Use of 5-Fluorouracil to Analyze the Effect of Macrophage Inflammatory Protein-1α on Long-Term Reconstituting Stem Cells In Vivo

By Valerie F.J. Quesniaux, Gerry J. Graham, Ian Pragnell, Deborah Donaldson, Stephen D. Wolpe, Norman N. Iscove, and Barbara Fagg

A macrophage-derived inhibitor of early hematopoietic progenitors (colony-forming unit-spleen, CFU-A) called stem cell inhibitor was found to be identical to macrophage inflammatory protein-1α (MIP-1α). We investigated the effect of MIP-1α on the earliest stem cells that sustain long-term hematopoiesis in vivo in a competitive marrow re-population assay. Because long-term reconstituting (LTR) stem cells are normally quiescent, an in vivo model was first developed in which they are triggered to cycle. A first 5-fluorouracil (5-FU) injection was used to eliminate later progenitors, causing the LTR stem cells, which are normally resistant to 5-FU, to enter the cell cycle and become sensitive to a second 5-FU injection administered 5 days later. Human MIP-1α administered from day 0 to 7 was unable to prevent the depletion of the LTR stem cells by the second 5-FU treatment, as observed on day 7 in this model, suggesting that the LTR stem cells were not prevented from being triggered into cycle despite the MIP-1α treatment. However, the MIP-1α protocol used here did substantially decrease the number of more mature hematopoietic progenitors (granulocyte-macrophage colony-forming cells [CFC], burst-forming unit-erythroid, CFU-gran, and pre-CFU-gran) recovered in the bone marrow shortly after a single 5-FU injection. In vitro, MIP-1α had no inhibitory effect on the ability of these progenitors to form colonies. This study confirms the in vivo inhibitory effect of MIP-1α on subpopulations of hematopoietic progenitors that are activated in myelodysplastic animals. However, MIP-1α had no effect on the long-term reconstituting stem cells in vivo under conditions in which it effectively reduced all later progenitors.

PERMANENT marrow recovery after cytotoxic drug therapy depends on the survival of hematopoietic stem cells having long-term reconstituting (LTR) potential. In the mouse, LTR stem cells normally reside in a quiescent state that renders them resistant to cycle-active drugs. However, little information is available on the regulation of their entry into active cycle and on the stimuli that might induce cycling. If marrow response to cytotoxic injury includes recruitment of LTR stem cells into cycle, then marrow toxicity of cycle active agents might be significantly reduced by factors that can selectively inhibit stem cell cycling.

An inhibitor of cycling of spleen colony-forming cells (colony-forming unit-spleen [CFU-S]), originally described in the supernatant of murine bone marrow (BM) cells, was recently shown to be identical to the macrophage inflammatory protein-1α (MIP-1α). Murine MIP-1α was cloned by Davatelis et al. and the human MIP-1α homologue has been identified and cloned. Both proteins comprise 69 amino acids and are 74% identical. MIP-1α was termed stem cell inhibitor based on its ability to inhibit in vitro the cycling of CFU-S and CFU-A, a progenitor forming macroscopic colonies composed primarily of macrophages and sharing properties in common with CFU-S. In vivo, in mice treated once with phenylhydrazine or 5-fluorouracil (5-FU), a single inoculation of MIP-1α reduced significantly the proportion of CFU-S and CFU-A in S-phase, as assessed by the sensitivity of the cells to the cell-cycle-specific cytotoxic drug cytosine arabinoside (Ara-C). This effect resulted in the protection of a Chinese hamster ovary (CHO) cells and purified as described.

MATERIALS AND METHODS

Cytokines. Human recombinant (hr) MIP-1α was produced in Chinese hamster ovary (CHO) cells and purified as described.

From Sandoz Pharma Ltd, Basel, Switzerland; the Beatson Institute for Cancer Research, Glasgow, UK; the Genetics Institute, Cambridge, MA; and the Ontario Cancer Institute, Toronto, Canada. Submitted July 9, 1992; accepted November 16, 1992.

