Preparation of a recombinant chimaera of insulin-like growth factor II and interleukin 3 with high proliferative potency for haemopoietic cells

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We have found that a slightly modified insulin-like growth factor II (IGF II) consisting of a chimaera of bombyxin and human IGF II (BOMIGF) is properly secreted in insect cells by using the baculovirus expression system. Human interleukin 3 (IL-3) was attached to the C-terminal amino acid residue of BOMIGF with peptide linkers containing five or twelve residues. Only the chimaera with the 12-residue linker had biological activities of both IGF II and IL-3. The chimaera had a significantly higher mitogenic activity than IL-3 in cell cultures of the human haemopoietic cell line TF-1 and its effect could be observed even at femtomolar concentrations. It was also able to stimulate

thymidine incorporation in IGF II-dependent bovine fetal erythroid cells. The chimaera significantly increased the number of macroscopic haemopoietic colonies in cultures of human peripheral blood in comparison with IL-3 or mixtures of IL-3 and BOMIGF *in vitro*. Subcutaneous injection of a BOMIGF–mouse IL-3 chimaera in normal C57BL/6 mice resulted in a significant increase of the number of spleen stem cells producing macroscopic haemopoietic colonies. This new system for the biosynthesis of IGF–cytokine fusion proteins in insect cells might prove advantageous for the low-cost and high-yield production of molecules with complementary or synergistic biological activities.

INTRODUCTION

Insulin-like growth factors (IGFs) can stimulate cell proliferation or differentiation in a large variety of tissues [1]. Although the effects of IGFs in haemopoietic cells were discovered in cells of the erythroid lineage [2–4], it is now evident that their target cells include many other lineages of the haemopoietic system [5]. This is particularly evident when IGFs are administered *in vivo* [6]. One of the problems associated with the use of IGFs as therapeutic agents is the relatively high cost of production. This is due to the presence of three cystine bridges with almost identical free energies of formation [7,8]. The expression in conventional bacterial or yeast expression systems results in the production of biologically inactive proteins that have to be refolded to the proper conformation [9]. The same problem persists in expression systems with insect cells [10]. Nevertheless, if IGF II is expressed as a chimaera with the signal peptide and the N-terminal sequence of the insulin-like insect hormone bombyxin, a properly folded, biologically active molecule (BOMIGF) can be produced in a secreted form [11]. Processing and secretion persist with more complex constructs consisting of small peptides attached to the C-terminal region of IGF II with a polyglycine linker [12]. It has been shown that cytokine chimaeras such as the fusion protein between granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin 3 (PIXY321) show an enhanced biological effect when compared with the single cytokines alone or in combination [13,14]. Because IGFs act often concurrently with other growth factors in a synergistic way, they represent the potentially most advantageous candidates for the production of divalent growth factor chimaeras. In this paper we describe the production of a chimaera of BOMIGF and interleukin 3 (IL-3) with a high proliferative activity for haemopoietic cells, which surpasses in some target cells the additive effects of BOMIGF and IL-3. These chimaeras might be useful not only as therapeutic agents but also as tools

for the study of the supramolecular organization of membrane receptors, heterologous receptor transactivation [15] or synergistic signal transduction pathways.

EXPERIMENTAL

Materials

All materials for tissue culture were obtained from Gibco-Life Technologies (Burlington, Ontario, Canada) with the exception of bovine transferrin (ICN Biochemicals), BSA (fatty acid-free, tissue culture grade, Sigma, St. Louis, MO, U.S.A.) and the semisolid culture media for haemopoietic stem cells (Stem Cell Technologies, Vancouver, BC, Canada). *methyl*-[³H]Thymidine (50 Ci/mmol) was from ICN. Baculovirus transfer vectors, linearized baculovirus DNA and liposomes were obtained from Invitrogen (La Jolla, CA, U.S.A.). The water-soluble Tetrazolium/formazan reagent Alamar Blue used in cell proliferation assays, haemopoietic growth factors and the ELISAs for human and mouse IL-3 were obtained from Biosource International (Medicorp, Montreal, Quebec, Canada) and in some cases from R&D Systems (Minneapolis, MN, U.S.A.). The human IL-3 (hIL-3) and mouse IL-3 (mIL-3) synthetic cDNA vectors were purchased from R&D Systems. IL-6 was from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Oligonucleotides were synthesized by the Sheldon Biotechnology Centre (McGill University, Montreal, Quebec, Canada).

