

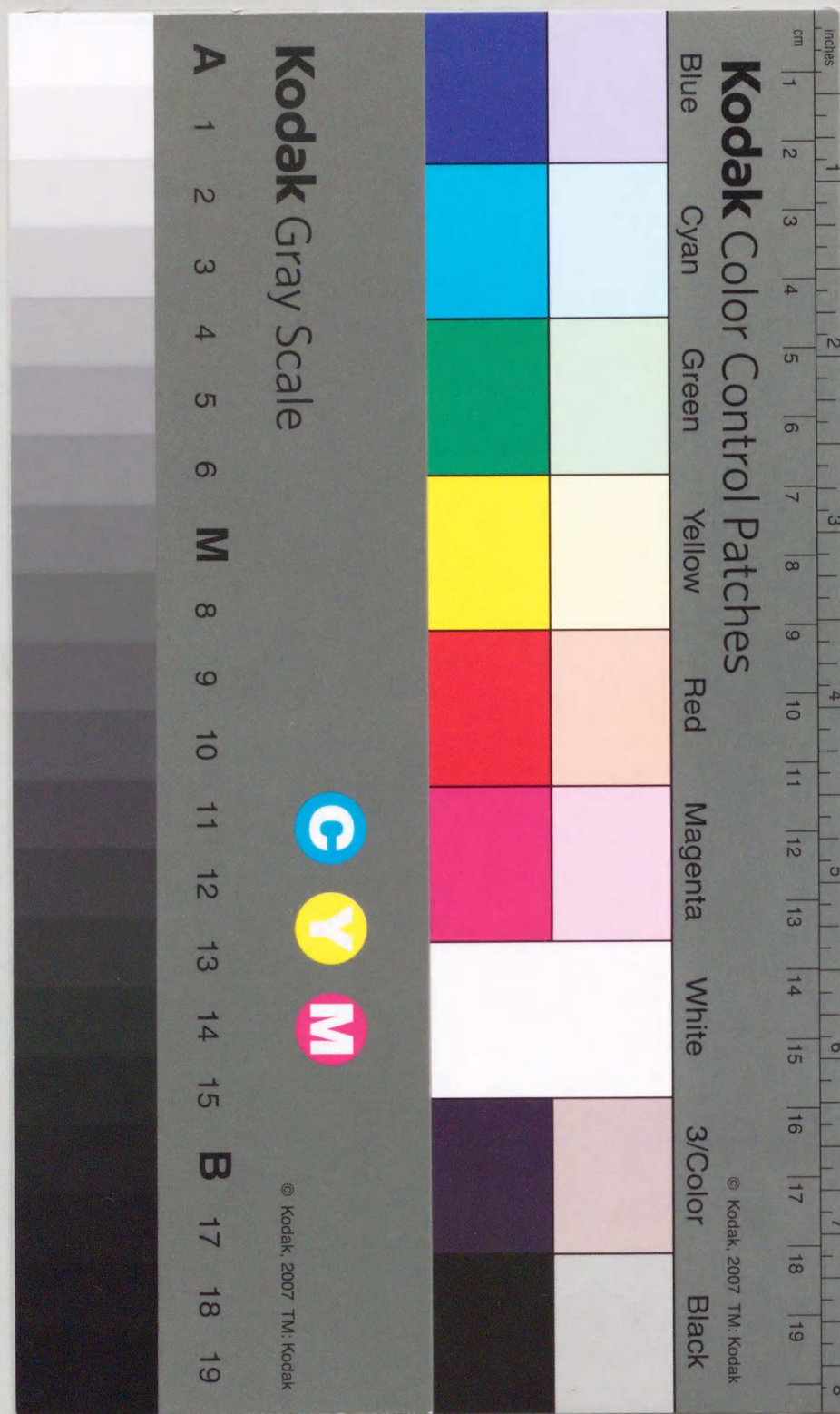
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<https://doi.org/10.11501/3086547>

出版情報：九州大学, 1991, 博士（医学）, 論文博士
バージョン：
権利関係：





LINEAGE NON-SPECIFIC DOWN REGULATION OF P210^{bcr/abl} IN THE CML CELL LINE, KU-812-F, DURING DIFFERENTIATION

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(Received 25 October 1989. Revision accepted 19 April 1990)

