Clinical Significance of Blood Cell Differentiation**

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It is a well known fact that the blood level of various hematopoietic factors and the states of hematopoietic stem cells are pathogenetically related to the development of various hematopoietic disorders. In this paper, the results of our studies relevant to this subject will be presented, putting emphasis on differentiation and growth factors, their mechanism of action, pathogenetic significance, and clinical application.

1) Differentiation of Erythroid Cells

i) Serum Erythropoietin Level in Various Hematological Disorders

We have developed a radioimmunoassay (RIA) for erythropoietin (EPO) to measure its serum levels in various hematopoietic disorders. With this RIA, it has become possible to measure the serum EPO level with much higher sensitivity and accuracy than with the conventional bio or *in vitro* assays. The results of our measurements confirmed that the serum EPO level increased in reverse proportion to the blood hemoglobin level. In the aged patients, degree of increase in the serum EPO level in response to anemia was less than that in young patients (data not shown).

An increase in the serum EPO level corresponding to the degree of anemia was also observed in patients with renal anemia. However, the EPO level was far lower than the level observed in patients with other anemias. It was also confirmed that in polycythemia vera (PV), the serum EPO levels were below normal, while in patients with secondary polycythemia, the serum EPO levels were significantly increased.

ii) Kidney and EPO

It is well documented that the kidney is intimately related to the production of EPO. However, the EPO activity in kidney extracts was

reported to be very low. The urinary excretion of EPO has also made explanation of this fact very difficult. To confirm the production of EPO in the kidney, we performed Northern hybridization of mRNA from the kidney using monkey EPO cDNA as a probe. This cDNA was provided by Kirin-Amgen company and was confirmed to hybridize with mouse as well as human genomic DNA.

As shown in Fig. 1, the presence of mRNA for EPO was confirmed in kidney tissue after hypoxic stimulation. This finding is considered to confirm the production of EPO in the kidney.

The site of EPO production in the kidney is still controversial. By using an anti EPO with high specificity which was raised against purified recombinant EPO, we have shown the presence of EPO in renal tubular cells. Purified rEPO was prepared from the culture medium of Chinese hamster ovary cells integrated with human EPO

Fig. 1. Induction of EPO-message in hypoxic mouse kidney shown by the Northern hybridization of mRNA from the kidney with EPO cDNA. Highest production of mRNA for EPO was observed after 4 hours of hypoxia as shown by photodensitometry on the right side of the figure.

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gene and supplied by Kirin-Amgen company. It has a specific activity of more than 10,000 units/mg protein with a purity of more than 98%, and it increased the hematocrit of recipient mice to more than 75%. The finding on the localization of EPO in renal tubular cells is contradictory to our previous report¹⁾ as well as other reports on the production of EPO by glomerular cells, although it can explain the inhibition of EPO production by an agent such as HgCl_2 which destroys the renal tubules and EPO production in some cases of kidney carcinomas which are derived almost exclusively from the proximal convoluted tubule. A study to identify the EPO production site by in situ hybridization with EPO cDNA is in progress.

iii) Polycythemia vera, EPO and EPO Receptor

The decreased serum EPO level in PV can be clinically useful for the differential diagnosis of PV from secondary polycythemia. This finding is also considered to show the autonomous production of erythroid cells in PV. The increased sensitivity of erythroid precursor cells (CFU-E) in PV had been demonstrated *in vitro* by spontaneous erythroid colony formation in the absence of EPO addition. CFU-E in PV responds to the small amount of EPO present in serum contained in the culture medium. To study whether this hypersensitivity to EPO is due to increased binding of EPO to CFU-E in PV, we performed the following experiments. Recombinant EPO was labeled with ^{125}I by the chloramine-T method. ^{125}I -rEPO was biologically active, and its binding to mouse erythroleukemia TSA 8 cells, which form erythroid colonies in response to EPO, was inhibited only by cold rEPO. Various other growth factors did not inhibit the binding. The binding was dependent on temperature, time, and hydrogen-ion concentration and inhibited by trypsin treatment. These findings indicate the presence of EPO receptors on EPO responsive cells. The EPO receptors were demonstrated by incubating the DMSO-treated mouse Friend leukemia B8 cells with ^{125}I -rEPO. After cross-linking, cell membrane proteins were analyzed by polyacrylamide-gel electrophoresis and autoradiography. As shown in Fig. 2, two kinds of proteins with molecular weights of 130,000 and 145,000 were demon-

