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Direct Transplantation of Chronic Myelogenous Leukemia Cells into Nude Mice and Establishment of a Leukemic Stem Cell (Ph^{1+}, CD34^{+}) Line Dependent on Mouse Bone Marrow Stromal Cells *in vitro*

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Peripheral blood cells from a female patient with Ph^1-positive chronic myelogenous leukemia (CML) in blast crisis were serially transplanted in BALB/c nude mice for 16 passages. This *in vivo* cell line, designated CML-N-1, had Ph^1 chromosome abnormality and BCR gene rearrangement. The cells expressed CD11b, CD13, CD33, CD34, CD38, and HLA-DR antigens until the 11th passage and subcutaneous tumors produced by these passages were composed of admixtures of immature and maturing cells that differentiated to basophils when cultured *in vitro*. From the 12th passage on, the tumors became composed mainly of immature cells expressing CD13, CD34, and HLA-DR, and no longer differentiated to basophils even upon *in vitro* culture. In contrast to the vigorous proliferation *in vivo*, CML-N-1 cells from any passage failed to proliferate *in vitro* under standard liquid culture conditions with or without growth factors, such as granulocyte-macrophage colony-stimulating factor, granulocyte colony-stimulating factor, monocyte colony-stimulating factor, interleukin 3, interleukin 6 and stem cell factor. However, a continuously growing cell line, designated CML-C-1, was established by culturing CML-N-1 cells on feeder layers of mouse bone marrow stromal cells. This mouse bone marrow stromal cell-dependent cell line showed immature cell morphology and expressed early myeloid phenotype positive for CD13, CD34, and HLA-DR. These results indicate that mouse bone marrow stromal cells provide a certain growth factor(s) active on human leukemia cells.

Key words: CML cell line — Stromal cell-dependent cell line — Transplantation

Chronic myelogenous leukemia (CML) is a multipotent hematopoietic stem cell disease characterized by an excessive clonal proliferation and differentiation of a transformed stem cell, resulting in an extreme accumulation of mature granulocytes and their progenitors in the bone marrow and blood. The mechanisms of proliferation and differentiation of CML cells are poorly understood. Heterotransplantation of human neoplastic cells has been a useful method to study the biology of tumor cells and has been successfully applied to CML cells.[1–7] In this study, we transplanted peripheral blood leukemia cells from a patient with CML in blast crisis into BALB/c nude mice and established a new serially transplantable CML cell line, CML-N-1, *in vivo*. These CML-N-1 cells repeatedly failed to grow *in vitro*. This observation suggested that the mouse provided a factor required for the growth of CML cells that was absent in culture. Thus, we attempted to culture CML-N-1 cells on feeder layers of BALB/c mouse bone marrow stromal cells and were able to establish a stromal cell-dependent CML cell line, CML-C-1, in culture. Several CML cell lines were established *in vitro*,[8] but most of them were factor-independent. Oez *et al.*[9] established a granulocyte-macrophage colony stimulating factor-dependent CML cell line, which should be useful for the study of the mechanism of proliferation of CML cells. We describe here the establishment and characteristics of CD34-positive CML cell lines that can be maintained both *in vivo* (CML-N-1) and *in vitro* (CML-C-1).

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CASE REPORT

A 39-year-old female visited a local hospital because of general malaise and dyspnea on exertion. She was thought to have leukemia and was transferred to our hospital on March 12, 1987. On physical examination, she was pale and her spleen was palpable 8.0 cm below the left costal margin. The red blood cell count was $256 \times 10^4/\mu l$, Hb 5.5 g/dl, Ht 29%, and platelets $54.2 \times 10^4/\mu l$. The white blood cell count (WBC) was $277{,}700/\mu l$ with 2% myeloblasts, 3% promyelocytes, 19% myelocytes, 18% metamyelocytes, 27% bands, 24% segmented neutrophils, 6% eosinophils, and 1% basophils. Bone marrow was hypercellular with a predominance of myeloid cells at various stages of maturation. Low neutrophil alkaline phosphatase activity and the presence of 46,XX,t(9;22)(q34;q11) abnormality in bone marrow cells established the diagnosis of CML. She was treated

with busulfan and did well until January 1990, when she was noted to be in blast crisis. In spite of combination chemotherapy of daunorubicin, cytarabine, 6-mercaptopurine and prednisolone, the number of blasts increased and the patient died on May 25, 1990.

