

Stromal Cells Provide Signals Different from Cytokines for STAT5 Activation in Hematopoietic Cells

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ABSTRACT. After detachment from the stromal cells, hematopoietic stem cells are thought to differentiate to the cytokine-dependent stages where their growth and differentiation are promoted by these cytokines. To examine the stromal regulation of hematopoietic stem cells, we previously established a primitive hematopoietic stem-like cell line, THS119, whose growth was dependent on the bone marrow stromal cell line, TBR59, and from which IL-3- (THS119/IL-3) or IL-7- (THS119/IL-7) dependent cell lines were then generated. Using these cell lines, we examined the difference in signals mediated by the stromal cells and cytokines.

The cytokine-dependent cell lines (THS119/IL-3 and THS119/IL-7) showed induction of STAT5 phosphorylation and target genes for STAT5 such as CIS, pim-1, p21 and bcl-xL upon addition of IL-3 or IL-7. IL-3 or IL-7 also induced STAT5 phosphorylation and STAT5 target genes of the stromal cell-dependent cell line, THS119, in the absence of stromal cells at levels similar to the cytokine-dependent cell lines. However, quite interestingly, TBR59 stromal cells could not induce STAT5 phosphorylation of THS119 cells, although they did induce STAT5 target genes in THS119 cells. In addition, the mRNAs for STAT5 target genes in THS119 cells on the stromal cells seemed to be more stable than those in the cytokine-dependent cell lines. Expression of the antiapoptotic genes bcl-2 and bcl-xL was higher in the stromal cell-dependent cell line than in the cytokine-dependent cell lines. These results suggested that stromal cells and cytokines may provide different signals for growth and differentiation of the hematopoietic cells.

Key words: stromal cell/IL-3/IL-7/hematopoietic stem cell

Studies on long-term bone marrow culture have shown that hematopoietic stem cells can be maintained for a long-time in culture on the stromal cell layers of bone marrow, in which the self-renewal and differentiation of the hematopoietic stem cells and their progenitors are regulated by stromal cell-stem cell communication via factors secreted from the stromal cells, extracellular matrices and adhesion molecules (Dexter *et al.*, 1977; Itoh *et al.*, 1989, 1996; Kodama *et al.*, 1984; Mauch *et al.*, 1980).

To study how the stem cells are regulated by the stromal cells, we have established many stromal cell lines from bone marrow of temperature-sensitive (ts) SV40 large T-

antigen gene transgenic mice (Kameoka *et al.*, 1995) and reported that cobblestone areas containing myeloid and B-lymphoid progenitors were formed from the sorted stem cells depending on a bone marrow stromal cell line, TBR59 (Iguchi *et al.*, 1997; Koguma *et al.*, 1998; Okuyama *et al.*, 1995). Using this stromal cell line as feeder layer, a novel stromal cell-dependent primitive hematopoietic cell line, THS119, was established from the sorted Lin⁻/Sca-1⁺ cells of bone marrow of ts SV40 large T-antigen gene transgenic mice after long-term passages (Yanai *et al.*, 1999).

THS119 cells expressed c-Kit and Sca-1, but did not express any surface markers (Gr-1, TER119, Mac-1, CD3, or B220) of differentiated hematopoietic cells, and showed multiple expression of lineage-restricted hematopoietic transcription factors, indicating that they retained immature phenotypes of the fractionated hematopoietic stem cells after immortalization. We examined whether known cytokines could be replaced from the stromal cells for maintenance of THS119 cells, but none of the cytokines supported

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Abbreviations: STAT, transducer and activators of transcription; IL, interleukin; CIS, cytokine-inducible SH2-containing protein; OSM, oncostatin M.

maintenance when IL-3, IL-6, IL-7, G-CSF, GM-CSF, M-CSF, Epo, or SCF was added to THS119 cells without stromal cells. However, when these cytokines were continuously added to the cocultures of THS119 cells with TBR59 cells for 2 months, the cells were able to grow in the presence of either IL-3 or IL-7 without TBR59 stromal cells and, finally, IL-3- (THS119/IL-3) or IL-7- (THS119/IL-7) dependent cell lines were established (Yanai *et al.*, 1999).