Fig. 2. Cross-linking of ^{125}I -labeled rEPO to its cell surface receptor on DMSO-treated mouse Friend leukemia B 8 cells. Cell membrane protein was chromatogrammed by polyacrylamide-gel electrophoresis and autoradiogrammed after incubation of B 8 cells with ^{125}I -rEPO for 1.5 hours at 15°C .

strated. The molecular weights of EPO receptors were calculated as 95,000 and 110,000. Since these EPO receptors showed the same electrophoretic pattern in reduced as well as in non-reduced states, it was speculated that it had no subunit structure.

The molecular weights of the EPO receptors of TSA8 cells and fetal mouse liver cells (FMLC) were identical with those of the above B8 cells. To identify the cells with EPO receptors in the normal hematopoietic organs, FMLC were fractionated by gravity. When the number of CFU-E and the binding capacity to EPO were measured for each fraction, these values showed a very good correlation. FMLC which had differentiated to erythroblasts after 2 days of incubation with EPO showed no binding capacity to EPO. These results are considered to indicate the presence of EPO receptor on CFU-E and not on differentiated erythroblasts. A Scatchard curve of the EPO receptors of the FMLC showed 2 phases; the K_d of the high-affinity receptor was 0.46 nM and that of low-affinity receptor was 3.96. The number of the low-affinity receptors per cell was

Fig. 3. EPO binding capacity of bone marrow cells from 7 patients with polycythemia vera. It was measured per number of nucleated cells, of erythroblasts and of CFU-E.

Table 1. Incidence of an increase of hematopoietic cells in peripheral blood of patients with various malignancies.

	Incidence of Cases with			
	Neutro- philia ($>10^4/\text{cmm}$)	Mono- cytosis ($10^3/\text{cmm}$)	Erythro- cytosis ($>14.5\text{g/dl}$)	Thrombo- cytosis ($>50 \times 10^4/\text{cmm}$)
Lung	9/122	4/122	3/146	4/141
Stomach	10/187	1/187	3/211	1/166
Colon	2/67	2/67	1/88	0/72
Liver	3/111	3/111	5/120	2/112
Pancreas	4/20	1/20	0/25	1/25
Kidney	1/16	0/16	1/18	0/16
Oral Cavity	3/34	1/34	1/36	1/35
Skin	1/26	0/26	0/13	2/13
Others	2/127	1/124	2/143	1/129
Total	35/710 (4.9%)	13/707 (1.8%)	16/800 (2.0%)	12/709 (1.7%)

calculated as 1,300.

The EPO binding capacity of bone marrow cells from 7 patients with PV was compared with the normal control. As shown in Fig. 3, the EPO binding capacity of the bone marrow cells of the PV patients was significantly increased per number of bone marrow non-phagocytic cells, of erythroblasts and of CFU-E. This finding shows that the high sensitivity of bone marrow cells from PV patients to EPO is due to an increased binding of EPO receptors on CFU-E of PV patients. Studies are now being conducted to detect activation of an oncogene in the hematopoietic cells of PV patients with the purpose of clarifying the pathogenesis of this disorder and relations of EPO receptors to the oncogene.

iv) EPO Producing Tumors

As shown in Table 1, 16 patients among 800 with various malignancies showed erythrocytosis. The highest percentage of secondary erythrocytosis was observed in patients with hepatoma.

Fig. 4. Expression of EPO mRNA in human hepatoma cells obtained from a patient with erythrocytosis and maintained in culture.

