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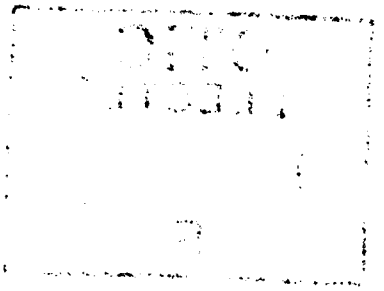

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Mast cell growth factor (C-*Kit* Ligand) in combination with granulocyte-macrophage colony-stimulating factor and interleukin–3: *in vivo* hemopoietic effects in irradiated mice compared to *in vitro* effects

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Abstract

In the presence of hemopoietic cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin–3 (IL–3), mast cell growth factor (MGF; also known as steel factor, stem cell factor, and c-*kit* ligand) has proven to be a potent hemopoietic regulator *in vitro*. In these studies, we examined the *in vivo* effects of MGF in combination with GM-CSF or GM-CSF plus IL–3. Effects were based on the ability of these cytokines to stimulate recovery from radiation-induced hemopoietic aplasia. Female B6D2F1 mice were exposed to a sublethal 7.75-Gy dose of ^{60}Co radiation followed by subcutaneous administration of either saline, recombinant murine (rm) MGF (100 μg/kg/day), rmGM-CSF (100 μg/kg/day), rmIL–3 (100 μg/kg/day), or combinations of these cytokines on days 1–17 postirradiation. Recoveries of bone marrow and splenic spleen colony-forming units (CFU-s), granulocyte macrophage colony-forming cells (GM-CFC), and peripheral white blood cells (WBC), red blood cells (RBC) and platelets (PLT) were determined on days 14 and 17 during the postirradiation recovery period. MGF administered in combination with GM-CSF or in combination with GM-CSF plus IL–3 either produced no greater response than GM-CSF alone or down-regulated the GM-CSF-induced recovery. These results sharply contrasted results of *in vitro* studies evaluating the effects of these cytokines on induction of GM-CFC colony formation from bone marrow cells obtained from normal or irradiated B6D2F1 mice, in which MGF synergized with GM-CSF or GM-CSF plus IL–3 to increase both GM-CFC colony numbers and colony size. These studies demonstrate a dichotomy between MGF-induced effects *in vivo* and *in vitro* and emphasize that caution should be taken in attempting to predict cytokine interactions *in vivo* in hemopoietically injured animals based on *in vitro* cytokine effects.

Abbreviations: GM-CSF; Granulocyte-Macrophage Colonly-Stimulating Factor; IL–3: Interleukin–3; MGF: Mast Cell Growth Factor; SCF: Stem Cell Factor; rm: Recombinant Murine; CFU-s: Colony Forming Unit-Spleen; GM-CFC: Granulocyte Macrophage Colony-Forming Cell; WBC: White Blood Cells; RBC: Red Blood Cells; PLT: Platelets; SLF: Steel Factor; G-CSF: Granulocyte Colonly-Stimulating Factor; IL–1: Interleukin–1; IL–6: Interleukin–6; Epo: Erythropoietin; CFC: Colony-Forming Cell; Sl: Steel; BFU-e: Erythroid Burst Forming Units; s.c.: Subcutaneous; PEG: Polyethyleneglycol; PIXY321: GM-CSF/IL–3 Fusion Protein.

Introduction

One of the most recent cytokines implicated in hemopoietic regulation is c-*kit* ligand, also known as mast cell growth factor (MGF), steel factor (SLF), and stem cell factor (SCF) [1–3]. C-*kit* ligand has been ascribed numerous hemopoietic and nonhemopoietic effects, although it was initially identified and purified based on its ability to stimulate mast cell growth [2–5].

Multiple studies have focused on the *in vitro* effects of this factor, demonstrating that alone it has limited hemopoietic activity, but when combined with other hemopoietic cytokines, including granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin–1 (IL–1), interleukin–3 (IL–3), interleukin–6 (IL–6), and erythropoietin (Epo), it synergizes to increase both the number and size of colonies generated from hemopoietic progenitors [3–11], and in some instances, to increase the replating potential of primitive progenitors [12]. Furthermore, in combination with such factors, c-*kit* ligand also synergistically enhances the *in vitro* expansion of hemopoietic progenitors grown in liquid cultures [13–15]. These effects are thought to result not only from the ability of c-*kit* ligand to potentiate progenitor cell proliferation but also from its ability to enhance progenitor cell survival [14, 16]. The observations that c-*kit* ligand in combination with other cytokines appears to generate large numbers of both committed colony-forming cells (CFC) and pre-CFC suggest that this factor may act earlier than other hemopoietic factors described to date [9, 17].

The most notable evidence that c-*kit* ligand is involved in hemopoietic regulation *in vivo* is the fact that mice with mutations at the *Steel (Sl)* locus, which encodes c-*kit* ligand, are defective in hemopoietic cell development [18–21]. Furthermore, the macrocytic anemia, deficiencies in tissue mast cells, abnormalities in megakaryocytopoiesis, and reduced granulocytopoiesis that occur in *Steel* mice can be partially corrected by the administration of c-*kit* ligand [22]. In addition to the data accumulated in *Steel* mice, a limited number of studies have recently reported the ability of c-*kit* ligand to stimulate hemopoiesis in normal mice, rats, canines, and nonhuman primates [23–26]. Among the effects reported following c-*kit* ligand administration *in vivo* are increases in peripheral blood erythrocyte, neutrophil, lymphocyte, monocyte, eosinophil, and basophil numbers, as well as increases in bone marrow cellularity, GM-CFC, and erythroid burst-forming units (BFU-e), and (in mice) increases in splenic GM-CFC and CFU-s. In addition to these studies performed with c-*kit* ligand in normal animals, the ability of this factor to moderately stimulate hemopoietic regeneration in a more clinically relevant condition of radiation-induced hemopoietic aplasia has also recently been demonstrated [26–27].

