In Vitro Establishment and Characterization of a Human Megakaryoblastic Cell Line

By Douglas A. Fugman, David P. Witte, Cindy L.A. Jones, Bruce J. Aronow, and Michael A. Lieberman

A human megakaryoblastic cell line, designated CHRF-288-11, has been established in vitro through the use of adherent stromal cells in long-term human bone marrow culture. Long-term bone marrow cultures were required for the initial adaptation of the megakaryoblastic cells to culture conditions; however, once adapted, the cells were weaned from the stromal layer until they proliferated in the complete absence of any feeder layers. The seed cells for the establishment of this line were derived from a solid tumor; the cloned cell line derived from this tumor exhibits markers characteristic of megakaryocytes and platelets. Specifically, the cells express platelet peroxidase, platelet factor 4, the platelet Ca++-adenosine triphosphatase (ATPase), glycoprotein IIb-lla (CDw411), factor VIII antigen, and the MY7 (CD13) and MY9 (CD33) antigens. The cells do not express the erythroid markers glycophorin A and hemoglobin, the myeloid marker myeloperoxidase, nor markers specific for T and/or B cells. The established cell line produces both basic fibroblast growth factor and transforming growth factor-β, properties demonstrated previously for the solid tumor. The clonal cell population exhibited a unique, singular karyotype, indicating cellular homogeneity. The cells display a doubling time of approximately 33 hours in either 25% horse or calf serum. Treatment of the cells with \( 1 \times 10^{-8} \) mol/L phorbol 12-myristate 13-acetate (PMA) leads to the induction of multi-nucleation and hyperploidy in the cells, with approximately 35% of the cells exhibiting two or more nuclei per cell, and greater than 80% of the cells enlarging in size. The establishment of this unique cell line under defined culture conditions will be beneficial for the future study of megakaryocytic properties expressed by this cell line. © 1990 by The American Society of Hematology.

THE STUDY OF platelet formation from megakaryocytes has been hampered in the past by the difficulty in obtaining large numbers of pure megakaryocyte populations for analysis. Recently, various technologic advances have been made in isolating relatively pure populations of megakaryocytes, although the cell numbers obtained are low. This makes biochemical analysis of megakaryocytes very difficult. Non-human megakaryocytes have been used to develop assays to identify thrombopoietic activity in human serum, but the results cannot be extrapolated to human cells. It is becoming apparent that the study of megakaryopoiesis and the factors that regulate it will require the development of either long-term culture systems derived from normal megakaryocytes or permanent cell lines derived from transformed megakaryocytes. A few human megakaryocytic in vitro cell lines have been established from patients with various hematologic disorders, including megakaryoblastic leukemia, and four of these cell lines have been shown to apparently undergo some differentiation along the megakaryocytic lineage by the addition of phorbol esters. It is thus apparent that useful information concerning megakaryopoiesis, and megakaryocytes in general, can be obtained through the study of such cell lines.

We have recently described a megakaryoblastic cell line that has been successfully passaged in nude mice for over 3 years. This line, designated CHRF-288, was originally established from a biopsy of a metastatic tumor in a 17-month-old infant with acute megakaryoblastic leukemia. We have been able to demonstrate that this line synthesizes a unique subset of growth factors, some of which are normally associated with platelets. In order to facilitate the study of platelet-specific proteins in this cell line, it would be advantageous to establish the CHRF-288 cell line in culture. We now report that this effort has been successful through the use of a feeder layer consisting of adherent stromal cells in long-term human marrow cultures. In normal marrow these are the cells that support hematopoiesis, and the conditions for establishing such cultures have been available for a number of years. The tumor cells were weaned from the stromal layer, cloned, and were shown to express characteristics of the cells grown in athymic nude mice (as determined morphologically, biochemically, immunologically, and karyotypically). Exposure of these cells to phorbol 12-myristate 13-acetate (PMA) leads to multinucleation in the absence of cytokinesis, indicating that the cells may be induced to differentiate along the megakaryocytic lineage. This cell line could, therefore, prove to be a useful system in which to study aspects of megakaryocytic differentiation and the regulation of various platelet-specific functions. In addition, this in vitro system may also provide for a unique approach to study important interactions between the supportive elements of the bone marrow and megakaryoblasts.