Construction of new pBluebac–BOMIGF transfer vectors

PCR directed mutagenesis was performed using as template a modified pBluebacI-BOMIGF with the polyglycine linker GGGGT at the C-terminal end of the BOMIGF sequence, identical with that of the heparin-binding BOMIGF previously described [12]. The first transfer vector used in this paper contained the restriction sites *Nco*I and *Hind*III introduced by

Abbreviations used: BOMIGF, chimaera of bombyxin and human IGF II [NQPQMVHTY-hIGF II(9–67)]; BOMIGF-(His)₆, BOMIGF-GGGGTGDDDDKSMGPDIEGRHHHHHH; BOMIGF–IL-3, chimaera of BOMIGF and human IL-3; GM-CSF, granulocyte/macrophage colony-stimulating factor; h, human; IGF, insulin-like growth factor; IL-3, interleukin 3; m, mouse; SCF, stem cell factor; TFA, trifluoroacetic acid.

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PCR with the *Pfu* DNA polymerase (Stratagene, La Jolla, CA, U.S.A.) after annealing and amplifying the BOMIGF template indicated above with a synthetic oligonucleotide encoding both restriction sites immediately after the polyglycine linker. These restriction sites were used to integrate the IL-3 gene, as described below and in Figure 1(A). The second transfer vector was constructed from the original BOMIGF gene [11]. The vector containing BOMIGF was annealed with an oligonucleotide of 88 bases (AAGCTTAATGGTGGTGATGATGGTGTGCGACCC-TCAATCAGATCTGGGCCCATGGACTTATCGTCATC-GTCGCCGACAGTTCCGCCACC), which contains, in order, a polyglycine linker region, a sequence coding for an enterokinase cleavage site, a multiple cloning region including sites for *NcoI*, *Apal* and *BglII*, a region encoding a factor Xa cleavage site, a hexahistidine tail and finally a terminal *HindIII* site with a stop codon (see Figure 1B). After elongation with the *Pfu* DNA polymerase, the new BOMIGF-(His)₆ was amplified by using two oligonucleotide primers coding for the N-terminal region (including a *BamHI* site) and the C-terminal region (with the *HindIII* site) of the modified molecule. The PCR product was cloned into pCR-Script SK(+) (Stratagene). DNA from selected clones was digested with *BamHI* and *HindIII* to detect inserts of the expected size. The sequence integrity of positive clones was verified by T7 sequencing (Pharmacia Biotech, Baie d'Urfe, Quebec, Canada). PCR-Script-BOMIGF-(His)₆ was digested with *BamHI* and *HindIII* and ligated with the pBluebacIII vector (Invitrogen, Sorrento Valley, CA, U.S.A.).

Construction of pBluebacIII-BOMIGF-IL-3 transfer vectors

The hIL-3 vector (R&D Systems) was modified by PCR with the *Pfu* DNA polymerase so as to introduce an *NcoI* site at the 5' end and a *BglII* site at the 3' end. The amplified PCR fragment was cloned into pCR-Script SK. The resulting pCRSK-hIL-3 clone was sequenced to verify the integrity of the modified hIL-3 gene. It was then digested with *NcoI* and *BglII* and inserted into the multiple cloning region of the pBluebac-BOMIGF-(His)₆ vector described above (see Figure 1C).

The mIL-3 vector was modified by PCR in the same fashion as the hIL-3 gene except that an *NcoI* site at the 5' end and a *HindIII* site at the 3' end were added in order to insert it in pBluebacIII-BOMIGF-(His)₆. Because of the use of a *HindIII* site the pBluebacIII-BOMIGF-mIL3 vector did not contain the factor Xa site and the hexahistidine tail.

Production and purification of recombinant chimaeras

The pBluebacIII-BOMIGF-IL-3 vectors were transfected with linear wild-type baculovirus DNA in Sf9 cells by using the cationic liposome transfection kit of Invitrogen. Recombinant viruses were purified as previously described [11] and selected by measuring the presence of recombinant protein in infection supernatants, using as bioassay the thymidine-incorporation-stimulating activity in fetal bovine liver cells [16] or IL-3 ELISAs. Pure recombinant virus was used to infect 5×10^8 Sf9 cells at a multiplicity of infection of 2 for the production of BOMIGF-hIL3, and a multiplicity of infection of 5 for the BOMIGF-mIL3. Collection of 200–250 ml of culture supernatants was done 3 days after infection. The purification of recombinant proteins was performed by three reverse-phase HPLC separations [12]. Supernatants acidified to 1% (v/v) trifluoroacetic acid (TFA) were first loaded on a 50 mm $\times$ 300 mm Bondapak C₁₈ column (Waters-Millipore, Milford, MA, U.S.A.) and eluted with a linear gradient of 0–80% (v/v) acetonitrile in 0.1% (v/v) TFA in water over 20 min at a flow rate of 100 ml/min. Pooled fractions with biological activity were subsequently loaded on a

