# Manipulations of Cholinesterase Gene Expression Modulate Murine Megakaryocytopoiesis In Vitro

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Megakaryocytopoiesis was selectively inhibited in cultured murine bone marrow cells by a 15-mer oligodeoxynucleotide complementary to the initiator AUG region in butyrylcholinesterase mRNA. Furthermore, conditioned medium from *Xenopus* oocytes producing recombinant butyrylcholinesterase stimulated megakaryocytopoiesis. These observations implicate butyrylcholinesterase in megakaryocytopoiesis and suggest application of oligodeoxynucleotides for modulating bone marrow development.

The *CHE* and *ACHE* genes, encoding the acetylcholine-hydrolyzing enzymes butyrylcholinesterase (BuChE; EC 3.1.1.8) and acetylcholinesterase (AChE; EC 3.1.1.7), are expressed in various developing cell types, including embryonic (12), hematopoietic (2), and germ (10, 13) cells; their functions in these cells remain obscure. We have cloned BuChE cDNA (17) and mapped the human *CHEI* locus (21) to the 3q21-ter chromosomal domain, which is subject to aberrations in leukemias, accompanied by abnormal megakaryocytopoiesis and platelet counts (16). Coamplification of the *ACHE* and *CHE* genes was subsequently observed in leukemias and platelet disorders (11). In this report, we demonstrate selective inhibition of megakaryocytopoiesis in cultured murine bone marrow cells challenged with a 15-mer antisense oligonucleotide directed against a region spanning the initiator AUG in human BuChE mRNA. Furthermore, we demonstrate stimulation of megakaryocytopoiesis in culture by conditioned medium from *Xenopus* oocytes microinjected with recombinant BuChE mRNA (OCM). These observations implicate BuChE in the yet unclarified process of megakaryocyte growth, proliferation, and differentiation and suggest the application of synthetic oligonucleotides to selectively modulate bone marrow cell development.

Bone marrow cultures were used to assess the etiological involvement of BuChE in megakaryocytopoiesis. Cells from the marrow of the femurs and tibias of 8- to 12-week-old endotoxin-resistant C3H/HeJ mice were cultured in LPM synthetic medium (Biological Industries, Bet-Haemek, Israel) containing 10% conditioned medium from WEHI-3 cells, 1% bovine serum albumin, $10^{-4}$ M thioglycerol, and 1% methylcellulose. Cells at a density of $0.5 \times 10^5$ to $1.0 \times 10^5$ cells/ml were plated in 35-mm petri dishes (Falcon 1008) or 24-well tissue culture Costar plates and incubated for 4 days at 37°C under 5% $CO_2$ with high humidity. The cultured murine bone marrow cells were then incubated with an antisense oligodeoxynucleotide (AS-CHEφ; Fig. 1a) targeted to human BuChE mRNA (17), which is almost identical to mouse BuChE mRNA in nucleotide sequence (H. Soreq et al., unpublished observations). AS-CHEφ was

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found to block translation of recombinant BuChE mRNA in microinjected *Xenopus* oocytes (20) up to 80% and to hybridize in situ with mouse cell suspensions (not shown). To permit optimal uptake of the oligonucleotide into the cells, the cells were incubated in methylcellulose supplemented with the LPM synthetic serum substitute and conditioned medium from WEHI-3 cells (WEHI), a rich source of the multilineage hematopoietic growth factor interleukin-3 (IL-3) (22). After 4 days in culture, AS-CHEφ was found to reduce the total number of colonies (Fig. 1c) and the relative proportion of megakaryocytes in the culture population (Fig. 1b). In addition, megakaryocyte colonies grown in the presence of AS-CHEφ appeared considerably smaller than controls (not shown). Pulse-labeling with 50 μCi of [$^{35}$S]methionine (1,000 Ci/mmol) per culture demonstrated that gross rates of nascent protein synthesis at day 4 remained unaffected by either of these oligonucleotides showing a level of $7 \times 10^4 \pm 3 \times 10^4$ cpm per culture per 24 h. This finding implied that the AS-CHEφ effect reflected a specific suppression of a gene product required for development and not a general toxicity. A stable phosphorothioate (5) analog of AS-CHEφ elicited a similar depression of colony formation in the cultures (Fig. 1c), whereas both phosphate and phosphorothioate complementary sense oligodeoxynucleotides (S-CHEφ; Fig. 1a) had no effect on either colony counts or cellular composition (Fig. 1b).