MATERIALS AND METHODS

Propagation of the solid tumor line, CHRF-288. The CHRF-288 tumor line was propagated in nude mice as previously described.14,15

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Establishment of long-term bone marrow cultures from human bone marrow mononuclear cells. Two procedures have been developed to enable human bone marrow mononuclear cells (BMMC) to be salvaged from unused fractions of T-cell depletion procedures. BMMC were salvaged by repeating the gelatin red blood cell sedimentation step on the initial red blood cell sediment obtained. The reclaimed BMMCs were concentrated and washed twice with phosphate-buffered saline (PBS), 2% human serum albumin (HSA, Travenol Laboratories, Deerfield, IL), 1% penicillin/streptomycin, and resuspended at a final cell concentration of $5 \times 10^7$ cells/mL.

BMMC were also reclaimed from the soy bean lectin agglutinate fraction of the T-cell depletion procedure by disaggregating the cells with 0.2 mol/L galactose in PBS, washing the separated cells two times with Media 199, 1% penicillin/streptomycin, and resuspending them to a final cell concentration of $5 \times 10^7$ cells/mL. The cultures of stromal layers derived from the lectin agglutinate fraction would not give rise to hematopoietic cells as the stem cell population had been removed; however, the stromal cell layer obtained from this population of cells was equally effective as a feeder layer as were the cells salvaged from the red cell sediment. Both types of stromal cell populations were used in our experiments, although they were never mixed together before initiating the stromal cultures. Long-term bone marrow (LTBM) cultures were established by plating 3 to $5 \times 10^7$ BMMCs in 25 cm$^2$ tissue culture flasks (Corning, Corning, NY) with 10 mL of LTBM media, consisting of Fischer's complete media for leukemic cells, 25% horse serum, and 1% penicillin/streptomycin. Initially, 1 $\mu$mol/L hydrocortisone was added to the media and cells were incubated in a 37°C, 100% humidified incubator with 5% CO$_2$ in air. After 5 or 6 days, all nonadherent cells (which would contain hematopoietic stem cells) were removed; the adherent cells were washed with media, and fresh LTBM media was added. Cultures were then fed weekly by removal of all media and replacement with fresh LTBM media. Hydrocortisone was removed from the culture media after 4 weeks of culture, and always before a culture's use as a feeder layer. From these cultures, stromal layers developed that consisted of fibroblasts, adipocytes, and macrophages, as is evident in Fig 1A. The removal of nonadherent cells from the cultures was important to select against long-term development of hematopoietic cells.

In vitro establishment of the megakaryocytic leukemic cell line CHRF-288-11. While CHRF-288 cells grow well within the nude mouse as solid tumors, such growth precludes the use of the cell line for many valuable experiments. In order to adapt these cells to in vitro growth, the marrow microenvironment was mimicked as closely as possible by using an in vitro system of stromal cells obtained from long-term human bone marrow cultures for use as a feeder layer. All other attempted procedures that did not use LTBM feeder layers were unsuccessful. Two such LTBM cultures were inoculated with a