It is thought that after detachment from the stromal cells, hematopoietic stem cells differentiate to the cytokine-dependent stages where their growth and differentiation are promoted by these cytokines. In the present study, we examined the different responses to cytokines and stromal cells shown by the stromal cell-dependent THS119 cell line and the IL-3- or IL-7-dependent cell lines.

Since signal transducers and activators of transcription (STATs) are known to be indispensable for intracellular signaling after stimulation with cytokines, growth factors and hormones (Darnell, 1997), we examined the induction of STAT5 phosphorylation and STAT5 target genes. The results suggested that the stromal cells and cytokines may provide different signals for growth and differentiation of hematopoietic stem cells.

Materials and Methods

Culture of THS119 cells and the cytokine-dependent cell lines

Culture of THS119 cells and TBR59 stromal cells was performed as reported previously (Yanai *et al.*, 1999). Throughout the experiments, E-RDF medium (Kyokuto Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 5% FBS, 10 µg/ml transferrin, 5 µg/ml insulin, 4.3 ng/ml sodium selenite, 1.53 µg/ml ethanolamine, and 100 µM 2-mercaptoethanol (2-ME) was used as a basic medium. For the cytokine-dependent cell lines, THS119/IL-3 was maintained in the presence of 2 ng/ml of IL-3 and THS119/IL-7 was maintained in the presence of 2 ng/ml of IL-7. Recombinant murine IL-3 was generously provided by Kirin Co. (Tokyo), and recombinant IL-7 was purchased from Genzyme (Cambridge, MA).

For analysis of growth of THS119 cells on TBR59 cells, conflu-

ent cell layers of TBR59 stromal cells were first formed in 6-well plates, and 2×10^5 of THS119 cells were inoculated onto each well and cultured at 33°C. Cell numbers were counted after recovering THS119 cells by collagenase treatment.

Detection of phosphorylation of STAT5 protein by Western blotting

To determine their cytokine response, THS119 cells were removed from the coculture with TBR59 stromal cells, washed twice, and transferred to the culture medium. Similarly, the cytokine-dependent cell lines maintained in the presence of IL-3 or IL-7 were washed twice and transferred to the culture medium without cytokines. The transferred cells were cultured for 4 hr and then either IL-3 (10 ng/ml) or IL-7 (5 ng/ml) was added to the culture and incubated for 30 min to detect phosphorylation of STAT5 protein.

Cells were homogenized in lysis buffer containing 50 mM Tris-HCl pH 8.0, 0.5% NP40, 1 mM EDTA, 150 mM NaCl, 10% glycerol, 1 mM sodium vanadate, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, and proteinase inhibitor cocktail (Boehringer Mannheim, Germany). The extracts were clarified by centrifugation at 15,000 rpm at 4°C for 15 min. The samples were resolved in SDS-PAGE and proteins were detected by immunoblotting with anti STAT5 (C-17) (Santa Cruz Biotechnology, Santa Cruz, CA) (Matsumoto *et al.*, 1999). Slowly migrating bands were assigned as phosphorylated STAT5 proteins (Beadling *et al.*, 1996).

Detection of mRNA by RT-PCR

Total RNA was isolated by Isogen (Wako Pure Chemicals, Ltd., Tokyo) according to the manufacturer's protocol. Five µg total RNA was denatured for 10 min at 70°C and reverse transcribed for 50 min at 42°C in a final volume of 20 µl PCR buffer containing 6.6 units/ml SuperScript II RT, 0.5 mM dNTP, 25 ng/µg Oligo(dT) primer, 1.25 mM MgCl₂, 5 mM DTT (SuperScript™ Preamplification System, GIBCO BRL). The RT reactions were terminated by heating for 15 min at 70°C. RT products were amplified by PCR amplification using 0.25 U/ml taq polymerase (AmpliTaq Gold, Perkin Elmer) with the gene specific primers for different gene probes (Table I) in a final volume of 10 µl. Cycling conditions consisted of a melting step at 94°C (30 sec), an annealing step at an appropriate annealing temperature (30 sec) and an extension step

