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Effect of Thrombopoietin on *in Vitro* Production of Megakaryocytes from Fetal Mouse Liver Cells[1] (42142)

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Abstract. Plasma clots containing fetal mouse liver cells (FMLC) were used to study the effects of a thrombocytopoiesis-stimulating factor (TSF) from kidney cell culture medium on the proliferation and maturation of megakaryocytes. Cells in the megakaryocytic series were identified by the presence of acetylcholinesterase (AChE) and by their morphological and ultrastructural characteristics. For these experiments, 1×10^3 to 1×10^5 FMLC were cultured for 1–7 days with 0–5 μg of TSF; control cultures were treated with production medium (PMC) in which kidney cells had not been grown. The number of AChE+ cells that were observed depended upon the number of cells plated, i.e., after 6 days of culture with 5 μg of TSF, an average of 187 AChE+ cells was found after plating 1×10^4 cells and 1020 AChE+ cells were observed after plating 1×10^5 cells. In dose-response experiments, the number of AChE+ cells rose with increasing doses of TSF. Significantly elevated numbers of AChE+ cells were observed after the addition of 1–5 μg of TSF. The optimum time of culture, based upon the number of AChE+ cells found, was 3–5 days. Ultrastructural analysis of megakaryocytes in plasma clots showed evidence of platelet shedding on Day 5. After the culture of FMLC with TSF, a larger number of AChE+ cells was formed from a given number of cells plated than in previous studies that used adult bone marrow cells. Therefore, because of its greater sensitivity, FMLC may be useful for the assay of low levels of TSF, and may be a valuable tool for studying the effects of megakaryocytic regulatory factors on megakaryocytopoiesis.

A thrombocytopoiesis-stimulating factor (TSF or thrombopoietin) is now known to be a major controlling factor of thrombocytopoiesis (1, 2). Most studies have used TSF from two sources, i.e., from kidney cell culture medium (3) and from thrombocytopenic animals (4); TSF from these sources appears to be similar (1). This factor controls platelet production *in vivo* (1, 2), and has been shown to influence the stimulation (5–7) and maturation (8–10) of megakaryocytes *in vitro*. Most previous *in vitro* studies of megakaryocytopoiesis have utilized bone marrow cells (5–12). However, fetal mouse liver cells (FMLC) have been found to be more sensitive targets for the study of the effects of erythroid regulatory factors on erythropoiesis *in vitro* (13), but only a few reports have utilized FMLC for studying megakaryocytopoiesis (14, 15). In these previous studies, Vainchenker *et al.* (14), utilizing human fetal liver cells in plasma clot cultures that were stimulated with erythropoietin, demonstrated megakaryocytopoiesis with platelet shedding. Nicola and Johnson (15) showed that megakaryocytes can be grown from FMLC in a liquid culture system containing pokeweed-mitogen-stimulated-spleen-conditioned medium (PWM-SCM). Since neither of these previous studies utilized TSF, the present work investigates its effects on the proliferation and differentiation capabilities of FMLC for the production of acetylcholinesterase-positive (AChE+) cells in a plasma clot culture system.

Materials and Methods. For these experiments, young (7 week old) virgin female C3H mice weighing 22–23 g were placed in a specially constructed cage that separated the female mice from the adult males (16). After 4–5 days in this conditioning cage, a female mouse was placed with a young mature male that had been placed in a standard cage 3–4 days earlier. The female mice were checked

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at the same time daily for the presence of vaginal plugs. If positive, they were placed in a separate cage and dated (Day 1).

On Day 13 the pregnant mice were killed by cervical dislocation and the whole uterus was removed and placed in a petri dish containing sufficient amounts of Leibovitz (L-15) medium (GIBCO, N.Y.) to keep the embryo moist during dissection. The uterus was cut longitudinally through its length and each sac containing an embryo was teased away from the uterus. The sac was ruptured and the liver removed from the embryos. All the livers were then placed in a known volume of L-15 medium and dispersed into a single-cell suspension by gentle aspiration through a Pasteur pipet or a 14- to 16-gauge needle. The cell suspensions were prepared at a concentration of $1–2 \times 10^7$ cells/ml.