MATERIALS AND METHODS

Heterotransplantation On April 24, 1990, the WBC of the patient was 196,000/μl with 64% blasts, 2% stabs, 19% segmented neutrophils, 2% lymphocytes, 3% eosinophils, and 10% basophils (Fig. 1A). Ten ml of peripheral blood was taken into a heparinized syringe and cells were separated by Ficoll-Conray gradient centrifugation. After washing with RPMI 1640 medium, 1×10^8 cells in 1.5 ml RPMI 1640 medium were inoculated subcutaneously into the back of 5 BALB/c athymic nude mice, 3–4 weeks old, bred in our animal care unit. Tumors arising in nude mice were excised and cells were teased apart with forceps and scissors. Approximately 1×10^8 cells suspended in 1.5 ml of RPMI 1640 medium were subcutaneously inoculated in the back of animals for serial transplantation.

Morphology Peripheral blood and bone marrow cell smears of the patient, and cytospin preparations of nude-mouse tumors and cultured cells were stained with May-Grünwald-Giemsa and evaluated for peroxidase, Sudan black B, naphthol AS-D chloroacetate esterase, α-naphthyl butyrate esterase, alkaline phosphatase, periodic acid-Schiff, toluidine blue, and alcian blue. Histologic sections were prepared from subcutaneous tumors and various organs of nude mice including the liver, spleen, kidneys, lungs, and lumbar vertebrae. Sections were stained with hematoxylin and eosin.

Cell marker analysis Cell marker analysis of the patient's fresh leukemia cells, nude-mouse tumor cells at the 8th, 9th, 15th, and 16th passages, and cultured cells was performed by the indirect immunofluorescence technique using the following monoclonal antibodies: Leu-4 (CD3) (Becton Dickinson, Mountain View, CA) for T-cell antigen; Leu-12 (CD19) (Becton Dickinson) for B-cell antigen; OKM1 (CD11b) (Ortho, Raritan, NJ), My7 (CD13) and My9 (CD33) (Coulter, Hialeah, FL), and My10 (CD34) (Becton Dickinson) for myelomonocyte and stem cell antigens; OKT10 (CD38) (Ortho) for progenitor cell, activated lymphocyte and plasma cell antigen; OKIa1 (Ortho) for HLA-DR antigen; and IIbIIIa (CD41a) (Dakopatts, Glostrup, Denmark) for megakaryocyte antigen.

Cytogenetic and gene analysis Chromosome analysis was performed on the patient's bone marrow cells at diagnosis and blast crisis, nude-mouse tumor cells from the 5th, 8th, and 14th passages, and cultured cells by the standard Giemsa banding technique.[10] Rearrangement of BCR gene was examined by the method previously described.[11]

Cell culture Attempts were made to culture nude-mouse tumor cells from the 1st, 2nd, 4th, 6th, 8th, 9th, 12th, 14th, and 16th passages, using RPMI 1640 medium supplemented with 20% fetal calf serum (FCS) in 35-mm petri dishes. Five ng/ml recombinant human interleukin 3 (IL-3) (Genzyme, Cambridge, MA), 2 ng/ml recombinant human interleukin 6 (IL-6) (Ajinomoto, Kawasaki), 125 ng/ml recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) (Hoechst, Tokyo), 125 ng/ml recombinant granulocyte colony-stimulating factor (G-CSF) (Chugai, Tokyo), 500 U/ml monocyte colony-stimulating factor (M-CSF) (Green Cross, Tokyo) and 50 ng/ml stem cell factor (SCF), a gift from Dr. K. Zsebo (Amgen, CA), were used to see whether these growth factors enhance the proliferation of nude-mouse tumor cells. Pooled femoral bone marrow cells from young adult BALB/c mice were cultured at 2×10^6/ml with RPMI 1640 medium supplemented with 10% FCS in 35-mm petri dishes to obtain feeder layers of bone marrow stromal cells. The cells were fed twice a week. Nonadherent cells gradually declined, and sheets of adherent cells appeared after 3 weeks. Then, tumor cells of the 6th nude mouse passage were placed on these cultures. The cells were fed twice a week with RPMI 1640 medium supplemented with 20% FCS. The 3-day-old culture supernatant of bone marrow stromal cells was also tested at 30% and 50% concentrations for any growth-promoting effect on nude-mouse tumor cells. All cultures were incubated at 37°C in a 100% humidified atmosphere of 5% CO_2 in air. The cells were observed under an inverted microscope. The viable cell numbers were counted by trypan blue dye exclusion in a Bürker-Türk cell counting chamber.