Table I. OLIGONUCLEOTIDE PRIMERS USED IN THIS STUDY

Gene	5' primer	3' primer	Reference
CIS	CTGGAGCTGCCCCGGGCCAGCC	CAAGCCTGACCACATCTGGG	Bjorbaek <i>et al.</i> , 1999
OSM	CGGCACAATATCCTCGGCATAAGG	TGCTCCTGGAAGGTCTGATTTTGC	Lischke <i>et al.</i> , 1998
pim-1	CACCGACACCCTGGAGGT	GCTACTTGCTGGATCCCCG	Nosaka <i>et al.</i> , 1999
p21	ATGTCCAATCCTGGTGATGTCCG	CTTCAGGGTTTCTCTTGCAG	Wu <i>et al.</i> , 1996
bcl-xL	ATGTCTCAGAGCAACCGG	TCACTTCCGACTGAAGAGTG	Nosaka <i>et al.</i> , 1999
bcl-2	TCGCTACCGTCGTGACTTC	AAACAGAGGTCGCATGCTG	
bax	ATGCGTCCACCAAGAAGCTGAG	CCCCAGTTGAAGTTGCCATCAG	Tomayko <i>et al.</i> , 1998
GAPDH	CTTACCACCATGGAGAAGG	TGAAGTCGCAGGAGACAACC	

at 72°C (90 sec). The number of cycles was determined to be that which showed a linear trajectory before they reached the maximum plateau. Each amplification product was electrophoresed on 2% agarose gel and visualized by staining with ethidium bromide.

Results

Growth properties of stromal cell-dependent and cytokine-dependent cell lines

THS119 cells were grown depending on TBR59 stromal cells, and IL-7 or IL-3 did not support their growth in the absence of stromal cells, although TBR59 cells produced significant levels of IL-7 (Koguma *et al.*, 1998; Yanai *et al.*, 1999). The IL-3-dependent cell line (THS119/IL-3) and IL-7-dependent cell line (THS119/IL-7) which were generated after 2 months of coculture with TBR59 in the presence of these cytokines, showed cytokine-dependent growth (Yanai *et al.*, 1999).

Growth properties of these 3 cell lines were examined as shown in Fig. 1. Parental THS119 cells grew well on TBR59 stromal cells and IL-7 or IL-3 stimulated them only slightly on the stromal cells. The IL-3-dependent cell line, THS119/IL-3, could not grow in the absence of cytokines,

and IL-3 strongly supported its growth, while IL-7 weakly supported it. IL-7 strongly supported growth of the IL-7-dependent cell line, THS119/IL-7, while IL-3 did not stimulate its growth, but seemed to prevent apoptosis since its cell number was maintained. In both cytokine-dependent cell lines, simultaneous addition of IL-7 and IL-3 caused significantly higher growth stimulation than did single addition.

Both cytokine-dependent cell lines showed a very slow growth rate on the stromal cells in the absence of cytokines, but cytokines stimulated the growth of both lines when cultured on TBR59 stromal cells at levels similar to those in the absence of TBR59 cells. These results indicated that growth of THS119 cells was stromal cell-dependent, whereas THS119/IL-3 and THS119/IL-7 cell lines were cytokine-dependent.