An appropriate dilution of cells in 0.1 ml of L-15 medium was added to a solution containing 0.2 ml of fetal calf serum (FCS; GIBCO, N.Y. or Flow Labs, McLean, Va.), 0.1 ml of beef embryo extract (GIBCO, N.Y.) diluted 1:4 in L-15 medium, 0.05 ml of production medium control (PMC) or TSF-rich medium in varying concentrations (1–5 μg protein/culture) diluted into L-15 medium, and 0.45 ml of L-15 medium. Following addition of 0.1 ml of bovine citrated plasma (GIBCO, N.Y.) and thorough mixing, a 0.1-ml aliquot was added to each well of disposable microtiter plates (Cooke Engineering Co., Alexandria, Va.) that were cut to fit inside a 100 × 15-mm petri dish and allowed to clot. The cells were cultured for varying lengths of time in a water-jacketed CO_2 incubator (National Appliance Co., Portland, Oreg.) at 37°C in an atmosphere of high humidity with 5% CO_2 in air. In order to maintain a high humidity environment, a 10 × 35-mm petri dish was filled with sterile water and placed alongside each microtiter plate.

For these experiments, TSF-rich medium from human embryonic kidney cell growth (TSF Lot No. 68-041) was used as a TSF source; this TSF preparation had been shown previously to contain high levels of TSF (3) and to stimulate megakaryocytic growth, differentiation, and cytoplasmic maturation in plasma clot cultures (6). Production medium control (PMC), medium without kidney cell growth, treated in a manner similar to TSF-rich medium was used as control.

Cultures were examined for the total number of AChE+ cells at daily intervals for 1–7 days. At these times, the plasma clots were rimmed and transferred to glass slides. The clots were dehydrated, first by placing filter papers on their surfaces, and then by placing 5–7 drops of cold 5% glutaraldehyde (in 0.1 *M* phosphate buffer, pH 7–7.2) on each filter paper over the clots. After 10 min the filter papers were removed, the clots were fixed on glass slides, and rinsed in 0.1 *M* sodium phosphate for 1 min. The direct coloring thiocholine method of Karnovsky and Roots (17) was used to stain for acetylcholinesterase. The plasma clots were then counterstained with Harris' hematoxylin and "blued" for 2–3 min in tap water. Final preparations were examined microscopically at a magnification of 420× for AChE+ cells.

Data were evaluated by use of Student's *t* test.

The ultrastructural analysis of megakaryocytes was performed by use of a transmission electron microscope with routine techniques (18). In brief, plasma clots were processed in a buffered-glutaraldehyde solution and thick sections were examined by light microscopy to detect megakaryocytes. Megakaryocytes were identified by their morphology and particularly by their lobulated nuclei. Serial thin sections were then made on an LKB Ultratome III and were examined unstained.

Results. Figure 1 shows the relationship between the number of cells plated (1×10^3 to 1×10^5 FMLC/culture) and the number of AChE+ cells formed after incubation with PMC or TSF (5 μg/treatment). As shown, with TSF there was a good correlation between the number of cells plated and the number of AChE+ cells that developed 6 days later. Megakaryocytic colonies were observed, but only at cell concentrations of $>2 \times 10^4$.

In dose–response experiments, 1×10^5 FMLC/culture were plated with increasing doses of TSF (Fig. 2). At a dose of 1 μg of TSF/culture the total number of AChE+ cells after 6 days of culture was about twofold