We have previously demonstrated the ability of GM-CSF or GM-CSF plus IL–3 to accelerate hemopoietic recovery in irradiated primates [28–29]. Because *in vitro* studies have demonstrated synergistic hemopoietic stimulation produced by c-*kit ligand combined* with GM-CSF or GM-CSF plus IL–3 [6–8, 14–16, 30–31], we evaluated whether coadministration of c-*kit* ligand with these cytokines would further enhance their ability to accelerate hemopoietic regeneration following radiation-induced hemopoietic aplasia.

We report that MGF administered *in vivo* either does not affect, or in some cases even downregulates, regenerative responses induced by GM-CSF or GM-CSF plus IL–3 in irradiated mice, and that these effects sharply contrast the ability of MGF to synergize with these cytokines *in vitro*.

Materials and methods

Cytokines

Recombinant murine c-*kit* ligand, henceforth referred to as MGF, was provided by Immunex (Seattle, WA). Recombinant murine GM-CSF and IL–3 were provided by Behringwerke AG (Marburg, Germany). Cytokines were expressed in yeast and purified to homogeneity as previous-

ly described [10, 32]. Endotoxin contamination of cytokines was below the limit of detection using the limulus amebocyte lysate assay. Each cytokine was administered subcutaneously (s.c.) in a 0.1-ml volume at the dose of 100 μg/kg. Cytokine doses were based on preliminary dose-response studies performed in our laboratory [27; Patchen, unpublished]. In combination studies, mice received each cytokine at a separate injection site. All injections were initiated 1 day following irradiation and continued daily for 17 days. Control mice were injected with an equal volume of sterile saline.

Mice

B6D2F1 female mice (~20 g) were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were maintained in an AAALAC (American Association for Accreditation of Laboratory Animal Care) accredited facility in Micro-Isolator cages on hardwood-chip contact bedding and were provided commercial rodent chow and acidified water (pH 2.5) *ad libitum*. Animal rooms were equipped with full-spectrum light from 6 a.m. to 6 p.m. and were maintained at 70 °F ± 2 °F with 50% ± 10% relative humidity using at least 10 air changes per hour of 100% conditioned fresh air. Upon arrival, all mice were quarantined and samples from cage water bottles were cultured to detect any mice infected with oropharyngeal *Pseudomonas* sp. Only healthy mice were released for experimentation. All animal experiments were approved by the Institute Animal Care and Use Committee prior to performance.

Irradiation

The ^{60}Co source at the Armed Forces Radiobiology Research Institute was used to administer bilateral total-body gamma radiation. Mice were placed in ventilated Plexiglas containers and irradiated with 7.75 Gy at a dose rate of 0.4 Gy/min. Dosimetry was performed using ionization chambers [33] with calibration factors traceable to the National Institute of Standards and Technology. The tissue-to-air ratio was determined to be 0.96. Dose variation within the exposure field was < 3%.

Peripheral Blood Cell Counts

Blood was obtained from halothane-anesthetized mice by cardiac puncture using a heparinized syringe attached to a 20-gauge needle. White blood cell (WBC), red blood cell (RBC), and platelet (PLT) counts were performed using a Coulter counter.

Cell Suspensions

Cell suspensions for each assay represented tissues from three normal, irradiated, or irradiated and cytokine-treated mice at each time point. Cells were flushed from femurs with 3 ml of McCoy's 5A medium (Flow Labs, McLean, VA) containing 10% heat-inactivated fetal bovine serum (Hyclone Labs, Logan, UT). Spleens were pressed through a stainless steel mesh screen, and the cells were washed from the screen with 6 ml medium. The number of nucleated cells in the suspensions was determined by Coulter counter. Femurs and spleens were removed from mice euthanized by cervical dislocation.

Granulocyte-Macrophage Colony-Forming Cell Assay

For *in vivo* studies, hemopoietic progenitor cells committed to granulocyte and/or macrophage development were assayed using a double-layer agar granulocyte-macrophage colony-forming cell (GM-CFC) assay in which mouse endotoxin serum (5% v/v) was added to feeder layers as a source of colony-stimulating factors [34]. In studies evaluating the direct *in vitro* effects of cytokines on colony formation, a single-layer agar culture system was used in which normal bone marrow cells and cytokines were prepared in a 0.33% agar-media suspension at the concentration of 5×10^4 cells per ml and 2 ml of this suspension was plated into 35-mm culture dishes. Colonies (> 50 cells) were counted after 10 days of incubation in a 37 °C humidified environ-

ment containing 5% CO_2. Triplicate plates were cultured for each cell suspension.

Spleen Colony Forming Unit Assay

Exogenous spleen colony-forming units (CFU-s) were evaluated by the method of Till and McCulloch [35]. Recipient mice were exposed to 9 Gy of total-body radiation to reduce endogenous hemopoietic stem cells. Three to five h later, bone marrow or spleen cells were intravenously (i.v.) injected into the irradiated recipients. Twelve days after transplantation, the recipients were euthanized by cervical dislocation, and their spleens were removed. The spleens were fixed in Bouin's solution, and grossly visible spleen colonies were counted. Each treatment group consisted of five mice.

Statistics

Results of replicate experiments were pooled and are represented as the mean ± standard error (SE) of pooled data. Statistical differences were determined by Behrens-Fisher t-test analysis. Significance level was set at $p < 0.05$.

