**In Vitro** Cell Growth Stimulated by Recombinant Human Cytokines Can Help to Diagnose Transient Leukemia in Neonates

**Hsi-Che Liu,**1,2 **Shu-Huey Chen,**1 **Lin-Yen Wang,**1 **Ting-Chi Yeh,**1 **I-Jen Chai,**3 **Der-Cherng Liang**† *

**Background/Purpose:** In a previous study, we demonstrated that *in vitro* cell growth stimulated by human placental conditioned medium distinguished between transient leukemia (TL) and congenital acute myeloid leukemia (AML) in neonates. We then sought to determine whether the application can be expanded if *in vitro* cell growths are stimulated by recombinant human cytokines including granulocyte-macrophage colony-stimulating factor (rhGM-CSF), interleukin-3 (rhIL-3), stem cell factor (rhSCF) and thrombopoietin (rhTPO).

**Methods:** Eight neonates with features indistinguishable from AML were studied. Seven patients had Down syndrome and the eighth a normal phenotype. Bone marrow or peripheral blood mononuclear cells (MNC) were cultured in the presence of rhGM-CSF + rhIL-3 + rhSCF or of rhTPO alone. After incubation, granulocyte-macrophage colony-forming units (CFU-GM)-derived colonies and clusters were scored on an inverted microscope. Colony-forming units-megakaryocyte (CFU-MK)-derived colonies were counted with an *in situ* CD61 immunostained dish. Liquid suspension cultures of MNC were stimulated by rhGM-CSF and/or rhTPO.

**Results:** CFU-GM-derived colonies and clusters from bone marrow and peripheral blood MNC revealed normal patterns in seven patients. RhTPO-stimulated megakaryocyte colony formation was normal in one patient. Cytospin smears of liquid suspension cultures all showed good myeloid or megakaryocytic maturation consistent with TL rather than AML. One neonate died on the 2nd day of life, but in the seven remaining patients, blasts disappeared from the peripheral blood within 10 months. Among four patients followed long-term, one developed myelodysplastic syndrome at 21 months. This child was given tailored chemotherapy and had a disease-free survival >20 months.

**Conclusion:** *In vitro* cell growth stimulated by recombinant human cytokines can help to diagnose TL in neonates. [J Formos Med Assoc 2007;106(5):365–371]

**Key Words:** transient leukemia, Down syndrome, recombinant human cytokines, granulocyte-macrophage colony-stimulating factor, thrombopoietin

Transient leukemia (TL) in neonates, also referred to as transient myeloproliferative disorder or transient abnormal myelopoiesis, is characterized by an excessive accumulation of leukemic blasts and other hematologic abnormalities. It occurs commonly in neonates with Down syndrome or in phenotypically normal neonates with chromosome 21 mosaicism, although it has also been reported in neonates with a normal karyotype. There is a high incidence in TL of spontaneous disappearance of blasts within the first few months of life. However, in the first month of

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1Division of Pediatric Hematology-Oncology, Department of Pediatrics and 3Department of Medical Research, Mackay Memorial Hospital and 2Mackay Medicine, Nursing and Management College, Taipei, Taiwan.

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*Correspondence to:* Dr Der-Cherng Liang, Division of Pediatric Hematology-Oncology, Department of Pediatrics, Mackay Memorial Hospital, 92, Section 2, Chung-Shan North Road, Taipei 10449, Taiwan.  
E-mail: dcliang@ms2.mmh.org.tw
life, it is generally difficult to differentiate TL from congenital acute myeloid leukemia (AML). Previously, we demonstrated that *in vitro* cell growth stimulated by human placental conditioned medium (HPCM) can distinguish between these two disorders.6

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a cytokine that promotes the proliferation and maturation of neutrophilic precursors. The function of mature neutrophils as well as the number and function of monocyte-macrophage cells are also enhanced by GM-CSF. The molecular sequence of endogenous human GM-CSF was first identified in 1985. Since then, trials of recombinant human GM-CSF (rhGM-CSF) have demonstrated its diverse biologic effects as playing a vital role in various hematopoietic as well as immune functions.7,8