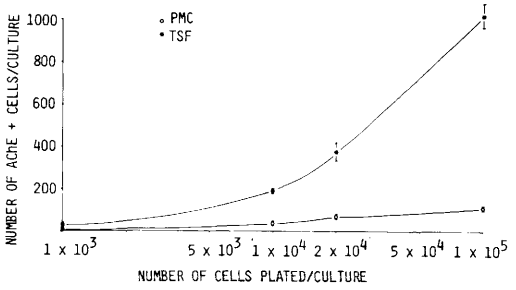

FIG. 1. Relationship between the number of fetal mouse liver cells plated and the number of acetylcholinesterase positive (AChE+) cells observed after 6 days of culture. Control production medium (PMC) and thrombocytopoiesis-stimulating factor (TSF) were added at 5 μg of protein per culture. Six to eight cultures were examined for each treatment; vertical lines represent the standard errors.

higher than for controls ($P < 0.0005$). Increasing the dose of TSF led to additional stimulation of AChE+ cells in the cultures ($P < 0.0005$); 5 μg of TSF/culture resulted in threefold increase over control. In addition, there was a linear correlation between the dose of TSF and the number and size of megakaryocyte colonies (Table I). Although not shown, TSF also increased the maturation of these cells, i.e., cells treated with TSF were morphologically more mature than cells treated with control media.

In an experiment to determine the optimum time of culture (Fig. 3), 2×10^4 FMLC were plated, along with 5 μg of TSF, and the AChE+ cells counted at daily intervals. When compared to controls, a significant increase in AChE+ cells (fourfold) was observed beginning at Day 3 of culture. The number of AChE+ cells remained at this level through Day 5 and declined thereafter. Cultures treated with PMC had fewer AChE+ cells than those treated with TSF, but showed a similar pattern with a 1 day earlier decline.

In this study we also noted some differences in the growth patterns of megakaryocytes from FMLC when compared to maturation of megakaryocytes in previous studies (6) using adult mouse bone marrow, i.e., FMLC began growing 1 day earlier, and the growth was more extensive than with cells from adult mouse marrow. Colonization of AChE+ cells was also greater and occurred approximately a day earlier than when adult mouse marrow cells were used. However, the size of FML megakaryocytes in plasma clot cultures was smaller (average 17 μm in diameter) than those of normal adult mouse marrow megakaryocytes (average 24 μm in diameter). Most of the FML megakaryocytes showed nuclei with only 1 or 2 lobes, with many of the cells showing detectable demarcation membranes with complete cytoplasmic maturation and visible platelet production. The ultrastructural characteristics of these megakaryocytes from Day 5 of culture showed all stages of megakaryocytes, i.e., immature, mature, and platelet producing megakaryocytes were seen.

The maturation of megakaryocytes from FMLC in plasma clot cultures was studied by electron microscopy. Figures 4, 5 show the ultrastructure of one mature megakaryocyte. Some parts of the megakaryocyte showed shedding of platelets, with highly developed demarcation membranes that were irregularly distributed. Electron microscopic analyses of control cultures on Day 5 showed degenerative changes and lack of cytoplasmic growth with no platelet formation.

Discussion. The results of this study demonstrate that TSF potentiates megakaryocyte growth of FMLC in plasma clot cultures;

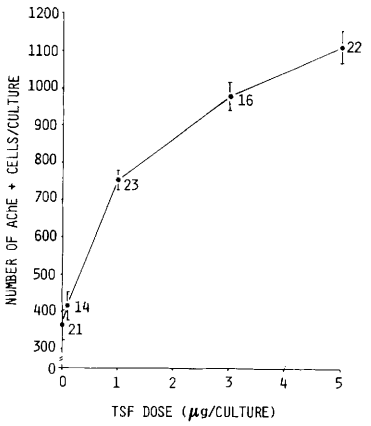

FIG. 2. Effects of various doses of TSF on the number of AChE+ cells found in plasma clots after 6 days of culture. 1×10^5 FMLC were plated/culture; vertical lines represent the standard errors and the numbers next to the points indicate the number of cultures/treatment.