Experimental Design for in Vivo Studies

The ability to accelerate hemopoietic regeneration in a murine model of severe radiation-induced hemopoietic hypoplasia was used to evaluate the potential of MGF to synergize with GM-CSF or GM-CSF plus IL–3 in inducing hemopoietic progenitor cell expansion *in vivo*. In preliminary studies (Table 1), it was determined that a sublethal 7.75 Gy ^{60}Co radiation exposure induced severe hemopoietic hypoplasia from which recovery (especially in the spleen) became evident between days 14 and 17 postirradiation. Based on these preliminary studies, subsequent studies evaluating the ability of cytokines to accelerate hemopoietic recovery focused on evaluation of bone marrow and splenic cellularity, CFU-s, and GM-CFC recoveries, as well as peripheral WBC, RBC, and PLT recoveries on days 14 and 17 postirradiation.

Results

In Vivo Studies in Irradiated Mice

The effects of MGF plus GM-CSF on bone marrow and splenic CFU-s, GM-CFC, and peripheral blood cell recoveries in sublethally irradiated mice are illustrated in Figures 1, 2, and 3, respectively. MGF alone, at the dose used in these studies, had no effect on CFU-s recovery. In contrast, GM-CSF alone induced accelerated marrow and splenic CFU-s recoveries, which were evident as early as day 14 postirradiation. MGF administered in combination with GM-CSF, however, had no further effect than GM-CSF alone on bone marrow CFU-s recovery (Fig. 1A), and even down-modulated the splenic CFU-s recovery (Fig. 1B) induced by GM-CSF alone. Though less significant, similar patterns were observed for GM-CFC (Fig. 2) and peripheral WBC (Fig. 3A) recoveries in mice treated with MGF plus GM-CSF. RBC and PLT recoveries in these mice, however, were not down-modulated (Fig. 3B and 3C).

In an additional study, MGF was administered to sublethally irradiated mice in combination with GM-CSF plus IL–3 and CFU-s (Fig. 4) and GM-CFC (Fig. 5) recovery evaluated on day 17 postirradiation. The appropriate obligatory single- and double-factor controls were also simultaneously evaluated. Administration of MGF alone or MGF plus GM-CSF induced recovery patterns identical to the results described above. IL–3 alone produced a stimulation of only splenic CFU-s and GM-CFC recovery. However, when IL–3 was administered in combination with GM-CSF, it enhanced bone marrow and splenic CFU-s and GM-CFC recoveries over those induced by GM-CSF alone; this stimulatory interaction was more pronounced in the spleen than in the bone marrow. In contrast, mice administered MGF in combination with GM-CSF plus IL–3 exhibited CFU-s and GM-CFC recoveries that were again reduced to the same level or below the level of recovery induced by GM-CSF alone. Interesting, however, was the observation that MGF in combination with IL–3 did enhance CFU-s and GM-CFC recoveries beyond those induced by MGF or IL–3

Table 1. Recovery of bone marrow and splenic cellularity, CFU-s and GM-CFC following a 7.75 Gy ^{60}Co exposure.

	Unirradiated	Irrad Day 7	Irrad Day 10	Irrad Day 14	Irrad Day 17	Irrad Day 21
Cells/Femur (millions)	12.4 ± 0.6	1.2 ± 0.1	1.8 ± 0.3	3.6 ± 0.4	3.8 ± 0.6	5.1 ± 0.5
CFU-s/Fumur	6,837 ± 198	9 ± 1	13 ± 2	328 ± 29	531 ± 37	1,084 ± 118
GM-CFC/Femur	11,478 ± 406	0 ± 0	89 ± 11	370 ± 83	1,219 ± 186	1,498 ± 136
Cells/Spleen (millions)	155.8 ± 6.1	12.7 ± 0.7	12.9 ± 0.7	35.9 ± 4.4	144.9 ± 11.4	242.5 ± 40.7
CFU-s/Spleen	2.878 ± 188	0 ± 0	8 ± 3	383 ± 42	2,135 ± 309	2,614 ± 256
GM-CFC/Spleen	3,036 ± 180	0 ± 0	0 ± 0	357 ± 40	3,053 ± 521	17,543 ± 982

Data represent the mean ± SE of values obtained from two replicate experiments.

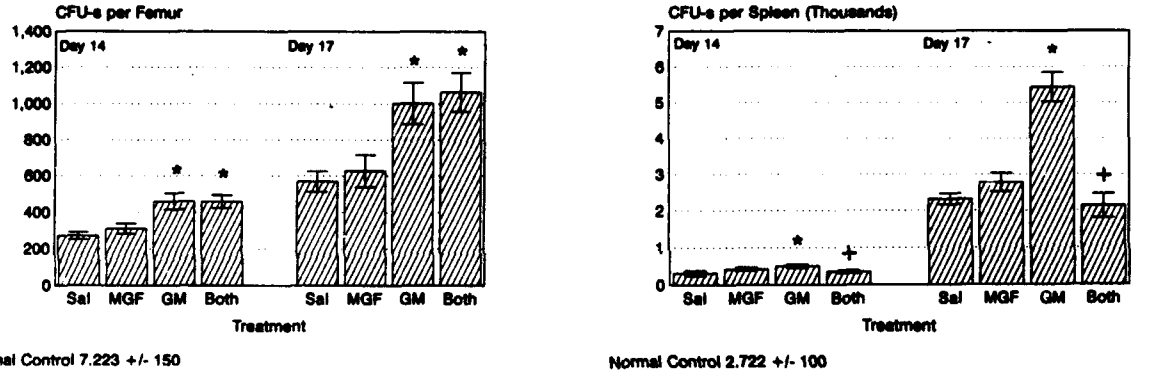

Fig. 1. Effect of MGF plus GM-CSF (each 100 μg/kg/d, s.c.) on postirradiation recovery of bone marrow and splenic CFU-s recovery in sublethally irradiated (7.75 Gy) B6D2F1 mice. Data represent the mean ± SE of values obtained from three replicate experiments. * $p < 0.05$, with respect to saline controls; + $p < 0.05$, with respect to GM-CSF values.