I.P. and G.J.G. are supported by grants from the Cancer Research Campaign (UK).

Address reprint requests to V.F.J. Quesniaux, PhD, Preclinical Research 386/155, Sandoz Pharma Ltd, 4002 Basel, Switzerland.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1993 by The American Society of Hematology.
In vivo treatments. 5-FU (150 mg/kg intravenously [IV] in isotonic balanced salt solution [IBSS]) was injected into CBA/J or C57Bl/6 mice on day 0 or days 0 and 5. MIP-1α was dissolved in IBSS containing 1% syngeneic mouse serum at the indicated concentration. The daily doses were divided into two subcutaneous (SC) injections administered at 9 AM and 5 PM. Control animals received vehicle alone. The MIP-1α treatments caused neither inflammation at the injection sites nor histologic alteration of the main organs (B. Ryffel, unpublished observation).

Competitive BM repopulation. Short- and long-term reconstituting cells were quantitated using a competitive repopulation assay adapted from Harrison.1,12 Lethally irradiated (7.5 Gy) female C57Bl/6 mice carrying the glucose phosphate isomerase Gpi-la allele (Gpi-la) were used as recipients. They were reconstituted by IV injection of BM cells from in vivo-treated male mice carrying the Gpi-la allele (Gpi-la), together with a fixed amount of congenic competitor BM cells from untreated male Gpi-la. The equivalent of 105 of a femur of untreated Gpi-la competitor cells (1.5 to 2.0 × 106 cells) was injected together with BM cells from 1/5, 1/10, or 1/50 of a femur from the treated Gpi-la donor. The Gpi-la repopulation was proportional to the number of Gpi-la cells injected and the results from 1/5, 1/10, and 1/50 of a femur were therefore pooled within each experimental group. The use of fractional proportions of the BM allowed a direct comparison of the repopulating ability of 5-FU-treated marrow, regardless of the diminished cell number, to that of untreated marrow. Between 1 and 12 months after transplantation, peripheral blood of the recipient was analyzed for the relative contribution of the experimental and treated marrow to circulating cells. Aliquots of blood were lysed in hypotonic (Gpi-lb) was injected into CBA/J or C57Bl/6 mice on day 0 or days 0 and 5. MIP-1α was dissolved in IBSS containing 1% syngeneic mouse serum at the indicated concentration. The daily doses were divided into two subcutaneous (SC) injections administered at 9 AM and 5 PM. Control animals received vehicle alone. The MIP-1α treatments caused neither inflammation at the injection sites nor histologic alteration of the main organs (B. Ryffel, unpublished observation).

Competitive BM repopulation. Short- and long-term reconstituting cells were quantitated using a competitive repopulation assay adapted from Harrison.1,12 Lethally irradiated (7.5 Gy) female C57Bl/6 mice carrying the glucose phosphate isomerase Gpi-la allele (Gpi-la) were used as recipients. They were reconstituted by IV injection of BM cells from in vivo-treated male mice carrying the Gpi-la allele (Gpi-la), together with a fixed amount of congenic competitor BM cells from untreated male Gpi-la. The equivalent of 105 of a femur of untreated Gpi-la competitor cells (1.5 to 2.0 × 106 cells) was injected together with BM cells from 1/5, 1/10, or 1/50 of a femur from the treated Gpi-la donor. The Gpi-la repopulation was proportional to the number of Gpi-la cells injected and the results from 1/5, 1/10, and 1/50 of a femur were therefore pooled within each experimental group. The use of fractional proportions of the BM allowed a direct comparison of the repopulating ability of 5-FU-treated marrow, regardless of the diminished cell number, to that of untreated marrow. Between 1 and 12 months after transplantation, peripheral blood of the recipient was analyzed for the relative contribution of the experimental and treated marrow to circulating cells. Aliquots of blood were lysed in hypotonic (Gpi-lb) was injected into CBA/J or C57Bl/6 mice on day 0 or days 0 and 5. MIP-1α was dissolved in IBSS containing 1% syngeneic mouse serum at the indicated concentration. The daily doses were divided into two subcutaneous (SC) injections administered at 9 AM and 5 PM. Control animals received vehicle alone. The MIP-1α treatments caused neither inflammation at the injection sites nor histologic alteration of the main organs (B. Ryffel, unpublished observation).