RESULTS

Heterotransplantation Ph^1-positive CML cells were initially inoculated into the back of 5 nude mice. Subcutaneous tumors developed after 14 days and grew progressively in all 5 animals. The tumors were serially transplanted for 16 passages and this *in vivo* cell line was designated CML-N-1. The overall incidence of takes was 93% (76 of 82) and tumor-bearing mice were killed or died 32 to 105 days after implantation. At autopsy, the tumors, 2 to 3 cm in diameter, were localized at the site of inoculation without invasion or metastasis to other organs. Histologically, the tumors were composed of primitive cells containing prominent nucleoli and frequent mitoses. Among these immature cells, cells having distinct granules and lobulated nucleus were observed (Fig. 1B) until the 11th passage. Cytospin preparations of tumor cells revealed that these maturing cells were

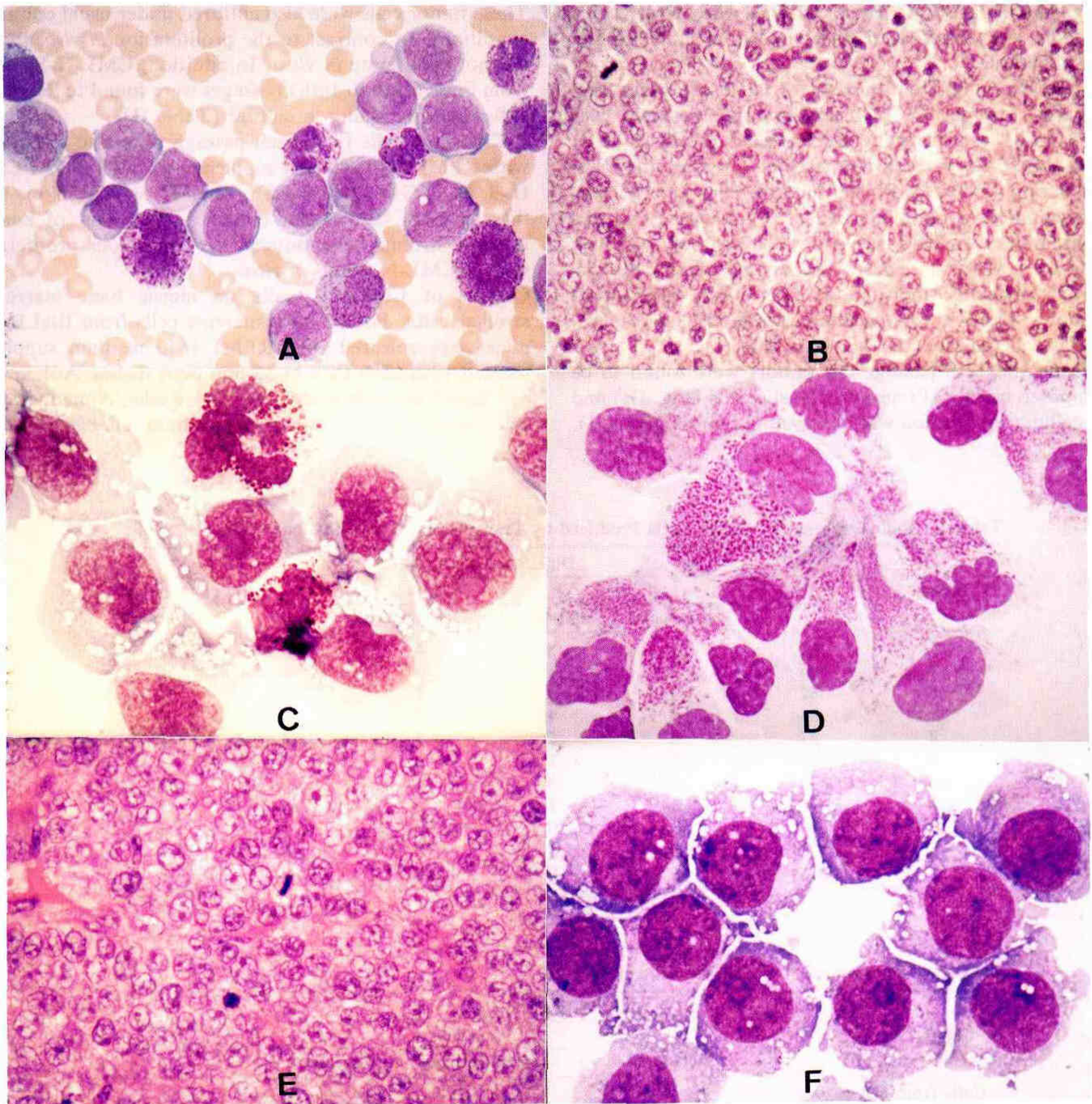

Fig. 1. A. Smear of patient's peripheral blood used for transplantation. Many blasts with prominent nucleoli and basophils at various stages of maturation are present. May-Grünwald-Giemsa staining (×500). B. Histologic section of a nude-mouse tumor in the 8th passage of CML-N-1 cells. Note the tumor consists of immature cells with prominent nucleoli and scattered maturing cells with cytoplasmic granules or band-form nuclei. Mitotic figures are also seen. Hematoxylin and eosin (×160). C. Cytospin smear of the tumor shown in Fig. 1B. Mature basophils are present among immature cells with many vacuoles. May-Grünwald-Giemsa staining (×800). D. Smear of CML-N-1 cells from the 8th passage cultured for 14 days. Most of the cells show differentiation to basophils. May-Grünwald-Giemsa staining (×800). E. Histologic section of a nude-mouse tumor in the 14th passage of CML-N-1 cells. There are sheets of immature cells with prominent nucleoli, but no maturing cells are seen. Hematoxylin and eosin (×160). F. Cytospin smear of the CML-C-1 cell line. All the cells exhibit immature morphology with abundant basophilic cytoplasm, round nuclei, and prominent nucleoli. No sign of maturation is seen. May-Grünwald-Giemsa staining (×800).