Abstract—CML cell line, KU-812-F, originally established from a patient with Philadelphia-chromosome-positive chronic myelocytic leukemia has maintained the ability to differentiate into both granuloid (basophilic) and erythroid lineages. The expression of P210^{bcr/abl} in KU-812-F cells during differentiation was studied by immunoblotting and immunoprecipitation. Immunoblotting with anti-phosphotyrosine sera revealed the down-regulation of P210^{bcr/abl} in both granuloid and erythroid lineages. Immunoprecipitation with anti-*abl* antibodies of ³⁵S-methionine-labelled cells revealed a reduced rate of synthesis of P210^{bcr/abl} protein. Cytotoxic agents that caused growth inhibition of the cells did not alter the expression of P210^{bcr/abl}. These results indicate that the down regulation of P210^{bcr/abl} protein is a lineage non-specific event accompanied by differentiation.

Key words: chronic myelocytic leukemia, P210^{bcr/abl}, KU-812-F, differentiation.

INTRODUCTION

THE PHILADELPHIA chromosome (Ph) is the cytogenetic hallmark of chronic myelocytic leukemia (CML) [1] whose translocation results in the production of chimeric *bcr-abl* mRNA. The resulting mRNA encodes a novel tyrosine specific kinase P210^{bcr/abl} [2, 3] that appears to be crucial in the pathogenesis of CML [4, 5]. However, the role of P210^{bcr/abl} in the manifestation of this disease has not been fully clarified.

The primary hematologic manifestation of CML is the marked accumulation of granuloid cell components. Little is known about the relationship between the expression of P210^{bcr/abl} and the differentiation of granuloid lineages in Ph positive stem cells.

The CML cell line, KU-812, was established by Kishi from a patient in the basophilic crisis of Ph positive CML [6]. His colleagues showed that KU-812-F cells, a subline of KU-812, exhibited a bipotentiality for cell differentiation into basophils and macrophage-like cells [7]. Recently, evidence of an erythroid differentiating capacity of these cells was demonstrated [8, 9]. These cells can now be considered as the closest model for pluripotent Ph posi-

tive stem cells. In this study, to clarify the mechanism of overgrowth of granuloid cells in CML, we compared the expression of P210^{bcr/abl} following both granuloid and erythroid differentiation in the CML cells, KU-812-F, using anti-phosphotyrosine sera and an immunoblotting method.

MATERIALS AND METHODS

Culture and treatment of the cells

KU-812-F cells were provided by the Japanese Cancer Research Resources Bank (Tokyo, Japan). Cells were maintained in RPMI-1640 medium (GIBCO Laboratories, Grand Island, NY) supplemented with 15% fetal calf serum (FCS, Sera-lab, Sussex, U.K.). Induction of erythroid lineage was performed through the addition of hemin (Wako Pure Chemical, Osaka, Japan) at a concentration of 0.1 mM. Cytoentrifuged smears were scored for hemoglobin-producing cells by staining with benzidine [10]. Transcription of γ -globin gene was estimated by Northern blot analysis [11]. The probe used in this study was a 0.5 kb Bam HI-Hpa II fragment containing the 5' end of the A γ -globin gene [12]. Induction of granuloid lineage was performed with Iscove's modified Dulbecco's medium (GIBCO) supplemented with 5 μ g/ml insulin (Sigma Chemical Co., St Louis, MO), 5 μ g/ml transferrin (Sigma), 10 ml/l nonessential amino acids (GIBCO) and 10 ml/l sodium pyruvate (GIBCO) [7]. Morphologic examination of the cytoentrifuged smears was done following staining with May-Grünwald-Giemsa. The positivity of Toluidine Blue (TB) staining of the basophil granules was estimated by metachromasia. Analysis of surface granuloid antigens was accomplished by indirect immunofluorescence staining

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and flow cytometric analysis. The antibodies used were CD13, CD14 and CD33.

