Comparative Effects of Insulin-Like Growth Factor II (IGF-II) and IGF-II Mutants Specific for IGF-II/CIM6-P or IGF-I Receptors on in Vitro Hematopoiesis

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Abstract. This report presents the results of studies investigating the effect of insulin-like growth factor II (IGF-II) on the proliferation and differentiation of CD34$^+$ bone marrow cells in serum-substituted liquid cultures. Bone marrow cells were enriched for CD34$^+$ cells and then placed in liquid cultures supplemented with either interleukin 3 (IL-3) or IL-3 and c-*kit* ligand with and without the addition of IGF-II. When CD34$^+$ cells were incubated with IL-3, cellularity increased throughout four weeks of culture. Cellularity was twofold greater when cultures also contained IGF-II. IGF-II also promoted an increase in cellularity in cultures with IL-3 and c-*kit* ligand. In combination with IL-3 or IL-3 and c-*kit* ligand, IGF-II promoted an earlier differentiation of granulocytes, as well as an increase in the number of megakaryocyte lineage cells. There were approximately twofold more colony-forming units for granulocytes and macrophages (CFU-GM) and burst-forming units for erythroid cells (BFU-E) in cultures containing both IL-3 and IGF-II than in cultures with IL-3 alone. These results demonstrate that in cytokine-supplemented media, physiological concentrations of IGF-II augmented both the proliferation and differentiation of CD34$^+$ bone marrow cells while maintaining a greater number of progenitor cells. To identify the receptors through which IGF-II enhances in vitro hematopoiesis, IGF-II was substituted with one of the mutant forms of IGF-II that selectively interacts with either IGF-II/CIM6-P receptors or with IGF-I and insulin receptors. The results with the mutant forms of IGF-II demonstrate that IGF-II augments in vitro hematopoiesis primarily through its interaction with IGF-I and possibly insulin receptors, rather than IGF-II/CIM6-P receptors. *Stem Cells 1996;14:337-350*

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Introduction

The mature form of insulin-like growth factor II (IGF-II) has a molecular weight of 7500 and is a single-chain polypeptide of 67 amino acids [1-2]. IGF-II, alone and in combination with other growth factors, has been shown to be a potent mitogen for a variety of cell lines as well as for cells in primary cultures [1-10]. Results from in vivo and in vitro studies also indicate that IGF-II is important for stimulating the clonal expansion of embryonic cells and in regulating the growth and differentiation of embryonic and fetal tissues [2, 6, 8-13]. IGF-II has a high binding affinity for its own receptor, the IGF-II/cation-independent mannose 6-phosphate (IGF-II/CIM6-P) receptor, and also binds to IGF-I and insulin receptors with moderate to high affinity [1, 2]. The mitogenic effects of IGF-II on many cell types have been shown to be mediated through the IGF-I receptor [1, 2, 7]. Results from other studies indicate that binding of IGF-II to IGF-II/CIM6-P receptors on embryonic or fetal cells may be important in mediating differentiation, in regulating

the availability of IGF-II and in modulating the activation of other proteins that also bind to IGF-II/CIM6-P receptors [1, 2, 10, 13-15]. Others demonstrated that interaction of IGF-II with IGF-II/CIM6-P receptors stimulated the proliferation of some transformed cell lines, as well as mediated motility of human rhabdomyosarcoma cells [11, 16]. The data suggest that differential binding of IGF-II to the IGF-I or IGF-II/CIM6-P receptors may be important in regulating the proliferation and differentiation of developing tissues.

The continuous replacement of mature blood cells and platelets in normal adults is maintained by committed and primitive progenitor cells that are heterogeneous with respect to their self-renewal, proliferative and differentiation capacities [17]. In vitro studies demonstrated that physiological concentrations of IGF-II in combination with other growth factors stimulated the proliferation of hematopoietic progenitor cells that form colonies of granulocytes and macrophages (i.e., colony-forming units for granulocytes and macrophages or CFU-GM) or erythroid cells (i.e., colony-forming units for erythroid cells or CFU-E and burst-forming units for erythroid cells or BFU-E) [18-22]. IGF-II also promoted an increase in the number of CFU-GM in interleukin 3 (IL-3) supplemented liquid cultures of peripheral blood cells [23]. In liquid cultures, IL-3 has been shown to stimulate the proliferation and progressive differentiation of hematopoietic progenitor cells [24-27]. Liquid cultures of bone marrow cells provide a model to investigate the early effects of IGF-II on the proliferation and differentiation of hematopoietic progenitor cells and the possible role of the IGF-I and IGF-II/CIM6-P receptors in mediating those responses.

The purpose of the studies in this report was to determine the effect of IGF-II on the clonal expansion, maintenance and differentiation of progenitors for megakaryocytes, granulocytes and macrophages, and erythroid cells. Bone marrow cells were enriched for hematopoietic progenitors by selection of cells expressing the CD34 cell surface antigen. $CD34^+$ cells were placed in liquid cultures supplemented with IL-3 or IL-3 and c-*kit* ligand, and the effect of the addition or absence of IGF-II on cellularity, the production of megakaryocyte lineage cells and the number of CFU-GM and BFU-E was determined. The results demonstrate that in cytokine-supplemented media, IGF-II enhanced the proliferation and differentiation of granulocyte-macrophage and erythroid progenitor cells. To identify the receptors through which IGF-II enhanced in vitro hematopoiesis, IGF-II in liquid cultures of $CD34^+$ bone marrow cells was replaced with one of the mutant forms of IGF-II that binds preferentially to either IGF-II/CIM6-P receptors or IGF-I and insulin receptors [7].

Materials and Methods

Preparation of Cell Suspensions

Bone marrow cells were obtained from cadaveric vertebral bodies (LifeNet Transplant Services; Virginia Beach, VA) or from bone marrow cells aspirated from the iliac crest of normal donors after informed consent under human use protocols approved by the National Cancer Institute and Walter Reed Army Medical Center. Ten to 15 ml of bone marrow aspirate were collected in a 20 cc syringe containing preservative-free heparin (Sigma; St. Louis, MO) as an anticoagulant and then diluted in Dulbecco's phosphate buffered saline (DPBS) without Ca^{+2} or Mg^{+2} (GIBCO; Grand Island, NY) that contains the penicillin-streptomycin (GIBCO). Cells with a density of ≤ 1.077 g/cm^3 were collected and washed three times after separation on a Ficoll-sodium diatrizoate gradient (Lymphocyte Separation Medium; Organon Teknika Corp.; Durham, NC).