Phosphorylation of STAT5 in 3 cell lines

It is well known that STATs are transcription factors indispensable for intracellular signaling after stimulation with cytokines, and that they form homo- or heterodimers upon phosphorylation of tyrosine residues, usually by Janus kinase (JAKs). Dimerized STAT proteins immediately enter the nucleus and bind to the specific DNA sequences in the

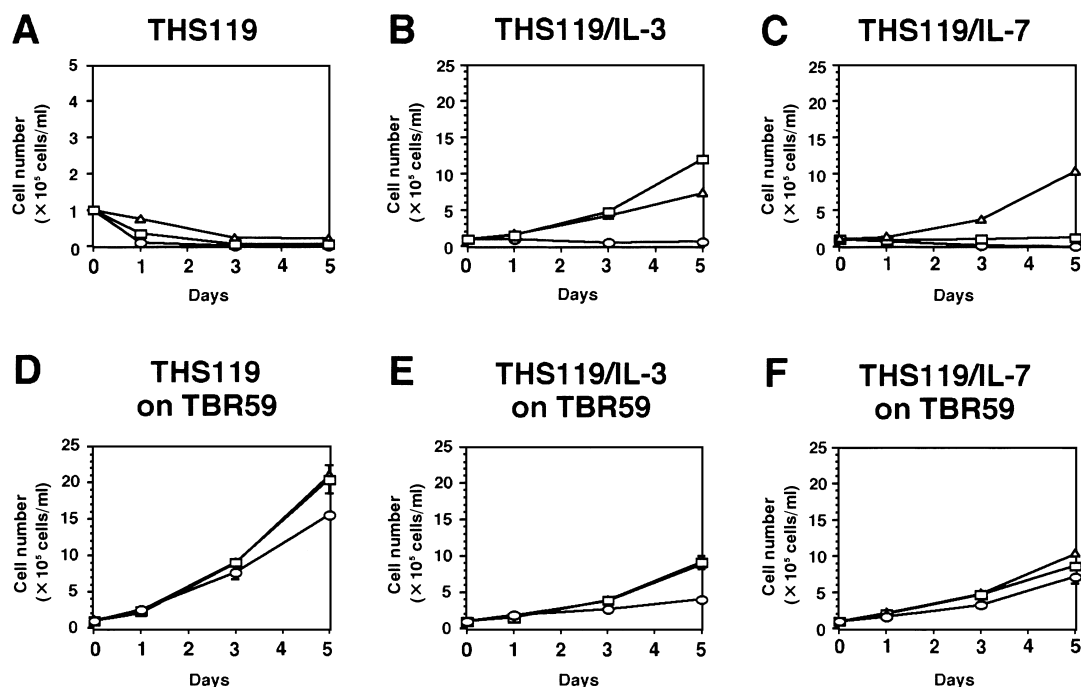


Fig. 1. Stromal cell-dependent and cytokine-dependent growth of THS119, THS119/IL-3, THS119/IL-7 cell lines. For culture of the cell lines on TBR59 cells, confluent cell layers of TBR59 stromal cells were first formed in 6-well plates, 2×10^5 of the cells were inoculated onto each well and the cultures were incubated at 33°C. In the absence of stromal cells, the cells were cultured in the absence of cytokines, or in the presence of 10 ng/ml of IL-3 or 5 ng/ml of IL-7, respectively. After culture on the stromal cells, the hematopoietic cells were removed from the stromal cells by collagenase treatment and cell numbers were counted. A, B and C: The cells were cultured without TBR59 stromal cell layers, but in the absence of cytokines (○) or in the presence of either IL-3 (□) or IL-7 (△). D, E and F: The cells were cultured on the TBR59 stromal cell layers in the absence of cytokines (○) or in the presence of either IL-3 (□) or IL-7 (△).

promoter regions of various genes, resulting in gene activation or repression. Since upon addition of IL-3 or IL-7, signals mediated by their receptors were known to induce phosphorylation of STAT5 proteins (Brown *et al.*, 1999; Mui *et al.*, 1995, 1996; Rosenthal *et al.*, 1997; van der Plas *et al.*, 1996), phosphorylation of STAT5 was examined as shown in Fig. 2. IL-3 or IL-7 induced strong phosphorylation of STAT5 in both THS119/IL-3 and THS119/IL-7 and also induced its phosphorylation in THS119 cells at levels similar to THS119/IL-3 or THS119/IL-7, when these cytokines were added to the culture in the absence of stromal cells. Interestingly, THS119 cells on TBR59 stromal cells, however, showed only barely detectable levels of STAT5 phosphorylation. These results suggested that the support of THS119 cells by the stromal cells may be mediated by signals other than the activation of STAT5, although THS119 cells have the responsibility for the cytokine-dependent STAT5 activation pathways.