Fig 1. In vitro establishment of CHRF-288 cells. (A) Control LTBM culture before addition of CHRF-288 cells. An adherent layer of stromal cells is evident without significant hematopoietic cell development. (B) Isolated group of stromal cells in an LTBM culture 3 hours after the addition of CHRF-288 cells (the round, refractile cells). CHRF-288 cells attached to the adherent stromal cells in a pattern that coincides with the cytoplasmic processes of the stromal cells (arrows). Several nonadherent CHRF-288 cells are present in the upper area of this field. (C) LTBM culture 3 days after the addition of CHRF-288 cells. Numerous, viable CHRF-288 cells are present and attached to the adherent stromal cells. (D) LTBM culture 12 days after the third transfer of CHRF-288 cells into new LTBM cultures. This is approximately 4 months after initial establishment of CHRF-288 cells in culture. All photographs were taken using Hoffman modulation optics (original magnification $\times 245$).
megakaryocyte tumor cell suspension. Tumor cells rapidly attached to the LTBM feeder layer and proliferated, forming large colonies of tumor cells attached to the adherent stromal cells (Figs 1B, C, and D). Cell growth was vigorous, and a nearly confluent monolayer of tumor cells attached to the adherent stromal cells (Figs 1B, C, and D). Cell growth was vigorous, and a nearly confluent monolayer of tumor cells developed over the stromal layer. The doubling time of the cells was 67 hours when grown on the stromal layer (data not shown). Cultures were fed weekly by complete replacement of LTBM media with fresh media. Once confluent, tumor cells were shed into the medium, and could be replated on new LTBM cultures without any cessation of cell proliferation. CHRF-288 cells were weaned from the stromal layer over a period of 4 months. Cells were spontaneously released from the stromal layer and collected and plated into a new dish in the absence of stromal cells. Some stromal cells were carried over by this procedure, however, and attached to the flask and proliferated. However, many of the CHRF-288 cells remained unattached to the stromal cells, and the process of replating was repeated. This continued until no further stromal cells were being carried over, and the CHRF-288 cells had obtained the capacity to grow in the absence of such cells, providing that the initial seeding density was high (10^6 cells per flask). At low densities, cell proliferation did not occur. Thus, in order to clone the cells, it was necessary to perform the procedure (limiting dilution) on LTBM cultures. Fourteen independent clones were isolated, removed from the stromal layer, and grown in culture. One clone, clone 11 (designated CHRF-288-11), was chosen for the experiments described below. The CHRF-288-11 cells are passaged in the absence of feeder layers by biweekly feeding in Fishers medium supplemented with either horse or bovine calf serum. 

Immunocytochemistry. In situ photomicrographs of unstained viable cultures were taken on a Zeiss inverted stage microscope using Hoffman modulation optics. Cyto centrifugate preparations of the cultured tumor cells were stained with Wright's stain on an Ames Hema-Tek slide stainer (Miles) followed by a manual 5 minute Giemsa stain for the cells was 67 hours when grown on the stromal layer (data not shown). Cultures were fed weekly by complete replacement of LTBM media with fresh media. Once confluent, tumor cells were shed into the medium, and could be replated on new LTBM cultures without any cessation of cell proliferation. CHRF-288 cells were weaned from the stromal layer over a period of 4 months. Cells were spontaneously released from the stromal layer and collected and plated into a new dish in the absence of stromal cells. Some stromal cells were carried over by this procedure, however, and attached to the flask and proliferated. However, many of the CHRF-288 cells remained unattached to the stromal cells, and the process of replating was repeated. This continued until no further stromal cells were being carried over, and the CHRF-288 cells had obtained the capacity to grow in the absence of such cells, providing that the initial seeding density was high (10^6 cells per flask). At low densities, cell proliferation did not occur. Thus, in order to clone the cells, it was necessary to perform the procedure (limiting dilution) on LTBM cultures. Fourteen independent clones were isolated, removed from the stromal layer, and grown in culture. One clone, clone 11 (designated CHRF-288-11), was chosen for the experiments described below. The CHRF-288-11 cells are passaged in the absence of feeder layers by biweekly feeding in Fishers medium supplemented with either horse or bovine calf serum. 

Microscopy. Aliquots of this extract were added to high density, exponentially growing CHRF-288 cells. After hybridization, filters were washed four times at room temperature with 0.25 mol Sucrose, 1 mmol/L EDTA, 0.1 mmol/L phenylmethylsulfonyl fluoride [PMSF], pH 7.0) or in an acidic buffer (4.8 x 10^6 cells in acidic ethanol).15 Neutral homogenates were clarified by centrifugation, and aliquots were added directly to serum-depleted 3T3-NR6 cells as previously described.15 Measurement of analysis of the [3H]-thymidine incorporation into DNA was performed as described.15 Acidic ethanol extracts were concentrated by ether precipitation16 and dissolution of the pellet in 200 μL 4 mol/L HCl. Aliquots of this extract were added to high density, exponentially growing A549 cells as described,15 and the extent of inhibition of DNA synthesis was determined.