Isolation of $CD34^+$ Bone Marrow Cells

A positive immunomagnetic selection procedure was used to select for bone marrow cells that expressed the CD34 antigen [28, 29]. Low density (≤ 1.077 g/cm^3) bone marrow cells were rosetted to magnetic Dynabeads (Dynal Inc.; Great Neck, NY) coated with K6.1, a monoclonal antibody for CD34 and separated by three or four cycles of attraction to a rare earth magnet. $CD34^+$ cells were detached and the beads were magnetically separated from the cells. Isolated $CD34^+$ cells were used after overnight incubation in Iscove's modified Dulbecco's medium (IMDM), (GIBCO) with 2% fetal bovine serum (FBS). $CD34^+$ cells were also isolated by positive immunomagnetic selection using a high-gradient magnetic separation column and MiniMacs CD34 progenitor cell isolation kit (Miltenyi Biotec Inc.; Sunnyvale, CA). The purity of the recovered cells was determined by staining with CD34 (anti-HPCA-2) phycoerythrin (PE), (Becton Dickinson;

San Jose, CA), which binds to a distinct epitope on the CD34 molecule, and flow cytometry analysis demonstrated that purity was 95% to 99%. $CD34^+$ cells were suspended in 7.5% dimethyl sulfoxide (DMSO) (Sigma) in 47% FBS and IMDM, cryopreserved in a controlled-rate freezer and stored in the vapor phase of liquid nitrogen. Cells were quickly thawed in a 37°C water bath, washed twice with excess IMDM, incubated for 1.5 to 2 h at 37°C, 5% CO^2 and washed again.

Source of Cytokines and rIGF-II

Recombinant human IL-3 and c-*kit* ligand (R & D Systems; Minneapolis, MN) were resuspended in DPBS with 0.1% bovine serum albumin (BSA) and diluted with serum-substituted medium. Human recombinant IGF-II, and four mutants of IGF-II (kindly provided by Daiichi Pharmaceutical, Ltd.; Tokyo, Japan) were prepared for use in liquid cultures by placing an aliquot of each in acidified ethanol, drying by speed vac and resuspending to a concentration of 10 μg/ml in 0.1 M acetic acid. Further dilutions were prepared in serum-substituted medium on the day they were added to the liquid cultures. The mutant forms of IGF-II had been prepared by site-directed mutagenesis [7]. The two mutants, [Leu43]IGF-II and [Leu27]IGF-II were shown to bind with high affinity to the IGF-II/CIM6-P receptors and with low affinity to IGF-I and insulin receptors. In contrast, [Arg54,Arg55]IGF-II and [Thr48,Ser49,Ile50]IGF-II were shown to bind with high affinity to IGF-I receptors and with low affinity to IGF-II/CIM6-P receptors.

Preparation of Serum-Substituted Liquid Culture Medium

Serum-substituted liquid culture medium used in these studies was basically as previously described [23]. The basal medium used in these studies was an enriched IMDM supplemented with sodium pyruvate (GIBCO), nonessential amino acids (GIBCO), L-glutamine (GIBCO), penicillin-streptomycin antibiotic solution (GIBCO) and sodium bicarbonate solution (Quality Biologics, Inc.; Gaithersburg, MD) [27]. At the time of culture, IMDM basal medium was further supplemented with 20 μg/ml L-asparagine (Sigma), 1% deionized RIA grade Cohn Fraction V BSA (Sigma) [30], 5×10^{-5} M β-mercaptoethanol, 300 μg/ml iron-saturated transferrin, 1% Excyte III (Miles Diagnostic; Kankakee, IL) and 10 μg/ml each of adenosine, cytidine, uridine, guanosine, 2′-deoxyadenosine, 2′-deoxycytidine, 2′-deoxythymidine and 2′-deoxyguanosine (all from Sigma). Iron-saturated transferrin [31] and the riboside mixtures [32] were prepared as 100× solutions, stored at –20°C, and frozen and thawed once. A 1% solution of Excyte III provided 96 to 99 μg/ml cholesterol.

Establishment of Serum-Substituted Liquid Cultures

Liquid cultures of $CD34^+$ bone marrow cells were established in 15 mm Gel-Well culture chambers (Costar; Cambridge, MA) [23, 27]. In this culture system, close cellular interactions of a small number of cells are maintained and the cells do not adhere to the culture surfaces. A single Gel-Well was placed in the center of a 35 mm gridded tissue culture dish and 1.5 ml medium was pipetted outside the Gel-Well. Factors (IL-3 and IGF-II) were added to the outside medium and gently swirled to mix. Either 1 or 2×10^4 $CD34^+$ bone marrow cells were added to the trough inside the Gel-Well and allowed to settle, then an additional 100 μl of medium were added. In some studies, 6.5 mm nontissue culture-treated 0.1 μm pore Transwells (Costar) in wells of 24-well plates were used instead of Gel-Wells. The outside media and factors were replaced weekly. After one to four weeks of incubation at 37°C, 5% CO^2 and 95% room air, the entire contents of individual Gel-Wells or Transwells were removed, washed and diluted to a final volume of 0.5 ml.

Cell concentrations were determined by hemacytometer counts and cell viability was determined by trypan dye exclusion. Slides for cell differential determinations were prepared by placing 1×10^5 cells in 0.1 ml medium with 0.1% BSA into a chamber of a cytocentrifuge (Shandon, Inc.; Pittsburgh, PA) and centrifuging at 800 rpm for 10 min. The slides were air-dried and then fixed and stained with Hema 3 (Curtin-Matheson, Inc.; Houston, TX).

Colony Assays for Detection of CFU-GM and BFU-E

GM-CFC and BFU-E determinations were made by subculturing 25 to 100 μl of cells from liquid cultures in 4.0 ml of 1.2% A4M Methocel (Dow Chemical Company; Midland, MI) [30] containing basal IMDM, 1% deionized Fraction V BSA (Sigma) [30], 30% heat inactivated FBS (Hyclone; Logan, UT), 5×10^{-3} M

β-mercaptoethanol (Sigma) and 20 μg/ml L-asparagine (Sigma). To stimulate colony growth from CFU-GM, 10% serum-free PHA-LCM (Stem Cell Technologies; Vancouver, Canada) was added to the methylcellulose medium. To stimulate colony growth from BFU-E, 2 U/ml human recombinant erythropoietin (R & D Systems) was also added to the colony-growth medium. One ml of methylcellulose medium containing cells was placed in a 35 mm gridded tissue culture dish (Nunc; Naperville, IL) and then maintained in a humidified 37°C incubator with 5% CO^2 and 95% room air. In one experiment, cells were plated in 0.3% agarose containing 2.5% 10 × 5637 cell-conditioned media [27]. After 14 days, colonies containing ≥50 granulocytes or macrophages or both, or ≥100 erythroid cells were counted as derived from CFU-GM and BFU-E, respectively.