Induction of STAT5 target genes in 3 cell lines by cytokines and stromal cells

To examine the induction of STAT5 target genes after STAT5 phosphorylation, RNAs were isolated from the 3 cell lines that had been cultured under different conditions and the induction of these mRNAs was monitored by RT-PCR method. CIS is one of the STAT5 target genes and was isolated as the immediate response gene for IL-3 (Yoshimura *et al.*, 1995). In both cytokine-dependent cell lines, IL-3 or IL-7 induced CIS mRNA, but IL-3 showed a stronger effect. Interestingly, THS119 grown on the TBR59 stromal cells maintained significantly high levels of CIS mRNA, although STAT5 phosphorylation was barely detectable and IL-3 or IL-7 induced it more strongly (Fig. 3). When THS119 cells were transferred to the culture in the absence of TBR59 cells and IL-3 or IL-7 was added to this culture, CIS mRNA was rapidly (within 1 hr) induced at levels sim-

ilar to the cytokine-dependent cell lines, although neither cytokine promoted growth of THS119 cells in the absence of TBR59. These results suggested that the stromal cell-dependent cell line, THS119, retained the signaling pathways responding to cytokines, similar to the cytokine-dependent cell lines.

In addition to CIS mRNA, we examined expression of oncostatin M, pim-1, bcl-xL, and p21 that are known to be the target genes for STAT5 (Noasaka *et al.*, 1999). The results showed that an essentially similar expression of pim-1, bcl-xL, and p21 was induced to that of CIS mRNA. Oncostatin M is known to be induced by IL-3 through STAT5 (Yoshimura *et al.*, 1996), however, to our surprise, oncostatin M was constitutively expressed even in the absence of cytokines among these cell lines.

mRNAs for STAT5 target genes except oncostatin M were rapidly reduced (within 4 hr) when the cytokine-dependent cell lines were cultured in the absence of cytokines, whereas they were stably expressed in THS119 cells even without stromal cells. These results suggested that the mRNA for STAT5 target genes (CIS, bcl-xL, pim-1, p21) were more stable in THS119 cells after detachment from the stromal cells than those in the cytokine-dependent cell lines after withdrawal of cytokines.

Expression of bcl-2 and bax genes

Maintenance of hematopoietic cells may require antiapoptotic activity. Since stromal cells and cytokines induced antiapoptotic gene bcl-xL which was regulated as a target for STAT5 in these cell lines, we investigated the expression of another antiapoptotic gene bcl-2 and the proapoptotic gene bax (Gibson *et al.*, 1996; Oltvai *et al.*, 1993). While bax was expressed constitutively, bcl-2 gene was highly expressed in THS119 on the stromal cells, whereas neither IL-3 nor IL-7 induced its mRNA at significant levels in either of the cytokine-dependent cell lines.