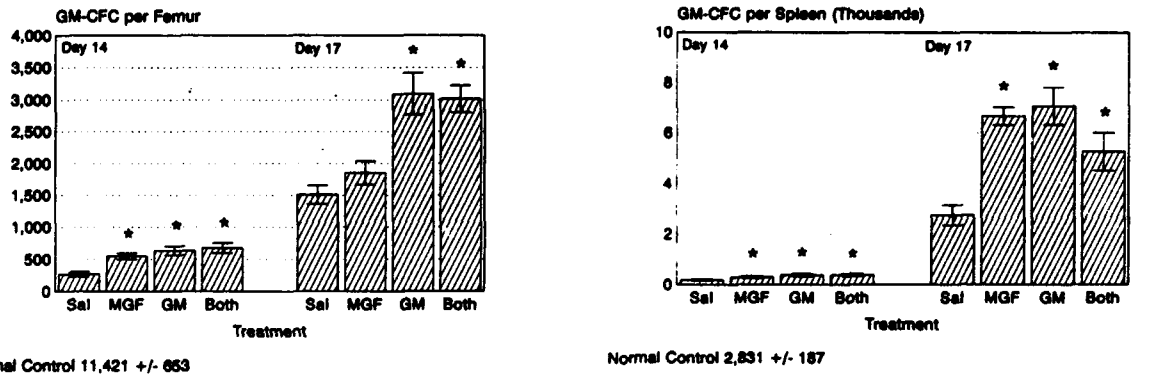

Fig. 2. Effect of MGF plus GM-CSF (each 100 μg/kg/d, s.c.) on postirradiation recovery of bone marrow and splenic GM-CFC recovery in sublethally irradiated (7.75 Gy) B6D2F1 mice. Data represent the mean ± SE of values obtained from three replicate experiments. * $p < 0.05$, with respect to saline controls; + $p < 0.05$, with respect to GM-CSF values.

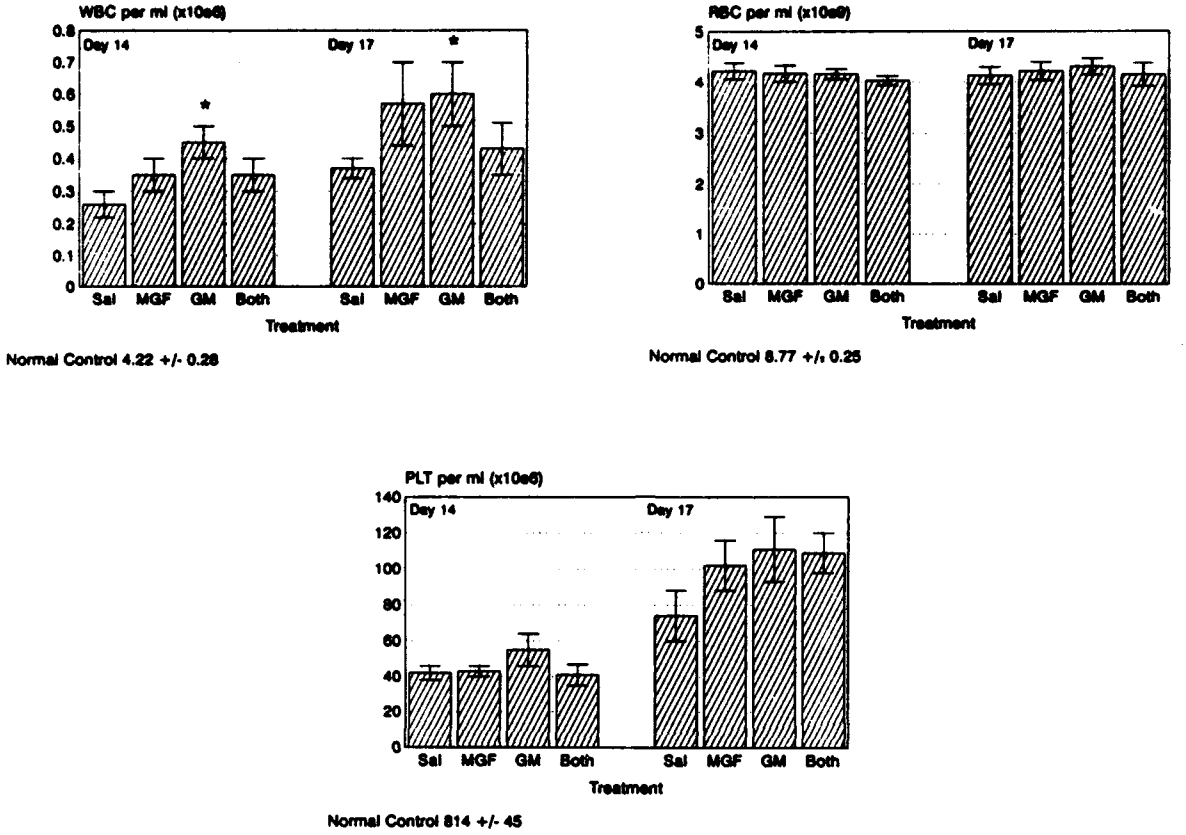

Fig. 3. Effect of MGF plus GM-CSF (each 100 μg/kg/d, s.c.) on postirradiation recovery of peripheral blood white blood cells, red blood cells, and platelets in sublethally irradiated (7.75 Gy) B6D2F1 mice. Data represent the mean ± SE of values obtained from three replicate experiments. * $p < 0.05$, with respect to saline controls; + $p < 0.05$, with respect to GM-CSF values.

alone. Splenic effects were more significant than bone marrow effects; however, MGF plus IL–3 treatment did not induce recovery surpassing that induced by GM-CSF plus IL–3.