Enzyme-Linked Immunosorbant Assay (ELISA) for Detection of Megakaryocyte Lineage Cells

To quantitate production of megakaryocytes from $CD34^+$ cells, the cells were placed in liquid culture with serum-substituted medium containing 2 ng/ml IL-3 and 20 ng/ml c-*kit* ligand to stimulate proliferation and lineage commitment. After 10 to 11 days of culture, megakaryocyte lineage cells were identified by expression of GP IIb/IIIa (CD41a) and quantitated using an enzyme-linked immunosorbant assay (ELISA) as previously described [33, 34]. The cells were washed three times by centrifugation of the 96-well plates, then all of the cells from each well were transferred to new wells in 96-well plates that had been pretreated with 1% glutaraldehyde, and centrifuged again. A glutaraldehyde-paraformaldehyde mixture (0.004% and 0.1%, respectively) was added for two hours. The wells were rinsed with water, filled with a saline solution containing 0.1% BSA and 0.1% azide, and then the plates were stored at 4°C for up to one week.

Quantitation of the megakaryocyte-specific GP IIb/IIIa and GP Ib antigens was done by ELISA, using the 10E5 and AV6 antibodies, respectively (a generous gift of *Dr. Barry Coller*, Mt. Sinai, NY). The fixed cells were blocked with 20% goat serum, followed by incubation with primary antibody (10E5 or a negative control antibody). After washing, a biotin-labeled secondary antibody was added (goat anti-mouse IgG; Pierce; Rockford, IL) followed by streptavidin-conjugated β-galactosidase (GIBCO). The soluble substrate chlorophenol red-β-D-galactopyranoside (CPRG; Boehringer Mannheim; Indianapolis, IN) was then added. Thirty minutes later, color development was read on a spectrophotometer (Molecular Devices; Menlo Park, CA) at a wavelength of 570 nm. Results are expressed as absorbance (means ± SD of $n = 3$ or 4 wells). For immunocytochemical staining of positive cells, an insoluble substrate was used following CPRG. The CPRG reaction product was washed off, and the substrate bluo-gal (GIBCO) was added to the ELISA wells. A blue precipitate identified GP IIb/IIIa positive cells [33].

Statistics

The paired *t*-test was used to test for significant differences in the number of CFU-GM and BFU-E in cultures with and without IGF-II. Paired samples were compared at each time point. The Student's two-tailed *t*-test was used to test for significant differences within an experiment. Values between different groups were considered to be significantly different for $p < 0.05$. *p* values were calculated using Microsoft Excel 4.0 statistical functions.

Results

Effect of IGF-II on Proliferation and Differentiation of $CD34^+$ Cells in Serum-Substituted Liquid Cultures

$CD34^+$ bone marrow cells were incubated for one to four weeks in serum-substituted liquid culture medium. The number of cells decreased in cultures that were not supplemented with IL-3 (Fig. 1). For example, after two weeks, the number of cells was less than 50% of the number added at day 0. When cultures were supplemented with IL-3, there was a progressive increase in the number of cells. For example, after two weeks, the cultures contained 3- to 11-fold more cells than added at day 0. IGF-II alone did not promote an increase in cellularity (data not shown), however, when added to cultures supplemented with IL-3, 100 and 150 ng/ml of IGF-II promoted a further twofold increase in cellularity. The number of cells in cultures supplemented with both IL-3 and c-*kit* ligand was greater than in cultures with IL-3 alone (Table 1). Similar to the results with IL-3, there was a further increase in

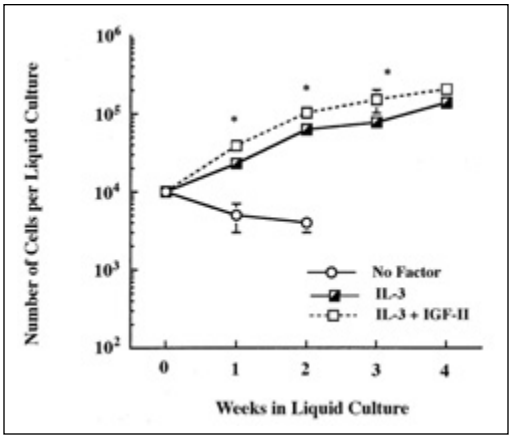

Figure 1. Effect of IL-3 and IL-3 plus IGF-II on the number of cells in serum-substituted liquid cultures of CD34⁺ bone marrow cells. *Cells (1×10^4) were placed in an enriched serum-substituted medium containing 2 ng/ml IL-3 (no insulin or IGF-II added to the medium), in medium containing IL-3 plus either 100, or 150 ng/ml IGF-II, or in medium without either IL-3 or IGF-II. Weekly, all of the cells from individual Gel-Wells or Transwells were removed, and the number of viable cells per well was determined. Values from cultures with no factors are the mean ± SEM from five experiments. Values for cultures with IL-3 and IL-3 plus IGF-II at one and two weeks are the mean ± SEM calculated from nine experiments and at three and four weeks from four and one experiments, respectively. *By paired t-test, the number of cells was significantly different from the number with IL-3 alone.*

the number of cells when the cultures were also supplemented with IGF-II.

The stages of granulocytic cells were evaluated after the culture of CD34⁺ cells with IL-3 alone or with both IL-3 and IGF-II. No band or segmented forms of differentiated granulocytes were detected in the purified preparations of CD34⁺ cells that were added to the liquid cultures at day 0. After one week, 76% to 88% of the granulocytic cells in cultures with IL-3 alone and with IL-3 + IGF-II were immature (i.e., blast and myelocyte stages) and 12% to 24% were of the more mature metamyelocyte, band and segmented stages (Fig. 2). After three weeks, the percentage of mature granulocytic cells in cultures supplemented with both IL-3 and IGF-II had already increased to 61%, but was only 47% in cultures with IL-3 alone. There was also an earlier differentiation of granulocytic cells when IGF-II was substituted with [Arg54,Arg55]IGF-II, a mutant form of IGF-II with a high affinity to insulin and IGF-I receptors, but a low affinity for IGF-II/CIM6-P receptors.