Although the incidence of erythrocytosis associated with malignancies is low, as shown in Table 1, when these tumors were transplanted into nude mice, the incidence of development of erythrocytosis in the nude mice increased to 4 out of 25 tumors. This finding indicates a fairly high incidence of latent secondary erythrocytosis in malignancies.

Hepatoma and renal cell carcinoma cells from 2 patients with marked erythrocytosis were maintained in culture. The culture medium was shown to contain erythropoietin activity by *in vitro* bioassay using FMLC²⁾. The activity was neutralized by anti-EPO antibody and as shown in Fig. 4, Northern blotting with EPO cDNA showed the presence of mRNA for EPO in hepatoma cells, thus demonstrating the production of EPO by these tumors.

2) Differentiation of Granulocytic Cells

i) Colony Stimulating Factors (CSFs) Producing Tumor

The presence of granulocyte-macrophage (GM), granulocyte (G), and macrophage (M) CSF stimulating the differentiation of CFU-GM, G and M respectively has been well documented. As in the case with EPO producing tumors, some tumors have been reported to produce these CSFs. Table 1 compiles numbers of malignant cases with unexplained neutrophilia or mono-

much more serious than after the 1st cycle, the administration of CSF-HU was effective in preventing neutropenia as well as accelerating the recovery from neutropenia in 39.5% of 53 evaluable cases of gynecological malignancies, and the CSF-HU was effective in 17 (50%) of 34 cases with neutrophil counts of less than 500/mm³. In patients with urological malignancies with neutropenia, the CSF-HU was effective in 50% of 36 evaluable cases. Only minimal side effects such as fever, nausea and vomiting were observed in about 10% of the cases. Preliminary data had shown that the CSF-HU was also effective in promoting the recovery from neutropenia in patients who received allogenic bone marrow transplantation for acute leukemia or severe aplastic anemia. 15.3 ± 3.7 days were necessary in 13 CSF-HU treated patients for recovery of the peripheral blood leukocyte counts to 1,000/mm³, while in 37 untreated patients, the time was 26.0 ± 9.9 days. This difference was statistically significant (p<0.01). Days for recovery of the granulocyte counts to 500/m³ were 21.54 ± 4.86 in CSF-HU treated group, while in untreated group, they were 28.54 ± 9.13 (p<0.05). The CSF-HU was considered to accelerate the recovery from neutropenia by stimulating the local production of G-CSF by the residual macrophages in the bone marrow of these patients.

iii) Leukemia Cells and Oncogenes

We reported activation of the N-ras oncogene in leukemic cells of acute lymphocytic leukemia (ALL) and chronic myelocytic leukemia (CML) patients⁵⁾. This activation was demonstrated by *in vitro* transfection assay using NIH 3T3 cells. Cloning and nucleotide analysis of the activated N-ras oncogene showed that the 34th nucleotide was changed to thymine from guanine by a point mutation. Recently, we studied the transforming gene in bone marrow cells of patients with various hematopoietic malignancies, including myelodysplastic syndrome, by transplanting the transfected NIH 3T3 cells into nude mice. This method was used to increase the sensitivity of the *in vitro* transfection assay. As shown in Table 3, in 11 of 12 leukemia cases, the presence of a transforming gene was demonstrated. Activation was observed in 5 cases in the N-ras gene and 1 in the K-ras

Table 3. Transforming activity of DNA obtained from bone marrow cells of patients with various leukemias and myelodysplastic syndrome. Transforming activity was assayed by *in vitro* and *in vivo* transfection method as described in the manuscript.