basophils (Fig. 1C) (Table I) that were positive for periodic acid-Schiff, acid phosphatase, alcian blue and toluidine blue.

CML-N-1 cells from these (1st to 11th) passages were cultured with RPMI 1640 medium supplemented with 20% FCS. In contrast to the vigorous proliferation *in vivo*, the CML-N-1 cell line did not grow *in vitro*. The cell number increased during the first week and the cells differentiated to basophils (Table II). Most of the cells differentiated within 4 weeks *in vitro* and only very few cells were alive thereafter. The differentiated cells displayed basophilic morphology (Fig. 1D) with typical granules positive for periodic acid-Schiff, acid phosphatase, alcian blue and toluidine blue.

From the 12th passage on, tumors were found to be composed mainly of immature blastic cells (Fig. 1E) and no cellular maturation was observed in tumors (Table I). These tumor cells were also cultured under usual culture conditions. In contrast to the proliferation *in vivo*, they did not proliferate *in vitro*. In addition, CML-N-1 cells from these (12th to 16th) passages were found to lose the differentiation ability in culture (Table II).

CML-N-1 cells from each passage were also cultured with RPMI 1640 medium supplemented with 20% FCS, IL-3, IL-6, GM-CSF, G-CSF, M-CSF, or SCF. None of these growth factors nor culture supernatant of bone marrow stromal cells induced the continuous proliferation of CML-N-1 cells *in vitro*.

Culture of CML-N-1 cells on mouse bone marrow stromal cells Pooled bone marrow cells from BALB/c mice were cultured with RPMI 1640 medium supplemented with 10% FCS in 35-mm petri dishes. Adherent cell layers were obtained after 3–4 weeks. Nonadherent cells were removed at every medium change. Then,

Table I. Differentiation of Tumor Cells Produced by Transplantation of CML-N-1 Cells

Passage	Differential count (%)					
	Blast	Basophilic				
		Promyelocyte	Myelocyte	Metamyelocyte	Band	Segmented
1	40	16	3	4	8	29
4	66	2	1	0	1	30
5	62	9	3	1	10	15
7	88	9	1	0	2	0
8	77	6	2	3	3	9
9	85	6	1	0	3	5
10	79	5	3	1	0	12
11	95	3	1	0	0	1
12	100	0	0	0	0	0
15	100	0	0	0	0	0
16	100	0	0	0	0	0