Effect of IGF-II on Number of CFU-GM and BFU-E in Liquid Cultures of CD34⁺ Bone Marrow Cells

The number of CFU-GM was determined after the incubation of CD34⁺ bone marrow cells in liquid cultures with serum-substituted medium. When CD34⁺ bone marrow cells were incubated for one and two weeks without growth factors, the number of CFU-GM was less than 5% of the number added at day 0 (Table 2). There was still a decrease in the total number of CFU-GM when CD34⁺ cells were incubated with either IL-3 alone or with both IL-3 and c-*kit* ligand, however, there were significantly more CFU-GM than in cultures of cells incubated without the addition of growth factors. In these studies, the decrease in the number of CFU-GM after one and two weeks was associated with a 1.3- to 11-fold increase in cellularity (Fig. 1).

Table 1. Effect of IGF-II on proliferation of CD34⁺ bone marrow cells incubated with IL-3 and *c-kit* ligand[a]

Factors in Cultures	Number of Cells/Well	Percentage of Number of Cells in Wells with:	
		IL-3 Alone	IL-3 + c-*kit* ligand
IL-3	2.1 ± 0.7 ($\times 10^4$)	100	
IL-3 + c-*kit* ligand	3.9 ± 0.7 ($\times 10^4$)[b]	210 ± 32	100
IL-3 + c-*kit* ligand + IGF-II	7.4 ± 1.9 ($\times 10^4$)[b,c]	382 ± 91	180 ± 28

[a]CD34⁺ bone marrow cells were incubated for one week, and then all of the cells from each well were collected and the number of viable cells was determined. The values are the mean ± SE values from four different donor marrows. IL-3 (2 ng/ml), c-*kit* ligand (20 ng/ml), and IGF-II (100 ng/ml) were added to the cultures at day 0.
[b]By paired *t*-test significantly different ($p < 0.05$) from the number of cells in cultures with IL-3.
[c]By paired *t*-test significantly different ($p < 0.05$) from the number of cells in cultures with IL-3 + c-*kit* ligand.

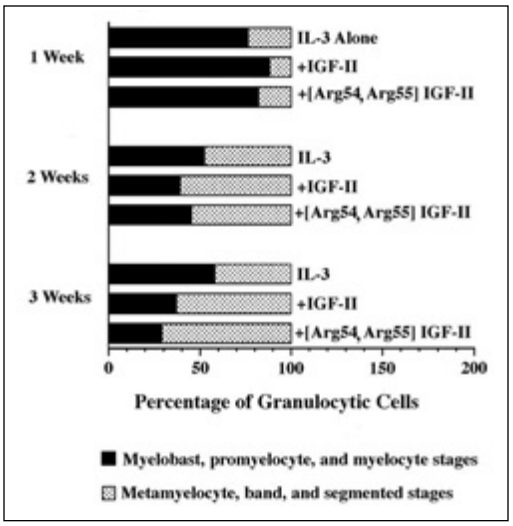

Figure 2. Effect of IL-3 and IL-3 plus IGF-II on the differentiation of granulocytic cells in serum-substituted liquid cultures of CD34⁺ bone marrow cells. *Cells were placed in an enriched serum-substituted medium containing 2 ng/ml IL-3 (no insulin or IGF-II added to the medium), in medium containing IL-3 plus 100 ng/ml IGF-II or IL-3 plus 100 ng/ml [Arg54,Arg55]IGF-II. Weekly, cytospin preparations of cells from individual liquid cultures were prepared and stained. Stages of 100 myeloid cells per culture were determined, and values at one and two weeks were calculated from two liquid cultures and at three weeks from one liquid culture each.*

The number of CFU-GM in cultures of CD34⁺ bone marrow cells incubated with IGF-II in addition to IL-3 or a combination of IL-3 and c-*kit* ligand was also determined. Similar to the response observed with IL-3 alone, there was a decrease in the total number of CFU-GM when CD34⁺ cells were incubated with both IL-3 and IGF-II (Fig. 3). Even though there was some heterogeneity in the response of cells from different donors (for example, Figs. 3A and 3B), the number of CFU-GM in cultures with both IL-3 and IGF-II was approximately twofold greater than the number in cultures with IL-3 alone (Table 3). The number of CFU-GM in cultures with a combination of IL-3, c-*kit* ligand and IGF-II was similar to the number with IL-3 and c-*kit* ligand. A greater number of BFU-E was also maintained in cultures supplemented with IGF-II in addition to IL-3 (Fig. 4). The decrease in the number of BFU-E (colonies of ≥100 erythroid cells) in cultures with IGF-II was associated with an increase in the number of smaller colonies consisting of 20 to 50 erythroid cells that was not observed in cultures with IL-3 alone. These results demonstrate that the increase in cellularity observed with IGF-II did not promote an earlier decrease in the number of CFU-GM or BFU-E.

Effect of Mutant Forms of IGF-II on Cellularity and CFU-GM Numbers in Liquid Cultures of CD34⁺ Marrow Cells

Studies were performed to identify the receptors through which IGF-II augments in vitro hematopoiesis. In liquid cultures of CD34⁺ bone marrow cells, one of the mutant forms of IGF-II that binds with normal or high affinity to either IGF-IICIM6-P receptors (i.e.,

Table 2. Effect of IL-3 and c-*kit* ligand on the number of CFU-GM in liquid cultures of CD34⁺ bone marrow cells[a]

Factors in Culture	Percentage of Number Added at Day 0[b]	
	After 1 Week of Incubation (*n*)[c]	After 2 Weeks of Incubation (*n*)[c]
No Factor	2 ± 1 (5)	1 ± 1 (5)
IL-3	61 ± 25 (11)	55 ± 29 (8)
IL-3 + c-*kit* ligand	129 ± 33 (4)[d]	N.D.[e]

[a]CD34⁺ bone marrow cells were placed in liquid culture with either no factor, 2 ng/ml IL-3 or IL-3 and 20 ng/ml c-*kit* ligand. One or two weeks later, cells were subcultured in methylcellulose containing PHA-LCM to stimulate colony growth from CFU-GM
[b]Values are the mean ± SE calculated from the mean number of colonies from three plates for each experiment.
[c]Number of different donor marrows.
[d]By paired *t*-test significantly different ($p = 0.02$) from the number in cultures with IL-3 alone.
[e]Not determined.