Immunoblotting

The preparation of cell lysates and the immunoblotting technique using anti-phosphotyrosine sera were performed as described by Richardson *et al.* [13]. Cells were washed twice with phosphate-buffered saline, pH 7.4 with 50 μ M Na_3VO_4 , solubilized at $1.0 \times 10^7/\text{ml}$ in sodium dodecyl sulfate (SDS) sample buffer (60 mM Tris-HCl, pH 6.8, 10% glycerol, 10 mM EDTA, 2% SDS, 2% 2-mercaptoethanol and 0.03% bromophenol blue) and were then boiled for 10 min. The cell lysates (4.0×10^5 cells) were subjected to 7.5% SDS-polyacrylamide gel electrophoresis and the proteins were transferred to a 0.45 μ m nitrocellulose filter (Schleicher & Schuell, Dassel, F.R.G.). The filters were preincubated in blocking buffer (3% bovine serum albumin in Tris-buffered saline) overnight at room temperature and then probed with anti-phosphotyrosine sera in blocking buffer for 3 h at room temperature. After extensive washing with 0.2% Nonidet P-40 in Tris-buffered saline, [^{125}I] protein A (30 mCi/mg, Amersham International, Bucks, U.K.) was added to the filter for 1 h. Filters were then washed extensively and autoradiographed. Anti-phosphotyrosine sera were prepared as described previously [14]. Anti-*abl* sera (pEX-5) were kindly provided by Dr O. N. Witte (University of California, Los Angeles, CA) [15].

Metabolic labelling and immunoprecipitation

Cells were washed three times with phosphate free Hanks balanced salt solution or methionine-free minimal essential Eagle medium (GIBCO) containing 5% dialyzed FCS and metabolically labelled for 2 h at 37°C with 200 μ Ci $^{32}\text{PO}_4$ (the Japan Atomic Research Institute, Tokyo, Japan) or 20 μ Ci ^{35}S -methionine (Amersham International, Bucks, U.K.). The cells were washed three times with 0.15 M NaCl, 10 mM Tris-HCl, pH 7.4 and solubilized in 1 ml of ice cold lysis buffer (0.15 M NaCl, 50 mM Tris-HCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EGTA, 2 mM MnCl_2 , 0.1 mM ZnCl_2 , 50 μ M Na_3VO_4 , 1 mM phenylmethylsulfonyl fluoride, pH 7.4) for 15 min. The lysate was clarified by centrifugation at 10,000 *g* for 5 min and protein A Sepharose slurry (Pharmacia, Uppsala, Sweden) was added. After a 1 h incubation at 4°C, the beads were removed. Immunoprecipitation was performed with 5 μ l of antisera for 2 h at 4°C and the antigen-antibody complex was collected with protein A Sepharose following 1 h incubation at 4°C. The beads were washed three times with lysis buffer. Finally, the proteins bound to the beads were solubilized in 2% SDS, 2% 2-mercaptoethanol, 10% glycerol and 62.5 mM Tris-HCl, pH 6.8, boiled for 3 min and then subjected to 7.5% SDS-polyacrylamide gel electrophoresis followed by autoradiography.

RESULTS

Differentiation induction

Approximately 2% of the uninduced cells showed positive results for benzidine staining. After culture

for 4 days, 64.2% of the cells showed positive staining for benzidine. Northern blot analysis of the total cellular RNA isolated from KU-812-F cells demonstrated that the transcription of the γ -globin gene increased during the differentiation (Fig. 1).

The serum-free culture (SFC) was maintained for over 4 weeks. During SFC, the number of cells containing cytoplasmic granules increased. After 14 days, the cells appeared as mature forms with cytoplasmic granules and a mature nucleus (Fig. 2). The number of cells showing metachromasia for TB increased proportionally from 18 to 48%. We confirmed the increase in the expression of granuloid surface markers, CD13, CD14 and CD33, during the SFC as described previously [7] (Table 1).

Effects of granuloid and erythroid differentiation on the expression of $\text{P210}^{\text{bcr/abl}}$

Metabolic labelling using $^{32}\text{PO}_4$ and immunoprecipitation revealed that 210,000 mol. wt protein which reacted with both anti-*abl* sera (pEX-5) and anti-phosphotyrosine sera was confirmed to be a CML-specific protein $\text{P210}^{\text{bcr/abl}}$ (Fig. 3).

There are several methods for detecting $\text{P210}^{\text{bcr/abl}}$. Because the immunoblotting method is thought to reflect an intracellular state of the protein as compared to either immunoprecipitation or immune complex kinase assay, we chose the immunoblotting method for detecting $\text{P210}^{\text{bcr/abl}}$ in the differentiating cells. By immunoblotting using anti-phosphotyrosine sera, the expression of $\text{P210}^{\text{bcr/abl}}$ decreased markedly during the differentiation both of granuloid and erythroid lineages. In addition, other tyrosine phosphorylated proteins considered to be substrates for $\text{P210}^{\text{bcr/abl}}$ tyrosine kinase were shown to decrease (Fig. 4 lanes 1–4). However, such cytotoxic agents as cytosine arabinoside (Ara-C) (0.5 nM) and vincristine (100 nM), which inhibit cell growth did not alter the expression of $\text{P210}^{\text{bcr/abl}}$ (Fig. 4 lanes 5–7).