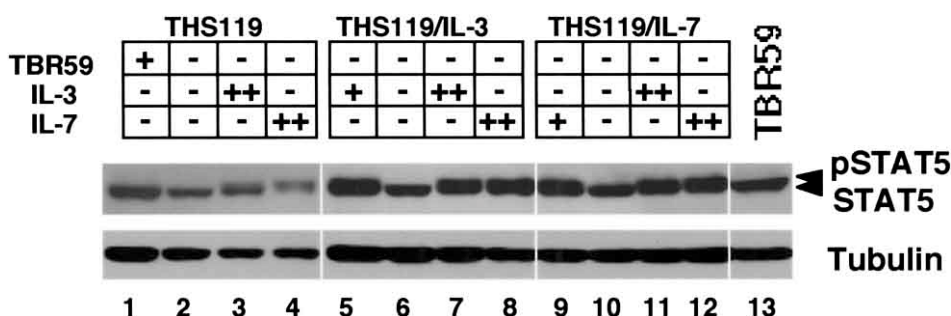


Fig. 2. STAT5 phosphorylation. STAT5 phosphorylation was determined as described in *Materials and Methods*. In lanes 1, 5, and 9, samples were obtained from cells continuously cultured in the presence of TBR59 stromal cells for THS119, in the presence of IL-3 for THS119/IL-3, and in the presence of IL-7 for THS119/IL-7, respectively. In lanes 2, 6, and 10, samples were obtained from cells cultured in the absence of growth stimuli for 4 hr (depleted culture). In lanes 3, 7, and 11, IL-3 was added to the depleted culture and incubated for 30 min. In lanes 4, 8 and 12, IL-7 was added to the depleted culture and incubated for 30 min. The samples (25 µg of protein/lane) were resolved in SDS-6% PAGE and proteins were detected by immunoblotting with anti STAT5 (C-17). Slowly migrating bands (upper arrow: pSTAT5) showed phosphorylated STAT5 proteins. Tubulin antibody was used for monitoring the amount of protein loaded in the samples.

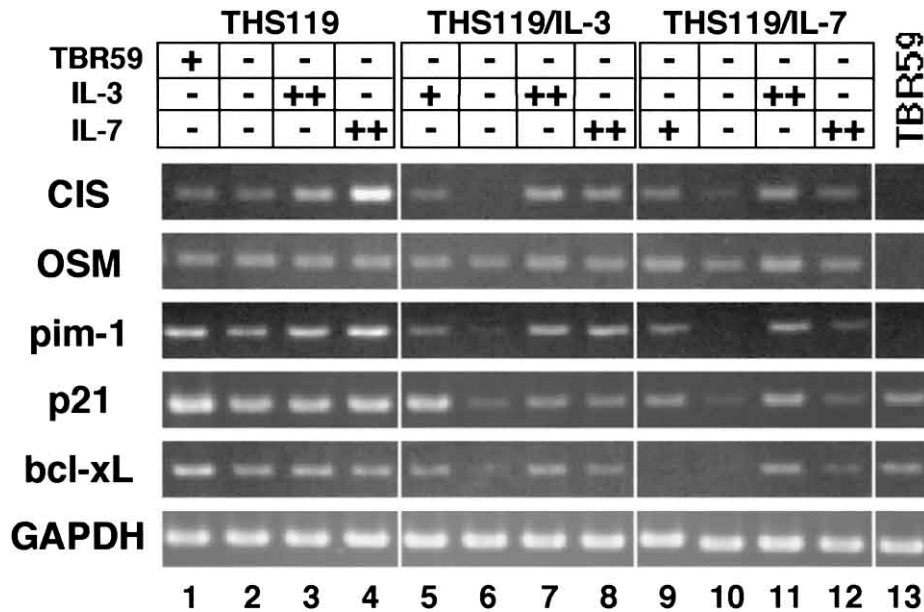


Fig. 3. Analysis of expression of genes downstream of STAT5. Levels of expression were monitored by RT-PCR as described in *Materials and Methods*. RNA samples in each lane are the same as in the Fig. 2, but the samples were taken from the cells incubated for 1 hr after addition of cytokines.