RNA analysis. The methods for all procedures are described below. Total RNA was isolated from 1 x 10^6 (or 1 g of CHRF-288 solid tumor tissue)15 as described previously.16 For Northern analysis, the total RNA was passed through an oligo-dT column to purify polyA+ RNA. Northern analysis of transforming growth factor-β1 (TGF-β1) mRNA was performed according to a modification of the previously described procedure.17 After electrophoresis, RNA was transferred to nitrocellulose and prehybridized for 4 hours at 42°C in 20 x SSC (1 x SSC contains 0.15 mol/L NaCl, 0.015 mol/L sodium citrate), 50 mol/L sodium phosphate, 2 x Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), and 50% formamide. The TGF-β1 probe (pBas; Genentech, San Francisco, CA)16 was labeled using the random primer technique.18 Hybridization with [32P]-labeled cDNA probe, at 1 x 10^6 cpm/mL, was carried out for 16 hours at 42°C in 4 x SSC, 40 mol/L sodium phosphate, 2 x Denhardt's solution, 0.1% SDS, and 40% formamide.

Karyotype analysis. Karyotype analysis of the growing cells was performed using standard procedures, which have been described previously.15

Western analysis. Western analysis for the presence of glycoprophorin A was carried out as described19 using monoclonal antibody 10F7 at a dilution of 1:200. Growth factor analysis. For the determination of growth factor activity in CHRF-288-11 cell extracts, the cells were extracted in either a neutral buffer (1 x 10^6 cells in 1.0 mL of 20 mol/L Tris, 0.25 mol Sucrose, 1 mol/L EDTA, 0.1 mol/L phenylmethylsulfonyl fluoride [PMSF], pH 7.0) or in an acidic buffer (4.8 x 10^6 cells in acidic ethanol).21 Neutral homogenates were clarified by centrifugation, and aliquots were added directly to serum-depleted 3T3-NR6 cells as previously described.15 Measurement of analysis of the [3H]-thymidine incorporation into DNA was performed as described.15 Acidic ethanol extracts were concentrated by ether precipitation16 and dissolution of the pellet in 200 μL 4 mol/L HCl. Aliquots of this extract were added to high density, exponentially growing A549 cells as described,15 and the extent of inhibition of DNA synthesis was determined.

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MEGAKARYOCYTIC CELL LINE

der, CO.10 The insert was excised as an Xba I-HindIII fragment and inserted into a similarly cut pBluescript SK+ transcription vector (Stratagene Cloning Systems, Inc. La Jolla, CA). RNA transcripts were generated from vectors linearized at each end of the insert and purified from agarose gels. Antisense probes were made per manufacturers’ recommendations, except that α-[32P]-cytidine triphosphate (CTP) was used exclusively at a final CTP concentration of 20 μmol/L (50 μCi/0.4 nmol). Unlabeled sense strand RNA, used as internal quantitation standards, were made in large quantity, purified by agarose gels, and quantitated spectrophotometrically. Hybridization and quantitation were performed as previously described,11 except that they were performed at 53° for 15 hours in 15 μL of buffer containing 400,000 cpm of antisense probe, 30 μg of either cellular RNA or carrier tRNA, and for standard quantitation, 0 to 200 pg of sense-strand RNA. Unhybridized RNA was digested by addition of 185 μL of an ice-cold solution that contained 0.45 mmol/L NaCl, 0.1 mmol/L LiCl, 10 mmol/L Tris HCl, pH 7.4, 1 mmol/L EDTA, and 4 μg/mL RNase A. The solution was then incubated at 16°C for 30 minutes, processed without denaturation, and electrophoresed on a 3.8% polyacrylamide gel that contained 1× TBE (0.089 mol/L Tris-borate, 0.025 mol/L EDTA).12 Autoradiography of the gel was for 24 hours at −70°C.

RESULTS

Characteristics of the cloned CHRF-288-11 tumor cells. Once the CHRF-288-11 cells were established in culture, it was necessary to demonstrate that they still retained the megakaryocytic features observed in the solid tumor. This was accomplished in a variety of ways. Karyotype analysis of the cloned cells (50, XY, +6q−, +8, +17, +21, 12p+, −10, +19p+, −15, 6p−, exhibited by 10 of 13 cells examined) demonstrated that the cells still exhibited similar chromosomal markers as described in the original tumor line.10 The karyotype demonstrates trisomy for chromosomes 6, 8, 17, 19, and 21. The extra chromosome 6 has a large deletion, and trisomy for chromosome 19 is variable, although greater than 90% of the megakaryocytic tumor cells carry this alteration. The only differences between the cloned cells and the original tumor cells as grown in nude mice was a small 6p deletion (p23). and the loss of chromosome 10. Thus, long-term culturing of the cells has retained the significant features of the karyotype and suggests that the cells are indeed similar to the cells passedage in nude mice.