Competitive BM repopulation. Short- and long-term reconstituting cells were quantitated using a competitive repopulation assay adapted from Harrison.1,12 Lethally irradiated (7.5 Gy) female C57Bl/6 mice carrying the glucose phosphate isomerase Gpi-la allele (Gpi-la) were used as recipients. They were reconstituted by IV injection of BM cells from in vivo-treated male mice carrying the Gpi-la allele (Gpi-la), together with a fixed amount of congenic competitor BM cells from untreated male Gpi-la. The equivalent of 105 of a femur of untreated Gpi-la competitor cells (1.5 to 2.0 × 106 cells) was injected together with BM cells from 1/5, 1/10, or 1/50 of a femur from the treated Gpi-la donor. The Gpi-la repopulation was proportional to the number of Gpi-la cells injected and the results from 1/5, 1/10, and 1/50 of a femur were therefore pooled within each experimental group. The use of fractional proportions of the BM allowed a direct comparison of the repopulating ability of 5-FU-treated marrow, regardless of the diminished cell number, to that of untreated marrow. Between 1 and 12 months after transplantation, peripheral blood of the recipient was analyzed for the relative contribution of the experimental and treated marrow to circulating cells. Aliquots of blood were lysed in hypotonic (1 mg/mL EDTA) solution, frozen and thawed, and were submitted to electrophoresis to determine the separation of Gpi-la and Gpi-lb isoenzymes by electrophoresis on cellulose acetate membranes and the colorimetric detection of the enzymatic product was performed essentially as described.13 The unit of contribution of repopulating cells was calculated as the ratio of Gpi-la to Gpi-lb isoenzyme activity, defining 1 U as the amount of reconstituting activity in γ50 of a competing Gpi-la femur. This measure has been shown to be linearly related to the number of Gpi-la marrow cells injected (N.N.I., unpublished observation, and confirmed within the present study).

Murine BM suspension culture. Mouse BM cells were cultured in 1 mL Iscove’s Modified Dulbecco’s Medium (IMDM; GibCO, Life Technologies AG, Basel, Switzerland) containing mIL-3 (10 ng/mL), hrIL-1 (10 ng/mL), α-thyoglycerol, 5% fetal calf serum (FCS), and 0.1% bovine serum albumin (BSA; Sigma, St Louis, MO) in 35 mm bacteriological petri plates, according to Iscove et al.14 After 4 days of incubation at 37°C, the cells were gently resuspended, harvested, and the content of colony-forming cells was determined in methylcellulose cultures (see next section). BM cells from untreated mice were seeded at 0.5 to 1 × 106 cells/mL and cells from 5-FU-treated animals were usually titrated from 0.1 to 1 × 106 cells/mL to obtain a sufficient number of colonies per plate upon replating.

Murine BM colony assay. Methylcellulose cultures of murine BM were prepared in IMDM containing mIL-3 (10 ng/mL), hrIL-1 (10 ng/mL), erythropoietin (Epo; 0.1 U/mL), 4% FCS, bovine serum fraction V (Sigma), transferrin, and lipids, according to Iscove et al.14 Macroscopic (>1 mm) multilineage colonies comprising erythroid, megakaryocytic, and other myeloid cells (CFU-mult); erythroid colonies, either pure or mixed with megakaryocytes (E); and granulocyte-macrophage (GM) colonies were counted on day 9. The colonies were identified by their morphology and were periodically picked, spread, and stained (Wright/Giemsa) for confirmation. BM cells from untreated mice were seeded at 4 to 8 × 106 cells/mL and cells from 5-FU-treated animals were titrated from 0.1 to 1 × 106 cells/mL to ensure a sufficient number of colonies per plate.