Table II. Maturation of CML-N-1 Cells in Culture

Culture day	Differential count (%)				Cell yield[a] ($\times 10^5$/ml)
	Blast	Basophilic			
		Promyelocyte	Myelocyte-band	Segmented	
Cells from 8th passage					
2	77	10	7	6	2.0
7	83	13	2	2	7.3
16	36	19	15	30	2.3
23	12	13	43	32	0.1
Cells from 16th passage					
0	100	0	0	0	2.0
7	100	0	0	0	6.2
14	100	0	0	0	4.1
21	100	0	0	0	2.9
33	100	0	0	0	3.3
40	100	0	0	0	1.2

a) The cultures were started at 2×10^5/ml cell density initially.

CML-N-1 cells from the 6th passage were cultured on these stromal feeder layer cells. Some of the CML-N-1 cells clustered around the stromal cells, and could be transferred onto new stromal cell layers every 2 to 3 months. After seven months of feeder-layer culture, surviving cells began to grow gradually. Definite cell growth was confirmed after one year of culture. This cell line, designated CML-C-1, grew in suspension with some cells attached to the stromal cells. Adhered CML-C-1 cells were easily detached by pipetting. The cell line was composed of primitive blastic cells and contained no maturing cells (Fig. 1F). The CML-C-1 cells were positive for acid phosphatase and periodic acid-Schiff, but negative for peroxidase, Sudan black B, naphthol ASD chloroacetate esterase, α-naphthyl butyrate esterase, toluidine blue, and alcian blue. The CML-C-1 cell line continued to be dependent on bone marrow stromal cells for growth (Fig. 2).

Cell marker analysis As shown in Table III, the patient's original CML cells were positive for My7 (CD13) and OKIa1 (HLA-DR). Although My10 (CD34) was not tested on the original CML cells, both CML-N-1 and

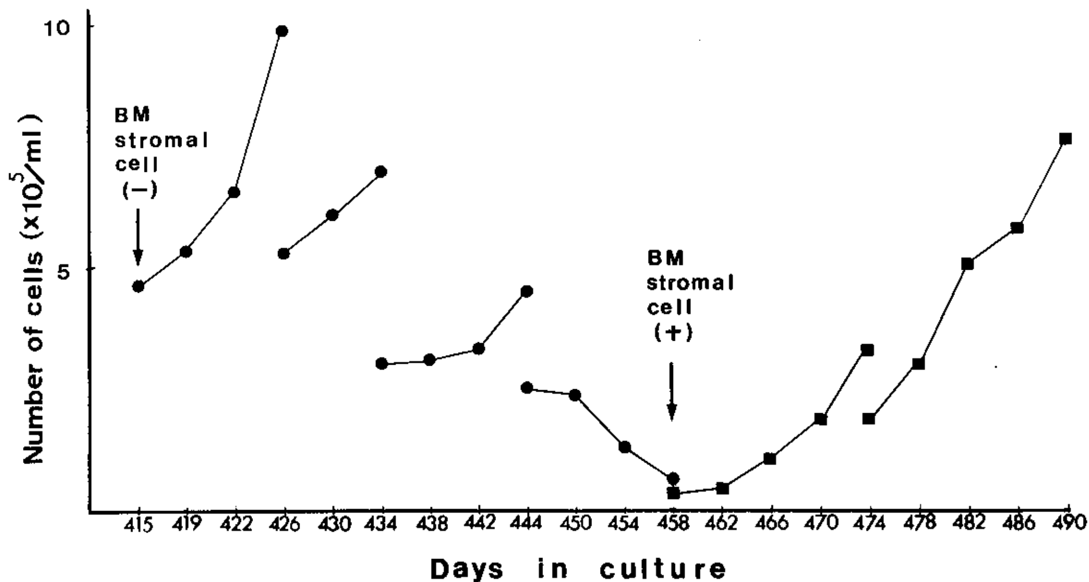

Fig. 2. Cell growth curve of the CML-C-1 cell line. The cells cultured without mouse bone marrow stromal cells (●) declined and gradually decreased in numbers. The cells started to grow again (■) after having been transferred onto murine bone marrow stromal cell layers. The cell numbers were counted every 3–4 days.

