Expression of bcr-abl Abrogates Factor-Dependent Growth of Human Hematopoietic M07E Cells by an Autocrine Mechanism

By Christian Sirard, Pierre Laneuville, and John E. Dick

The introduction of a retrovirus vector expressing p210\textsuperscript{bcr-abl} (P210) into the human factor-dependent cell line M07E resulted in the rapid outgrowth of factor-independent cells. Early after infection, four factor-independent clones were isolated and analyzed in greater detail along with mass populations obtained from separate infections. High levels of P210 tyrosine kinase activity were measured in the factor-independent cells. The mass populations and three of the four clones remained responsive to exogenous growth factors. Concentrated conditioned media isolated from the factor-independent populations and from all clones contained biologically active granulocyte-macrophage colony-stimulating factor (GM-CSF); interleukin-3 (IL-3) was detected at low levels in the mass population and in two of the clones. Neutralizing antibodies to IL-3, GM-CSF, and mast cell growth factor inhibited proliferation of the factor responsive clones by 60% to 90%. These results indicate that the growth autonomy of the P210-expressing M07E cells was acquired via an autocrine mechanism. In addition to factor-independent growth, P210-expressing M07E cells readily acquired a more mature megakaryocytic phenotype compared with control M07E cells. These data provide experimental evidence that expression of P210 tyrosine kinase in human hematopoietic cells induced growth factor secretion resulting in a pleiotropic effect on growth factor dependence and differentiation.

The developmental program of normal hematopoiesis is tightly regulated by cytokines and cellular interactions within the bone marrow microenvironment. De-regulated expression of these cytokines or of the genes involved in their signal transduction pathway contributes to the multistep progression of leukemogenesis. Chronic myelogenous leukemia (CML) provides an important model system to examine this multistep process because the disease follows a highly predictable time course from chronic phase to blast crisis. The Philadelphia chromosome (Ph), a translocation between chromosomes 9 and 22, is the hallmark of CML and results in a chimeric transcript translated into a novel p210\textsuperscript{bcr-abl} (P210) fusion protein with an enhanced tyrosine kinase activity. CML is a clonal disorder of hematopoietic stem cells and the expression of an activated abl proto-oncogene by fusion with the bcr locus is believed to be responsible for the abnormal granulopoiesis observed in the chronic phase. Reconstitution of mice with murine bone marrow infected with a p210\textsuperscript{bcr-abl} retrovirus results in a wide spectrum of leukemias, including a CML-like granulopoiesis. Although there is controversy as to whether bcr-abl expression predominately acts on stem cells, colony-forming progenitors, or mature cells, cell culture studies have identified alterations in the proliferation and differentiation of CML progenitors and in the response of immature long-term culture initiating cells (LTC-IC) to negative growth regulators. CML patients contain increased numbers of circulating LTC-IC and colony-forming progenitors relative to normal individuals and these progenitors cycle more frequently than normal progenitors. In addition, CML colony-forming progenitors have altered adherent properties, resulting in their premature release from the bone marrow into the peripheral blood. Such premature release could deprive the Ph-positive cells from short range positive or negative growth signals. Together, these studies imply that CML cells have acquired some growth autonomy; however, the biologic role of bcr-abl expression and the mechanism by which it perturbs the hematopoietic developmental program are unclear.

Several mechanisms have been described that reduce or eliminate the requirement for growth factors. One possibility is disruption of the signal transduction pathway leading from a growth factor receptor, resulting in its constitutive activation even in the absence of ligand binding. Another possibility involves the inappropriate expression and secretion of growth factors or their receptors, establishing an autocrine loop. In some cases of acute myeloid leukemia (AML) and acute lymphocytic leukemia (ALL), leukemic cells constitutively express growth factor genes and in many cases the leukemic cells remain responsive to growth factor stimulation. Furthermore, abnormal production of growth factors has been associated with some cases of CML. However, the relationship between the expression of tyrosine kinases, cellular phosphotyrosine protein levels, and autocrine growth factor stimulation has not been systematically explored in human neoplasia.