TABLE I. THE EFFECTS OF VARIOUS DOSES OF TSF ON THE AVERAGE NUMBER AND SIZE OF MEGAKARYOCYTE COLONIES AFTER CULTURING FMLC

Dose of TSF (μg)	Number of cultures	Average number of megakaryocyte colonies/culture ± SE	Average number of megakaryocytes/ colony ± SE
0	7	5.85 ± 1.01	2.12 ± 0.07
0.1	8	11.87 ± 1.87*	2.96 ± 0.33*
1.0	5	20.80 ± 2.63***	3.02 ± 0.18**
3.0	6	52.66 ± 11.35**	3.07 ± 0.36*
5.0	4	59.25 ± 22.58*	3.53 ± 0.13***

Note. FMLC were cultured in plasma clots for 6 days using 1×10^5 cells/culture. Values were signficantly increased over control: $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$.

significant megakaryocytopoiesis was found when TSF was added at 1–5 μg/l $\times 10^4$ to 1 $\times 10^5$ FMLC and cultured for 3–5 days. Not only did TSF increase the number of AChE+ cells, but also the number and size of megakaryocyte colonies and the rate of differentiation and cytoplasmic maturation of megakaryocytes, thus leading to the observed platelet production. Therefore, megakaryocyte progenitors from FMLC can be used in plasma clot cultures to measure megakaryocytopoiesis; TSF stimulation caused platelets to be released from the megakaryocytes.

Several recent studies (8–10) have shown that there may be two humoral regulators important in the control of megakaryocytopoiesis, and probably thrombocytopoiesis, in *in vitro* systems, i.e., the megakaryocyte-colony-stimulating activity (Meg-CSA) and TSF. Meg-CSA is believed to exert its effect on the colony-forming-unit megakaryocyte (CFU-M), causing these cells to proliferate and undergo partial maturation. The second regulator, TSF, has been claimed not to support CFU-M growth by itself *in vitro* but is thought to finalize the maturation process of megakaryocytes by influencing nuclear endoreduplication and completing cytoplasmic maturation for platelet production (19). However, a recent study (7) showed that TSF extracted from the plasma of thrombocytopenic rabbits will stimulate megakaryocytopoiesis *in vitro;* whereas, earlier studies using this source of TSF showed negative effects (20). In agreement with the results of the present work, other studies (5, 6) showed that crude preparations of TSF from human embryonic kidney cells will support the growth of megakaryocytopoiesis *in vitro,* indicating that these crude TSF preparations probably contain, in addition to TSF, Meg-CSA. Alternatively, the source of progenitor cells and/or culture techniques may have influenced, to a large degree, the role of these stimulators on megakaryocytopoiesis *in vitro.*

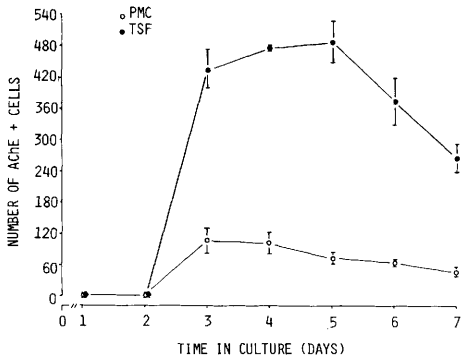

FIG. 3. Effects of culture time on the number of AChE+ cells after addition of PMC or TSF (5 μg of TSF/culture). In this experiment, 2×10^4 FMLC were plated per culture. Vertical lines represent the standard errors and six to eight cultures were used for each treatment.

Previously, studies of *in vitro* differentiation of megakaryocyte progenitors (CFU-M) were made using bone marrow cells from a variety of species (5–12), and in a few instances fetal liver cells were used (14, 15). Several different culture techniques, cell concentrations, and stimuli, to include erythropoietin and PWM-SCM, have been used (5–12, 14, 15, 19, 20).