preparative Vydac C₄ column (22 mm $\times$ 250 mm; SPE, Concord, Ontario, Canada) and eluted at a rate of 16 ml/min with a gradient of 0–48% (v/v) acetonitrile in 0.1% (v/v) TFA in water for 20 min followed by a 5 min gradient of 48–80% (v/v) acetonitrile in 0.1% (v/v) TFA. Fractions with thymidine-incorporating activity were applied to a Vydac C₄ semipreparative column (10 mm $\times$ 250 mm) and eluted at a rate of 3 ml/min with a gradient of 0–21% (v/v) acetonitrile in 0.1% (v/v) TFA in water for 15 min, 21–72% (v/v) acetonitrile in 0.1% (v/v) TFA for 60 min and 72–80% (v/v) acetonitrile in 0.1% (v/v) TFA for 5 min. Fractions with thymidine-incorporation-stimulating activity were tested for purity by SDS/PAGE and silver staining (Phast system, Pharmacia). The preparations were quantified by amino acid analysis, ELISAs or the Bradford protein assay [17].

Cell proliferation assays

The haemopoietic cell line TF-1 was maintained in culture in the presence of GM-CSF (0.1–1 ng/ml) in RPMI 1640 supplemented with 10% fetal bovine serum [18]. Before the addition of cytokines, cells were starved for 18 h at a density of 2×10^5 cells/ml in RPMI 1640 with either (1) 0.5% (v/v) fetal bovine serum or (2) 300 μ g/ml albumin supplemented with 30 μ g/ml bovine transferrin. Plates with 96 wells were precoated with albumin by the addition of 150 μ l of a 1% (w/v) BSA solution in RPMI 1640 medium followed by incubation at 37 °C for at least 2 h. Wells were washed twice with 0.2 ml of RPMI medium. Serial dilutions of factors were made in the wells in 150 μ l of RPMI 1640 medium supplemented with albumin-transferrin as indicated above. TF-1 cells (10^5) in 50 μ l were added to each well to give a final incubation volume of 0.2 ml. Proliferation of cells was assessed after 3 days of incubation at 37 °C by the addition of 22.5 μ l of Alamar Blue solution containing 1.5 μ Ci of [³H]thymidine. The absorbances at 570 and 630 nm were determined after 4 h of incubation at 37 °C (Amalar Blue quantification). An aliquot of 180 μ l was taken from each well, mixed with ice-cold HBSS and further processed for the determination of thymidine incorporation as previously described [16].

Fetal bovine erythroid cells were prepared and cryopreserved until use as previously indicated [19]. Incubations and thymidine incorporation were done by following the procedures for the determination of BOMIGF dose-response curves [11].

Assay for haemopoiesis *in vitro* with human peripheral blood

Haemopoietic colony-forming cells present in human peripheral blood cells from normal donors were prepared by the Ficoll-Paque method [20] and plated at a cell density of 4×10^5 cells/ml in methylcellulose medium containing 0.9% methylcellulose, 30% (v/v) fetal bovine serum, 1% (w/v) BSA, 100 mM 2-mercaptoethanol, 2 mM glutamine in Iscove's modified Dulbecco's minimum essential medium (Methocult HCC-4230; Stem Cell Technologies, Vancouver, BC, Canada). The medium contained 3 units/ml erythropoietin, 25 ng/ml human stem cell factor (SCF), 20 ng/ml human IL-6 and 20 ng/ml human GM-CSF. The cells were incubated with the different recombinant chimaeras, BOMIGF and IL-3 as indicated in the figures. Colonies were scored after 10–15 days in culture. Macroscopic colonies (more than 0.5 mm in diameter) were counted directly without a microscope.

Quantification of colony-forming cells in spleen and bone marrow after administration of BOMIGF-mIL-3 *in vivo*

The methods described here are based, with a few modifications, on the protocol described by Tzarfaty et al. [6] for the quantification of the haemopoietic effects of IGF I in normal mice.

C57BL/6 mice (20 g, older than 7 weeks) were injected subcutaneously with 2 μ g of BOMIGF–mIL-3 chimaera in 200 μ l of PBS daily for 6 days. Control mice received the same volume of PBS. At day seven the mice were killed by cervical dislocation. Bone marrow cell suspensions were obtained by flushing femurs and tibia with heparinized alpha medium supplemented with 2% (v/v) fetal bovine serum. Spleen cell suspensions were prepared after flushing the cells through 22-gauge needles. Red blood cells were eliminated by the NH_4Cl method (Stem Cell Technologies). The cells were plated in methylcellulose medium as indicated by Tzarfaty et al. [6], with 9×10^5 and 9×10^4 cells/ml for spleen and bone marrow cells respectively. The medium contained 0.9% methylcellulose, 15% (v/v) fetal bovine serum, 100 mM 2-mercaptoethanol, 2 mM L-glutamine, 1% (w/v) BSA, 10 μ g/ml bovine insulin, 200 μ g/ml human transferrin, 3 units/ml erythropoietin, 10 ng/ml mouse recombinant IL-3, 10 ng/ml human recombinant IL-6 and 50 ng/ml mouse recombinant SCF (Methocult M3434). Colonies were counted after 10 and 14 days of incubation.