Although CML does not naturally occur in mice, experiments in the murine system show that the expression of bcr-abl leads to a loss in the requirement for growth factors in myeloid cell lines and to tumorigenicity in pre-B-lymphoid cell lines. Similarly, expression of the v-abl oncogene usually abrogates the growth factor requirement and transforms a variety of murine cell lines, including fibroblasts, mast cells, erythroid cells, pre-B-lymphoid cells, and myeloid cells. Although most of...
these studies conclude that factor independence arises via a nonautocrine mechanism without any alteration in the differentiation program, other studies, using more sensitive assays, have reported that granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) messages can be induced in murine cells by expression of either bcr-abl or v-abl. Infection of murine fibroblasts with v-abl also induces secretion of transforming growth factor α (TGFα). However, these latter studies have not shown that factor independence occurs via an autocrine loop. Expression of src-related oncogenes in v-myc or v-myb transformed avian myeloid cells results in autocrine growth via activation of the endogenous chicken myelomonocytic growth factor (cMGF).

To determine the biologic consequence of expressing bcr-abl in human cells, we have introduced a retrovirus expressing the P210 tyrosine kinase into a human megakaryoblastic leukemia cell line, M07E, that is dependent on IL-3 or GM-CSF. We report that M07E cells expressing P210 lose their requirement for exogenous growth factor stimulation and exhibit a more differentiated phenotype than the parental line. An autocrine mechanism is involved in the growth autonomy of the factor-independent cells.

MATERIALS AND METHODS

Isolation of amphotropic packaging cell lines. The retroviral construct used in this study containing the P210 cDNA, designated pJW-RX, was described in detail by Daley et al. The neomycin (neo) bacterial gene is expressed from the herpes-virus thymidine kinase (tk) promoter and the P210 gene is transcribed from the 5' long terminal repeat (LTR). Amphotropic packaging cell lines, expressing a functional P210, were obtained by infecting PA317 cells with viral supernatants (provided by O. Witte, Howard Hughes Medical Institute, Los Angeles, CA) derived from ψ-2 ecotropic cells transfected with the pJW-RX vector. Infected PA317 cells were selected in 400 µg/mL of active G418 (GIBCO-BRL, Gaithersburg, MD) and clones were tested for high virus titers. Control amphotropic packaging cell lines produced the N249 or pYN99 neo viruses.

Cell lines, culture conditions, and gene transfer. All cells were routinely grown in a minimum essential media (MEM) without antibiotics containing 10% fetal bovine serum (FBS) and incubated at 37°C in a 5% CO2 atmosphere. M07E cell cultures (provided by S. Clark, Genetics Institute, Cambridge, MA) were supplemented with 2 mmol/L L-glutamine (GIBCO) and 10 U/mL of recombinant human IL-3 (rhIL-3; provided by D. Williams, Immunex, Seattle, WA). The karyotype of M07E cells is relatively normal and has an unlimited life span (>2 years). These cells also have a very low reversion frequency. Retrovirus infections of M07E were performed at a cell density of 1 × 10^5 cells/mL with 3 consecutive changes of filtered viral supernatant supplemented with 8 µg/mL of polybrene at 3-hour intervals. Infected cells were maintained in the presence of rhuIL-3 for 5 days, after which they were washed twice and plated in methylcellulose cultures at a cell density of 1 × 10^4 cells/mL in LUX suspension plates (Nunc, Roskilde, Denmark) (47% methylcellulose, 30% FBS, 2 mmol/L L-glutamine, and 10^4 mol/L 2-mercaptoethanol with or without G418 and/or rhuIL-3 to assess gene transfer efficiency). Infected, washed M07E cells were also placed in liquid culture containing 10% FBS but deprived of growth factors at a cell density of 1 × 10^6 cells/mL to generate a mass population of factor-independent cells. The absence of helper
The negative control was neo-infected M07E cells (M-NEO) and the positive control was the CML-derived cell line, K562, that expresses a functional P210 kinase. Cell lysates were immunoprecipitated either with an anti-ab/ antiseraum (+lanes) or with a preimmune sera (-lanes) before in vitro autophosphorylation. The labeled proteins were separated on SDS-polyacrylamide gels. Shown are autoradiograms exposed for a period of 20 minutes with an intensifying screen.