To further investigate the role of BuChE as a regulatory element in the megakaryocytopoietic pathway, incubation medium from *Xenopus* oocytes microinjected with recombinant BuChEmRNA (20) (BuChE-OCM) was added to bone marrow cultures. BuChE-OCM significantly augmented colony formation in a variety of semisolid and liquid culture conditions (Table 1). A 2-fold dilution of the BuChE-OCM reduced the effect to 33% of the initial level, whereas a 10-fold dilution completely abolished it (not shown). Conditioned medium from control oocytes injected with Barth saline solution (Brt-OCM) also elicited an enhancement of colony formation, in concurrence with reports on the presence of platelet-derived growth factor (PDGF) (15) and other growth-stimulating factors (24) in OCM. However, the Brt-OCM effect was significantly smaller than that observed
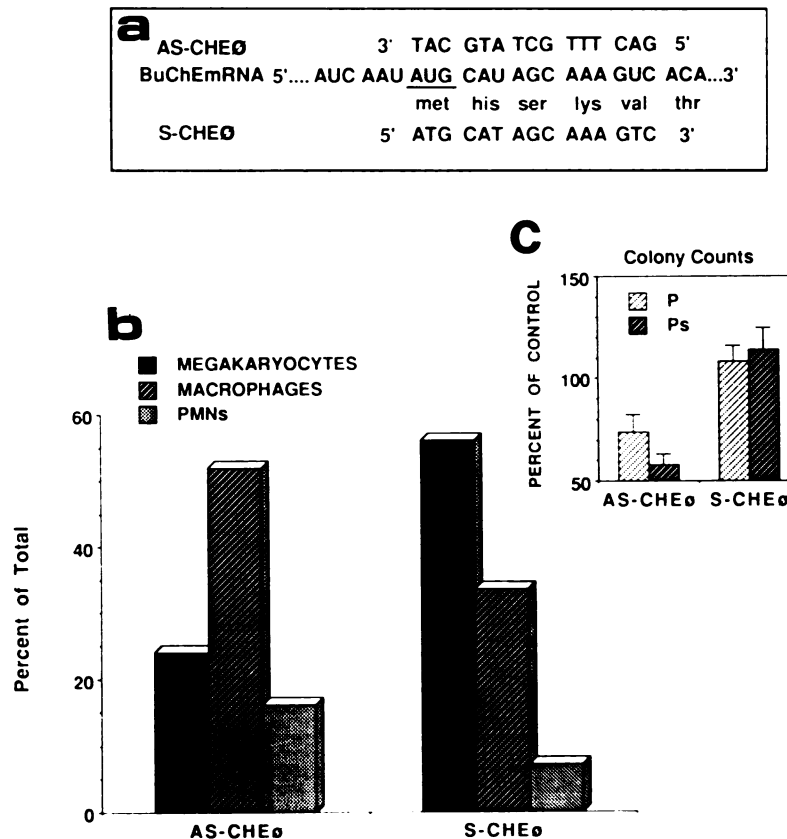
FIG. 1. Antisense *CHE* oligonucleotide inhibition of megakaryocytopoiesis. (a) Oligonucleotides. Phosphorothioate- and phosphate-containing sense (S-CHEφ) and antisense (AS-CHEφ) 15-mer oligodeoxynucleotides were synthesized by using an Applied Biosystem 380B DNA synthesizer. The phosphate and phosphorothioate oligonucleotides were synthesized by the phosphoramidite and H-phosphonate methods, respectively. Both were purified by reverse-phase high-performance liquid chromatography, the phosphorothioate oligonucleotide before and after removal of the dimethoxytrityl group. The region spanning the initiator AUG in BuChE mRNA (17) and the oligonucleotide concentrations used were selected in view of the experience of others (28), and AS-CHEφ was confirmed to be potentially effective at the examined final concentration of 5 μM (expressed as oligomer concentration) on the basis of its ability to block translation of synthetic BuChE mRNA when coinjected at this concentration into *Xenopus* oocytes. Also, when tested in culture, 2.5 μM AS-CHEφ was not effective at all, whereas 10 and 15 μM concentrations of both AS-CHEφ and S-CHEφ were increasingly toxic to the cultured cells. (b) Differential cell analysis of antisense oligonucleotide-treated, semisolid bone marrow cultures. Colonies grown in serum-free methylcellulose cultures containing a sense or antisense phosphorothioate *CHE* oligonucleotide were picked with drawn-out Pasteur pipettes, concentrated (5 min at 500 × g) by Cytospin (Shandon, 2) centrifugation in phosphate-buffered saline, stained with May-Grünwald Giemsa stain, and analyzed microscopically. Megakaryocyte colonies contained at least 4 cells; macrophage or granulocyte colonies consisted of 50 cells or more. The relative fraction of each cell type represented among the total cells recovered from two independent experiments is shown. Almost all colonies were pooled from each plate, and at least 500 cells were counted for each datum set. The distribution obtained with S-CHEφ was essentially identical to that observed in control (no oligonucleotide) cultures. (c) Colony counts. The total number of all types of colonies, including megakaryocytes, granulocytes, and macrophages, was scored by using a Zeiss Stereozoom binocular after 4 days of growth in the presence of phosphorothioate (Ps) or phosphate (P) oligonucleotides. Plots present percentage of control (no oligonucleotide) culture counts. Data represent averages of two (phosphorothionate) or three (phosphate) independent experiments ± standard error of the mean.