The detection of $\text{P210}^{\text{bcr/abl}}$ in mature granulocytes cannot be performed successfully because of the inhibitory activity associated with these cells [16, 17]. Therefore, the effect of differentiated basophilic cells on the detection of $\text{P210}^{\text{bcr/abl}}$ was studied by immunoblotting. The amount of $\text{P210}^{\text{bcr/abl}}$ in the mixture of blastoid cells and differentiated basophilic cells was unchanged (Fig. 5), which means that the reduction in protein must result from an intracellular event related to differentiation.

Metabolic labelling using ^{35}S methionine and anti-*abl* sera (pEX-5) showed that the decreased expression of $\text{P210}^{\text{bcr/abl}}$ was mainly due to a decrease in protein synthesis rather than to a decrease in phosphorylation (Fig. 6).



FIG. 1. Northern blot analysis of γ -globin mRNA from KU-812-F cells. KU-812-F cells (lane 1), KU-812-F cells cultured with hemin (0.1 mM) after 4 days (lane 2).

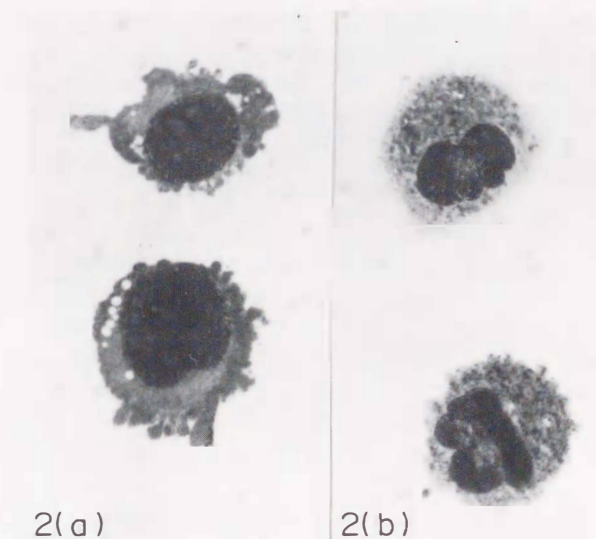


FIG. 2. KU-812-F cells before and after induction of basophilic differentiation. (a) Uninduced KU-812-F cells show blastic appearance. (b) KU-812-F cells cultured in serum-free conditions for 14 days have a mature granulocytic appearance with fine cytoplasmic granules. (May-Grünwald-Giemsa stain.)

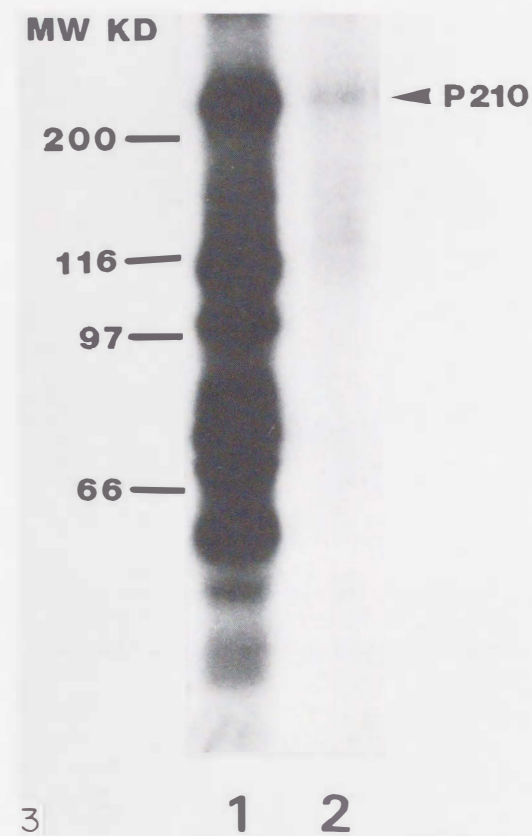


FIG. 3. Identification of P210^{bcr/abl} in KU-812-F cells. The cells were labelled with ³²PO₄ and the proteins were immunoprecipitated with anti-phosphotyrosine sera (lane 1) and anti-*abl* sera (pEX-5) (lane 2).