In Vitro Studies Performed with Bone Marrow Cells Obtained from Normal Mice

Because results of our *in vivo* studies contrasted dramatically with previously published results of *in vitro* studies demonstrating synergistic stimulatory effects of MGF combined with GM-CSF or GM-CSF plus IL–3 [6–8, 14–16, 30–31], we performed *in vitro* studies to verify our cytokine activities. Initial *in vitro* studies focused on determining the ability of MGF alone or in combination with GM-CSF or GM-CSF plus IL–3 (as well as the respective single- and double-factor obligatory controls) to alter GM-CFC colony formation when directly cultured with bone marrow cells obtained from normal mice. Results are presented in Table 2 and Figure 6.

In these initial studies, colony-stimulating effects of MGF alone were evaluated at concentrations of 0.125 ng/plate, 12.5 ng/plate, and 25 ng/plate. No colony formation was evident at the 0.125-ng concentration; however, sporadic cluster (< 50 cells) and colony formation became evident at higher MGF concentrations, with 12.4 ± 0.7 colonies per plate being observed at the 25-ng concentration. IL–3 alone (0.125 ng/plate) induced no colony formation, and GM-CSF at the

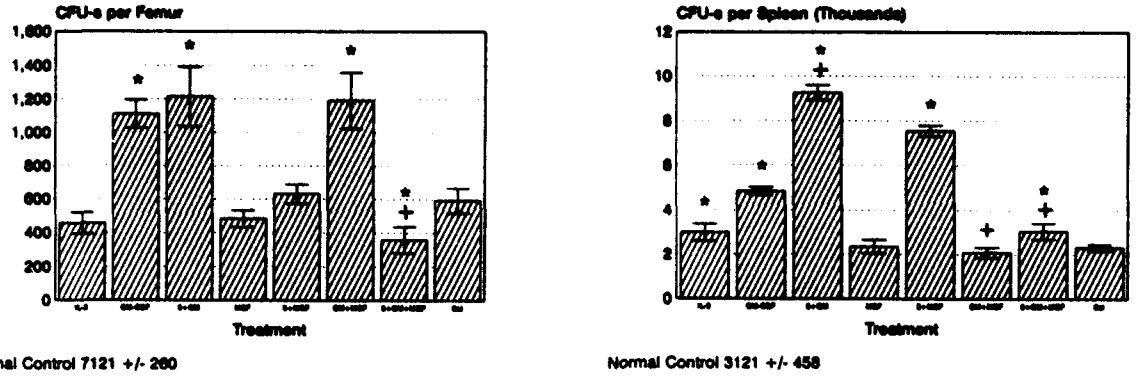

Fig. 4. Effects of MGF plus GM-CSF and IL–3 (each 100 μg/kg/d, s.c.) on postirradiation recovery of bone marrow and splenic CFU-s recovery in sublethally irradiated B6D2F1 mice. Data represent the mean ± SE of values obtained from one experiment. * $p < 0.05$, with respect to saline controls; + $p < 0.05$, with respect to GM-CSF values.

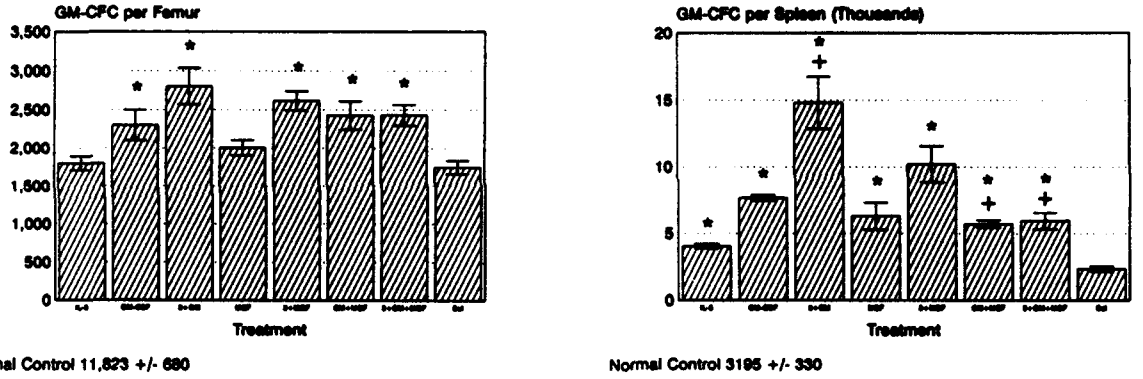

Fig. 5. Effects of MGF plus GM-CSF and IL–3 (each 100 μg/kg/d, s.c.) on postirradiation recovery of bone marrow and splenic GM-CFC recovery in sublethally irradiated (7.75 Gy) B6D2F1 mice. Data represent the mean ± SE of values obtained from one experiment. * $p < 0.05$, with respect to saline controls; + $p < 0.05$, with respect to GM-CSF values.