Mouse stem cells with high proliferative potential produce very large colonies. Those with diameters larger than 1 mm were considered to be macroscopic and were counted directly without a microscope.

RESULTS

Figure 1 is a schematic diagram of the BOMIGF–IL-3 chimaeras used in this work. The first construct (Figure 1A) was made from a BOMIGF sequence containing a small polyglycine linker as described in the Experimental section. The baculovirus vector coding this fusion protein was sequenced to confirm the in-frame insertion of the IL-3 gene. This vector was transfected together with the linearized wild-type baculovirus DNA; after transfection the recombinant virus was identified by the expression of the β -galactosidase gene (metabolism of 5-bromo-4-chloroindol-3-yl β -D-galactopyranoside) and the production of IL-3 (ELISA). Unfortunately this construct did not have any IGF activity as

identified by the thymidine-incorporation assay of fetal bovine erythroid cells. Nevertheless the HPLC fractions containing the fusion protein could be identified by using the IL-3 ELISA. It was found that the fractions containing the purified protein could stimulate the proliferation of the IL-3-dependent TF-1 cells but were unable to enhance thymidine incorporation into fetal bovine erythroid cells (results not shown). We concluded that this chimaera was properly secreted but that the attachment of IL-3 very close to the IGF moiety was somehow inhibiting the action of BOMIGF in bovine erythroid cells. Therefore a second construct was engineered (Figures 1B and 1C) that not only provided a greater distance between BOMIGF and IL-3 (12 residues), but also introduced new properties to the transfer vector that can be used in the future for the construction and easy isolation of hybrids between IGF and cytokines or other growth factors. These modifications consisted of the introduction, after the polyglycine linker, of an enterokinase cleavage site followed by a multiple cloning site for the insertion of the desired protein. At the C-terminal end, the amino acid sequence IEGR is recognized by the restriction protease factor Xa. This is followed by a hexahistidine tail as a tool for affinity chromatography isolation. An example of one of these chimaeras is the BOMIGF–hIL-3 fusion protein (Figure 1C).

The BOMIGF–IL-3 chimaera shown in Figure 1(C) could be produced in a secreted form in insect cells and isolated by HPLC with three chromatographic steps in a single day. The chimaera had the right amino acid composition and a molecular mass of 32 kDa (SDS/PAGE), which could be cut down to 27 kDa after treatment with peptide:N-glycosidase F (New England Biolabs, Mississauga, Ontario, Canada), a glycosidase that cleaves N-linked carbohydrates of glycoproteins. The comparison of the results of amino acid analysis and ELISAs indicated that the antibodies used in the Biosource ELISA kit for human IL-3 were appropriate for the quantification of the chimaera, whereas the antibody supplied in the R&D kit gave a value that was 1/27 of the results of amino acid analysis. Therefore the use of enzyme or radiolabelled immunoassays for chimaeric molecules have to

Figure 1 Schematic diagram of the fusion proteins used in this work

(A) Chimaera of BOMIGF (fusion protein of bombyxin and hIGF II [11]) and IL-3 joined by a short linker of five residues (protein 239). (B) New expression vector for BOMIGF with a multiple cloning site for the insertion of sequences coding growth factors, cytokines or other proteins. The expression product of this gene is the peptide BOMIGF-(His)₆ or protein 265. (C) Chimaera of BOMIGF and human IL-3 with the long linker of 12 residues and an hexahistidine structure at the C-terminus (protein 337). The corresponding fusion protein with mouse IL-3 but with a C-terminus devoid of factor Xa cleavage site plus hexahistidine will be called 406. This protein was used for experiments *in vivo* (see text).

Figure 2 Comparison of the thymidine-incorporation-stimulating activity of IL-3, BOMIGF and the BOMIGF-IL-3 chimaera into fetal erythroid cells

Fetal bovine erythroid cells (3×10^5) were incubated with increasing concentrations of hIL-3 ($\blacktriangle$), BOMIGF(His)₆ ($\bullet$), the chimaera BOMIGF-IL-3 with the structure shown in Figure 1(C) ($\blacksquare$) and equimolar mixtures of BOMIGF(His)₆ and hIL-3 ($\square$). The cells were incubated for 16 h at 37 °C followed by a 1 h incubation with [3 H]thymidine as previously described [16]. The results are representative of five similar experiments that indicated that the stimulation of thymidine incorporation in cells treated with the high concentrations of the chimaera (60 nM) were significantly higher than those observed with equimolar mixtures of IL-3 and BOMIGF(His)₆ ($P < 0.002$, paired t test).

be evaluated carefully because of possible steric hindrance of the antibodies used.