It was then determined, both microscopically and immunologically, that the megakaryocyte-specific genes that were expressed in the solid tumor were still expressed in the cultured cells; ie, the cells were phenotypically unchanged. Light microscopic analysis of Wright-Giemsa stained cells (Fig 2) demonstrated that the cells ranged in size from 15 to 20 μm in diameter, contained a highly basophilic, slightly granular cytoplasm with a prominent eosinophilic perinuclear Golgi zone. Most of the cells had a single oval or rounded nucleus with one to three prominent nucleoli. Cells with bilobed nuclei were occasionally present (2% to 3% of the total), and a few cells had complex multilobed nuclei (less than 0.5% of the population). Several cells with cytoplasmic protrusions were also observed, although the majority of cells were rounded. Immunohistochemistry demonstrated the presence of platelet glycoprotein IIb-IIIa and factor VIII antigen, characteristics of both platelets and megakaryocytes (Fig 3A and B). Greater than 90% of the cells express GPIIb-IIIa, while 30% to 40% of the population expressed factor VIII antigen. The factor VIII positive cells were generally the larger, more mature cells but also included many immature cells that showed little cytologic evidence of maturation. Immunohistochemistry on acetone permeabilized cells identified a diffuse staining for PF4 antigen in the cytoplasm (Fig 3C), as well as platelet Ca2+-ATPase immunoreactivity (Fig 3D). In contrast to the factor VIII stain, PF4 was generally expressed in most cells, although there was more cell to cell variability in intensity, and the PF4 signal was focally concentrated in many of the cells. Myeloperoxidase immunoreactivity was not detected (not shown).

Electron microscopy (Figs 4A, B, C, and D) showed most of the cells had a single nucleus with a single prominent nucleolus, and the chromatin was moderately condensed. Most of the cells contained few cytoplasmic organelles other than large numbers of polyribosomes. The more mature cells (Fig 4A) generally had extensive rough endoplasmic reticulum, frequent mitochondria, and a well-developed Golgi complex associated with numerous small vesicles, some of which were coated. There were also numerous granules that varied in size and electron density. Small round granules (100 to 300 nm) were evident, and contained an electron dense matrix that either completely filled the granule or was surrounded by a clear halo in the more differentiated cells. These granules could generally be seen budding near the Golgi complex but occasionally could be seen to arise from areas of the endoplasmic reticulum in the periphery of the cells (Fig 4C). Ultrastructural features of the smaller dense...
Fig 3. Immunohistochemistry of CHRF-288-11 cultured cells. (A) Positive fluorescence of a group of cultured tumor cells after incubation with monoclonal antibody T-10, which identifies platelet GP Ib-llla antigen. (B) Photomicrograph showing immunofluorescence of a group of cells after incubation with anti-factor VIII-related antigen primary antibody. The positive reaction is primarily cytoplasmic in distribution. (C) PF4 immunoreactivity. (D) The platelet-specific Ca$^{2+}$-ATPase. Before incubation with antibody to factor VIII, the Ca$^{2+}$-ATPase, or PF4, the cells were briefly permeabilized in acetone. Controls using nonspecific antibodies as the primary label were negative for fluorescence (data not shown) (original magnification, all panels at ×300).