Table III. Cell Marker Analysis of Patient's Leukemia Cells, CML-N-1, and CML-C-1

Monoclonal antibody	CD	Reactivity (%)					
		Patient's leukemia cells	CML-N-1				CML-C-1
			8th passage	9th passage	15th passage	16th passage	
Leu-4	(CD3)	1	0	0	0	0	0
Leu-12	(CD19)	1	0	0	0	0	0
OKM1	(CD11b)	nt	19	12	0	0	0
My7	(CD13)	47	79	50	45	65	100
My9	(CD33)	9	26	12	0	0	0
My10	(CD34)	nt	65	80	100	100	100
OKIa1		68	53	78	100	100	100
OKT10	(CD38)	nt	37	23	0	0	0
IIbIIIa	(CD41a)	nt	0	0	0	0	0

nt: not tested.

Table IV. Representative Karyotypes of Patient's Leukemia Cells, CML-N-1, and CML-C-1

Patient's leukemia cells	
At diagnosis (March 1987)	46,XX,t(9;22)(q34;q11)
Three months before experiment (January 19, 1990)	46,XX,t(9;22)(q34;q11)
Two weeks before experiment (April 10, 1990)	46,XX,t(9;22)(q34;q11)
	48,XX,t(9;22)(q34;q11),+8,+8
In vivo cell line	
CML-N-1 cells from 5th passage	50,X,–X,del(3)(q21q23),+8,+8,+8, t(9;22)(q34;q11),+21,+der(22)t(9;22)(q34;q11)
CML-N-1 cells from 8th passage	50,X,–X,del(3)(q21q23),+8,+8,+8, t(9;22)(q34;q11),+21,+der(22)t(9;22)(q34;q11)
CML-N-1 cells from 14th passage	49,X,–X,del(3)(q21q23),der(7;8)(p22;q22), +8,+8,t(9;22)(q34;q11),+21,+der(22)t(9;22)(q34;q11)
	50,X,–X,del(3)(q21q23),+8,+8,+8, t(9;22)(q34;q11),+21,+der(22)t(9;22)(q34;q11)
In vitro cell line	
CML-C-1 cell line	49,XX,del(3)(q21q23),+8,+8,t(9;22)(q34;q11),+21

CML-C-1 cell lines were positive for CD34 as well as CD13 and HLA-DR. CML-N-1 cells from the 8th and 9th passages were also shown to be positive for OKM1 (CD11b), My9 (CD33), and OKT10 (CD38); however, the cells from the 15th and 16th passages, and the CML-C-1 cell line were negative for CD11b, CD33, and CD38 antigens.

Cytogenetic and gene analysis Ph[1] chromosome was found as the sole abnormality at diagnosis in March 1987. Additional chromosome abnormalities, +8,+8, were noted at blast crisis in April, 1990. In CML-N-1 cells from passages 5 and 8, a karyotype of 50,X,−X, del(3)(q21q23),+8,+8,+8,t(9;22)(q34;q11),+21, +der(22)t(9;22)(q34;q11) was found (Table IV). Another chromosome abnormality, der(7)t(7;8)(p22;q22), was added in CML-N-1 cells from passage 14 (Table IV). The karyotype of CML-C-1 cell line was 49,XX, del(3)(q21q23),+8,+8,t(9;22)(q34;q11),+21 (Table IV, Fig. 3). Both CML-N-1 cells from the 6th and 9th passages and the CML-C-1 cell line were shown to have rearrangement of the BCR gene.