The CFU-A and CFU-S assays were performed as previously described.6,15

Human BM colony assay. Human BM cells were obtained from informed and consenting allogenic transplant donors, frozen, and stored in liquid N2. The cells were thawed and adhered overnight to tissue culture plates containing 25% FCS in IMDM. Nonadherent cells (5 × 105 cells/mL) were cultured in 1% methylcellulose (Fluka, Buchs, Switzerland) in IMDM containing hrIL-3 (2.4 ng/mL) or mKL (50 mg/mL) and hrEpo (0.1 U/mL) plus 0.8% BSA, 380 μg/mL human transferrin, and 16% FCS.16 Neutrophil, macrophage, eosinophil, and erythroid colonies were counted after 14 days.

RESULTS

Effect of a single 5-FU injection on colony-forming cells tested ex vivo either directly or after expansion in suspension culture. Mice were injected with 5-FU (150 mg/kg) on day 0 and killed after 2, 5, 8, and 10 days. Control mice (day 0) received vehicle alone. Femoral BM cells were recovered and incubated either directly in methylcellulose cultures containing IL-3, IL-1, and Epo or in suspension cultures containing IL-3 and IL-1 for 4 days before methylcellulose culture. The number of BM cells per femur was reduced by greater than 90% 5 days post–5-FU and gradually increased on days 8 and 10 (day 0: 14.0; day 2: 4.5; day 5: 0.8; day 8: 1.9; day 10: 3.1 million nucleated cells per femur). The number of all direct CFC (Fig 1A) as well as numbers of pre-CFC recovered from suspension culture (Fig 1B) were similarly reduced at 2 and 5 days post–5-FU. Most precursor numbers returned to normal on day 8, with an overshoot on day 10 post–5-FU.

Thus, both pre-CFC and direct CFC were profoundly reduced by a single treatment with 5-FU and followed similar kinetics of recovery.

Effect of a single 5-FU treatment on short-term and long-term reconstituting cells. The BM cells recovered from Gpi-la mice before or 2, 5, 8, and 10 days after a single 5-FU treatment were assayed for their content of reconstituting cells in an in vivo competitive BM repopulation assay.11,12 Marrow cells from the treated Gpi-la mice were mixed with a reference dose (1/10 femur) of untreated Gpi-la cells and injected into lethally irradiated Gpi-la recipients. The competitive marrow ensured the prompt hematopoietic recovery and survival of the recipient mice. In mice reconstituted with 1/10 of a Gpi-la femur alone, host Gpi-la erythrocytes decreased to 20% of the total after 1 month, and were undetectable after 2 months.

Figure 2 shows that the cells providing very short-term hematopoietic repopulation at 1 month were reduced 7 to 12-fold 2 and 5 days post–5-FU and returned to normal levels by 8 to 10 days. In contrast, the cells sustaining repopulation at 2 months and beyond were more resistant to 5-FU. Two- and 3-month repopulating cells were only twofold to fivefold decreased at 2 and 5 days post–5-FU. As shown in Fig 3, LTR stem cells capable of sustaining greater than 6 months
Fig 1. Effect of a single 5-FU injection on BM colony-forming cells tested either directly or after expansion in suspension culture. Groups of three Gpi-1\(^+\) mice were killed 2, 5, 8, or 10 days after a single 5-FU injection (150 mg/kg, IV). Untreated mice served as control (day 0). The femoral BM cells were harvested, counted, and pooled within each group. The cell suspensions were tested either (A) directly in methyl cellulose cultures or (B) in suspension cultures before colony assay. GM colonies (●), erythroid colonies (○), and macroscopic CFC\(_{\text{max}}\) (△) were counted after 9 days of culture. The results are expressed as the number of colonies per femur and are the means from two independent experiments (4 plates per point each).

of repopulation were essentially unaffected on day 7 after 5-FU.