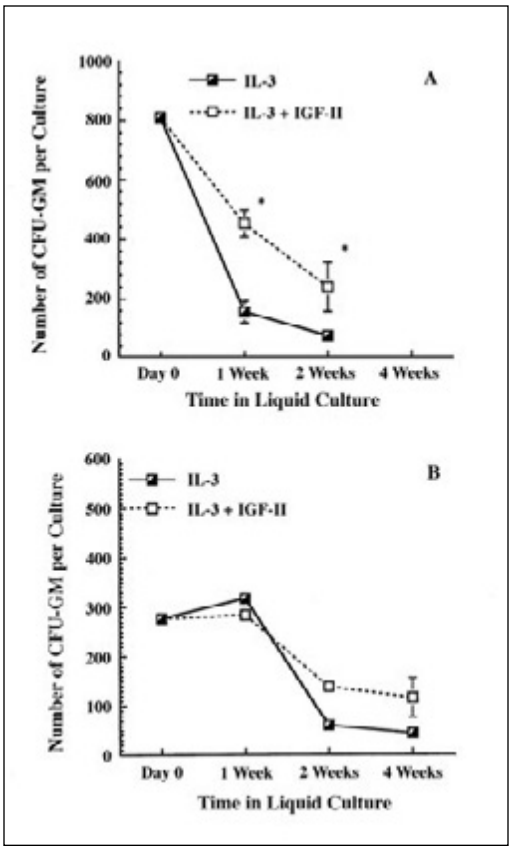

Figure 3. Comparison of the effect of IL-3 and IL-3 plus IGF-II on the number of CFU-GM in serum-substituted liquid cultures of CD34⁺ bone marrow cells. *Cells (1 × 10⁴) were placed in an enriched serum-substituted medium containing 2 ng/ml IL-3 (no insulin or IGF-II added to the medium) or in medium containing IL-3 plus 100 ng/ml IGF-II. Weekly, cells were subcultured in methylcellulose containing 10% PHA-LCM for the detection of colonies derived from CFU-GM. The mean number of colonies from three plates observed after 14 days was used to calculate the total number of CFU-GM per liquid culture. Values for IL-3 + IGF-II are the mean ± SE of the mean values from two Gel-Well cultures or the mean value from one Gel-Well culture for cells incubated with IL-3 alone. A and B are the results from two different donor marrows.*

[Leu43]IGF-II and [Leu27]IGF-II) or IGF-I and insulin receptors (i.e., [Arg54,Arg55]IGF-II and [Thr48,Ser49,Ile50]IGF-II) was substituted for IGF-II. Similar to the results observed with IGF-II, both [Arg54,Arg55]IGF-II and [Thr48,Ser49,Ile50]IGF-II promoted an increase in cellularity that was greater than with IL-3 alone (Fig. 5A). In contrast, neither [Leu43]IGF-II nor [Leu27]IGF-II promoted an additional increase in cellularity over that observed with IL-3 alone (Fig. 5B). The number of CFU-GM in cultures supplemented with a combination of IL-3 and either [Arg54,Arg55]IGF-II or [Thr48,Ser49,Ile50]IGF-II was greater than the number in cultures supplemented with IL-3 alone or a combination of IL-3 and [Leu43]IGF-II or [Leu27]IGF-II (Table 4). Similar results were observed for BFU-E (data not shown). In these studies, neither [Leu27]IGF-II nor [Leu43]IGF-II appeared to have an influence on the proliferation and maintenance of CFU-GM in cultures of CD34⁺ bone marrow cells. These results are consistent with the possibility that the increased production or maintenance of CFU-GM with IGF-II was mediated through the IGF-I or insulin receptors rather than through the IGF-II/CIM6-P receptors.

Effect of IGF-II and Mutant Forms of IGF-II on Production of Megakaryocytes

Initial studies demonstrated that 6 to 200 ng/ml IGF-II, [Arg54,Arg55]IGF-II and [Thr48,Ser49,Ile50]IGF-II, but not [Leu43]IGF-II or [Leu27]IGF-II, enhanced the proliferation of the CMK cell line established from human megakaryoblastic leukemia cells (data not shown). In a preliminary study, when 1 × 10⁴ CD34⁺ bone marrow cells were incubated for one week with IL-3 alone there was a 2.2-fold increase in cellularity. Mature megakaryocytes similar to the large cells with at least four tethered nuclei shown in Figure 6 were observed in those cultures. In cultures with both IL-3 and 100 ng/ml IGF-II, there were 1.4-fold more mature megakaryocytes (i.e., 350 with IL-3 alone versus 500 with IL-3 + IGF-II) associated with a similar further increase in total cellularity.

In order to determine the effect of IGF-II on immature and mature megakaryocyte lineage cells produced by CD34⁺ cells in liquid cultures an ELISA was used to detect platelet glycoproteins GP IIb/IIIa and GP Ib. CD34⁺ cells were cultured with IL-3 and c-*kit* ligand with and without the addition of IGF-II. Ten to eleven days later an ELISA was used to detect platelet glycoproteins GP IIb/IIIa and GP Ib which were expressed as absorbance units. IGF-II in com-

Table 3. Effect of IGF-II on the number of CFU-GM in liquid cultures of CD34$^+$ bone marrow cells[a]

Factors in Culture	Percentage of Number in Cultures with IL-3 Alone[b]	
	After 1 Week of Incubation (n)[c]	After 2 Weeks of Incubation (n)[c]
IL-3 + IGF-II	186 ± 21 (11)[d]	201 ± 36 (7)[d]
IL-3 + c-*kit* ligand	255 ± 36 (4)[d]	N.D.[e]
IL-3 + c-*kit* ligand + IGF-II	255 ± 50 (4)[d]	N.D.[e]

[a]CD34$^+$ bone marrow cells were placed in liquid culture. One or two weeks later, cells were subcultured in methylcellulose containing PHA-LCM to stimulate colony growth from CFU-GM. At day 0, the cultures were supplemented with either 2 ng/ml IL-3, IL-3 and 100 ng/ml IGF-II, IL-3 and 20 ng/ml c-*kit* ligand, or a combination of IL-3, c-*kit* ligand, and IGF-II.
[b]Values are the mean ± SE calculated from the mean number of colonies from three plates for each experiment.
[c]Number of different donor marrows.
[d]By paired t-test significantly different ($p < 0.02$) from the number in cultures with IL-3 alone.
[e]Not determined.