Diagnosis	In Vitro			In Vivo		
	Total	Positive	N-ras	Total	Positive	N-ras
Myeloid Leukemia						
AML	8	1	0	2	2	2
AMoL	2	0	0	3	3	1
AMMoL	2	0	0	1	1*	0
CML	8	1	1	2	2	0
Lymphoid Leukemia						
T-ALL	3	2	1	2	2	1
B-ALL	1	0	0	1	0	0
Null-ALL	4	0	0	1	1	1
B-CLL	2	0	0	0	0	0
	32	4	2	12	11	5
MDS						
PARA	8	0	0	2	1	0
PASA	3	0	0	2	1	0
RAEB	8	0	0	4	2	1
CMML	1	0	0	2	1	0
	20	0	0	10	5	1

* K-ras

gene. It was noted that in cases of primary acquired refractory anemia, primary acquired sideroblastic anemia, refractory anemia with excess of blasts and chronic myelomonocytic leukemia, the presence of the transforming gene was demonstrated in their bone marrow cells. These cases are considered to be in a state of leukemia even though they are diagnosed as preleukemia by the hematological findings.

The presence of bcr-abl gene in Ph¹ positive CML is well documented. We also could demonstrate by Northern blotting, the presence of mRNA for the bcr-abl gene in the bone marrow cells of all cases of CML. The demonstration of mRNA for bcr-able was useful for the differential diagnosis of CML from chronic myelomonocytic leukemia and acute blastic transformation of CML from acute leukemia.

c-abl proto-oncogene belongs to the src family, and its product is speculated to be related to a growth factor or its receptor. Recently Cook, et al reported that the interleukin 3 (IL-3) dependent leukemic cell line cells became independent of IL-3 following the infection with Abelson mouse leukemia virus⁶⁾. Since they could not demonstrate the presence of IL-3 in the culture medium of the cells infected with this virus, it is speculated that c-abl is intimately related to

IL-3 receptor and its activation by fusion with the bcr gene caused an increase in IL-3 receptor on CML leukemia cells. It is well known that in CML thrombocytosis and erythrocytosis are often observed in addition to granulocytosis. Since IL-3 stimulates multipotential hematopoietic stem cells to differentiate to various lineages, the hematological findings in CML can be explained by the increase of IL-3 receptors induced by activation of the c-abl oncogene.

It is well documented that the products of some oncogenes are identical to growth factor or its receptor. Data on CSFs, which strongly suggest the production of GM-CSF and M-CSF receptors by c-fps and c-fms oncogene, respectively, have been reported by Sherr. et al and Feldman, et al^{7,8)}.

To study the change of these oncogenes during hematopoietic cell differentiation, we cultured HL 60 leukemia cells with TPA or DMSO to differentiate the leukemia cells into macrophages or granulocytes. The changes in the expression of c-myc, c-fms and c-fps genes were followed by Northern blotting after the addition of those differentiation factors. As we expected, differentiation of HL 60 cells to macrophages by TPA induced an apparent increase in mRNA for c-fms, while the mRNA for c-fps was increased during the process of differentiation to macrophages as well as granulocytes induced by TPA and DMSO, respectively. The expression of c-myc was decreased after addition of both inducers. This decrease was considered to reflect a reduction in the mitotic activity of leukemic cells accompanying the differentiation of leukemic cells by various kinds of inducers.

iv) Growth Factors Derived from Leukemic Cells

To study the autocrine system for the proliferation of leukemic cells, we established several sublines of human leukemia derived K-562 cells which proliferate under serum-free and protein-free conditions. We demonstrated the presence of 2 kinds of growth factor (leukemia-derived growth factor (LGF) 1, 2) in the culture medium of one of those subline cells⁹⁾. LCF-1 stimulated the proliferation of several leukemia cell line cells, including K 562 *in vitro* but had no effect on the growth of fibroblasts. LGF-2, on the other hand,

Met-Gln-Ile-Phe-Val-Lys-Thr-Leu-Thr-Gly-
Lys-Thr-Ile-Thr-Leu-Glu-Val-Glu-Pro-X-
Asp-X-Ile-X-Asn-Val-X-Ala-X-Ile-

LGF-1 MW 20000

UBIQUITIN 8600	
In nuclear	Regulation of chromatic structure
In cytoplasm	ATP-dependent proteolysis