These results showed that short-term erythroid reconstituting cells were depleted by a single injection of 5-FU and followed similar kinetics of recovery as CFC and pre-CFC, whereas most of the longer-term reconstituting cells were resistant to 5-FU. Because the LTR stem cells thus appeared to be normally at rest, it would be necessary to induce them into active cycle to study the potential effects of negative regulators such as MIP-1\(\alpha\).

Effect of a single 5-FU injection on LTR stem cells. The fast recovery of the hematopoietic progenitors 8 to 10 days after 5-FU treatment suggested that earlier stem cells might be stimulated to enter into cycle and to rapidly proliferate and differentiate. To test this, we used one 5-FU injection as a stimulus and, 5 days later, a second 5-FU injection to kill cells that were induced to proliferate. The femoral BM cells were harvested 2 days after the second 5-FU injection and tested in the competitive repopulation assay. The results clearly showed that, although 3- to 12-month reconstituting cells are resistant to a single 5-FU injection, pretreatment of animals with 5-FU renders LTR stem cells highly susceptible to a second dose administered 5 days later (Fig 3). As expected, the number of nucleated BM cells and all later progenitors, including CFC and pre-CFC, were substantially depleted in the harvested BM (similarly to what was seen 2 days after a single 5-FU injection).

These experiments indicated that LTR stem cells did enter into cycle 5 days after 5-FU injection and defined an experimental window for studying the effect of negative regulators on the cycling of primitive LTR stem cells.

Effect of MIP-1\(\alpha\) on LTR stem cells. The experimental model described above provided an opportunity to investigate the effects of MIP-1\(\alpha\) on cycling reconstituting stem cells. If MIP-1\(\alpha\) could inhibit the onset of cycling of LTR stem cells, these should remain resistant to the second dose of 5-FU.

MIP-1\(\alpha\) used at a dose active on colony-forming cells (3 \(\mu\)g/d, SC, see below) or vehicle was administered from day 0 to 7 to mice that received 5-FU on days 0 and 5. Marrow cells were harvested on day 7 and tested in the competitive repopulation assay. Figure 4 shows the numbers of reconstituting units recovered as a function of length of reconstitution in recipient animals. The lower levels of short-term (1 month) reconstituting activity confirms the greater vulnerability of these cells to kill by 5-FU compared with cells with longer-term reconstituting potential. The later assay time points indicate that the second dose of 5-FU reduced marrow reconstituting activity to only 10% of normal, and that MIP-1\(\alpha\)
had no significant protective effect in this system. MIP-1α did not affect the reconstituting activity of marrow in control mice receiving no 5-FU. It also had no effect in mice that received only a single dose of 5-FU on day 0 (not shown), suggesting that MIP-1α was not toxic to either resting or cycling LTR stem cells.

These results indicate that MIP-1α did not prevent the induction of active cycling of LTR stem cells in mice previously injected with 5-FU.

Effect of MIP-1α on the recovery of hematopoietic progenitors after 5-FU injection. To control that the MIP-1α treatment was effective in vivo at the dose used in the preceding experiments, marrow cells were also tested for colony formation. Because it was established that both CFC and pre-CFC cycle in the normal steady state, the effect of MIP-1α on these progenitors could be tested before or after a single dose of 5-FU. If MIP-1α could inhibit their cycling without additional adverse effects, then protection against 5-FU should be observed. On the other hand, inhibition of cycling can be noxious to cells that normally cycle, and would also be expected to block their numerical recovery after initial exposure to 5-FU, so that the net effect of MIP-1α on CFC numbers could be negative.