Interleukin-3 (IL-3) is known to be involved in the growth and the differentiation of CD34+ progenitor cells into basophils, mast cells, dendritic cells (myeloid- or nonmyeloid-derived), and, to a lesser extent, eosinophils and monocyte-macrophages.8 *In vitro* studies have shown that stem cell factor (SCF; also known as kit ligand, mast cell growth factor, or steel factor) acts on hematopoietic stem cells and progenitor cells. Recombinant human SCF (rhSCF), in concert with rhIL-3 or rhGM-CSF has been demonstrated to encourage growth of granulocyte-macrophage colony-forming units (CFU-GM) in both semi-solid and liquid culture media.9

Thrombopoietin (TPO), the ligand for the c-mpl receptor (Mpl ligand, ML), is a major regulator of platelet production *in vivo*. Recombinant human TPO (rhTPO) alone can stimulate proliferation and maturation of megakaryocytes in liquid suspension and in colony-forming units-megakaryocyte (CFU-MK) progenitor assays.10 We have studied the effects of rhTPO on megakaryocyte colony formation from control human bone marrow cells and human leukemia cells at diagnosis. Our previous work showed that rhTPO alone effectively stimulates megakaryocyte colony formation in histologically normal-looking bone marrow. In contrast, rhTPO stimulates leukemic colony formation only in patients with acute megakaryocytic leukemia (AMKL).11

In this study, our goal was to demonstrate whether *in vitro* cell growth stimulated by recombinant human cytokines can differentiate TL from AML in the neonatal period.

**Materials and Methods**

Between March 1995 and July 2003, eight consecutive neonates with hematologic and clinical findings suggestive of AML were studied. Seven patients had Down syndrome and the eighth had a normal phenotype. Congenital infection and hemolytic anemia were carefully excluded in all eight.

Bone marrow and peripheral blood smears were stained with a modified version of the Romanowsky method.12 Immunophenotyping was performed by flow cytometry using monoclonal antibodies against human leukocyte antigen (HLA)-DR, CD34, CD19, CD10, sIg (κ and λ), CD2, CD3, CD4, CD8, CD13, CD14, CD33 and CD61 as previously described.13 For the assessment of a megakaryocytic proliferative disorder, at least 15% of the blast cells need to be positive for the platelet-specific monoclonal antibody, CD61. Samples were defined as positive for any of the other cell markers if >30% of the cells were fluorescence-reactive. Leukemic cells were cytogenetically analyzed using banded chromosomes from unstimulated peripheral blood or direct and 24-hour-cultured preparations of fresh bone marrow, as described previously.14 Chromosome abnormalities were designated by using the 1995 International System for Human Cytogenetics Nomenclature.15

**Cells**

Bone marrow samples were centrifuged over Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) at 2600 rpm for 20 minutes at room temperature. The mononuclear cells (MNC) recovered from the interface were washed three times by centrifugation at 2000 rpm for 10 minutes and then resuspended in Iscove’s modified Dulbecco’s medium.
Adherent cells were then removed according to previously described methods.11

**Recombinant human cytokines**

The recombinant human cytokines used included rhGM-CGF, rhIL-3, rhSCF and rhTPO (provided by Kirin Brewery, Tokyo, Japan).

**Cell culture**

Bone marrow MNC were cultured at a concentration of $2 \times 10^5$ cells and peripheral blood MNC at $1 \times 10^6$ cells in a single layer system with 1 mL α-medium containing 0.3% agar, 20% fetal calf serum (FCS), rhGM-CSF 12.5 ng, rhIL-3 50 ng, and rhSCF 100 ng. After 7 days of incubation in 5% CO$_2$ and fully humidified air at 37°C, GM-CFU-derived colonies and clusters on agar plates were scored on an inverted microscope. Aggregates of 8–40 cells were counted as clusters and >40 cells as colonies.