**Southern blot analysis.** High molecular weight DNA was isolated from lysed cells in the presence of 2% sodium dodecyl sulphate (SDS). After digestion for 2 hours at 37°C with 0.15 mg/mL of proteinase K, the DNA was extracted sequentially with phenol and chloroform and precipitated with ethanol in the presence of 0.2 mol/L sodium chloride. DNA was transferred to nylon membranes (Hybond N; Amersham, Arlington Heights, IL) and hybridized with DNA probes labeled with [γ-32P]ATP by random-primed DNA labeling (Boehringer Mannheim Biochemicals, Laval, Quebec, Canada). Hybridization was performed in 7% SDS, 1 mmol/L EDTA, 0.5 mol/L NaHPO4 (pH 7.2), and 1% bovine serum albumin. The blots were washed under stringent conditions in which the last wash was performed at 50°C for 20 minutes in 0.1× SSC and 0.1% SDS. Radiolabeled probes were stripped from the membrane by boiling twice in 0.1× SSC and 0.5% SDS.

In vitro kinase assay. The autophosphorylation assay was performed as described by Konopka et al. Briefly, a total of 3 × 106 exponentially growing cells was washed in cold phosphate-buffered saline and lysed in 1 mL of cold phosphate lysis buffer (PLB; 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.01 mol/L NaHPO4, pH 7.5, 0.1 mol/L NaCl, 5 mmol/L EDTA) plus 10 μg/mL of fresh leupeptin. Extracts were clarified by centrifugation and immunoprecipitated for 2 hours at 4°C with 5 μL of either normal or anti-ab rabbit sera pEX-5′ (provided by A.M. Pendergast and O. Witte) in the presence of protein A-agarose beads (Oncogene Inc, Uniondale, NY). Immunoprecipitates were washed twice with PLB, once with 40 mL of PLB, once with 20 mL of PIPES (1,4-piperazinediethanesulfonate), pH 7.5, and once with 20 mL of PIPES (pH 7.0) containing 20 mmol/L MnCl2 and resuspended in 40 μL of the same buffer. Autophosphorylation reactions were performed for 5 minutes at 30°C with 5 μCi of [γ-32P]ATP (3,000 Ci/mmol; Amersham). The reaction was terminated with 1 mL of cold PLB containing an additional 5 mmol/L EDTA and the protein substrate was eluted into SDS gel sample buffer by incubation for 5 minutes at 95°C and separated by electrophoresis on a 7% SDS-polyacrylamide gel. Radiolabeled proteins were analyzed by autoradiography.

**Proliferation bioassay.** Cell proliferation was assessed by either MTT or XTT assays. Unless indicated otherwise, 2 × 104 washed M07E cells/mL were incubated in quadruplicates in 100 μL of medium in flat-bottomed 96-well microtiter plates in 10% serum in either GM-CSF or IL-3. Five days after retroviral infection, the cells were plated in methylcellulose in the presence or absence of exogenous growth factors. On average, 10% of the P210-infected cells gave rise to colonies in the absence of growth factors (Table 1). Consistently, there was a rapid outgrowth of factor-independent cells within 7 days after the

**RESULTS**

**Expression of P210 in M07E cells abrogates growth factor dependence.** To determine if expression of the bcr-abl oncogene alters the growth factor requirement of human hematopoietic cells, M07E factor-dependent cells were infected with a retroviral vector expressing the P210 cDNA. The M07E cell line was established from a patient with megakaryoblastic leukemia and is dependent for growth on either GM-CSF or IL-3. Five days after retroviral infection, cells were plated in methylcellulose in the presence or absence of exogenous growth factor. On average, 10% of the P210-infected cells gave rise to colonies in the absence of growth factors (Table 1). Consistently, there was a rapid outgrowth of factor-independent cells within 7 days after the
withdrawal of growth factors from the liquid cultures in presence of 10% serum. Control experiments involving infection of M07E cells with vectors expressing only the neo gene, designated as M-NEO cells, failed to grow in the absence of growth factors (Table 1). When placed in the presence of G418 supplemented with growth factors, the neo-infected population displayed an average gene transfer efficiency of 22%, which was only double the efficiency of factor-independence observed after P210 infection (Table 1). This slightly reduced frequency was probably caused by low virus titer of the much larger size (13 kb) P210 vector. A low frequency of G418-resistant colonies (1.6%) was observed in P210-infected cells (Table 1), largely because of poor expression of the neo gene from the tk promoter in M07E cell lines (data not shown), as occurs frequently in retroviral vectors with two promoters. The high frequency of factor-independent colonies implied that no additional genetic alterations, other than expression of the P210 gene, were needed to abrogate the growth factor requirement of M07E cells.