Fig. 6. Photographs of normal B6D2F1 bone marrow GM-CFC colonies grown *in vitro* in the presence of various cytokines. Cultures contained 1×10^5 cells and 0.125 ng/plate of GM-CSF, 0.125 ng/plate of IL–3, 25 ng/plate of MGF, or combinations of the respective cytokine concentrations. All photos at 11X magnification. The "lines" apparent in the pictures represent culture plate grid-lines and were purposely photographed in each shot to give perspective to the colony size. A: MGF; B: GM-CSF; C: IL–3; D: GM-CSF plus IL–3; E: MGF plus GM-CSF; F: MGF plus IL–3; G: MGF plus GM-CSF plus IL–3.

Table 2. Effects of MGF, GM-CSF, and IL–3 on normal bone marrow GM-CFC colony formation when added *in vitro* to GM-CFC cultures[a].

	No MGF	0.125 ng MGF	12.5 ng MGF	25.0 ng MGF
—	—	0.0 ± 0.0	2.1 ± 0.6	12.4 ± 0.7
+ GM-CSF	29.8 ± 2.6	32.5 ± 2.3	61.0 ± 8.0 ($p < 0.04$)	75.8 ± 7.0 ($p < 0.002$)
+ IL-3	0.0 ± 0.0	0.0 ± 0.0	8.2 ± 1.9 ($p < 0.04$)	25.3 ± 2.2 ($p < 0.0001$)
+ GM-CSF + IL-3	35.7 ± 3.6	40.2 ± 5.7	73.5 ± 7.5 ($p < 0.008$)	91.3 ± 7.7 ($p < 0.0001$)

[a]GM-CFC numbers per 1×10^5 normal B6D2F1 bone marrow cells cultured for 10 days in 0.33% soft agar. GM-CSF and IL–3 concentrations were always 0.125 ng/plt; MGF concentration varied and is indicated for each group. Data represent the mean ± SE of values obtained from four replicate experiments. Endotoxin serum control cultures had 84.3 ± 4.6 colonies.

same concentration induced suboptimal colony formation compared to control colony formation generated by endotoxin serum (29.8 ± 2.6 colonies vs 84.3 ± 4.6 colonies, $p < 0.0001$). When these concentrations of IL–3 and GM-CSF were used in combination, a slight enhancement in GM-CFC colony number and a clear enhancement in colony size were observed in comparison to GM-CSF-induced growth alone (Fig. 6).

To investigate potential synergistic effects of MGF, bone marrow cells were cultured with the 0.125-ng/plate concentrations of GM-CSF and IL–3 in the presence of increasing concentrations of MGF. MGF interacted with GM-CSF and IL–3 to increase both GM-CFC colony number and colony size (Table 2; Fig. 6). The effects were directly proportional to MGF concentration, with the lowest concentration producing no significant interactive effects. When all three cytokines were combined, an even more dramatic synergy was observed, especially with respect to colony size (Table 2; Fig. 6).

An additional interesting observation was an increase in the appearance of obvious "doublet" colonies which were detected at a frequency of 7.5 ± 2.5 per 10^5 bone marrow cells in cultures stimulated with endotoxin sera (Table 3). These colonies appeared to arise from a single division of a primitive cell, from which each daughter cell then proliferated to form large overlapping individual colonies. In cytokine cultures, such colonies were only observed in cultures containing the higher concentrations of MGF in combination with GM-CSF, or in combination with GM-CSF plus IL–3; the addition of IL–3 to MGF plus GM-CSF significantly increased the incidence of these characteristic "doublet" colonies in the cultures (e.g., 30.0 ± 4.1 vs 14.5 ± 0.5, $p < 0.005$).

In Vitro Studies Performed with Bone Marrow Cells Obtained from Irradiated Mice

Because the possibility existed that hemopoietic progenitor cells in irradiated mice may respond differently to cytokines than hemopoietic progenitor cells in normal mice, additional *in vitro* studies were performed using bone marrow target cells obtained from mice 17 days after exposure to 7.75 Gy ^{60}Co. In these studies, bone marrow cells were cultured in the presence of the cytokine concentrations inducing the most dramatic effects on normal bone marrow cells i.e., 25 ng/plate of MGF, 0.125 ng/plate of GM-CSF, 0.125 ng/plate of IL–3, and the respective combinations (Table 4). Although the total number of colonies obtained from 10^5 irradiated bone marrow cells was in all instances less than the total number of colonies obtained from the same number of normal bone

Table 3. Effects of MGF, GM-CSF, and IL–3 on normal bone marrow "Doublet" GM-CFC colony formation when added *in vitro* to GM-CFC cultures[a].

	No MGF	0.125 ng MGF	12.5 ng MGF	25.0 ng MGF
—	—	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
+ GM-CSF	0.0 ± 0.0	0.0 ± 0.0	6.5 ± 1.5 ($p < 0.02$)	14.5 ± 0.5 ($p < 0.0001$)
+ IL-3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
+ GM-CSF + IL-3	0.0 ± 0.0	0.0 ± 0.0	9.0 ± 2.0 ($p < 0.02$)	30.0 ± 4.1 ($p < 0.0001$)

[a]GM-CFC numbers per 1×10^5 normal B6D2F1 bone marrow cells cultured for 10 days in 0.33% soft agar. GM-CSF and IL–3 concentrations were always 0.125 ng/plt; MGF concentration varied and is indicated for each group. Data represent the mean ± SE of values obtained from four replicate experiments. Endotoxin serum control cultures had 7.5 ± 2.5 doublet colonies.

marrow cells, results were qualitatively similar in that the addition of MGF to cultures containing GM-CSF, IL–3, or GM-CSF plus IL–3 clearly enhanced the colony formation stimulated by these factors alone.