CFU-MK culture was performed using an agarose-serum free system,16 1 mL containing rhTPO 100 ng. After incubation for 14 days, the whole dish was fixed and refrigerated until immunocytochemical staining with CD61 in situ. A cluster of three or more CD61(+) cells seen under light microscope was counted as a CFU-MK-derived colony.11

The bone marrow or peripheral blood MNC were also suspended in 1 mL α-medium containing 15% FCS and rhGM-CSF 12.5 ng and/or rhTPO 100 ng. Cells were harvested 7 days later and cytospin preparations were made and stained with the modified Romanowsky system (rhGM-CSF) or immunostain by CD61 (rhTPO).

Since it is often difficult to obtain enough cells from bone marrow aspiration in neonates, peripheral blood cells, which comprised 16–80% of blasts, were also used. CFU-GM and CFU-MK assays, and liquid suspension cultures of bone marrow or peripheral blood were performed to explore the characteristics of hematopoiesis in our patients.

**Control**

The bone marrow samples from 14 non-Down syndrome children with AML were collected as positive controls of CFU-GM assay stimulated by rhGM-CSF + rhIL-3 + rhSCF. According to the FAB (French-American-British) classification of AML,17–19 there were one M0, two M1, three M2, two M3, one M4, three M5 and two M7 (AMKL). The genetic abnormalities included two t(8;21), two t(15;17), one t(1;22) and one trisomy 8 and eight normal karyotype. Two AMKL patients were also used in the study of CFU-MK assay stimulated by rhTPO.

**Results**

The characteristics of all eight neonates at diagnosis are summarized in Table 1. The hematologic values varied substantially, with white blood cell counts ranging from $11.5$ to $180 \times 10^9$/L and blasts from 16% to 89%. Patients 4, 6, and 7 had

<table>
<thead>
<tr>
<th>Table 1. Characteristics of patients at presentation</th>
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<tr>
<td>Patient</td>
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<tr>
<td>2</td>
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*Patient with normal phenotype. WBC = white blood cell; PB = peripheral blood; Hb = hemoglobin.*
myelofibrosis, with little yield on bone marrow aspiration. Immunophenotyping was performed for six patients, including three in whom it was done using peripheral blood. The immunophenotypic data varied, with CD61 positivity (>15%) in four of the six patients. The positive results were 40% in Patient 4, 28% in Patient 6, 25% in Patient 7 and 16% in Patient 8. The remaining Patients 2 and 5 expressed positivity (>30%) of CD34, HLA-DR and CD33. They showed 5% and 6% CD61 stained cells, respectively.

The results of CFU-GM and CFU-MK assays are shown in Table 2. CFU-GM assays of five bone marrow samples (Patients 1, 4, 5, 6 and 8) disclosed normal ratios of clusters to colonies as compared with normal historical controls.6 In contrast, lack of colony formation (median 0, range 0–6.5) with poor (down to 0) or extensive cluster formation (up to 1315), with a median of 8.5, were observed in 14 positive controls of AML bone marrow samples. These results generated the ratios of clusters to colonies to an abnormally wide range of 4.25 to infinity. The number of CFU-MK-derived colonies from Patient 2 was similar to those of normal historical controls,11 rather than the extensive megakaryoblast proliferation seen in two positive controls with AMKL. The results of cytospin smears of liquid suspension cultures are summarized in Table 3. After stimulation by rhGM-CSF alone, cytospin smears of liquid suspension cultures from five bone marrow and four peripheral blood samples showed good myeloid and macrophage maturation without an increase of myeloblasts. Liquid suspension cultures of bone marrow and peripheral blood cell from Patient 8 were also stimulated by rhTPO and the cytospin smears from both samples showed an increase in mature megakaryocytes. Bone marrow cells from Patients 4 and 6 were co-stimulated with rhGM-CSF and rhTPO, with the resulting cytospin smears demonstrating good myeloid, macrophage and megakaryocytic maturation without an increase of myeloblasts or megakaryoblasts. In patients with an increased number of CFU-GM-derived colonies or clusters, liquid suspension culture results were all normal.