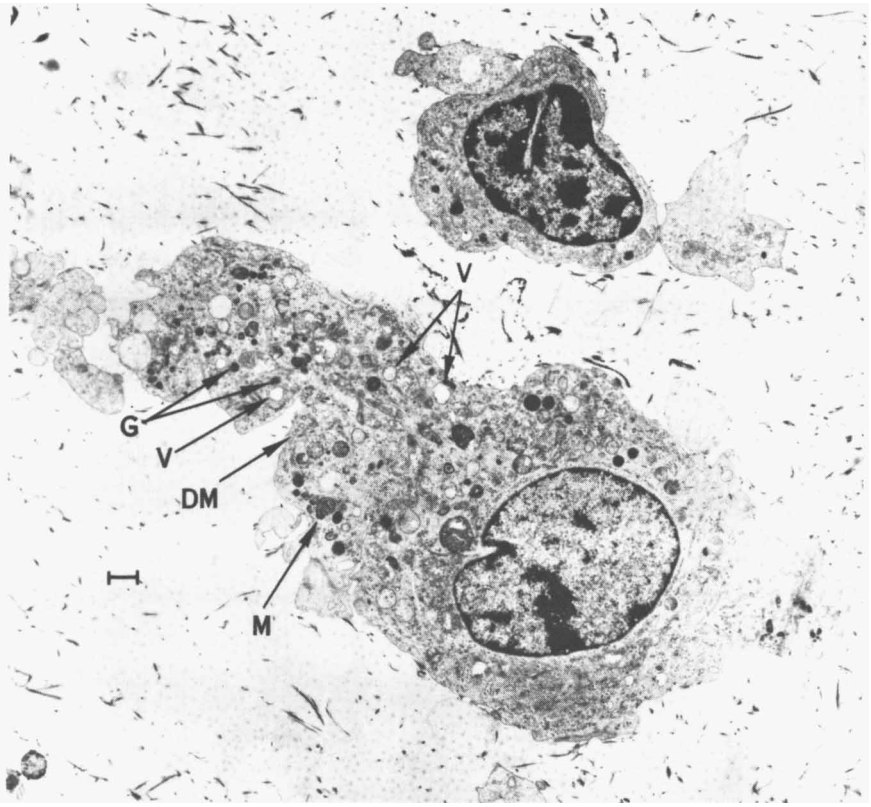

FIG. 4. Photomicrograph of a mature megakaryocyte after 6 days of culture with granules (G), vacuoles (V), small vacuoles for forming demarcation membranes (DM), and mitochondria (M) present, horizontal solid bar represents one μm, 5400×.

Plating efficiencies of CFU-M from murine bone marrow varied from 1:5000 to 1:1500 (5–12), probably because of the large variation in techniques used. In the present study, using a plasma clot culture technique and stimulation of FMLC with TSF, the number of AChE+ cells observed was markedly increased over controls, i.e., at optimum conditions (5–6 days after plating 1×10^5 FMLC and adding 5 μg of TSF) greater than 1000 AChE+ cells were found compared to less than 100 AChE+ cells for control cultures. Therefore, a 10-fold increase in the number of AChE+ cells was found after adding TSF to these cultures. TSF also stimulated an increase in AChE+ cell colonies (Table I) when compared to control cultures. The size and number of these colonies, however, depended upon the number of cells plated and the dose of TSF.

Previously, Vainchenker *et al.* (14) have shown that erythropoietin will cause an increase in the number of mixed colonies of fetal human liver cell cultures. Nicola and Johnson (15) showed that PWM-SCM stimulated CFU-M in FMLC cultures, but other stimulants, such as mouse lung-conditioned medium or L-cell-conditioned medium had no effect. We are unaware of a previous study showing that TSF will stimulate FMLC in culture to produce AChE+ megakaryocytes; however, the present study showed a dose–response relationship between the number of AChE+ cells observed at Day 6 and the dose of TSF.