To establish rigorously the early biologic consequence of P210 expression in M07E cells, three independent infection experiments were performed and clones were isolated from the factor-independent population within several doublings after retroviral infection. Southern analysis performed on EcoRI-digested DNA extracted from the factor-independent mass populations (a representative population, MBA-M, is shown) and clones indicated that all infected cells contained an intact EcoRI fragment of 7.2 kb corresponding to the P210 cDNA of the parental vector (Fig 1B). The lower bands observed in Fig 1 represent endogenous abl sequences within M07E cells. Because no other EcoRI sites were present in the vector, the clonal origin was determined by re-probing the Southern blots with the neo gene (Fig 1A). DNA from the MBA-M cell population was examined early (9 days) after infection (MBA-ME) and after 2 months in cul-

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Fig 3. Responsiveness of the factor-independent cells to increasing amount of exogenous growth factors. Infected M07E cells (2 × 10⁶/well) were stimulated with increasing amounts of IL-3 (A and C) or GM-CSF (B and D). After 4 days of incubation, the cell density was measured using the XTT bioassays. Similar dose-response curves to (A) and (B) were obtained with all 3 mass populations obtained from separate infections (data not shown). The data shown are representative of at least three independent experiments in which each data point represents the mean of quadruplicate measurements ± SD.
Autocrine Growth of Cells Expressing bcr-abl.

The absence of visible bands in the MBA-ME cells indicated that the population was polyclonal, whereas a few prominent bands were observed in the MBA-ML cells (Fig 1A), indicating that an oligoclonal expansion occurred within a few months in culture. This clonal expansion was not restricted to P210-expressing cells, because M-NEO-infected cells also evolved into a clonal population within 1 month after G418 selection (Fig 1A). Each of the clones (MBA.1 through MBA.4) had different retroviral integration sites (Fig 1A). The double band observed with MBA.1 clone represents 2 retroviral integration sites because the bcr-abl band is twice the intensity of the endogenous abl gene (Fig 1B).

The activity of the P210 kinase in these factor-independent cells was assessed by in vitro autophosphorylation. Immunoprecipitations were performed with antibodies specific to the abl protein using equivalent numbers of lysed cells. The results shown in Fig 2 illustrate that the autophosphorylation of P210 was as efficient in the factor-independent cells as in the control cell line, K562. The K562 cell line was originally derived from a patient with CML and contains multiple copies of the P210 gene. These results indicate that all factor-independent cells overexpress functional P210 protein.

Factor-independent cells remain responsive to growth factors. One of the mechanisms by which P210 expression could abrogate factor dependence in M07E cells was by activation of an autocrine loop. In most instances in which an autocrine mechanism has been described, the autocrine factor was produced at suboptimal levels and cells remained responsive to exogenously added growth factors. To assess if the P210-expressing factor-independent cells had retained the ability to respond to exogenous growth factors, the growth stimulation was monitored at low cell density using the colorimetric XTT bioassay in the presence of varying concentrations of GM-CSF or IL-3. A minimum of four independent experiments were conducted and representative curves are depicted in Fig 3. Analysis of the clonal populations showed that three clones, MBA.2, MBA.3 and MBA.4, remained as responsive to IL-3 and GM-CSF (Fig 3A and B, respectively) as the M-NEO cells; whereas one clone, MBA.1, was maximally stimulated in the absence of growth factors (Fig 3C and D). All 3 mass population analyzed also remained responsive to GM-CSF and IL-3 as determined by proliferation and clonogenic assays (data not shown). The responsiveness of nonresponsive cells was stable throughout a 4-month period in culture. These results suggest that some clones within the mass population are fully autonomous for their growth requirements, whereas most others are suboptimally stimulated.