In a first series of experiments, mice were treated with various amounts of MIP-1α (0, 0.3, 1, or 3 μg/d, SC) 1 day before and 2 days after a single injection of 5-FU. Figure 5 shows that, without MIP-1α, marrow CFC and pre-CFC were reduced to 10% to 30% of normal 3 or 4 days after administration of 5-FU. Treatment with MIP-1α consistently led to an additional 3- to 10-fold reduction in recovered precursor numbers. The number of day 10 CFU-S was also reduced to 25% of normal 3 to 4 days after 5-FU and was reduced four-fold further by MIP-1α (72% to 79% decrease, n = 6 to 10 per group). These effects were reversible as colony formation was essentially normal in all MIP-1α-treated groups 10 days after 5-FU (data not shown). MIP-1α had no effect in control mice that were not treated with 5-FU.

In the preceding set of experiments described in Fig 4, MIP-1α administration from day 0 to 7 in mice that received one injection of 5-FU on day 0 led to a 70% to 90% reduction in the numbers of marrow CFC, pre-CFC, and CFU-A recovered at day 7 (68%, 89%, and 87%, respectively).

These results established that administration of MIP-1α at the dose used led to measurable reduction of CFC, pre-CFC, CFU-S, and CFU-A populations in animals treated with 5-FU, although it had no effect on these progenitors in normal animals.

Effect of human MIP-1α on murine and human BM progenitors in vitro. The direct effect of human MIP-1α on murine BM cell colony formation was tested to check the possible ex vivo effect of the MIP-1α carried over with the BM cells after in vivo treatment. MIP-1α at 0.01 to 100 ng/mL had no effect on the formation of erythroid, GM, or multilineage colonies from murine BM cells (Fig 6A). Since murine MIP-1α has been reported to inhibit both murine and human BM cell colony formation in response to either IL-3 or KL plus Epo, we further tested the effect of the human MIP-1α on human BM cells (Fig 6B). The formation of erythroid bursts in response to IL-3 or KL plus Epo was unaffected by concentrations of MIP-1α ranging from 1 to 1,000 ng/mL. Thus, in our culture conditions, human MIP-1α had little effect on the cycling of either human or murine marrow CFC driven by various cytokines, including IL-1, IL-3, KL, and Epo.

DISCUSSION

In vivo treatment with 5-FU preferentially eliminates proliferating cells, which in BM comprise all the late progenitors ranging from CFC to CFU-S and cells sustaining 8 week erythroid reconstitution in vivo. This leads to 90% depletion of the BM cells. However, it is not lethal and the hematopoietic system of the treated animals recovers within 2 weeks of treatment. 5-FU has been shown to spare earlier "pre-CFU-S" and 3-month repopulating stem cells. In agreement with these earlier studies, we show here that one injection of 5-FU spared the LTR stem cells and reduced all later cell populations, including pre-CFC, CFU-S, pre-CFC, and CFC. These results suggest that the more mature progenitors normally cycle and that pre-CFC are distinct from LTR stem cells.

Although LTR stem cells were resistant to one injection of 5-FU, and therefore solidly at rest within a 48-hour window after the injection, a second dose of 5-FU at 5 days reduced LTR activity by 10-fold. Thus, the depletion of the late BM progenitors initiated mechanisms that recruited LTR stem
MIP-1α DOES NOT PROTECT LTR STEM CELLS IN VIVO

30

241

Fig 3. Effect of two 5-FU injections on the repopulating cells. Groups of two to three Gpi-1+ mice were either untreated (●), or injected with 5-FU (150 mg/kg) on day 0 (■) or on days 0 and 5 (▲) and the repopulating capacity of their BM cells harvested on day 7 was tested as in Fig 2. The results are pooled from four independent experiments (mean ± SEM, n = 19 to 25 mice per point).

cells into active cycle. These results are in agreement with a recent study reported by Harrison and Lerner in which a similar experimental model was used to study the kinetics of LTR activation after 5-FU injection. Such an experimental model provides the opportunity to test for agents that might inhibit the entry of LTR stem cells into cycle.