Since none of the eight neonates had in vitro cell growth patterns suggestive of AML, TL was diagnosed in all eight. Accordingly, no chemotherapy was given. Patient 7, who had respiratory failure,

### Table 2. CFU-GM or CFU-MK assays stimulated by recombinant human cytokines

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<tr>
<th>Patient</th>
<th>Bone marrow</th>
<th>Peripheral blood</th>
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<tr>
<td></td>
<td>Cluster</td>
<td>Colony</td>
</tr>
<tr>
<td>CFU-GM assay stimulated by rhGM-CSF + rhIL-3 + rhSCF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>82</td>
<td>19.5</td>
</tr>
<tr>
<td>3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>1224</td>
<td>1868</td>
</tr>
<tr>
<td>5</td>
<td>282.5</td>
<td>193</td>
</tr>
<tr>
<td>6</td>
<td>437</td>
<td>422</td>
</tr>
<tr>
<td>7</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>440</td>
<td>144</td>
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<tr>
<td>Positive controls (14 AML)*</td>
<td>0–1315 (8.5)</td>
<td>0–6.5 (0)</td>
</tr>
<tr>
<td>CFU-MK derived colonies stimulated by rhTPO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>–</td>
<td>0.5</td>
</tr>
<tr>
<td>Positive controls (2 AMKL)</td>
<td>–</td>
<td>Numerous</td>
</tr>
</tbody>
</table>

*Data for positive controls are presented as range (median). The number of colonies and clusters for bone marrow cells is given per $2 \times 10^5$ mononuclear cells and for peripheral blood cells per $1 \times 10^6$ mononuclear cells. CFU-GM = colony-forming units-granulocyte-macrophage; CFU-MK = colony-forming units-megakaryocyte; rh = recombinant human; GM-CSF = granulocyte-macrophage colony-stimulating factor; IL-3 = interleukin-3; SCF = stem cell factor; ND = not done; TPO = thrombopoietin.
acutely renal failure, and liver dysfunction, died after withdrawal of life support on the 2nd day of life. In the other seven patients, the blasts disappeared from the peripheral blood within 10 months. Three patients subsequently died because of other conditions (tetralogy of Fallot at surgery, annular pancreas after surgery, and acute renal failure). Among the four patients (three with Down syndrome and one with a normal phenotype) followed long-term, Patient 6 developed myelodysplastic syndrome at the age of 21 months. He was given chemotherapy with lower doses tailored for Down syndrome, consisting of cytarabine and idarubicin (7 + 3) as induction therapy and three courses of cytarabine sequentially combined with VP-16, L-asparaginase and VP-16 as post-remission therapy. As of June 30, 2005, disease-free survival was longer than 20 months. The other three patients aged 24, 56, and 66 months were alive without leukemia.

**Discussion**

Children with Down syndrome have a 10- to 20-fold increased risk of developing leukemia. Based on the screening of peripheral blood smears in a small cohort of newborns with Down syndrome, Doyle and Zipurksy estimated that approximately 10% of such infants have TL. When fetal cases are taken into consideration, the incidence could be as high as 20%. Spontaneous disappearance of blasts from TL is observed in the majority of cases within the first few months of life. However, of those who enter spontaneous remission, 13–33% subsequently develop a hematologic disorder, most frequently AMKL, usually diagnosed at less than 3 years of age (mean age, 16 months). TL may also develop in neonates with mosaic trisomy 21 karyotypes and infrequently in those without a known chromosomal abnormality.

Though our sample size was limited, the clinical features at diagnosis, the diverse results of surface antigen expression, and the relapse rate before 3 years of age were quite similar to the characteristics of patients in published large-scale reviews. Based on the results of in vitro cell growth patterns, all the eight patients were diagnosed with TL. In the seven patients who survived the first week of life, blasts disappeared from the peripheral blood within months. Only one patient who subsequently relapsed with myelodysplastic syndrome responded well to chemotherapy.