To determine whether the P210-expressing cells secreted growth factors, CM from the factor-independent populations was assayed for stimulatory activity using parental M07E cells as an indicator. Although the bioassay can detect picogram levels of GM-CSF and nanogram levels of IL-3 (Fig 3), no significant amounts of M07E-stimulatory activity could be detected. To increase the sensitivity of the assay, lethally irradiated MBA-ME and MBA-ML cells were incubated at varying cell densities together with M07E indicator cells. The results shown in Fig 4A illustrate that the irradiated factor-independent cells can support M07E cell proliferation whereas M-NEO irradiated cells cannot. The irradiated factor-independent cells alone did not grow in the MTT bioassay. These results indicate that the p210-expressing cells produced small amounts of factor capable of supporting M07E cell growth. Accordingly, CM from various factor-independent populations was concentrated 25-fold and reassayed for the presence of M07E-stimulatory activity. All three independent mass populations examined secreted small amounts of factor capable of stimulating M07E cell proliferation, whereas no stimulatory activity was detected in concentrated CM derived from M-NEO cell populations (Fig 4B). Similarly, CM from each of the four clones tested also contained M07E-stimulatory activity, with clone MBA.2 producing the most factor (Fig 4C).

GM-CSF and IL-3 are present in the CM derived from the factor-independent cells. The identity of the biologically active growth factor from the CM of the factor-independent cells was determined by incubating the CM with neutralizing antibodies directed against either GM-CSF or IL-3. A 30-fold reduction (using ED 50% values) of the stimulatory activity in MBA-ME CM was observed with anti-GM-CSF neutralizing antibodies, whereas no inhibition of stimulatory activity was detectable with anti-IL-3 neutralizing antibodies (Fig 5A). However, when both anti-IL-3 and anti-GM-CSF neutralizing antibodies were used, all the M07E-stimulatory activity was inhibited, indicating that excess amounts of GM-CSF precluded the detection of small amounts of IL-3. The presence of GM-CSF was also detected by enzyme-linked immunosorbent assay (ELISA) at a concentration of 5 ng/mL in the concentrated CM of factor-independent cells; no detectable IL-3 was found (data not shown). Two different patterns of stimulatory activity were observed with CM of the clonal populations. Similar to the mass populations, the CM of the MBA.1 and MBA.4 clones needed both anti-GM-CSF and anti-IL-3 to completely neutralize the M07E-stimulatory activity (a representative clone is shown in Fig 5B), whereas only anti-GM-CSF was needed to fully neutralize the stimulatory activity found in the CM media of the MBA.2 and MBA.3 (a representative clone is shown in Fig 5C). Growth inhibition of M07E cells was not caused by the toxicity of the neutralizing antibody because the addition of mast cell growth factor (MGF), at the minimum concentration required to support M07E cell growth, fully restored cell proliferation in the presence of both neutralizing antibodies (data not shown). These results establish that the expression of P210 in M07E cells induced the secretion of GM-CSF in all cells while a lower proportion of cells also secreted IL-3; GM-CSF was always secreted in greater amounts than IL-3.

Growth inhibition of the factor-independent cells using neutralizing antibodies. In the classical model of autocrine growth, cells are stimulated by the secretion of growth factors that then bind to their cognate membrane surface receptors. Consequently, if such a mechanism was responsible for the autonomous growth of the factor-independent cells, their growth should be inhibited by the addition of neutralizing antibodies. The ability of anti-GM-CSF, anti-
IL-3, and anti-MGF antibodies to inhibit proliferation was tested alone or in combination on each of the clonal populations. The proper cell density was first determined for each factor-independent clone to obtain logarithmic growth during the time of the inhibition assay. Neutralizing antibodies were added at a concentration that was 1,000-fold in excess over the concentration that we had previously shown could neutralize all the stimulatory activity detected in the concentrated CM. In all clones, the strongest inhibition obtained with single neutralizing antibodies was with anti-IL-3 (Fig 6). Anti-MGF or anti-GM-CSF antibodies had no significant effect on the growth of most clones except for MBA.4, in which cell growth was inhibited by 30% with either one of these antibodies. The addition of IL-3 together with anti-GM-CSF or anti-MGF and of GM-CSF with anti-IL-3, at concentrations sufficient for maximum stimulation, restored cell proliferation, indicating that the effects of the neutralizing antibody were specific (data not shown). These results suggest that, although IL-3 was present in low or undetectable amounts in the CM of the factor-independent clones, it is an important secreted factor for growth stimulation. It is possible that the M07E bioassay of the CM was not sensitive enough to detect small amounts of factor that could still synergize with IL-3 and GM-CSF. Because in vitro studies have shown that MGF synergizes very well with IL-3 and GM-CSF, all 3 neutralizing antibodies were added to the factor-independent cells. Clones MBA.3, MBA.4, and MBA.2 showed growth inhibition of 60%, 70%, and 90%, respectively, indicating that MGF must also be secreted (Fig 6). These results confirm that expression of P210 in M07E cells results in growth autonomy via an autocrine loop for at least three of the four factor-independent clones.