Previous studies showed that short-term exposure to MIP-1α affected the cell-cycle control of CFU-S and CFU-A in regenerating murine BM. This effect was seen in vitro in BM from phenylhydrazine-treated mice and seemed to be more pronounced on day 12 CFU-S than on more mature day 8 CFU-S. In vivo in phenylhydrazine-treated mice, a single inoculation of MIP-1α (10 µg/mouse, intraperitoneally [IP]) reduced the proportion of day 12 CFU-S in S-phase from 38% to 2% of control within 4 hours. Similarly, 8 days after a single 5-FU injection, the proportion of CFU-A in S-phase was reduced from 48% to 10% of controls within 4 hours of MIP-1α treatment. Two recent studies further investigated whether the inhibition of cell cycling would result in the protection of CFU-S and CFU-A from a second cy-

Fig 4. In vivo effect of MIP-1α on repopulating BM cells. Groups of two to three Gpi-1+ mice were injected twice with 5-FU on days 0 and 5 and were treated either with MIP-1α (3 µg/d; ●) or with vehicle (SC twice daily; ■) from day 0 through day 7. When the animals were killed and their BM cells tested for repopulating capacity as in Fig 2. The results are pooled from two independent experiments (mean ± SEM, n = 9 to 17 recipient mice per point). Normal untreated BM contained 35.5, 18.5, and 17.0 RU/femur at 3, 6, and 12 months of reconstitution in these experiments.

0 2 4 6 8 10 12

0.1 0.3 1 3

0 2 4 6 8 10 12

Months of reconstitution

Reconstituting units per femur

Reconstituting units per femur

MIP-1α

Control
In mice treated with Ara-C at 0 and 7 hours, MIP-1α injected at 3, 6, and 10 hours increased the number of CFU-S recovered after 2 days from 56% to 81% of control and the number of CFU-A recovered after 1 day from 46% to 56% of untreated control. Similarly, MIP-1α (5 to 15 μg/mouse, IV), administered in between two doses of hydroxyurea given 7 hours apart, was also shown to increase the number of CFU-S recovered after 3 to 7 days.

Recently, the physical separation of CFU-S from the LTR stem cells has clearly demonstrated that CFU-S are distinct from the most primitive stem cells. Therefore, it remained to test the effects of MIP-1α directly on the very early stem cells capable of sustaining long-term hematopoiesis in vivo. We investigated here whether LTR stem cells could respond to MIP-1α and be protected from chemotherapy. For that, we used a quantitative assay specific for LTR stem cells and first devised a method to recruit the LTR stem cells into cycle. We failed to see any effect of a sustained 7-day exposure to MIP-1α on the LTR stem cells in these conditions. In contrast, the number of late precursors recovered after 5-FU were significantly reduced in the MIP-1α-treated animals, clearly indicating that MIP-1α had measurable effects in vivo at the doses used.

Treatment of normal mice with the same amounts of human MIP-1α did not alter the CFC and pre-CFC content of their BM. Therefore, in vivo in normal mice, the cycling CFC, pre-CFC, and the quiescent LTR stem cells were unaffected by MIP-1α, although after treatment with 5-FU, MIP-1α had negative effects on cycling CFC and pre-CFC, but not on cycling LTR stem cells.

Our observations seem to be in apparent contrast to the results reported recently by Dunlop et al and Lord et al, in which MIP-1α gave some protection of the CFU-S or CFU-A compartment against treatment with Ara-C or hydroxy-
MIP-1α does not protect LTR stem cells in vivo

The effect of MIP-1α on the cell cycling of CFU-S and CFU-A, which is stimulated shortly after chemotherapeutic insult, and to explore the usefulness of such agents in extending the range of anticancer chemotherapy.