with BuChE-OCM ($P < 0.025$ to 0.001) (Table 1). Furthermore, antibodies elicited against purified human PDGF (7) completely prevented the Brt-OCM augmentation effect, whereas BuChE-OCM enhancement was only partially blocked (Table 1).

Analysis of differential cell counts attributed the increase in CFU in BuChE-OCM-treated cultures to enhanced megakaryocyte proliferation and maturation, whereas the increase in Brt-OCM-treated cultures appeared in the macrophage population (Fig. 2a). A substantial increase in the number of late as opposed to early megakaryocytes was also observed in BuChE-OCM-treated cultures as compared with both control and Brt-OCM-treated cultures. This shift was accompanied by a significant increase in the average diam-

eter of megakaryocytes (Fig. 3), indicating that BuChE-OCM played a specific role in megakaryocyte maturation. Colonies cultured in horse serum (HS)-containing media were larger, developed faster, and contained a higher proportion of megakaryocytes than did those grown on LPM. Therefore, each type of experiment was evaluated separately. In both HS-containing and HS-free media, cultures deprived of WEHI gave rise to colonies that were small, poorly developed, and disintegrated after day 3, indicating that OCM affected megakaryocyte colony formation by synergizing with the IL-3 in WEHI. Substitution of recombinant murine purified IL-3 for WEHI confirmed this conclusion (Table 1).

BuChE-OCM exerted a more pronounced enhancement

TABLE 1. Effect of OCM on megakaryocyte colony formation in semisolid cultures[a]

| Medium | Day | No. of expts | Avg no. of colonies/culture dish ± SEM | | |
|--------|-----|-------------|------------------|------------------|------------------|
| | | | No OCM | + BuChE-OCM | + Brt-OCM |
| HS/WEHI | 3 | 8 | 1.00 ± 0.03 (59–276)[b] | 1.81 ± 0.06 (<0.001)[c] | 1.24 ± 0.09 (<0.001) |
| | 4 | 11 | 1.16 ± 0.07 (72–252) | 2.03 ± 0.12 (<0.001) | 1.50 ± 0.08 (<0.001) |
| HS/WEHI + αPDGF | 4 | 1 | 1.14 ± 0.08 (417–646) | 1.26 ± 0.08 (ND)[d] | 1.01 ± 0.09 (ND) |
| HS/IL-3 | 3 | 1 | 1.00 ± 0.10 (61–277) | 2.02 ± 0.06 (<0.008) | 1.25 ± 0.05 (<0.085) |
| | 4 | 1 | 1.69 ± 0.05 (122–276) | 2.34 ± 0.17 (<0.01) | 1.90 ± 0.30 (<0.055) |
| LPM/WEHI | 4 | 4 | 1.00 ± 0.07 (221–386) | 1.43 ± 0.04 (<0.007) | 1.28 ± 0.03 (<0.02) |
| LPM/WEHI + αPDGF | 4 | 2 | ND | ND | 0.60 ± 0.04 (<0.001) (466)[b] |