Fig. 7. Amino acid sequence of N-terminal of purified leukemia derived growth factor (LGF)-1 obtained from K562 T-1 cells maintained in protein from culture medium.

stimulated the proliferation of only fibroblasts. LGF-1 purified to homogeneity on SDS gel-electrophoresis had a molecular weight of about 20,000 daltons. A part of the N-terminal amino acid sequence of LGF-1 which is shown in Fig. 7 was identical to the sequence of ubiquitin. Ubiquitin is a protein which is present in the cytoplasm as well as in the nucleus, and its functions are proteolysis and control of the function of chromosomal DNA. We also confirmed that several LGF-2s purified so far are different from platelet derived growth factor (PDGF) and fibroblast growth factor (FGF). It is speculated that in the development of the bone marrow fibrosis which is sometimes seen in patients with leukemia, leukemia cell-derived growth factor of fibroblasts, such as LGF-2, could be playing a role. LGF-1 stimulates the growth of leukemic cells, while LGF-2 stimulates the growth of fibroblasts which are surrounding leukemia cells, thus making the hematological features of leukemias.

3) Differentiation of Lymphoid Cells

All lymphoid cells of B-cell malignancies so far studied had rearrangement of the Ig gene, and those of T cell malignancies showed rearrangement of T cell antigen receptor (TCR) gene. These results indicate that all lymphoid malignancies are derived from one malignant lymphoid cell, and that these rearrangements are very useful tumor markers of lymphoid malignancies.

The following is the results of our study on Ig and TCR gene rearrangement analysis in leukemic cells of common ALL and acute non-lymphocytic leukemia.

i) Rearrangement of Ig and TCR Genes in Com-

mon ALL

Common ALL is defined as the one type of ALL whose leukemic cells are negative for surface Ig, Leu 1 and Leu 4 antigens, negative for E-rosette formation and positive for J 5. When we analyzed the gene rearrangement of leukemic cells of 9 cases of common ALL, 3 cases were positive for rearrangement of both the Ig and TCR genes, 2 case were positive for TCR gene rearrangement and 4 cases were positive for Ig gene rearrangement. Korsmeyer reported in 1983 that in all common ALL cases, leukemia cells showed rearrangement of the Ig gene¹⁰⁾, indicating that malignant lymphoid cells of common ALL are partially differentiated into the B cell lineage. However, our results showed that common ALL cells had characteristics of both T and B cells. The lymphoid cells showing rearrangement of both genes are considered to have once tried to differentiate to one lymphoid cell lineage but failed to achieve an effective rearrangement of the gene. They then had to switch to another lineage, thus demonstrating rearrangement of both genes. This finding means that the differentiation of lymphoid cells is stochastic and not predetermined.

ii) Rearrangement of Ig and TCR Genes in Acute Non-lymphocytic Leukemia (ANLL)

We analyzed the rearrangement of Ig and TCR genes in leukemic cells of 15 cases of ANLL. Leukemic cells from 12 cases did not show any rearrangement of these genes, as had been expected from the diagnosis of ANLL. However, in the 3 remainings, their leukemic cells showed rearrangement of the TCR gene or both. This finding seems to indicate an unreliability of the FAB classification. It is also possible to speculate from this finding that some leukemic cells of ANLL had changed the cell lineage during their differentiation from myeloid to lymphoid or vice versa.

One 22-year-old case of ANLL whose leukemic cells were diagnosed as FAB M 1 by their morphology was treated very intensively by combination of cytosine arabinoside (Ara-C), including high dose Ara-C, and anthracyclines. The response to this treatment was unsatisfactory. After we found the Ig and TCR genes in his leukemic cells

were rearranged, we switched to a treatment including vinka alkaloids. He then showed a good response to this treatment and achieved a complete remission. We also experienced a case of CML whose leukemic blasts showed a morphological characteristic of lymphatic blasts on the first blastic transformation. He then returned to the chronic phase after treatment including vinka alkaloid. On the second blastic transformation, his leukemic blasts had the morphological features of myeloid blasts. Throughout these 2 blastic transformations, the blasts showed rearrangement of the Ig gene. These findings were explained as lineage infidelity. However, it is possible to explain these findings by the presence of multipotential stem cells capable of differentiating into both myeloid and lymphoid cell lineages and by a stochastic model of differentiation of the hematopoietic stem cells.