Expression of P210 indirectly affects M07E cell differentiation. Examination of Wright-stained factor-independent cells showed increased megakaryocytic differentiation compared with parental M07E cells. The features that delineated this differentiation included (1) acquisition of a
greater cytoplasmic/nuclear ratio; (2) multi-lobe nucleus; (3) the appearance of blebs on the cell surface maybe responsible for platelet formation; and (4) condensed chromatin (Fig 7). Two factor-independent populations (M3.16 and MBA-ME) were further examined by cytochemical stains to assess cell differentiation. Both factor-independent populations gave similar results, but only results from the MBA-ME population are shown. In contrast to M-NEO cells, factor-independent cells stained much more intensely for acid phosphatase or for nonspecific esterase (with or without fluoride), or with Periodic Acid Schiff stain. In addition, there was a twofold to threefold increase in the proportion of positively staining cells relative to the mock-infected cells (Table 2). These stains assay for either the enzymatic activity or the glycogen storage acquired during cell differentiation. Four P210-expressing clones were also analyzed by immunohistochemistry staining for glycophorin IIIa and factor VIII, whose expression is increased with megakaryocytic differentiation (Table 3). All of the clones show increased staining intensity compared with parental cells, indicating that the expression of P210 affects the developmental program of M07E cells and induces differentiation along the megakaryocytic lineage.

**DISCUSSION**

This study provides evidence that the expression of P210 in human leukemic M07E cells abrogates their growth dependence on exogenous GM-CSF and IL-3. The rapid appearance and high frequency of factor-independent clones after retrovirus infection suggests that P210 expression alone is sufficient to obtain growth autonomy without additional genetic alterations. Although P210-expressing M07E cells no longer require exogenous factors, the mass population and most clones still remain responsive to these growth factors. This result implies that the P210 tyrosine kinase, although strongly activated in the factor-independent cells (Fig 2), does not completely bypass the ability of exogenous growth factor to deliver mitogenic signals. This observation seems unique to our model system because expression of tyrosine kinases, including P210, in murine cells results in factor-independent growth but the cells lose their responsiveness to exogenous growth factors.

Biologically active GM-CSF and IL-3 were detected in the concentrated CM using neutralizing antibodies directed against each growth factor. The fact that the P210-expressing M07E cells retained their ability to respond to exogenous IL-3 and GM-CSF and also produced these same factors indicates that autocrine growth stimulation may be a mechanism whereby P210 induces factor-independence. Conclusive proof that an autocrine mechanism could be responsible for the factor-independent cell proliferation was provided by the significant inhibition obtained with neutralizing antibodies directed against GM-CSF, IL-3, and MGF. Three of four clones were inhibited by 60% to 90%. Consistent with the autocrine hypothesis, the clone MBA.2, which produced the highest amount of stimulatory activity, was inhibited the greatest. Paradoxically, although GM-CSF was secreted in higher amounts in the CM derived from the responsive clones, the greatest growth inhibition was seen with anti-IL-3 neutralizing antibodies. M07E cells are more responsive to IL-3 than GM-CSF (Fig 3) and small amounts of other factors, such as MGF, can synergize with IL-3 to stimulate cell growth. Furthermore, IL-3 and MGF could be membrane-bound and would not be detected in the concentrated CM of the factor-independent populations.

Autocrine growth stimulation may occur by two different mechanisms: the secretion of growth factor and binding to its receptor at the cell surface or by internal binding as the cognate receptor assembles in the endoplasmic reticulum. The fact that three clones were strongly inhibited by the addition of neutralizing antibodies indicates that the secreted growth factor must bind to the cell surface receptor. However, none of these clones could be completely inhibited and the non-growth factor-responsive clone, MBA.1, showed no inhibition. These results suggest that internal binding may also play an important role. There are examples with other systems that growth factors can functionally bind to their receptors intracellularly. Transformation of normal rat kidney (NRK) cells by the v-sis oncogene occurs through internal binding to, and activation of, the platelet-derived growth factor (PDGF) receptor. The induction of factor independence in mouse hematopoietic cell lines by expression of either GM-CSF or IL-3 cDNAs also occurred through internal binding to their respective receptors. When a sequence was added at the carboxy-terminal of the v-sis and IL-3 cDNA, to retain the molecules in the endoplasmic reticulum, autonomous growth was obtained without...
Fig 7. Morphologic comparison of the M-NEO and MBA-ME cells. Cells were prepared by cytopsin and Wright stained. The M-NEO control cells (A) have features consistent with blast-type cells, whereas MBA-ME cells (B) show a more mature megakaryocytic phenotype such as higher cytoplasmic/nuclear ratio, multi-lobe nucleus, and bleb formation. Micrographs were taken at 1,000 X magnification.