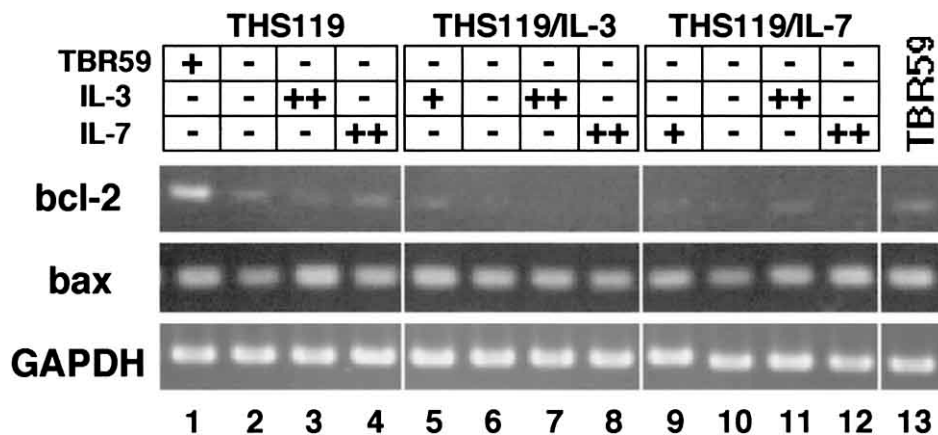


Fig. 4. Expression of bcl-2 and bax genes. RNA samples were obtained from the culture of THS119 in the presence (+) or absence (-) of TBR59 stromal cells, THS119/IL-3 in the presence (+) or absence (-) of IL-3, and THS119/IL-7 in the presence (+) or absence (-) of IL-7, respectively.

Discussion

Within the bone marrow, self-renewal and differentiation of the hematopoietic stem cells may be regulated by the contact with the stromal cell layers (Dexter *et al.*, 1977; Itoh *et al.*, 1989, 1996; Kodama *et al.*, 1984; Mauch *et al.*, 1980; Spooner and Dexter, 1997). After detachment from the stromal cells, hematopoietic stem cells might differentiate to the growth factor-dependent stages and its growth and differentiation may be promoted by these cytokines (Okubo *et al.*, 2000). To understand this process, we established stromal cell-dependent hematopoietic stem cell lines by using one of the bone marrow stromal cell lines, TBR59. We

succeeded in establishing the THS119 cell line, a novel TBR59 stromal cell-dependent primitive hematopoietic cell line from the bone marrow stem cells sorted as Lin⁻/Sca-1⁺ cells (Yanai *et al.*, 1999). Growth of THS119 cells was TBR59 stromal cell-dependent, and therefore they induced apoptosis gradually even in the presence of cytokines after detachment from the stromal cells. However, IL-3 or IL-7-dependent cell lines could be generated from THS119 cells after prolonged culture with cytokines on the stromal cells.

We compared the properties of the stromal cell-dependent THS119 cell line and the two cytokine-dependent cell lines, THS119/IL-3 and THS119/IL-7, to determine whether the stem cells acquire their response to cytokines after de-

tachment from the stromal cells.

Although THS119 and the two cytokine-dependent cell lines expressed mRNAs for the IL-3 or IL-7 receptors to similar extents (Yanai *et al.*, 1999), the growth of THS119 was highest on TBR59 stromal cells while IL-3 and IL-7 stimulated its growth only slightly, indicating that the stromal signals may be preferable for the maintenance of THS119 cells. On the contrary, IL-3 promoted growth of THS119/IL-3 and IL-7 promoted growth of THS119/IL-7, but both these cell lines showed only slight growth on TBR59 stromal cells, indicating that cytokines may be preferable for the growth signals of these cells.

We examined STAT5 phosphorylation since signals mediated by IL-3 or IL-7 receptors were known to induce phosphorylation of STAT5 proteins (Mui *et al.*, 1995, 1996; Rosenthal *et al.*, 1997). In both cytokine-dependent cell lines, IL-3 and IL-7 induced STAT5 phosphorylation to a similar extent, although both showed rather preferential growth stimulation to THS119/IL-3 or THS119/IL-7. In addition, CIS, bcl-xL, pim-1, and p21 as target genes for STAT5 were induced at levels corresponding to those of STAT5 phosphorylation in both cytokine-dependent cell lines. Although IL-3 did not stimulate growth of THS119/IL-7, it seemed to protect apoptosis; thus induction of bcl-xL may partly be involved in the antiapoptotic effect of IL-3. Thus, both cytokine-dependent cell lines may have the same signaling pathways through STAT5 proteins, but IL-3 or IL-7 may preferentially use the upstream signals in combination with some other costimulatory signals.