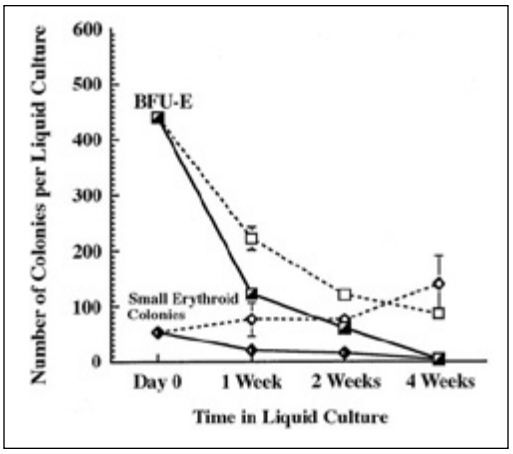

Figure 4. Comparison of the effect of IL-3 and IL-3 plus IGF-II on the number of BFU-E in serum-substituted liquid cultures of CD34$^+$ bone marrow cells. *Cells (1×10^4) were placed in an enriched serum-substituted medium containing 2 ng/ml IL-3 (no insulin or IGF-II added to the medium) or in medium containing IL-3 plus 100 ng/ml IGF-II. One and two weeks later, cells were subcultured in methylcellulose containing 10% PHA-LCM and 2 U/ml erythropoietin for the detection of colonies derived from BFU-E (≥100 erythroid cells) and small erythroid colonies (20 to 50 cells). The mean number of colonies from three plates observed after 14 days was used to calculate the total number of colonies per liquid culture. Solid lines are values from cultures supplemented with IL-3 alone and dashed lines are values from cultures supplemented with IL-3 and IGF-II. Values for IL-3 + IGF-II are the mean ± SE of the mean values from two Gel-Well cultures or the mean value from one Gel-Well culture for IL-3 alone. The data for this figure are from the same experiment as shown in Figure 3B.*

bination with IL-3 and c-*kit* ligand promoted an increase in both the total number of cells and the number of megakaryocyte lineage cells that was greater than with IL-3 and c-*kit* ligand alone (Fig. 7). The increase in the number of cells expressing GP IIb/IIIa in response to IGF-II was also detected by an increase in absorbance values from 0.286 ± 0.019 in cultures without IGF-II to 1.783 ± 0.136 (mean ± SD for $n = 3$) in cultures with both IL-3 and 100 ng/ml of IGF-II. Absorbance values for expression of GP IIb/IIIa and GP Ib were also increased when either [Arg54,Arg55]IGF-II or [Thr48,Ser49,Ile50]IGF-II was substituted for rIGF-II (Fig. 8). In contrast, neither [Leu27]IGF-II nor [Leu43]IGF-II promoted an increase in absorbance values that was greater than with IL-3 and c-*kit* ligand alone. These results are consistent with the possibility that the increased production of megakaryocyte lineage cells with IGF-II was mediated through the IGF-I or insulin receptors rather than through the IGF-II/CIM6-P receptors.

Discussion

The studies in this report investigated the effect of IGF-II on cellularity and the number of committed myeloid and erythroid progenitor cells in serum-substituted liquid cultures of CD34$^+$ bone marrow cells. In these studies, IL-3 alone (i.e., no IGF or insulin added to the medium) or IL-3 plus c-*kit* ligand promoted the proliferation of CD34$^+$ bone marrow cells. When the cultures were also supplemented with IGF-II there was a further twofold increase in the total number of cells. IGF-II also promoted an increase in the

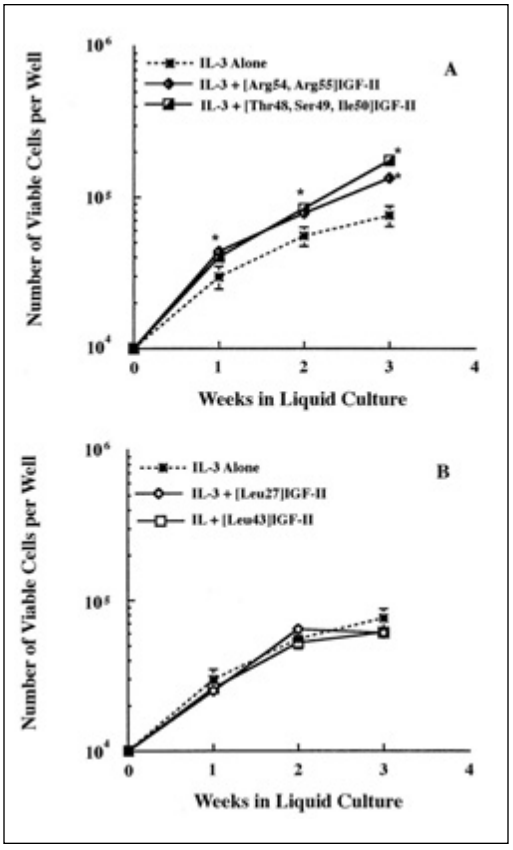

Figure 5. Effect of mutant forms of IGF-II on the number of cells in serum-substituted liquid cultures of CD34⁺ bone marrow cells. *Cells (1×10^4) were placed in an enriched serum-substituted medium containing 2 ng/ml IL-3 (no insulin or IGF-II added to the medium), in medium containing IL-3 plus 100 or 150 ng/ml of one of the mutant forms of IGF-II. For individual experiments the same concentration was used for all four mutant forms of IGF-II. Weekly, all of the cells from Gel-Wells or Transwells were removed, and the number of viable cells per well was determined. The number of cells recovered from cultures supplemented with mutant forms of IGF-II was compared to the number in cultures with IL-3 alone. IGF-II was substituted with: A) mutant forms of IGF-II with a normal to high affinity for the IGF-I and insulin receptors and a reduced affinity for IGF-II/CIM6-P receptors or B) mutant forms of IGF-II with normal affinity for IGF-II/CIM6-P receptors and a reduced affinity for IGF-I receptors. Values are the mean ± SE calculated from seven experiments for one and two weeks and from three experiments at three weeks. *By paired* t*-test, the number of cells was significantly different (*$p \leq 0.04$*) from the number with IL-3 alone.*

number of megakaryocyte lineage cells and an increase in the proportion of mature differentiated granulocytes. The increase in cellularity with the addition of IL-3 alone was associated with a decrease in the number of myeloid and erythroid colony-forming cells. The additional increase in cellularity observed with IL-3 and IGF-II was not associated with a further reduction in the number of colony-forming cells. These results suggest that IGF-II supported the maintenance of a greater number of committed progenitor cells. To identify the receptors through which IGF-II augmented in vitro hematopoiesis, one of the mutant forms of IGF-II that has a high affinity for either IGF-II/CIM6-P receptors or IGF-I and insulin receptors and a reduced affinity for the heterologous receptor was substituted for IGF-II [7]. The results indicate that the increase in cellularity and differentiation observed in cultures with IGF-II was mediated primarily through the IGF-I or insulin receptors rather than through the IGF-II/CIM6-P receptors.