The new BOMIGF-IL-3 chimaera (Figure 1C) stimulated thymidine incorporation into bovine liver erythroid cells (Figure 2). The dose-response curve is very similar to that obtained with BOMIGF(His)₆ (Figure 1B). The stimulation at higher concentrations (60 nM) was none the less significantly higher than that obtained with equimolar mixtures of BOMIGF and hIL-3, indicating that the linkage between IL-3 and BOMIGF had increased the activity despite the fact that human IL-3 is not active at this concentration (Figure 2, $\blacktriangle$). The high proliferative potential of the chimaera could be observed in the IL-3-dependent haemopoietic cell line TF-1. Preliminary experiments indicated that the best conditions to observe an increase in cell proliferation after treatment with IL-3 was to starve the cells of growth factors overnight in a medium with 0.5% (v/v) fetal bovine serum or with the albumin-transferrin mixture indicated in the Experimental section. The cells were subsequently incubated for 3 days in the presence of the growth factors. Under these conditions it was impossible to see any stimulation of thymidine incorporation or cell proliferation with BOMIGF(His)₆ within a concentration range of 10^{-7} to 5 nM. In only one of four experiments was there a marginal increase in cell numbers (6% of control cultures) at the highest concentration tested (5 nM). The method of starvation had an important effect on the dose-response curves of the chimaera compared with control cell cultures treated with IL-3 (Figure 3). Figure 3(A) shows that the BOMIGF-IL-3 chimaera ($\square$) is more potent than IL-3 ($\triangle$) in the stimulation of cell proliferation in those cell cultures previously starved in medium supplemented with albumin alone. The chimaera (average median effective dose 0.34 pM) was 23 times more active than IL-3 (average median effective dose 7.9 pM, paired t test, $n = 6$, $P < 0.05$). This effect could also be observed, to a smaller extent, when the cells were previously starved in the presence of fetal bovine serum (Figure 3A, $\blacksquare$, $\blacktriangle$). However, the differences

Figure 3 Comparison of the effects of IL-3 and the BOMIGF-IL-3 chimaera on TF-1 cell proliferation

TF-1 cells were incubated with increasing concentrations of hIL-3 ($\triangle$, $\blacktriangle$) or the BOMIGF-IL-3 chimaera shown in Figure 1(C) ($\square$, $\blacksquare$) for 3 days in cells that had previously been starved overnight in a medium supplemented with albumin-transferrin ($\square$, $\triangle$) or 0.5% (v/v) fetal bovine serum ($\blacksquare$, $\blacktriangle$). Then cell proliferation (A) and thymidine incorporation (B) were measured after a 4 h incubation with Amalar Blue and [3 H]thymidine, as indicated in the Experimental section. The results are representative of six different experiments.

between the chimaera and IL-3 at low concentrations (1 fM or less) are more pronounced in these cultures starved in the presence of fetal calf serum than albumin alone. This difference could be better appreciated after a short (4 h) incubation with thymidine at the third day of treatment with the cytokines (Figure 3B). Analysis of the cell proliferation dose-response curves, obtained after starvation with fetal bovine serum, with a curve-fitting program (Graphpad) indicated that the dose-response curve for BOMIGF-IL-3 best fits a double-sigmoid curve, whereas the IL-3 curve (or curves with equimolar mixtures of IL-3 and BOMIGF; results not shown) have a simple dose-response curve with no increases over a wide range of low concentrations (Figure 4). The average median effective dose at high concentrations (picomolar range) was of 11.4, 2.7 and 6.3 pM for IL-3, the BOMIGF-IL-3 chimaera and the equimolar mixture of BOMIGF and IL-3 respectively. The differences between the average median effective dose of the chimaera and IL-3 ($P < 0.01$) or between the chimaera and the equimolar mixture of IL-3 and BOMIGF ($P < 0.05$) were statistically significant ($n = 6$, paired t test). The median effective dose at low concentrations for cells starved in fetal calf serum could not be measured accurately. However, the increase in cell proliferation observed at a concentration of 1 fM was significantly higher in cell cultures treated with the chimaera than in those treated with IL-3 ($P < 0.05$, $n = 6$). No significant differences could be seen between both treatments at the same concentration if the cells

Figure 4 Dose–response curve for BOMIGF–IL-3 fitting a double-sigmoid equation

TF-1 cells were starved overnight in the presence of 0.5% (v/v) fetal bovine serum and incubated with increasing concentrations of BOMIGF–IL-3 (■) or IL-3 alone (●) for 3 days. Cell proliferation was measured by the Amalar Blue method. Results are means $\pm$ S.E.M. for three determinations. Curve fitting was with the program Graphpad. A total of six experiments similar to that illustrated in this figure were done and the results indicated that (A) the numbers of cells in cultures incubated with low concentrations of the chimaera (first plateau of the sigmoid curve, 1 fM) were significantly higher than those in cultures treated with IL-3 ($P < 0.05$); (B) the differences between the effects of the chimaera and IL-3 at high concentrations (second plateau of the sigmoid curve) were non-significant; and (C) the difference between the average median effective dose at high concentrations was significant ($P < 0.01$). Abbreviation: l.c., log concentration.

were previously starved with medium containing the albumin–transferrin mixture.