Fig 4. Electron photomicrographs of CHRF-288-11 culture cells. (A) An eccentric nucleus (N) and cytoplasm containing numerous mitochondria, polyribosomes, prominent Golgi apparatus (G), and numerous empty vesicles and small granules with a dense central matrix (arrowhead). (B) A higher magnification of a cell containing larger granules (300 to 600 nm). These granules (arrowheads) have a diffuse granular matrix with a dense nucleoid resembling α-granules. (C) A cell with numerous small round granules (100 to 300 nm) that contain an electron dense matrix surrounded by a clear halo. One of these dense granules can be seen arising from a Golgi complex (arrowhead). (D) The platelet peroxidase reaction. There is an intense perinuclear reaction (N, nucleus) as well as in the endoplasmic reticulum. The Golgi cisternae granules contained no reactivity (original magnification: A, ×12,000; B, ×17,000; C, ×13,100; D, ×13,100).
granules resemble the various stages of platelet dense granule formation as described by White.\textsuperscript{33} Larger granules (300 to 600 nm), resembling \(\alpha\)-granules, had a diffuse, finely granular matrix and, occasionally, a moderately dense nucleoid (Fig 4B). The platelet peroxidase reaction (Fig 4D) performed as described in the text and in reference 47. Erythrocyte ghosts were prepared as previously described.\textsuperscript{34} Lanes 1 and 2 are solubilized megakaryocyte cells (50 and 25 \(\mu\)g, respectively); lanes 3 and 4, solubilized erythrocyte ghosts (50 and 25 \(\mu\)g, respectively). No immunoreactivity is seen with the megakaryocytes.

Cloned CHRF-288-11 cells were also examined by flow cytometric analysis for the expression of cell surface markers characteristic of T cells, B cells, natural killer cells, lymphoblastic leukemia, monocytes, and megakaryocytes. As can be seen in Table 1, the CHRF-288-11 cells expressed the HLA-DR backbone antigen, GPIlb-IIIa, and epitopes recognized by MY7 (CD13) and MY9 (CD33) monoclonal antibodies. No T and B cell markers were evident on the cell, nor was there evidence for the expression of glycophorin A by Western blot analysis (Fig 5), immunocytochemistry (data not shown), or hemoglobin as determined by immunocytochemistry (not shown). Benzidine staining also failed to reveal the presence of heme (data not shown). These data would indicate that the line is expressing megakaryocytic markers, and that it is not biphenotypic for the erythroid lineage.

Clone 11 was also examined for the production of bFGF and TGF-\(\beta\), two factors shown to be synthesized in large quantity by the solid tumor from which these cells were derived.\textsuperscript{13} Northern blot analysis of the RNA from the cultured cells (Fig 6) using a probe specific for TGF-\(\beta\) showed a strong hybridization signal at 2.2 kb, identical to the signal in the megakaryocyte solid tumor RNA. This indicates that CHRF-288-11 cells produce TGF-\(\beta\) message, and the data in Table 2 indicate that a TGF-\(\beta\) activity is also present in cell extracts. Solution hybridization analysis of total RNA prepared from the cultured cells for bFGF transcripts indicates the presence of such transcripts (Fig 6). Full length bFGF mRNA protected a fragment 474 bases long, as well as a fragment 446 bases long in equal abundance. The 474 base band corresponds to the size of the control sense strand signal, which did not show any other bands (data not shown). This indicates that the 446 base band resulted from cellular processing of the mRNA. The quantities of each species were determined based upon densitometric comparison with the results of hybridization to synthesized sense strand RNA from the same riboprobe vector.\textsuperscript{31} The cultured cells contained 5 pg of the 474 base species per 30 \(\mu\)g total RNA, and 6.8 pg (per 30 \(\mu\)g of total RNA) of the 446 base species. Similarly, the tumor tissue contained 3.4 pg of the 474 base species and 4.6 pg of the 446 base species, both per 30 \(\mu\)g of total RNA. Total RNA from a mesoblastic nephroma, which produces aFGF but not bFGF,\textsuperscript{34} and total RNA from an osteogenic sarcoma, which does not produce bFGF, gave no signal in these assays indicating less than 0.03 pg/30 \(\mu\)g total RNA based on the signal intensity of the control sense strand (Fig 6). These data, coupled with the biologic activity demonstrated in Table 2, indicate that CHRF-288-11 cells are producing active forms of bFGF, as we have previously shown in the solid tumor.\textsuperscript{15}