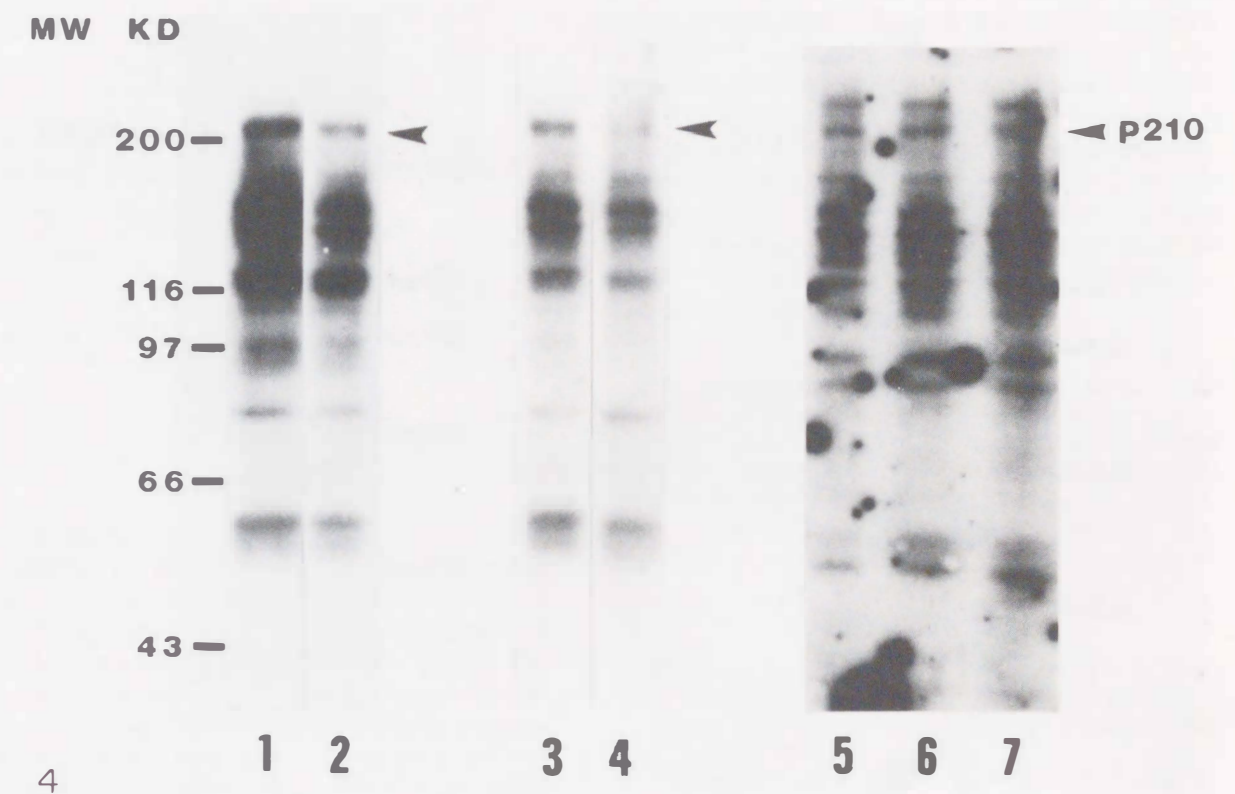


FIG. 4. Effect of differentiation and cytotoxic agents on P210^{bcr/abl} by using immunoblotting with anti-phosphotyrosine sera. Uninduced KU-812-F cells (lanes 1, 3, 5), KU-812-F cells cultured with hemin (0.1 mM) after 5 days (lane 2), KU-812-F cells cultured in SFC after 14 days (lane 4) and KU-812-F cells cultured with Ara-C (0.5 nM) (lane 6) and vincristine (100 nM) (lane 7).