Preliminary experiments on the effects of the recombinant chimaera and IL-3 on haemopoietic colony formation from human peripheral blood indicated that a concentration of 0.02 pmol/ml was more appropriate for observing changes in colony formation than the amounts usually considered to be optimal for this kind of assay (0.57 pmol/ml IL-3; StemCell Technologies) and therefore the low concentration was used for

Figure 5 Effect of the BOMIGF–IL-3 chimaera on colonies produced from haemopoietic progenitors from human peripheral blood

Haemopoietic progenitors from human blood were enriched by the Ficoll–Paque method and plated in a semisolid medium containing optimal concentrations of erythropoietin, GM-CSF, IL-6, SCF and IGF II [e.g. 30% (v/v) fetal bovine serum] but not IL-3. The recombinant growth factors indicated on the abscissa were added at 0.02 pmol/ml. The columns labelled BOMIGF relate to the BOMIGF(His)₆ of Figure 1(B). The number of colonies formed is expressed as the number originating from 1 ml of blood. Abbreviations: BFU-E, burst-forming units, erythroid; CFU-GM, granulocyte/macrophage colony-forming units. 'Macroscopic' refers to colonies with a diameter of at least 0.5 mm. Results are means $\pm$ S.E.M. for four determinations.

Figure 6 Effect of administration of BOMIGF–mIL-3 *in vivo* to haemopoietic progenitor cells

C57BL/6 were given 2 μ g of BOMIGF–mIL-3 daily for 6 days. Mice were killed at day 7, bone marrow and spleen cells were prepared and colony formation was scored as indicated in the text. Numbers of colonies (left; hatched bars) are means $\pm$ S.E.M. ($n = 3$) for a representative experiment. The average percentage stimulation over the number of colonies observed in cell cultures from control animals is indicated at the right (filled bars). The chimaera significantly increased the number of macroscopic colonies in the spleen ($P < 0.02$, $n = 6$). Abbreviations: BFU-E, burst-forming units, erythroid; CFU-GM, granulocyte/macrophage colony-forming units.

all the experiments with human peripheral cells. Figure 5 shows the effects of BOMIGF, IL-3, an equimolar mixture of BOMIGF and IL-3 and the chimaera on colony formation per ml of human peripheral blood. The cell culture medium had the concentrations considered to be optimal for all required cytokines with the exception of IL-3. The chimaera had the same effects as IL-3, BOMIGF or equimolar mixtures of both factors. The only significant differences between (1) the chimaera and IL-3 and (2) the chimaera and the mixture (IL-3 + BOMIGF) were observed in the increased number of macroscopic colonies, which suggests a preferential effect in cells of high proliferative potential ($P < 0.01$, analysis of variance, Tukey test [21]).

A recombinant factor active *in vitro* might be completely inactive *in vivo*. Therefore we decided to test the mouse equivalent of the BOMIGF–hIL-3 chimaera in normal mice. We compared the action of the recombinant and control mice receiving PBS alone. Figure 6 shows the number of haemopoietic colony-forming cells in spleens and bone marrow cells of animals treated with the recombinant BOMIGF–mIL-3 chimaera. The only significant increase was observed in the number of stem cells producing macroscopic colonies in the spleen, hinting again at a preferential effect in cells of high proliferative potential ($P < 0.02$, $n = 6$, paired *t* test). However, it seems that most colony-forming cells of the spleen and bone marrow increased after treatment with the BOMIGF–mIL-3 chimaera.

DISCUSSION

Chimaeric molecules containing two different cytokines or growth factors could have a practical clinical application as stimulators of growth or differentiation in those cells with receptors for both components of the fusion protein. The best known example of a successful fusion protein is PIXY321, a synthetic hybrid protein of human GM-CSF and IL-3 [13]. This molecule was developed as a potential haemopoietic growth factor with multilineage activity. It was found that some of its biological activities *in vitro* actually surpassed the action of GM-CSF and IL-3 given alone or in combination [13]. In addition, it enhanced granulocyte recovery *in vivo* in a way similar to GM-CSF, and platelet recovery as observed after IL-3 treatment [22].