[a] Average colony counts per culture dish (as a fraction of the value for the control culture on the first day counted) are shown for various culture media with and without the addition upon initiation of culture of 0.5% (vol/vol) BuChE-OCM or Brt-OCM. Oocyte microinjections were done as described previously (19, 20). HS/WEHI, 15% HS (GIBCO), 10% WEHI, $10^{-4}$ thioglycerol (Sigma), and 1% methylcellulose (DOW, A4M Premium) made up in Iscove modified Dulbecco medium (GIBCO); HS/WEHI + αPDGF, as above with a 1:40 dilution of rabbit anti-human PDGF antiserum; HS/IL-3, as above with purified recombinant murine IL-3 (20 U/ml) replacing WEHI; LPM/WEHI, LPM synthetic serum substitute containing 10% WEHI, 1% deionized bovine serum albumin (Sigma fraction V), $10^{-4}$ M thioglycerol, and 1% methylcellulose; LPM/WEHI + αPDGF, as above with 1:40 αPDGF.
[b] Numbers in parentheses are ranges of colony numbers counted in each experiment.
[c] Numbers in parentheses indicate statistical significance (P) of deviations, calculated by Student's t test versus no-OCM controls.
[d] ND, Not determined.

effect in methylcellulose cultures supplemented with 15% HS, a rich source of tetrameric BuChE (25), than that observed under serum-free conditions (Table 1). Given the negligible BuChE enzymatic activity contributed by OCM in these cultures (≤0.01% of serum contribution) (20), it appears unlikely that the stimulating factor in BuChE-OCM is catalytically active BuChE. Moreover, 5 min of heating to 45°C resulted in a 40% reduction in the BuChE-OCM effect,
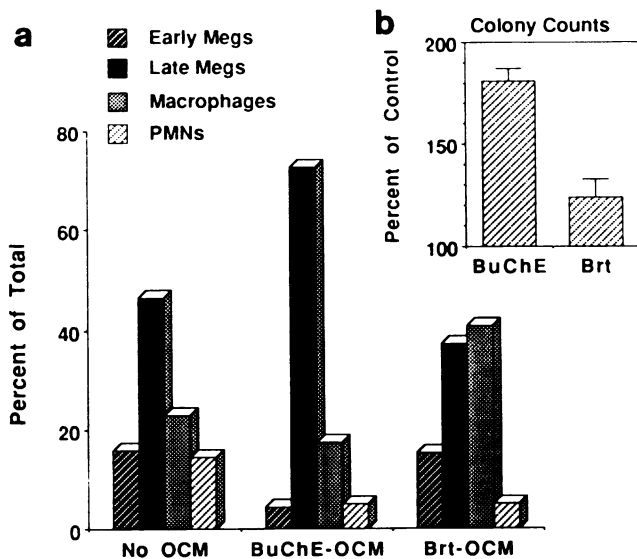


FIG. 2. BuChE-OCM augmentation of megakaryocytopoiesis. (a) A representative differential cell analysis (one of three experiments) of colonies picked from serum-fortified methylcellulose cultures. Cultures were grown with or without addition of 0.5% BuChE-OCM or Brt-OCM. Early megakaryocytes, defined as immature forms with one or two nuclei, were distinguished from late ones, characterized by their larger size and tendency to shed cytoplasmic fragments. Note the relative increase in late-stage megakaryocytes upon addition of BuChE-OCM and the increase in macrophages in Brt-OCM-containing cultures. (b) Colony counts. The total number of colonies observed after 3 days of incubation is plotted as a percentage of control (no OCM) cultures ± standard error of the mean (results for eight independent experiments, triplicate cultures per experiment).

whereas BuChE activity remained 95% intact. Therefore, these results suggest the presence of an as yet unknown heat-sensitive amphibian factor secreted by the oocytes in response to the intracellular accumulation of heterologous heat-stable, human BuChE, an enzyme not normally synthesized in Xenopus oocytes (19).