4) Differentiation of Megakaryocytes-Thrombocytes

It is well documented that Meg-CSF and thrombopoietin are hematopoietic factors regulating thrombopoiesis *in vivo*, although the difference of Meg-CSF from IL-3 has recently been discussed.

We occasionally encounter cases of malignancy associated with unexplained thrombocytosis. As shown in Table 1, we observed marked thrombocytosis in patients with lung, kidney, oral-cavity, skin and liver carcinomas. One patient with hepatoma showed marked thrombocytosis of $400 \times 10^4/\text{mm}^3$. The thrombocyte count decreased in parallel with the decrease of serum α -fetoprotein level after the treatment with mitomycin C.

One important role of thrombocytes in the vascular system is the repair of injured vascular wall. It is well documented that in the process of repair of the vascular wall, various growth factors derived from thrombocytes are participating. Among those factors, PDGF has been well known and extensively studied. We recently discovered a new growth factor from platelets¹¹⁾. This factor, which was named vascular endothelial proliferation factor (VEPF), stimulated the proliferation of vascular endothelial cells but not fibroblasts *in vitro*. We have been trying to purify VEPF

from 100 1 of human whole blood in collaboration with Dr. Heldin of Uppsala University. The purified VEPF has a molecular weight of 60,000 daltons and an isoelectric focusing point of 4.0 ~ 4.8, and it is unstable to heat, inactivated by trypsin and not absorbed by a column containing heparin. These characteristics of VEPF are different from those of PDGF. These 2 factors, PDGF and VEPF, are hypothesized to collaborate in the repair process of vascular injury.

In this paper, I have introduced the results of works relating to hematopoietic cell differentiation and performed by the Hematology Research Group in our Department of Medicine. In describing this work, I have emphasized the studies relating to growth and differentiation factors, their receptors, and genes in hematopoietic cells, since it is my belief that such studies based on cell biology will become a major trend in research on clinical medicine.

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REFERENCES

- 1) Mori S, et al: Glomerular epithelium as the main locus of erythropoietin in human kidney. *Jap J Exp Med* 55: 69, 1985.
- 2) Okabe T, et al: Production of erythropoietin-like activity by human renal and hepatic carcinomas in cell culture. *Cancer* 55: 1918, 1985.
- 3) Hatake K, et al: Purification of human urinary colony-stimulating factor by high-performance liquid chromatography. *J Chromat* 344: 339, 1985.
- 4) Ishizaka Y, et al: Mode of action of human urinary colony-stimulating factor. *Exp Hematol* 14: 1, 1986.
- 5) Hirai H, et al: Transforming genes in human leukemia cells. *Blood* 66: 1371, 1985.
- 6) Cook W, et al: Malignant transformation of a growth factor-dependent myeloid cell line by Abelson virus without evidence of an autocrine mechanism. *Cell* 41: 677, 1985.
- 7) Sherr C J, et al: The c-fms proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. *Cell* 41: 665, 1985.
- 8) Feldman R A, et al: Specific expression of the cellular fms/fes-encoded protein NCP92 in normal and leukemic myeloid cells. *Proc Natl Acad Sci* 82: 2379, 1985.
- 9) Finley D and Varshavsky A: The ubiquitin system: functions and mechanisms. *TIBS* September 1985, p. 343.
- 10) Korsmeyer S J, et al: Immunoglobulin gene rearrangement and cell surface antigen expression in acute lymphocytic leukemias of T cell and B cell precursor origins. *J Clin Invest* 71: 301, 1983.
- 11) Miyazono K, et al: A platelet factor stimulating the proliferation of vascular endothelial cells. *Exp Cell Res* 159: 487, 1985.