Discussion

Morbidity and mortality associated with high-dose irradiation can be directly attributed to infectious and hemorrhagic complications resulting from radiation-induced neutropenia and thrombocytopenia. Sustained hemopoietic recovery following chemotherapy or radiation exposure requires surviving pluripotent stem cells to self-renew as well as to differentiate into multipotent and committed progenitors capable of giving rise to functional mature cells. In recent years, administration of single hemopoietic growth factors, including G-CSF, GM-CSF, MGF, IL–1 and IL–6, has been shown to stimulate hemopoietic regeneration when administered following radiation- or chemotherapy-induced myelosuppression [13, 27, 29, 36–41]. In addition, some cytokine combinations have proven to surpass the effectiveness of single agents [28–29, 42]. In particular, the combination of GM-CSF plus IL–3 has proven to be quite effective in accelerating the postirradiation regeneration of both neutrophils and platelets [29], as has the recently developed GM-CSF/IL–3 fusion protein, PIXY321 [43]. Because c-*kit* ligand, *in vitro*, has been shown to synergize with GM-CSF or GM-CSF plus IL–3 in stimulating progenitor cell proliferation and expansion [6–8, 14–16, 30–31], we hypothesized that administration of MGF in combination with these cytokines *in vivo* may further improve hemopoietic regeneration beyond that obtained with only GM-CSF or GM-CSF plus IL–3. Unexpectedly, MGF did not further enhance the regenerative hemopoietic effects of GM-CSF or GM-CSF plus IL–3 in sublethally irradiated mice. Furthermore, in some instances, hemopoietic responses induced by these cytokines *in vivo* were actually down-regulated by coadministration of MGF.

Our *in vivo* results directly contrast both results of previous *in vitro* studies demonstrating synergistic hemopoietic stimulation with c-*kit* ligand in combination with GM-CSF or GM-CSF plus IL–3, and *in vitro* results generated in our own laboratory. The *in vitro* data presented in Tables 2–4 and Figure 6 clearly demonstrate that our MGF was capable of augmenting GM-CSF-stimulated or GM-CSF-plus-IL–3-stimulated GM-CFC colony formation from bone marrow cells obtained from either normal or irradiated mice, thus eliminating the possibility that regenerating bone marrow

Table 4. Effects of MGF, GM-CSF, and IL-3 on irradiated bone marrow GM-CFC colony formation when added *in vitro* to GM-CFC cultures[a].

	MGF Present	Regular Col	Doublet Col
+ GM-CSF	No	22.6 ± 2.2	1.0 ± 0.4
	Yes	38.3 ± 1.7	4.3 ± 0.9
		($p < 0.0001$)	($p < 0.05$)
+ IL-3	No	0.0 ± 0.0	0.0 ± 0.0
	Yes	2.0 ± 0.7	0.0 ± 0.0
+ GM-CSF + IL-3	No	27.4 ± 1.5	1.7 ± 0.2
	Yes	41.9 ± 2.3	5.2 ± 0.9
		($p < 0.0001$)	($p < 0.05$)
—	Yes	0.0 ± 0.0	0.0 ± 0.0

[a] GM-CFC numbers per 1×10^5 bone marrow cells obtained from 7.75-Gy irradiated B6D2F1 mice on day 17 postexposure and cultured for 10 days in 0.33% soft agar. GM-CSF and IL-3 concentrations were always 0.125 ng/plt; MGF concentration was 25 ng/plt. Data represents the mean ± SE of values obtained from four replicate experiments. Endotoxin serum control cultures had 35.2 ± 3.1 regular colonies and 1.9 ± 0.3 doublet colonies.

cells in irradiated mice are somehow unresponsive o MGF-mediated effects. Furthermore, since the ame MGF, GM-CSF, and IL-3 cytokine stocks were used for both our *in vivo* and our *in vitro* tudies, and since *in vitro* studies were performed t a later time than *in vivo* studies, differences in ytokine batches, or loss of cytokine activities can e excluded as explanations for the contrasting ffects observed in *in vivo* versus *in vitro* studies.

Although the possibility that the MGF dose sed in our *in vivo* studies induced suppression ecause it was too high cannot be eliminated, his seems unlikely since the 100 ug/kg/d MGF lose used was at the low end of the 100–200 g/kg/d MGF dose range which we have shown o be capable of accelerating hemopoietic recovry in myelosuppressed animals [27]. Our studies lo, however, suggest that cytokine dose ratios nay play a critical role in eliciting synergistic emopoietic responses. In our *in vitro* studies performed with normal bone marrow cells, no MGF-nduced synergy was observed in GM-CSF, IL-3, or GM-CSF plus IL-3 cultures when the MGF concentration was equal to other cytokine concentrations (i.e., 0.125 ng/plate of each cytokine); however, when MGF concentration exceeded the other cytokine concentrations by 100–200 fold (i.e., the 12.5 ng/plate and 25 ng/plate MGF concentrations), extremely significant enhancement in colony formation was observed. Hence, these results suggest that an excessive MGF concentration may be required in order to obtain synergy. Since our *in vivo* studies employed an equal dose of each cytokine (100 μg/kg/d), it may be that the MGF dose administered *in vivo* was simply insufficient to observe synergy. However, because individual cytokines differ pharmacokineticaly, and pharmacokinetics can dramatically alter the bioavailability of agents *in vivo*, it is difficult to directly compare *in vitro* and *in vivo* cytokine dose relationships. Although the MGF dose used in our *in vivo* studies was not synergistic with GM-CSF or GM-CSF plus IL-3, it was biologically active as evidenced by the fact that, in some instances,

this MGF dose actually *down-regulated* responses induced by GM-CSF alone or GM-CSF plus IL–3.