IGF-II has been shown to be important in promoting the proliferation and differentiation of fetal and embryonic cells including myoblasts, metanephros and transplanted embryos [2, 6, 8-10, 12-14, 35]. Others demonstrated that 0.2 to 1000 ng/ml IGF-II increased the number of erythroid and granulocyte-macrophage colonies formed in serum-substituted colony growth medium containing saturating concentrations of erythropoietin or other growth factors [18-22]. Colony size was also increased in cultures supplemented with IGF-II [21-22]. A previous report demonstrated that IGF-II increased IL-3-induced expansion of CFU-GM in serum-substituted liquid cultures of peripheral blood cells [23]. The present studies were expanded to investigate the effect of IGF-II on the proliferation and differentiation of granulocyte-macrophage, erythroid and megakaryocyte progenitors from CD34⁺ bone marrow cells.

In these studies, IGF-II alone (i.e., no other growth factors were added to the medium) had no effect on the number of cells in liquid cultures of CD34⁺ bone marrow cells. In contrast, IGF-II amplified the stimulatory effects of IL-3 and c-*kit* ligand on cellularity and differentiation. With IL-3 alone, cellularity increased throughout four weeks of culture and there was also a progressive development of mature band and segmented stages of granulocytes from the more immature blast and myelocyte stages. When the cultures were also supplemented with IGF-II, there was

Table 4. Effect of mutant forms of IGF-II on the number of CFU-GM in liquid cultures of CD34⁺ marrow cells[a]

Mutant Forms of IGF-II	Percentage of Number in Cultures with IL-3 Alone[b]	
	After 1 Week of Incubation (*n*)[c]	After 2 Weeks of Incubation (*n*)[c]
Reduced Affinity for IGF-II/CIM6-P Receptors		
IL-3 + [Arg54,Arg55]IGF-II	174 ± 28 (7)[d]	201 ± 53 (4)[d]
IL-3 + [Thr48,Ser49,Ile50]IGF-II	142 ± 16 (5)[d]	130 ± 33 (4)
Reduced Affinity for IGF-I and Insulin Receptors		
IL-3 + [Leu27]IGF-II	95 ± 11 (5)	81 ± 29 (4)
IL-3 + [Leu43]IGF-II	108 ± 33 (5)	121 ± 35 (4)

[a]CD34⁺ bone marrow cells were placed in liquid culture. One or two weeks later, cells were subcultured in methylcellulose containing PHA-LCM to stimulate colony growth from CFU-GM. At day 0, the cultures were supplemented with 2 ng/ml IL-3 and 100 or 150 ng/ml of one of the mutant forms of IGF-II.
[b]Values are the mean ± SE calculated from the mean number of colonies from three plates for each experiment.
[c]Number of different donor marrows.
[d]By paired *t*-test significantly different ($p < 0.05$) from the number in cultures with IL-3 alone.

a further twofold increase in total cellularity, and a greater proportion of granulocytic cells were of the mature band and segmented stages. Megakaryocyte lineage cells were detectable when CD34⁺ bone marrow cells were stimulated with IL-3 alone or IL-3 plus c-kit ligand, however, there was an increased number of megakaryocytes in cultures also supplemented with IGF-II. Results of studies by *Debili et al.* [36] indicated that megakaryocyte lineage cells express the platelet glycoprotein GP IIb/IIIa during early stages of differentiation and that GP Ib is expressed at more mature stages of megakaryocyte development. In the present studies, there was an increase in the expression of both GP IIb/IIIa and GP Ib. These results suggest that, in addition to stimulating an increase in the production of megakaryocytes, IGF-II also had stimulatory effects on megakaryocyte maturation. There were also more CFU-GM and BFU-E in cultures with both IGF-II and IL-3 than in cultures with IL-3 alone. The results of the present studies demonstrate that in cytokine-supplemented media, IGF-II augmented both the proliferation and differentiation of CD34⁺ bone marrow cells while maintaining a greater number of committed progenitor cells.

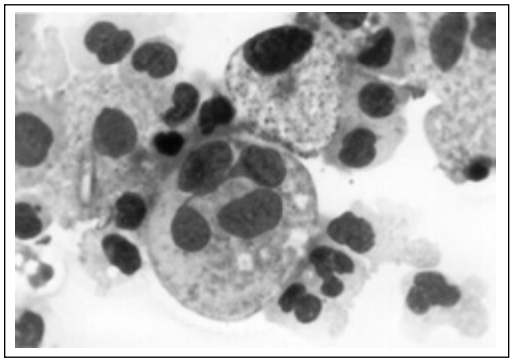

Figure 6. Photomicrograph of mature megakaryocyte lineage cells similar to those detected in liquid cultures of CD34⁺ bone marrow cells incubated with IL-3 and both IL-3 and IGF-II. *Figure is from 100× magnification.*

IGF-II binds with moderate to high affinity to IGF-II/CIM6-P, IGF-I and insulin receptors [1, 2]. The mitogenic effects of IGF-II on many types of cells have been shown to be mediated through the IGF-I receptor [1, 2, 7]. For example, studies by *Merchav et al.* [21, 22] demonstrated that the stimulatory effects of IGF-II on myeloid and erythroid colony formation were prevented when IGF-I receptors were blocked with the anti-IGF-I receptor antibody, αIR-3. The results of studies with K562 erythroleukemia cells, however, indicate that the binding of IGF-II to IGF-II/CIM6-P receptors may also stimulate cell proliferation [11]. In the present studies, the effect of the mutant