In the present study, it was shown that the AChE+ cells were absent in the cultures until Day 3 (Fig. 3), indicating that the observed cells could not have been residual cells in the plated material. Instead, the AChE+ cells

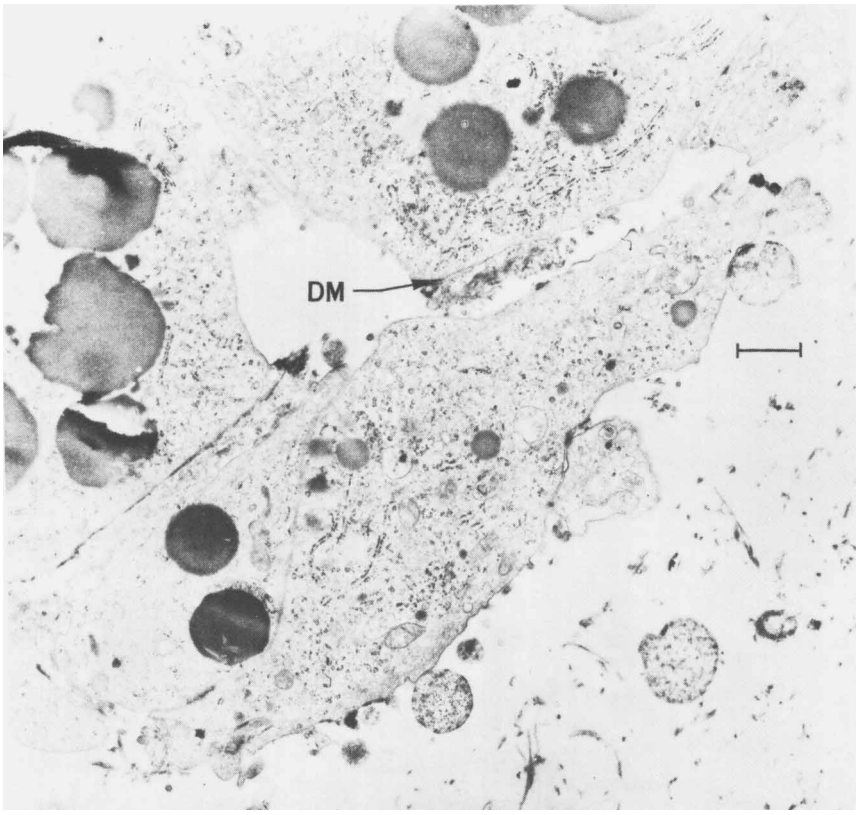

FIG. 5. Photomicrograph of the same cell as shown in Fig. 4 except at a higher magnification of the platelet shedding area with demarcation membranes (DM) and granules present; horizontal solid bar represents one μm, 13,500×.

observed herein must have been produced from precursor cells. As shown, after plating 2×10^4 FMLC and adding TSF the number of AChE+ cells observed in culture was significantly elevated by 3 days, the numbers then plateaued (to about 425–450 AChE+ cells/culture) through Day 5, and decreased thereafter. Only a few AChE+ cells (about 100) were observed on these days when control medium was used. Similar data have been published by Nicola and Johnson (15), who showed that Meg-CFC (CFU/M) from FMLC increases in culture with time after the addition of PWM-SCM.

The use of FMLC, when compared to adult mouse bone marrow, showed some differences in the patterns of megakaryocyte growth. FMLC showed shorter time requirements for the appearance of AChE+ cells than did adult mouse bone marrow cells (6). Fetal pluripotential stem cells have shorter generation times than adult cells (21), which could explain the difference. However, we noticed that unlike adult mouse tissue the number of nuclear lobes of the megakaryocytes failed to correlate with cytoplasmic maturation. In this study, one to three lobes seemed to be sufficient for platelet production by FMLC in culture. In agreement with this finding, Vainchenker *et al.* (14) noted smaller megakaryocytes from fetal liver cells than from adult specimens after stimulation with erythropoietin in plasma clots. Keleman (22) also noted that the sizes of human embryonic megakaryocytes, both in smears of liver and marrow, were smaller than megakaryocytes from adults, and showed that megakaryocytes from embryonic tissue had fewer lobes when compared to megakaryocytes from adults.

In the present work we have described the

use of FMLC for the production of mature platelet-producing megakaryocytes. FMLC appear to produce more AChE+ cells after TSF-treatment than adult murine bone marrow cells. A FMLC assay for TSF appears possible. This assay has potential for use in clinical cases where the levels of TSF produced are small.

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