The reason for the hemopoietic down-modulation observed *in vivo* following MGF coadministered with GM-CSF or GM-CSF plus IL–3 is uncertain. C-*kit* ligand is known to exist both as an integral membrane-associated protein possessing an extracellular domain, transmembrane domain, and intracytoplasmic domain, as well as to exist as a soluble protein produced by proteolytic cleavage of the membrane-associated form [3, 6, 10, 44]. The membrane-associated protein is readily produced by hemopoietic stromal elements [44–46]. In spite of the fact that hemopoietic precursor cells can clearly respond to soluble c-*kit* ligand [22], it has been suggested that the stromal membrane-bound form is perhaps more important than the soluble form in regulating *in situ* hemopoiesis [44–46]. The MGF used in our studies was a soluble c-*kit* ligand. Since irradiation alone has been shown to increase c-*kit* ligand expression [47], in conjunction with exogenous MGF administration, c-*kit* ligand concentration may have become sufficient in irradiated mice to saturate c-*kit* (i.e., the c-*kit* ligand receptors) present on hemopoietic precursor cells. Such receptor blockade could prevent the binding of hemopoietic precursor cells to hemopoietic stromal elements *via* membrane-associated stromal c-*kit* ligand, hence interfering with subsequent hemopoietic proliferation and differentiation signals that may be stromal mediated. However, since down-modulation of hemopoiesis by MGF was only observed when MGF was coadministered with the otherwise stimulatory GM-CSF or GM-CSF plus IL–3 treatments, it is perhaps more plausible that MGF may down-modulate GM-CSF receptor expression. IL–3 receptor expression, on the other hand, does not appear to be affected since control mice administered MGF plus IL–3 actually exhibited better hemopoietic recovery than the recovery induced by either of these cytokines administered alone.

Little information has been published on *in vivo* effects of c-*kit* ligand in combination with other cytokines. However, Ulich et al. did report the ability of coadministered c-*kit* ligand (SCF) and GM-CSF to synergistically increase bone marrow GM-CFC and neutrophil numbers in normal rats [48]. There are several major differences between Ulich's study and ours which may explain the contrasting effects observed in the two studies. First, Ulich utilized a polyethyleneglycol (PEG) modified c-*kit* ligand (PEGalated recombinant rat SCF). The MGF used in our studies was not PEGalated. PEG modification can increase the *in vivo* bioavailability of proteins by altering pharmacological properties such as extending plasma half-life and increasing resistance to proteolysis [49]. Whether PEGalation alone may account for some of the hematological differences observed between our study and that of Ulich is not certain. Second, *c-kit* ligand:GM-CSF dose ratios and cytokine administration protocols differed significantly between the two studies. The MGF:GM-CSF dose ratio administered in our study was 1:1, while a 5:1 SCF:GM-CSF dose ratio was administered in Ulich's study. Furthermore, in our study cytokines were administered subcutaneously for 17 days, while in Ulich's study cytokines were administered intravenously for only 7 days. Both of these differences may have altered the effects of the injected cytokines. The length of the treatment protocol, in particular, may have had a significant impact. With respect to c-*kit* ligand administration, in normal mice receiving s.c. injections of PEG-SCF (100 μg/kg/d) over a 21-day period, it has been noted that although leukocytosis initially occurs, by day 18 cell counts drop to subnormal levels despite continued SCF treatment [24]. Ulich's study evaluated responses only through 7 days of treatment; had treatment continued, suppressive effects may have also been observed in his study.

Another possible explanation for the differences observed in our two studies could relate to the fact that normal animals were used in Ulich's study, while irradiated animals were used in ours. Previous studies performed in our laboratory with IL–3, GM-CSF, and the combination of these cytokines have revealed conflicting responses elicited in normal versus irradiated primates, despite identical cytokine administration

protocols [28; MacVittie, unpublished]. In fact, Schuening et al. very recently have reported on the effects of coadministered PEG-SCF (recombinant canine SCF; 200 ug/kg/d × 21 days, s.c.) and G-CSF (recombinant canine; 10 ug/kg/d × 21 days, s.c.) in canines exposed to an otherwise lethal 5 Gy irradiation [26]; the effects were different from the synergistic effects observed by others when these two cytokines were coadministered to normal animals [24, 48]. Although in combination-treated irradiated canines Schuening et al. did not report a down modulaton of G-CSF-induced responses as we have observed in irradiated mice coadministered MGF and GM-CSF (or GM-CSF + IL–3), the granulocyte, platelet, and survival responses they observed were not better than those induced by the individual cytokines. Thus, Schuening's results further stress the variation in responses elicited following cytokine administration in compromised animals. Since we have recently demonstrated that endogenous production of several cytokines increases dramatically following radiation exposure such as that used in our studies presented in this paper [47, 50], it seems plausible that cytokine-cytokine interactions could be very different in radiation-induced aplastic animals than in normal animals.

In conclusion, our studies demonstrate that MGF administered *in vivo* either does not affect or in some cases even down-regulates regenerative responses induced by GM-CSF or GM-CSF plus IL–3 in sublethally irradiated mice, and that these responses sharply contrast the *in vitro* effects of these cytokines. Furthermore, these studies emphasize that caution must be taken in attempting to predict cytokine interactions *in vivo* in hemopoietically injured animals based on *in vitro* cytokine effects or cytokine effects in normal animals.

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