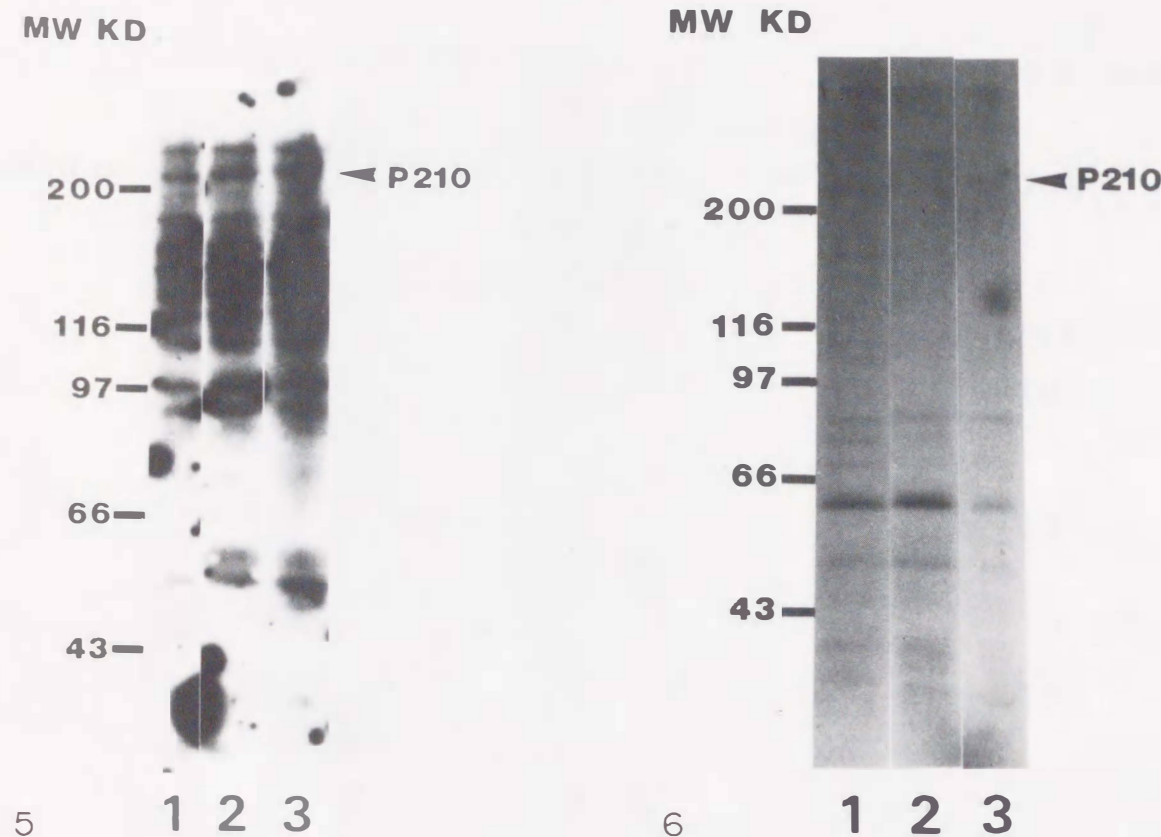


FIG. 5. Effect of differentiated basophilic cells on P210^{bcr/abl} detection by immunoblotting with anti-phosphotyrosine sera. Uninduced KU-812-F cells alone (lane 1), mixture of uninduced KU-812-F cells (1×10^7 cells) and differentiated basophilic cells (1×10^6 cells, lane 2, or 5×10^6 cells, lane 3).