In THS119, a stromal cell dependent cell line, STAT5 phosphorylation was induced by IL-3 or IL-7 in the absence of stromal cells at levels similar to those in the cytokine-dependent cell lines; thus THS119 cells may have the same signaling pathways via STAT5 proteins as do the cytokine-dependent cell lines. However, STAT5 phosphorylation in THS119 cells was barely detectable when cells were cultured on TBR59 stromal cells, even though a significant level of IL-7 was produced in TBR59 cells as reported elsewhere (Koguma *et al.*, 1998). Therefore, TBR59 stromal cells may use the dominant signals to support THS119 cells in processes other than STAT5 phosphorylation. In contrast to the absence of STAT5 activation, THS119 cells expressed CIS, bcl-xL, pim-1, p21 and oncostatin M, target genes for STAT5 proteins. It is likely that phosphorylation of the STAT family protein other than STAT5 and/or other signaling pathways mediated by the stimulus of TBR59 stromal cells may be required for the induction of expression of CIS, bcl-xL, pim-1, p21 and oncostatin M.

It is noted that mRNAs for STAT5 target genes (CIS, bcl-xL, pim-1, p21) were more stable in THS119 cells after detachment from the stromal cells than those in the cytokine-dependent cell lines after withdrawal of cytokines. These suggested that the stromal signals may be more persistent while the cytokine signals may be transient. Such signaling pathways may be important to determining the supporting

activity of the hematopoietic stem cells by the stromal cells in a bone marrow hematopoietic microenvironment.

On proapoptotic and antiapoptotic genes, stromal cells induced bcl-2 gene more strongly than cytokines, and bcl-xL mRNA persisted after detachment from the stromal cells, whereas bax, a proapoptotic gene, was expressed constitutively in all cell lines to a similar extent. These results suggest that stromal cells may provide stronger survival signals for the hematopoietic cells than cytokines. On regulation of bcl-2 gene, it was reported that insulin-like growth factor-I (IGF-I) induces a cAMP-response element (CRE) site-containing bcl-2 promoter through a novel signaling pathway involving mitogen-activated protein kinase kinase 6/p38beta mitogen-activated protein kinase/MAP kinase-activated protein kinase-3 (MAPK kinase 6/p38beta MAPK/MAPKAP-K3)/cAMP-response element-binding protein (CREB) (Pugazhenthir *et al.*, 1999). Furthermore, regulation of bcl-2 expression by IGF-I was shown to involve a signaling cascade mediated by PI3-kinase/PDK1/Akt/CREB (Pugazhenthir *et al.*, 2000). It would be interesting to determine whether stromal signals that induce bcl-2 gene expression are mediated by IGF-I secreted from the stromal cells.

Finally, we demonstrated that oncostatin M was constitutively expressed in all 3 cell lines even in the absence of stromal cells or cytokines, while it was isolated as a rapidly inducible gene which responded to IL-3 and was shown to be a target gene for STAT5. It is possible that THS119 could produce cytokines that stimulated its growth, but we favor the view that oncostatin M and/or cytokines may regulate the supporting ability of the stromal cells, because we demonstrated that supporting ability of the bone marrow stromal cells was stimulated by exogenously added oncostatin M (Yanai *et al.*, in preparation). Since THS119 cells were originated from Dexter's long-term bone marrow culture where the hematopoietic cells were maintained by a mixture of stromal cells, the continuous expression of oncostatin M by the hematopoietic cells may stimulate the supporting ability of the stromal cells during the establishment of cell lines, and hence may facilitate establishment of THS119 cell line as a stromal cell-dependent hematopoietic stem cell line. This mutual regulation between stromal cells and hematopoietic cells may also be important in the hematopoietic microenvironment.

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