean ± SD for quadruplicate cultures. (B) Effects of each HGF on total cell number after suspension culture. Arrows indicate input number on day 0. No GF, no growth factors.

**Fig 2.** Effects of IL-3 and GM-CSF on differentiation of TL blasts. (A and B) Morphology of TL blasts (Pt. 2) before (A) and after (B) culture in the presence of GM-CSF. (C and D) Electron micrograph of a cell (Pt. 3) after culture in the presence of GM-CSF (C) and higher magnification (D). Arrow indicates a cytoplasmic granule containing particulate material in the marginal area. (E and F) Morphology of TL blasts (Pt. 3) before (E) and after (F) culture in the presence of IL-3. (G-I) Cytochemistry of cells after culture in the presence of IL-3 (Pt. 3): peroxidase staining (G), toluidine blue staining (H) and immunocytochemistry for tryptase (I). Arrows indicate cells exhibiting weakly positive reaction for peroxidase (G) and tryptase (I). (J and K) Electron micrograph of a cell (Pt. 3) after culture in the presence of IL-3 (J) and higher magnification (K). Note the presence of various types of granules with different internal structures: homogeneous electron-dense material (1), particulate material (2), scroll-like inclusions (3) and both of (2) and (3) in the same granule (4). Bars (A, B, E-I), 20 µm; (C and J), 2 µm; (D and K), 1 µm.

**Fig 3.** Morphological quantification of proportion of each cell type after 7-day suspension culture. (A) Cultures in the presence of SCF, IL-3 or GM-CSF. (B) Cultures in the presence of TPO. Elongated cells with hybrid basophil/mast cell features are shown as “Baso/Mast” in both (A) and (B). †After 14-day culture of TL blasts from patient 4 in the presence of IL-3, baso/mast cells accounted for 24%, basophils 36%, immature myeloid cells 30%, blasts 2%
and others 8%, whereas in the presence of GM-CSF, baso/mast cells accounted for 9%,
basophils 63%, immature myeloid cells 13%, blasts 1% and others 14%.

**Fig 4.** Effects of TPO on differentiation of TL blasts. (A and B) Morphology of TL blasts (Pt. 4) before (A) and after (B) culture in the presence of TPO. (C) Flow cytometric analysis of CD41 expression in TL blasts (Pt. 4) on day 0 (d0) and day 7 (d7) in culture with TPO. (D-G) Electron micrograph of TL cells (Pt. 4) cultured for 7 days in the presence of TPO (D and F) and higher magnification (E and G). Both multi-lobed large cells (D and E) and mononuclear small cells (F and G) possess platelet demarcation membrane system (arrow) and platelet specific granules with electron-dense core (arrowhead). Bars (A and B), 20µm; (D and F), 2 µm; (E and G) 1 µm.

**Fig 5.** Western blot analysis of TL blasts. (A-C) Expression of GATA1 and GATA1s. (D and E) Expression of GATA2. Leukemic cell lines, K562, CMK and KU812 are controls for full-length GATA1, GATA1s and GATA2, respectively, and α-tubulin is a control for protein loading. Different parts of the same gel are placed side by side in the left panel of (B), as indicated by black bars, by removing unnecessary lanes between them.

**Fig 6.** Mutation analysis of Patient 4. (A) Direct sequencing of the PCR product. Genomic DNA was extracted from TL blasts before and after the culture with GM-CSF and TPO. GATA1 exon 2 was amplified by PCR and the product was directly sequenced. (B) Two distinct mutations found in patient 4. To separate two overlapping sequences shown in (A), the PCR product was sequenced after subcloning into pGEM-T Easy vector.
Table 1. Clinical profile and cytogenetic findings of patients with TL

<table>
<thead>
<tr>
<th>Pt.</th>
<th>Age</th>
<th>Sex</th>
<th>WBC (10⁹/L)</th>
<th>PB blast (%)</th>
<th>Karyotype</th>
<th>GATA1 mutation</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24 d</td>
<td>M</td>
<td>33.0</td>
<td>18</td>
<td>47, XY, +21</td>
<td>c.158_162delCCACCinsT*</td>
<td>Remission without treatment, died of unrelated illness (11 mon)</td>
</tr>
<tr>
<td>2</td>
<td>0 d</td>
<td>M</td>
<td>56.3</td>
<td>50</td>
<td>47, XY, +21</td>
<td>c.108_151del44</td>
<td>Remission without treatment, AMKL (1 y 7 mon)</td>
</tr>
<tr>
<td>3</td>
<td>2 d</td>
<td>M</td>
<td>184</td>
<td>75</td>
<td>47, XY, +21</td>
<td>c.2dupT</td>
<td>Remission without treatment, alive (6 y)</td>
</tr>
<tr>
<td>4</td>
<td>8 d</td>
<td>M</td>
<td>34.0</td>
<td>82</td>
<td>47, XY, +21</td>
<td>c.41_57del17, c.5_23del19insCCTCTGAG</td>
<td>Remission without treatment, AMKL (1 y 10 mon), alive (10 y)</td>
</tr>
<tr>
<td>5</td>
<td>4 d</td>
<td>F</td>
<td>34.5</td>
<td>40</td>
<td>47, XX, +21</td>
<td>wild type</td>
<td>Remission without treatment, AMKL (2 y 0 mon), died of cerebral hemorrhage (2 y 4 mon)</td>
</tr>
</tbody>
</table>

*: as per Genbank entry NM_002049.3: the A of the ATG of the initiator Met codon is counted as nucleotide +1.
Figure 1
Figure 4
Figure 5

A

Pt. 2  Pt. 1
K562 CMK d0  d3  d5  d7  d0  d3  d5  d7
GATA1
GATA1s
α-Tubulin

B

Pt. 3  Pt. 3
GM-CSF  TPO
K562 CMK d0  d3  d5  d7  d14  d0  d3  d5  d7
GATA1
GATA1s
α-Tubulin

C

Pt. 1
EPO + SCF
d0  d7  d14  d21
GATA1
GATA1s
α-Tubulin

D

Pt. 2  Pt. 3
KU 812  IL-3
K562 CMK d0  d3  d5  d7  d14  d0  d3  d5  d7  d14
GATA2
α-Tubulin

E

Pt. 4  Pt. 3
GM-CSF  TPO
KU
d0  d3  d5  d7  d14  812  d0  d3  d7  d14
GATA2
α-Tubulin
Figure 6