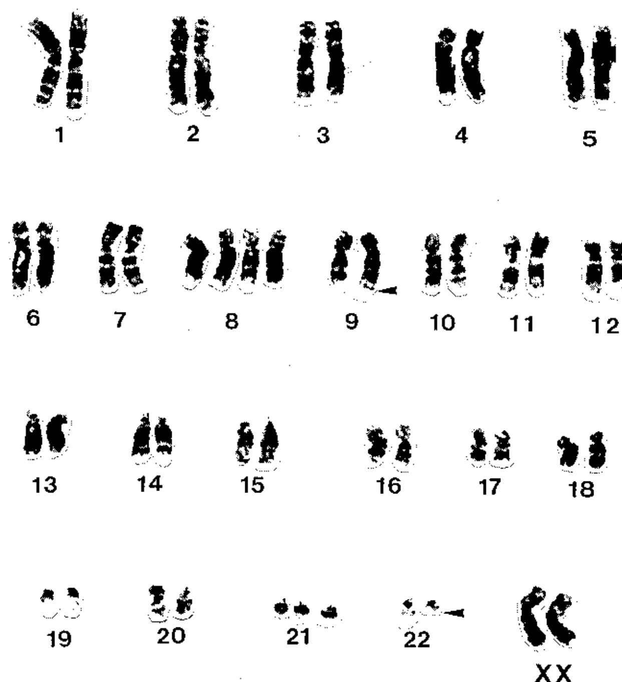

Fig. 3. Representative karyotype of CML-C-1 showing 49, XX,del(3)(q21q23),+8,+8,t(9;22)(q34;q11),+21. Ph[1] translocation is indicated by arrowheads.

DISCUSSION

The application of molecular technology has led to great progress in CML research. The ABL-BCR fusion protein arising from Ph[1] chromosome translocation was shown to play a crucial role in the pathogenesis of CML.[12–15] The mechanisms of cellular proliferation and maturation in CML, however, are unknown and many biological questions still remain to be solved.

Heterotransplantation study of CML cells would contribute to the understanding of biological properties of the disease and so we attempted the direct heterotransplantation of peripheral blood leukemia cells from a female patient with CML in blast crisis. This resulted in establishment of a new Ph[1]-positive cell line, CML-N-1, in nude mice. From the 1st through the 11th passages, the subcutaneous tumors in mice were composed of

admixtures of immature cells and mature basophils that were considered to express CD11b and CD38.[16, 17] *In vitro* culture of CML-N-1 cells from these passaged tumors in standard liquid culture conditions resulted in terminal differentiation to basophils followed by cell death. The maturation of CML-N-1 cells *in vivo* and *in vitro* during the 1st to 11th passages was reminiscent of basophilic differentiation of fresh CML cells[18–20] or a CML cell line,[21] indicating maturational potential of CML cells even from blast crisis. From the 12th passage on, however, the tumors were found to be composed solely of primitive blastic cells without CD11b and CD38 expression. In fact, CML-N-1 cells from the 12th through 16th passages no longer displayed maturation when cultured *in vitro*. Such maturation arrest and excessive proliferation appeared to reflect the characteristics of CML cells in patients with disease progression.

Although CML-N-1 cells grew vigorously *in vivo*, they failed to do so *in vitro*. This sharp contrast suggested that a suitable microenvironment and/or a growth factor(s) were absent under the culture conditions used. Accordingly, we cultured CML-N-1 cells on feeder layers of bone marrow stromal cells prepared from BALB/c mice. This method improved the survival of CML-N-1 cells *in vitro* and a continuously growing cell line, CML-C-1, was established. The cell line proved to be completely dependent on mouse bone marrow stromal cells for growth.

Recently, several human leukemia cell lines dependent on growth fators have been established.[9, 22–29] The establishment of these factor-dependent human leukemia cell lines suggests that the growth of certain human leukemia cells is regulated by lineage-specific growth factors. Hematopoietic stem cells were shown to grow for prolonged periods on appropriate stromal cells.[30–40] The present study has demonstrated that human leukemic (Ph^1-positive) stem (CD34-positive) cells can be induced to grow continuously on murine bone marrow stromal cells. The mechanism by which murine stromal cells support the growth of CML cells is not clear. Further studies are necessary to identify a possible growth factor(s) involved in this process.

Ph^1 chromosome translocation is usually observed as the sole abnormality in the chronic phase of CML. Additional 8, 21, or Ph^1 chromosome abnormalities that were found in CML-N-1 and CML-C-1 cell lines are known to occur with progression of the disease from the chronic phase to acute crisis.[41, 42] During the serial passage in nude mice, der(7)t(7;8)(p22;q22) abnormality newly emerged as a chromosomal evolution in CML-N-1 cells. The significance of this chromosome abnormality is unknown. Except for the ABL-BCR fusion protein resulting from Ph^1 chromosome translocation, little is known about other molecular genetic abnormalities associated with disease progression. p53 gene[43, 44] abnormalities are found in some cases of blast crisis of CML. No abnormal p53 expression, however, was found in either CML-N-1 cells from the 6th passage (showed maturation) or 14th passage (showed no maturation), or the CML-C-1 cell line (data not shown).

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