secretion of the growth factor, clearly demonstrating that the growth factors can activate the receptors within intracellular compartments. Thus, both autocrine mechanisms could be effective in the abrogation of the growth factor requirements in M07E cells expressing P210, thus extending to human hematopoietic cells the concept of internal autocrine growth stimulation. However, we cannot rule out the possibility that, in addition to autocrine growth stimulation, P210 also directly activates various components of the mitogenic signal, providing an alternate explanation for the ab-
sence of complete growth inhibition by neutralizing antibodies. Specific inhibition of the growth factor and their receptors by antisense oligonucleotides should directly address these questions.

Our results with M07E cells seem to be different from many of the studies that have examined the role of P210 or v-abl in murine cells such as FDCP-1 cells, 32D cells, and mast cells, which indicate that a nonautocrine mechanism was responsible for the autonomous growth. These studies suggested that P210 interferes with the normal signal transduction pathway, resulting in constitutive activation independent of ligand binding. These conclusions of a nonautocrine mechanism were based on the absence of growth stimulatory activity in the nonconcentrated CM from most factor-independent clones and the lack of growth factor message by Northern analysis. It is worth noting that more sensitive reverse transcription-polymerase chain reaction and S1-nuclease methods have detected low levels of IL-3 message in murine bcr-c-abl or v-abl transformed FDCP-1 cells and messages for IL-3, IL-4, and GM-CSF in v-abl transformed mast cell line, and also that a small amount of stimulatory activity was present in the CM of a few clones. However, the biologic relevance of these secreted factors for cell growth has not been demonstrated.

Some features of Ph-positive CML are consistent with autocrine growth stimulation. Urine samples of chronic-phase CML patients display higher levels of colony-stimulating factors than that found in urine from normal or acute-phase CML patients and increased levels of CSF-1 were measured in the serum of CML patients. G-CSF transcripts have been detected in purified promyelocytes/myelocytes from some chronic-phase CML patients and neutralizing antibodies specifically inhibited granulocytic colony formation. Although there are few differences between the growth of CML-derived progenitors and normal cells in semisolid cultures in the absence of growth factors, a proportion of CML samples produce small spontaneous colonies in the presence of FCS alone.

The factor-independent M07E cells also exhibited features consistent with increased megakaryocytic differentiation, suggesting that P210 expression in these cells also altered the differentiation status of M07E cells. In agreement with this result, P210 can induce mast cells to switch their differentiation to erythroid or myeloid lineages in vitro. In addition, the differentiation program of an IL-3-dependent murine cell line can be altered by the introduction of either v-abl or P210. Thus, the analysis of P210 gene expression in M07E cells provides a model system to study the molecular mechanisms that cause growth factor gene expression and altered differentiation.

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**Table 2. Cytochemical Staining of the Factor-Independent Cells**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Acid Phosphatase</th>
<th>PAS</th>
<th>NSE - Fluoride</th>
<th>NSE + Fluoride</th>
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<tbody>
<tr>
<td>M-NEOt</td>
<td>25.7</td>
<td>12.8</td>
<td>33.3</td>
<td>19.0</td>
</tr>
<tr>
<td>MBA-ME</td>
<td>85.2</td>
<td>41.3</td>
<td>69.0</td>
<td>52.2</td>
</tr>
</tbody>
</table>

* Cells were cytochemically stained with Periodic Acid Schiff (PAS), nonspecific esterase (NSE), or acid phosphatase stains. Percentages were determined by counting at least 200 cells per slide.
† The M-NEOt positive-staining cells were very weak for all stains compared with the MBA-ME cells.
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