**Table 1. Immunocytochemical Characteristics**

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<tr>
<th>Antibody Cluster Designation</th>
<th>Specificity</th>
<th>% Reactivity</th>
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<tbody>
<tr>
<td>Leu-1 CD-5</td>
<td>T cell</td>
<td>0.0(−−)</td>
</tr>
<tr>
<td>OKT3 CD-3</td>
<td>T cell</td>
<td>0.7(−−)</td>
</tr>
<tr>
<td>OKT11 CD-11</td>
<td>T cell</td>
<td>0.8(−−)</td>
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<tr>
<td>Leu-2A CD-8</td>
<td>T-cytotoxic/suppressor</td>
<td>0.4(−−)</td>
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<td>OKT8 CD-8</td>
<td>T-cytotoxic/suppressor</td>
<td>0.0(−−)</td>
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<tr>
<td>Leu-3A CD-4</td>
<td>T-helper inducer</td>
<td>2.0(−−)</td>
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<tr>
<td>OKT4 CD-4</td>
<td>T-helper inducer</td>
<td>1.0(−−)</td>
</tr>
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<td>Thymocyte</td>
<td>0.5(−−)</td>
</tr>
<tr>
<td>Leu-7</td>
<td>NK cell</td>
<td>0.0(−−)</td>
</tr>
<tr>
<td>Leu-9 CD-7</td>
<td>NK cell</td>
<td>0.3(−−)</td>
</tr>
<tr>
<td>Leu-12 CD-19</td>
<td>B cell</td>
<td>0.5(−−)</td>
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<td>IL-2 receptor</td>
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<td>CALLA CD-10</td>
<td>Common acute lymphoblastic leukemia antigen</td>
<td>0.4(−−)</td>
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<tr>
<td>OKM5 CD-36</td>
<td>Monocytes, platelets</td>
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<td>Platelet GP Iib-Illa</td>
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<tr>
<td>Tab CD-41</td>
<td>Platelet GP Iib-Illa</td>
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<td>MY7 CD-13</td>
<td>Early myeloid cells, monocytes</td>
<td>90.9(+)</td>
</tr>
<tr>
<td>MY9 CD-38</td>
<td>Early myeloid cells, monocytes</td>
<td>95.7(+)</td>
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Flow cytometric analysis of cultured CHRF-288-11 cells. Analyses were performed on an Ortho Spectrum III analytical flow cytometer, and labeling was performed as described in "Methods." CHRF-288 cells were positive for HLA-DR antigen, GPIib-IIa, and epitopes for monoclonal antibodies MY7 and MY9 were also present on the cells. This profile indicates cells committed to the megakaryocytic lineage.
appeared to be a secondary S phase (S2) containing 1.8% of the cells, which likely corresponds to the 2% binucleated cells observed morphologically. After 4 days of PMA treatment, the 2N peak decreased by 40%, whereas the S1 phase and 4N cells increased to 13.7% and 21%, respectively (Fig 7F). This occurred even though the cellular proliferation rate decreased by 60% (data not shown). In addition, 8.1% of the treated cells contained an 8N or greater DNA content (8N + S2).

DISCUSSION

A human megakaryocytic leukemia cell line, designated CHRF-288-11, has been successfully established in tissue culture starting with cells in a solid tumor. Establishment of the cell line required the presence of adherent bone marrow stromal cells, which are composed of macrophages, adipocytes, fibroblasts and endothelial cells.16,17,35 Such cells have been shown to produce hematopoietic growth factors,6,19 as well as producing a microenvironment sufficient for supporting hematopoiesis. Such an adaptation scheme may be beneficial for other cell types that are normally found in the marrow. This is a novel procedure, and may prove to be widely applicable to a variety of cell types. The fact that the CHRF-288-11 cells could be weaned from the stromal layer suggests that the importance of stromal cell derived growth factors in the proliferation of CHRF-288 cells has diminished with continuous culturing, and that the cells themselves are producing an autocrine growth factor.

The cloned cells appear to be of the megakaryocytic lineage as determined by a wide variety of criteria (Figs 3 through 7 and Table I). The only markers expressed by the line that one might not expect to find on megakaryocytes are the HLA-DR antigen and the MY7 (CD13) and MY9 (CD38) markers. HLA-DR antigens are found on a variety of cell types, including B cells, but they have also been identified on early stage megakaryoblasts as well.5,21 The MY7 marker (CD-13) is primarily expressed on peripheral blood monocytes and granulocytes, 5% to 40% of normal bone mononuclear cells, and 80% of all acute myeloid leukemia cases. It is not usually found on erythrocytes.