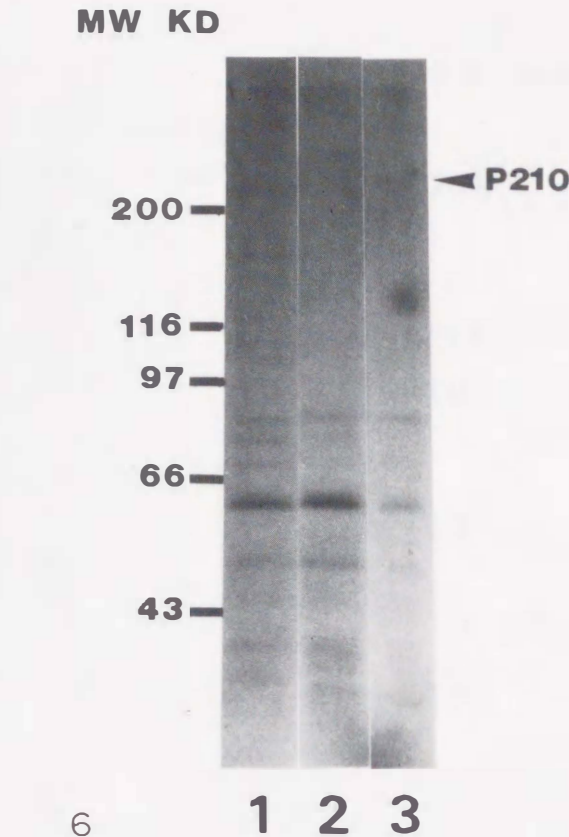


FIG. 6. Reduction of P210^{bcr/abl} protein synthesis in differentiated cells. The cells were labelled with ³⁵S-methionine and the proteins were immunoprecipitated with anti-*abl* sera (pEX-5). Uninduced cells (lane 1), hemin-treated cells (lane 2) and cells under SFC (lane 3).

TABLE 1. GRANULOID DIFFERENTIATION OF KU-812-F CELLS IN SERUM FREE CULTURE

Morphology	Percentage on each experimental day					
	0	5	7	10	12	14 day
Blast	72.0	50.5	22.0	0.0	0.5	0.0
Pro	5.5	15.5	27.5	24.5	5.0	0.0
Myel	4.0	13.0	23.5	42.0	49.5	56.0
Met	2.0	4.5	13.0	12.5	18.0	26.0
Band	0.0	0.0	10.5	8.0	15.0	8.5
Seg	1.0	6.0	0.5	12.0	11.0	9.5
Mitosis	7.0	3.5	0.0	0.0	0.0	0.0
Erythroid like	8.5	7.0	2.0	0.0	0.0	0.0
TB positive	18				48	
Surface marker						
CD 13	35.4					96.6
CD 14	<1.0					20.0
CD 33	38.8					98.3

DISCUSSION

Several studies on the transforming potential of CML-specific P210^{bcr/abl} protein in hematopoietic cells support the concept that the expression of P210^{bcr/abl} may contribute to the clonal outgrowth of Ph-positive stem cells over normal cells [18–21]. Ph-positive CML has a clonal origin in a pluripotent stem cell that can differentiate into whole lineages [22]. However, in CML, the predominant growth of the stem cell's progeny can be seen only in granuloid lineage, including basophilic lineage. Progenitor assay *in vitro* revealed that in CML patients, not only granuloid progenitors but also erythroid progenitors increased [23]. Although some aspects of hematopoietic cell kinetics must have been altered, the precise mechanism causing granuloid accumulation has not been fully elucidated.

Recently, Caracciolo *et al.* observed that the expression of *c-abl* was crucial to normal myelopoiesis but not to erythropoiesis [24]. It is possible that the predominant growth of granuloid lineage components is explained by the difference in the expression of P210^{bcr/abl} between granuloid and erythroid lineages. Using the CML cell line K-562, several studies have shown reduced levels of P210^{bcr/abl} during erythroid differentiation [13, 14, 25, 26]. In this study, we determined whether the expression of P210^{bcr/abl} is regulated in a lineage-specific manner using KU-812-F cells that possess a definite differentiating potency both in granuloid and erythroid lineages. In SFC, the cells differentiated through basophilic lineage and decreased the expression of P210^{bcr/abl}. The cells also decreased the expression of

P210^{bcr/abl} when they were incubated with hemin and differentiated into erythroid lineage. In both lineages, the reduction was mainly attributable to a decrease in protein synthesis rather than in protein phosphorylation. The inhibition of growth but not differentiation caused by cytotoxic agents was not associated with the down regulation of P210^{bcr/abl} as shown in this study, suggesting that the reduction of P210^{bcr/abl} is coupled with differentiation but not growth inhibition. These results suggest that a decrease in the expression of P210^{bcr/abl} occurs in a lineage non-specific fashion and plays a role in the differentiation pathways leading to both granuloid and erythroid lineages. Therefore it may be suggested that a mechanism other than the expression of P210^{bcr/abl} is crucial to the dominance of the granuloid lineage over the erythroid lineage.

Acknowledgements—We thank Dr O. N. Witte for the gift of pEX-5. This work was supported in part by a grant-in-aid for cancer research (0105076) from the Ministry of Education, Science and Culture in Japan and by a grant from the Fukuoka Cancer Society.

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