These cytokines are, however, structurally related and act through the activation of receptors that belong to the same haemopoietic receptor superfamily. We thought that it would be advantageous to produce chimaeric molecules between growth factors and cytokines with receptors of different families and acting through different signal transduction pathways. IGFs are good candidates as partners for cytokines in chimaeric molecules. They stimulate haemopoietic cell proliferation *in vitro* and *in vivo* [2–5] and act through the IGF I receptor, a tyrosine kinase receptor of the insulin receptor superfamily. There is indirect evidence indicating that the IGF I receptors can be *trans*-activated by other growth factor receptors. bFGF, a growth factor that does not stimulate DNA synthesis in certain BALB/3T3 cell lines, is able to do so in the same cells over-expressing the IGF I receptor [23]. In an analogous system of mouse embryo cells, it was found that EGF supported growth only in cells overexpressing the IGF receptors. Furthermore only the IGF receptors of EGF-treated cells were phosphorylated [15]. These experiments indicate that IGFs acting through the IGF I receptor are good candidates for the construction of chimaeric proteins. Unfortunately the large-scale synthesis of recombinant IGFs with conventional bacterial expression systems is cumbersome and expensive [9]. We have solved this problem in the baculovirus expression system by attaching the signal peptide and the N-terminal sequence of the insect insulin-like peptide bombyxin to hIGF II (BOMIGF). In this paper we describe the biological activity of the first IGF–cytokine chimaera produced by taking advantage of this new eukaryotic IGF expression system.

The new BOMIGF–IL-3 chimaera shown in Figure 1(C) was properly secreted by Sf9 insect cells infected with the recombinant baculovirus in a glycosylated form of 32 kDa, which could be converted into a 27 kDa form by peptide:N-glycosidase F, which cuts oligosaccharides from N-linked glycoproteins. The amount of protein produced is far greater than that previously observed for the production of BOMIGF alone [11]. Because glycosylation seems to facilitate the secretion of cytokines such as erythropoietin [24], it is possible that the glycosylated IL-3 might be improving the passage of the chimaera through the secretory pathway.

We have tested the biological activity of the IGF–IL-3 chimaeras *in vitro* with two different cell culture systems. The first consists of a bioassay in which the incorporation of thymidine into fetal bovine erythroid cells is stimulated by low concentrations of IGF or erythropoietin [4,11,12,25,26]. These cells did not respond to human IL-3 (Figure 2). Cells cultured with the chimaera had a significantly higher thymidine-incorporation-stimulating activity than equimolar mixtures of BOMIGF and IL-3. This represents a gain of function compared with IL-3 occurring at concentrations that are much higher than those expected to be required for a simple interaction with cytokine receptors. It is possible that the human IL-3 component of the chimaera might be able to interact with related bovine cytokine receptors at concentrations that might be physiological for IGFs but supraphysiological for cytokines. An alternative explanation would be an improved stability of the chimaera or improved access to the IGF receptors. This possibility is particularly attractive owing to the observation that the C-terminal-extended, glycosylated pro-IGF II is a more potent stimulator of granulocyte/macrophage colony formation of human peripheral haemopoietic stem cells than the mature, low-molecular-mass IGF II in serum-free media [27]. These potential mechanisms could be tested by introducing a stop codon after the first two IL-3 glycosylation sites or by eliminating the cytokine receptor binding through point mutations in IL-3 residues 21 and 22 [28].

The second experimental system *in vitro* was the IL-3-dependent human haemopoietic cell line TF-1. BOMIGF alone could not significantly stimulate growth or thymidine incorporation in these cells. Wang et al. [29] have also found that the stimulation of thymidine incorporation in TF-1 taking place in cells incubated with 1–100 ng/ml IGF I is marginal compared with the effects observed with other cytokines. However, the simultaneous addition of BOMIGF and IL-3 resulted in a shift of the median effective dose of the dose–response curve for cell proliferation from 11.4 to 6.3 pM, indicating a synergistic effect of both cytokines. The first chimaera of BOMIGF and IL-3 (Figure 1A) in which both cytokines were separated by a short linker (GGGGT) had IL-3 activity in TF-1 cells but was unable to stimulate thymidine incorporation into fetal bovine erythroid cells (results not shown). This observation indicated an absence of IGF-like activity of the chimaera. Therefore we designed a new BOMIGF vector (Figure 1B) that was useful for the production of a BOMIGF–IL-3 chimaera (Figure 1C) with activities in both fetal erythroid cells and TF-1 cells. The new vector consisted of the BOMIGF gene, driven by the baculovirus polyhedrin promoter, followed at the C-terminal residue by a flexible polyglycine linker, an enterokinase cleavage site, a multiple cloning site for the insertion of genes for growth factors or cytokines, a factor Xa cleavage site and finally a (His)₆ tail. The (His)₆ could be useful for the affinity purification of BOMIGF–growth factor chimaeras in which the IGF partner might be sensitive to the relatively harsh conditions used here for the purification of the fusion proteins. Because the IGF–IL-3 chimaeras could be isolated fast and efficiently by HPLC, we did not investigate, in this study, the functionality of the two restriction protease cleavage sites, or the utility of the (His)₆ tag for affinity purification.