There is no single conventional test which can differentiate congenital AML from TL in all cases. Although most patients with TL have a significant megakaryocytic component, the majority of blasts lack definitive identifiable features.

| Table 3. Liquid suspension cultures stimulated by recombinant human cytokines |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Patient | Bone marrow | Peripheral blood | Bone marrow | Peripheral blood | Bone marrow | Peripheral blood |
| 1 | Good myeloid maturation | Good myeloid maturation | ND | ND | ND | ND |
| 3 | ND | Good myeloid maturation | ND | ND | ND | ND |
| 4 | Good myeloid and macrophage maturation, blasts < 5% | ND | Good myeloid and macrophage maturation, blasts < 5%; mature megakaryocytes | ND | ND | ND |
| 5 | Good myeloid maturation | ND | ND | ND | ND | ND |
| 6 | Good myeloid maturation | ND | Megakaryoblast < 10% | ND | ND | ND |
| 7 | ND | Good myeloid and macrophage maturation | ND | Increase in mature megakaryocytes | Increase in mature megakaryocytes |
| 8 | Good myeloid and macrophage maturation | Good myeloid and macrophage maturation | ND | Increase in mature megakaryocytes | Increase in mature megakaryocytes |

$rh =$ recombinant human; GM-CSF = granulocyte-macrophage colony-stimulating factor; TPO = thrombopoietin; ND = not done.
Platelet-associated antigens, such as CD41 and CD61, are commonly expressed in TL. Morphologic, immunologic, and cytogenetic data suggest that TL may involve the proliferation of bone marrow cells that are a common progenitor for the erythroid and megakaryocytic lines.\textsuperscript{25–27} Regardless, these features are still not specific enough to distinguish between TL and congenital AML.

Using agar cultures of bone marrow and peripheral blood, Moore et al showed that none of 108 untreated AML cases had both colony and cluster growth patterns within the normal range.\textsuperscript{28} Shih and Chang demonstrated that cultures from bone marrow cells of 62 untreated AML were characterized by lack of normal colony formation with growth of varying numbers of clusters, ranging from no growth at all or excessive microcluster formation.\textsuperscript{29} In contrast, in reports of three neonates with Down syndrome and TL, normal colony formation was found in methylcellulose semisolid culture.\textsuperscript{30} A normal ratio of clusters to colonies was shown in two newborn babies with Down syndrome and TL.\textsuperscript{31} By extending the applicability of the semisolid agar system to liquid suspension cultures, our previous study demonstrated that \textit{in vitro} cell growth was helpful in differentiating TL from AML.\textsuperscript{6}

Before the emergence of recombinant human cytokines, HPCM was widely used as a source of colony-stimulating activity. We chose recombinant human cytokines instead because they are standardized preparations which can easily be titrated so that use in different laboratories can provide replicable results. With stimulation by rhGM-CSF + rhIL-3 + rhSCF, the CFU-GM assays from 14 positive AML controls consistently showed very abnormal \textit{in vitro} cell growth patterns which contrasted to the neonatal TL which invariably revealed a normal \textit{in vitro} cell growth pattern. The results in the one patient with a CFU-MK assay of bone marrow cells after stimulation by rhTPO were similar to those of normal historical controls. In contrast, both positive AMKL controls disclosed extensive CFU-MK derived colonies. Cytospin smears of liquid suspension cultures afford direct observation of the differentiation and maturation of hematopoietic cells. Our smears, either from bone marrow and peripheral blood, both showed good myeloid and megakaryocytic maturation without an increase in myeloblasts or megakaryoblasts.

For such neonates who had higher percentages of blasts in peripheral blood than those in the bone marrow, their bone marrow was hard to aspirate due to their smaller body size; but we successfully further expanded the capacity to differentiate TL from congenital AML previously by using HPCM stimulation\textsuperscript{6} to using recombinant human cytokine stimulation in this study. The methods reported here using peripheral blood as a substitute of bone marrow may provide an alternative diagnostic tool.

Recent studies discovered that acquired mutations of \textit{GATA1} are the most specific genetic markers linked to Down syndrome AMKL. Mutations in \textit{GATA1} are also shown in TL blasts.\textsuperscript{32,33} To date, the mutations have never been detected in samples from Down syndrome patients with other leukemias, including those with ALL or non-AMKL AML.\textsuperscript{34} Our method may provide a useful diagnostic adjunct especially for the neonatal TL cases whose status of \textit{GATA1} mutation is uncertain or unavailable.

In conclusion, our study demonstrates \textit{in vitro} cell growths stimulated by recombinant human cytokines, including rhGM-CSF, rhIL-3, rhSCF and rhTPO can help to diagnose TL in neonates.

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**References**