Although cholinergic mechanisms have been implicated in megakaryocytopoiesis (2, 11) and AChE is recognized as a specific marker of murine megakaryocytes (3), these data provide the first evidence for BuChE involvement in megakaryocyte development. To directly examine whether BuChE is expressed in these cells, we performed cytochemical cholinesterase staining (18) of megakaryocytes grown in LPM liquid cultures. Activity staining was pronounced and appeared sensitive to inhibition by both the AChE-specific inhibitor BW284C51 and the BuChE-specific inhibitor tetraisopropylpyrophosphamide (Fig. 3c), indicating the presence of both enzymes and directly revealing the previously undetected presence of BuChE in these cells.

Antisense oligodeoxynucleotides to viral (14) or cellular (28) mRNAs have been shown capable of arresting translation of their corresponding mRNAs in cultured cells (23). When targeted against growth-related genes such as c-myc, they also block cell proliferation in hematopoietic cell lines (26). Recently, a synthetic COOH-terminal PF4 peptide was demonstrated to inhibit growth of a human megakaryocytic cell line (6). However, to the best of our knowledge, this study provides the first demonstration of selective modulation of a single hematopoietic lineage in a mixed population of primary bone marrow cells and may foreshadow the application of synthetic oligonucleotides to bone marrow cell-specific growth modulation in vivo.