The BOMIGF–IL-3 chimaera was also a very powerful stimulator of proliferation of TF-1 cells (median effective dose 2.7 pM), indicating that fusion between both molecules represents an advantage over the combination of both cytokines given simultaneously. The hybrid molecule of IGF and IL-3 sequences thus resembles the synergistic effect of PIXY321 [13] compared with combinations of GM-CSF and IL-3. A surprising observation was that the dose–response curve of the chimaera in cells previously starved in the presence of fetal bovine serum corresponded to a double-sigmoid curve. The chimaera was able to increase cell numbers significantly at femtomolar concentrations, suggesting that only few molecules are still able to push the cells through a cell cycle successfully. Alternatively, the chimaera might be improving cell survival at these concentrations, because in many instances the number of cells was marginally greater than the number of cells plated, whereas non-treated cultures showed a decline in cell numbers. This effect could not be seen with IL-3 alone, or, to a smaller extent, with the chimaera in cells previously starved in a serum free medium. These results suggest the requirement of a serum co-factor that facilitates cell proliferation or survival at femtomolar concentrations. Although this action at very low concentrations is not typical for IL-3, other cytokines are active at femtomolar levels [30]. We consider this effect at femtomolar concentrations to be the most significant improvement of the fusion protein over IL-3; it is probably due to the improved survival of IL-3-dependent haemopoietic cells in the presence of IGFs [31]. There is increasing evidence for the protective effect of IGFs against apoptosis (programmed cell death) in different tissues, a property that is mediated by the IGF-I receptor [32,33].

Although artificial fusion proteins might turn out to be very active with established cell lines in an environment *in vitro*, they might be completely ineffective with normal cells *in vitro* or when

injected into intact animals *in vivo*. PIXY321 was particularly effective in intact animals in preclinical studies [22] and in phase I clinical trials [14]. In this paper we demonstrate that the BOMIGF–hIL-3 chimaera is active in the stimulation of haemopoietic stem cell colony formation in normal cells taken from peripheral blood. These experiments were done in a semisolid culture medium optimized for colony growth containing all essential factors with the exception of IL-3. Owing to the overlapping IL-3-like effects of GM-CSF and SCF present in the medium as well as the large amounts of IGFs in fetal bovine serum one should not expect very large differences between the treated cells and control cell cultures. Nevertheless the BOMIGF–IL-3 chimaera significantly and specifically enhanced the total number of macroscopic-colony-forming cells in the semisolid cultures. These large colonies are believed to be formed from stem cells with high proliferative potential [34,35]. It is possible that TF-1 cells belong to this population of primitive stem cells. Although they have receptors for erythropoietin and GM-CSF, they also have the CD34⁺ antigen typical of myeloid pluripotent stem cells [18].

To see whether the BOMIGF–IL-3 chimaera had a haemopoietic effect *in vivo*, we had to construct a new vector in which the species-specific hIL-3 was replaced by mIL-3. This BOMIGF–mIL-3 chimaera could be recognized with the antibodies of an mIL-3-specific ELISA (Biosource); it was biologically active in bovine liver erythroid cell bioassay (IGF-like activity) and in a cell proliferation assay of the mouse MC-9 haemopoietic cell line (IL-3-like activity, results not shown). This BOMIGF–mIL-3 fusion protein was then injected subcutaneously into normal C57BL/6 by following a very similar protocol to that previously used for testing the effects of IGF I in haemopoiesis [6]. The dose used (2 µg/day per mouse) was within the range used by other investigators for mIL-3 (0.2–2 µg per mouse [36–39]) but lower than that used for IGF I (5 per mouse [6]). After 7 days, spleen and bone marrow cells were prepared and the number of haemopoietic colony-forming cells was quantified with a semisolid culture medium. Although there was a general increase in all haemopoietic cell colonies, the only significant difference between controls and BOMIGF–mIL-3-treated cells was the number of macroscopic haemopoietic colony-forming cells present in the spleen. This result is in agreement with the results obtained with the colony-forming cells of human peripheral blood and suggests again a specific effect in cells with high proliferative potential. It is reasonable to suggest that the chimaera might be mobilizing bone marrow stem cells with high proliferative potential into the periphery, followed by lodging in the spleen. To define more closely the role of the chimaera on haemopoietic cells, we have recently administered BOMIGF–mIL-3 to mice treated with azidothymidine, a drug used for AIDS patients that causes a decrease in both white and red blood cell numbers. Mice treated with the chimaera gained the weight lost after treatment with azidothymidine and had an augmentation of all haemopoietic colony-forming cells in bone marrow and spleen. There was a preferential elevation of non-erythroid haemopoietic cells. This was manifested by a significant increase in circulating white blood cell numbers with daily injections of only 14 ng/mouse (M. DiFalco, L. Dufresne and L. F. Congote, unpublished work). These experiments were previously almost impossible to do, owing to the excessive cost of commercial cytokines. This new baculovirus expression system will facilitate many other related preclinical studies of IGF–cytokine fusion proteins.

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