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<tr>
<th>Table 2. Growth Factor Activity in CHRF-288-11 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth Factor Activity</td>
</tr>
<tr>
<td>Cell Extract</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Neutral extraction (μL)</td>
</tr>
<tr>
<td>0.7</td>
</tr>
<tr>
<td>3.4</td>
</tr>
<tr>
<td>17.2</td>
</tr>
<tr>
<td>Acidic-extraction (μL)</td>
</tr>
<tr>
<td>0.75</td>
</tr>
<tr>
<td>1.25</td>
</tr>
<tr>
<td>2.50</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>TGF-β1 (1 ng/mL)</td>
</tr>
</tbody>
</table>

CHRF-288-11 cells were extracted as described in Methods. From these data, one can calculate that these cells contain 59 units of mitogenic activity per 10^6 cells, and approximately 8.5 ng of TGF-β equivalents per 10^6 cells.
Fig 7. Morphologic and ploidy changes after PMA treatment of CHRF-288-11 cells. (A through D) Wright's stained cytocentrifuge preparations of CHRF-288-11 cells before and after treatment with $10^{-8}$ mol/L PMA. A, untreated controls; B, 2 days after PMA treatment; C, 4 days after PMA treatment; and D, 6 days after PMA treatment. All fields are at the same magnification, ×1,000. (E and F) Flow cytometric DNA analysis of untreated, control CHRF-288-11 cells (E) and cells treated with $10^{-8}$ mol/L PMA for 4 days (F). S1, S2, and S3 refer to cells in intermediate states of DNA synthesis between 2N and 4N, 4N and 8N, and above 8N, respectively. The mean fluorescent intensities of the 2N, 4N and 8N peaks are 48.3, 96.5, and 190.0, respectively. For each analysis, 50,000 cells were counted.
platelets, or lymphocytes.\textsuperscript{40,41} The presence of CD-13 on the CHRF-288-11 cells is not necessarily surprising, since a recent report\textsuperscript{42} has indicated that 5% to 10% of megakaryoblastic leukemia cases shown to be positive for GPIIb-IIIa were also positive for CD-13, indicating that cells of megakaryocytic origin often express both markers. In addition, two other recently described megakaryocytic cell lines, the DAMI line\textsuperscript{10} and T-33\textsuperscript{8} are positive for CD-13 as well. The presence of this antigen may indicate the immature aspect of the line,\textsuperscript{43} and it is possible that if the cells are induced to differentiate to a more mature form, the expression of this marker would decrease. The MY9 (CD-38) marker is similar in cell expression to MY7,\textsuperscript{44} but it too has been detected on other cells derived from megakaryoblastic leukemias,\textsuperscript{45} and the T-33 cell line,\textsuperscript{8} and may also be represented on immature forms of this cell type as well.

Unlike the K562\textsuperscript{26} and DAMI\textsuperscript{10} cell lines, the CHRF-288-11 cell line does not express erythroid characteristics, indicating that the cells are not biphenotypic. The cells are remarkably homogenous, in both karyotype and marker expression. Greater than 95% of the cells express GPIIb-IIIa and PF4. This is in contrast to the MEG-01S cell line,\textsuperscript{11} in which only 40% of the cells are positive for GPIIb-IIIa, and the DAMI cell line,\textsuperscript{10} which does not exhibit a constant karyotype. CHRF-288-11 cells also demonstrated a potential for hyperploidy in response to phorbol esters (Fig 7), similar to results reported for the EST-IU cell line,\textsuperscript{13} the MEG-01S cell line,\textsuperscript{12} the DAMI cell line,\textsuperscript{10} and the T-33 cell line.\textsuperscript{8} In a normal cell cycle pattern, the S phase and 4N cells would be expected to decrease if the rate of proliferation also decreased. However, under conditions of PMA treatment of CHRF-288-11 cells, in which the proliferation rate decreased by 60%, the number of cells in the S and 4N phases actually increased, and many cells exhibited ploidy values of greater than 4N as well. These data indicate that there has been a transgression from the classical cell cycle pattern, and that many of the cells fall into a pattern of nuclear replication without cytoplasmic division, which is consistent with megakaryocytic development. Thus, while further studies are required, the CHRF-288-11 cell line may prove to be a valuable model for the study of megakaryocytes and megakaryocyte-associated functions.

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DA Fugman, DP Witte, CL Jones, BJ Aronow and MA Lieberman

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