Whereas little is known about the molecular mechanisms regulating megakaryocytopoiesis in vivo, a variety of multilineage and lineage-specific native and recombinant growth factors have been shown to stimulate megakaryocytopoiesis (1, 8, 9). Nonetheless, no lineage-specific factor capable of driving this process has yet been purified. Abnormal megakaryocytopoiesis, resulting in platelet overproduction (thrombocytosis) or depletion (thrombocytopenia), is implicated in a number of debilitating bleeding disorders (4). The results presented here may suggest the use of AS-CHEφ to arrest thrombocytosis or the abnormal proliferation of other cell types dependent on CHE gene expression. Likewise,
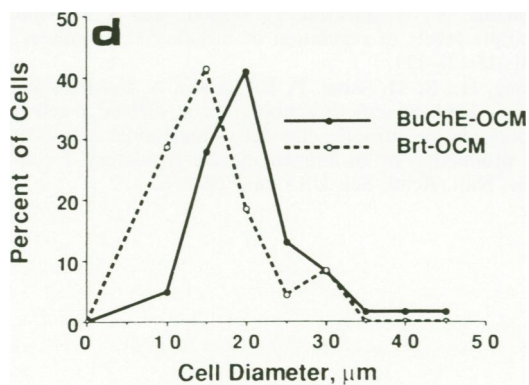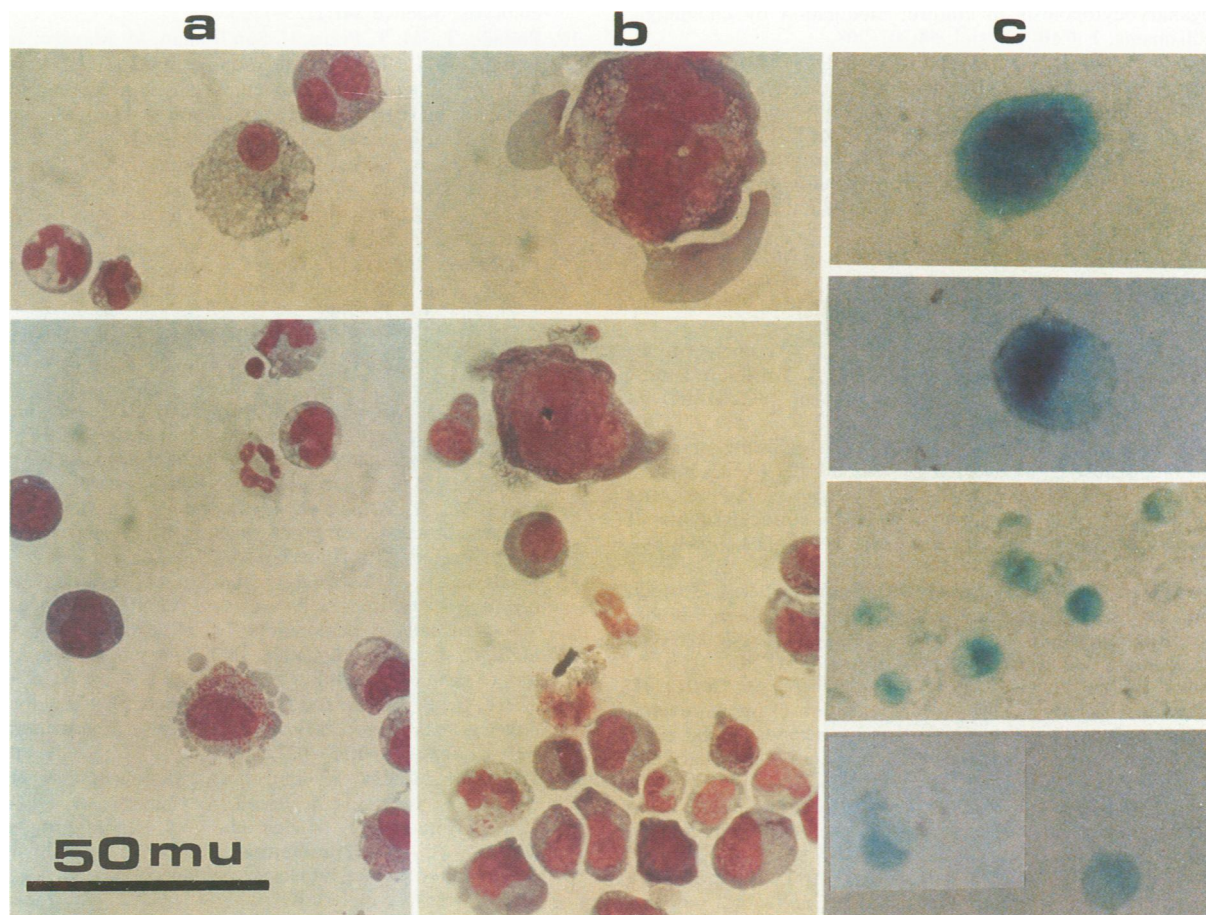
FIG. 3. Influence of BuChE-OCM on the morphology of hematopoietic cells. Liquid cultures were grown in LPM with or without BuChE-OCM; and cells were picked and stained as described in the legend to Fig. 1 and photographed in a Zeiss Axioplan microscope equipped with an HC100 camera. (a and b) Representative microscope fields. (a) Control cells. Note heterogeneity of cellular morphologies. (b) Megakaryocyte-enriched BuChE-OCM treated culture. Note the presence of multicell megakaryocyte colony (bottom) and mature megakaryocytes (top). (c) Cytochemical staining of megakaryocytes for cholinesterase activity. Fixed cells were stained for cholinesterase activity by indolyl acetate (18) in the absence of inhibitors (top panel) and in the presence of $10^{-5}$ M 1,5-bis (4-allyldimethylammoniumphenyl)-pentan-3-one dibromide (BW284C51), a specific AChE inhibitor (20; second panel), tetraisopropylpyrophosphoramide, a selective BuChE inhibitor (17; third panel), or both inhibitors (bottom panel). Note the partial sensitivity of staining to both inhibitors, indicating the presence of both AChE and BuChE in murine megakaryocytes. Bar = 50 μm. (d) Stimulation by BuChE-OCM of megakaryocyte growth. The percentage of cells in each size range (5-μm intervals) is plotted for BuChE-OCM- and Brt-OCM-treated cultures. A total of 60 to 70 cells were measured for each culture type. Average diameter ± standard error of the mean of megakaryocytes grown in the presence of BuChE-OCM (18.8 μm ± 0.63) was found to be significantly larger ($P < 0.025$) than for cells grown in the presence of Brt-OCM (14.4 μm ± 0.85), an increase comparable to that observed in the in vivo response to the stimulus of thrombocytopenia on megakaryocyte growth (4).

armed with a *Xenopus* oocyte bioassay for a putative effector of megakaryocytopoiesis, molecular cloning and purification of a direct modulator of this hemocytopoietic process may lead to a novel therapeutic approach to the treatment of thrombocytopenia.

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