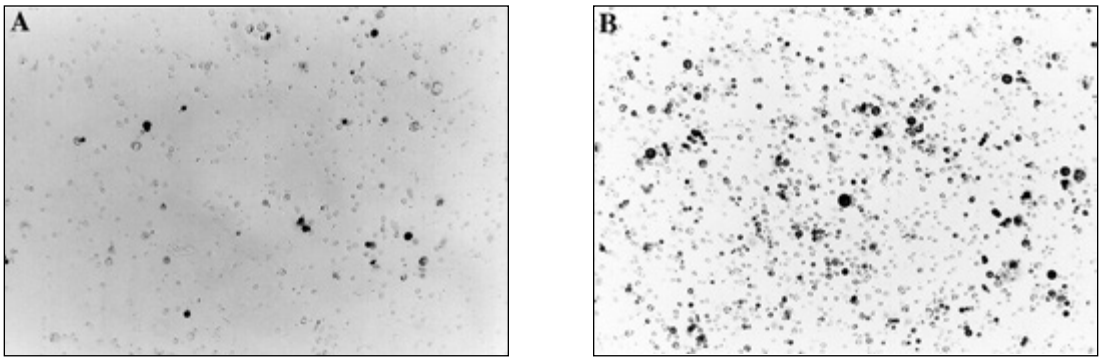

Figure 7. Immunocytochemical staining for the detection of GP IIb/IIIa positive cells. *The photomicrographs show that when compared to IL-3 plus c-*kit *ligand alone (A), 100 ng/ml IGF-II promoted an increase in cellularity and in the number of cells positive for GP IIb/IIIa (B) that corresponded to an increase in absorbance.*

forms of IGF-II on the proliferation of $CD34^+$ bone marrow cells was investigated. Analogs of IGF-II prepared by site-directed mutagenesis were designed to selectively interact with either IGF-I or IGF-II/CIM6-P receptors and have a greatly reduced affinity for the other receptor type [7]. For example, [Leu27]IGF-II and [Leu43]IGF-II have a normal affinity for the IGF-II/CIM6-P receptor, but an 80- to 220-fold reduced affinity for the IGF-I and insulin receptors. In contrast, [Thr48,Ser49,Ile50]IGF-II and [Arg54,Arg55]IGF-II have normal to high affinities for the IGF-I and insulin receptors, but low or no affinity for the IGF-II/CIM6-P receptors. Both [Arg54,Arg55]IGF-II and [Thr48,Ser49,Ile50]IGF-II promoted an increase in cellularity that was similar to that of recombinant IGF-II. In contrast, neither [Leu27]IGF-II nor [Leu43]IGF-II promoted an increase in cellularity that was greater than with IL-3 alone. These results along with those of *Merchav et al.* [21, 22] are consistent with the possibility that the stimulatory effects of IGF-II on the proliferation of $CD34^+$ bone marrow cells were mediated through the IGF-I or insulin receptors rather than through the IGF-II/CIM6-P.

Result by *Rogers et al.* [10] and *Rosenthal et al.* [13] demonstrated that in vitro differentiation of developing renal anlage and smooth muscle, respectively, was mediated through IGF-II/CIM6-P receptors [10, 13]. In the present studies, IGF-II promoted an increase in the production of megakaryocytes from $CD34^+$ bone marrow cells that was detected by an increase in expression of the platelet glycoproteins GP IIb/IIIa and GP Ib. A similar increase in platelet

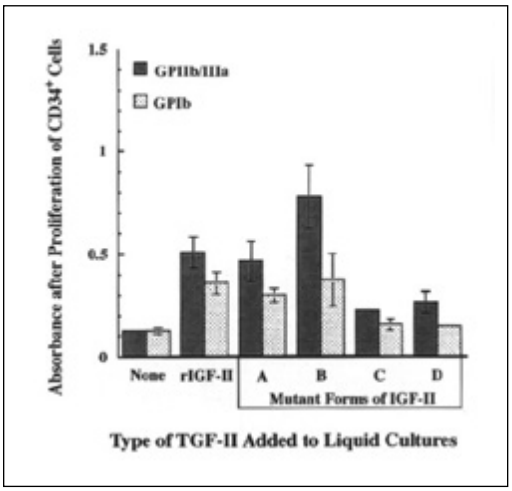

Figure 8. Effect of mutant forms of IGF-II on the production of megakaryocytes from $CD34^+$ bone marrow cells. *Cells (4×10^3) were placed in wells of 96-well plates containing an enriched serum-substituted medium containing 2 ng/ml IL-3 plus 20 ng/ml c-*kit *ligand (no insulin or IGF-II added to the medium) or in medium containing IL-3 plus 100 ng/ml of [Arg54,Arg55]IGF-II (A), [Thr48,Ser49,Ile50]IGF-II (B), [Leu27]IGF-II (C), or [Leu43]IGF-II (D). Eleven days later the cells were fixed and stained for platelet glycoproteins GP IIb/IIIa and GP Ib. The expression of GP IIb/IIIa and GP Ib was measured by ELISA and expressed as absorbance units. The values are the mean ± SD from three wells.*

glycoprotein expression was observed when IGF-II was substituted with either [Arg54,Arg55]IGF-II or [Thr48,Ser49,Ile50]IGF-II, but not with either [Leu27]IGF-II or [Leu43]IGF-II. Results in the present studies suggest that in addition to promoting an increase in megakaryocyte production and maturation, [Arg54,Arg55]IGF-II also increased the differentiation rate of granulocytes. These results suggest that stimulatory effects of IGF-II on the differentiation of granulocyte and megakaryocyte lineage cells was not mediated through IGF-II/CIM6-P receptors, but by binding of IGF-II to IGF-I or insulin receptors.

The results in the present studies demonstrate that physiological concentrations of IGF-II augmented cytokine-induced proliferation and differentiation of $CD34^+$ bone marrow cells. These results and those by others [18-23] suggest that in addition to its role in fetal development, IGF-II may have a role in the regulation of adult hematopoiesis. It is not clear whether the stimulatory effects of IGF-II on in vitro hematopoiesis are in response to direct effects on $CD34^+$ progenitor cells or to effects of IGF-II on other cells that are produced in response to IL-3 and c-*kit* ligand. In other studies, the cell suspensions were not necessarily depleted of accessory cells such as monocytes and lymphocytes or of relatively immature committed cells that may have responded to IGF-II. In the present studies, bone marrow cells with a relatively high purity of $CD34^+$ cells (≥95%) were used, however, studies of the effects of IGF-II on cell proliferation were not performed until at least one week of culture. During that time, IL-3 alone promoted cell proliferation and differentiation. IGF-II may have enhanced further differentiation or acted as a survival factor for cells produced in response to other stimulatory factors. Single cell experiments and studies at earlier times will be important in delineating the direct and indirect effects of IGF-II on $CD34^+$ cells. Multiple pathways appear to be important in regulating the biological functions and gene expression of IGF-II. Some effects of IGF-II are related to direct interactions of IGF-II with IGF-I or IGF-II/CIM6-P receptors [1, 2, 7, 10, 13, 14, 35] or regulated by its interaction with IGF-binding proteins [2]. IGF-II can also regulate the gene expression of IGF-II [12, 35], the expression of IGF-I receptors [12], the cell surface expression of IGF-II/CIM6-P receptors [37], and act as a second signal for proliferation [38]. The importance of these other aspects of IGF-II function and regulation on hematopoiesis are not known.

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