Fig. 6. In vitro effect of human MIP-1α on murine and human BM cell colony formation. (A) Murine BM cells (5 X 10⁶ CBA cells/mL) were tested in methylcellulose cultures containing increasing concentrations of rhMIP-1α together with rmIL-3 (10 ng/mL), rhIL-1β (10 ng/mL), and rhEpo (0.1 U/mL). Erythroid (A), GM (B), and CFC-mult (C) colonies were counted after 9 days in culture. (B) Non-adherent human BM cells (5 X 10⁶ cells/mL) were incubated in methylcellulose cultures containing either rhIL-3 (2.4 ng/mL) or KL (50 ng/mL; A) together with rhEpo (0.1 U/mL) and increasing concentrations of MIP-1α. Erythroid bursts were counted after 14 days in culture. The background stimulation obtained with Epo alone was 32 bursts/10⁶ cells. The results are pooled from two independent experiments (4 plates per point).

We are grateful to Susi Wehrli, Irma Ziegler, Carolina Steiner, and Beatrice Ward for their skillful technical assistance and to Drs Max Schreier, Gordon Keller, and Steven Clark for fruitful discussions.

REFERENCES

7. Dunlop DJ, Wright EG, Lorimore S, Graham GJ, Holyoake T, Kerr DJ, Wolpe SD, Pragnell IB: Demonstration of stem cell in-

urea. However, it is worth noting that different precursor cells were measured and that the experimental protocols used were therefore very different. The early studies investigating the effect of MIP-1α on the cell cycling of CFU-S and CFU-A, which is stimulated shortly after chemotherapeutic insult, used short-term protocols over hours. In contrast, a period of 5 days was required to recruit the LTR stem cells into cycle⁶ and we therefore used a prolonged exposure to MIP-1α over several days. MIP-1α was injected subcutaneously twice daily to ensure sustained bioavailability of the drug while avoiding any inflammatory response. We showed that no inflammation occurred at the site of injection or in the major organs and that daily doses of MIP-1α of 0.3 to 3 µg/mouse over 3 to 7 days were effective in reducing the number of colony-forming cells, including CFU-S and CFU-A, recovered after 5-FU injection.

MIP-1α was first reported to have no effect on later precursors such as GM-CFC in primary cultures of BM cells.² However, murine MIP-1α was independently shown to enhance GM-CFC colony formation on its own and to suppress GM-CFC, BFU-E, and multilineage colonies stimulated by pokeweed mitogen spleen-conditioned medium,¹⁹ suggesting a broader range of activities for MIP-1α on early and late progenitors. In the study presented here, human MIP-1α had no inhibitory effect on human or murine colony-forming cells in vitro (CFC-mult, erythroid, GM).

Our results and previous studies clearly show that LTR stem cells differ from all later progenitors tested, including CFU-S and CFU-A in their response to 5-FU in vivo. In addition, we show here that, even after activation by 5-FU, the LTR stem cells also differ from later progenitors in their response to MIP-1α. Therefore, our study demonstrates that previous models based on indirect assessment of stem cell response using assays detecting more mature progenitors are not necessarily predictive for LTR stem cells. The experimental model described here will be useful for identifying agents effective for maintaining the most primitive stem cells at rest, and to explore the usefulness of such agents in extending the range of anticancer chemotherapy.

ACKNOWLEDGMENT

We are grateful to Susi Wehrli, Irma Ziegler, Carolina Steiner, and Beatrice Ward for their skillful technical assistance and to Drs Max Schreier, Gordon Keller, and Steven Clark for fruitful discussions.
hition and myeloprotective effects of SCI/rhMIP-1α in vivo. Blood 79:2221, 1992
13. Eppig JJ, Kozak LP, Eicher EA, Stevens LC: Ovarian teratomas in mice are derived from oocytes that have completed the first meiotic division. Nature 269:517, 1977
Use of 5-fluorouracil to analyze the effect of macrophage inflammatory protein-1 alpha on long-term reconstituting stem cells in vivo

VF Quesniaux, GJ Graham, I Pragnell, D Donaldson, SD Wolpe, NN Iscove and B Fagg