

Transforming Growth Factor β : Is It a Downregulator of Stem Cell Inhibition by Macrophage Inflammatory Protein 1 α ?

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Summary

Transforming growth factor β_1 (TGF- β_1) and macrophage inflammatory protein 1 α (MIP-1 α) have recently been identified as potent inhibitors of hemopoietic stem cell proliferation. From previous studies, these molecules appear to have similar functions in the control of stem cell proliferation. This study was designed to investigate the relationship, if any, between these two negative regulators in an attempt to elucidate possible distinctive roles for each within the hemopoietic system. We report here that both MIP-1 α and TGF- β are capable of inhibiting the same stem cell population (colony-forming unit [CFU]-A/CFU-S) with similar potencies. We further show that TGF- β potently inhibits MIP-1 α gene expression in bone marrow-derived macrophages, the presumed source of MIP-1 α in the bone marrow. This inhibition is not specific to MIP-1 α in that expression of MIP-1 β , a related molecule that does not exhibit potent stem cell inhibitory properties, is inhibited in a similar manner. The inhibition of MIP-1 α gene expression is also seen as a reduction in MIP-1 α protein production, which markedly decreases 24 h after treating RAW 264.7 cells, a murine macrophage cell line, with TGF- β . These *in vitro* results suggest that in the presence of active TGF- β *in vivo*, and in the absence of upregulators of MIP-1 α transcription, very little MIP-1 α will be produced. To address how MIP-1 α 's target cells, the stem cells, would respond to TGF- β , and the consequently low levels of MIP-1 α produced, we analyzed the effect of TGF- β on MIP-1 α receptor levels on FDCP-MIX cells, a murine stem cell line. We show that TGF- β (100 pM) reversibly downregulates MIP-1 α receptor levels on these cells to a maximum of 50–70% after 24 h. This level of downregulation does not change upon increasing the concentration of TGF- β or the length of exposure of the cells to TGF- β . Scatchard analysis shows that TGF- β downregulates MIP-1 α receptor numbers with no change in affinity of the remaining receptors. These results suggest that TGF- β may be capable of interfering with MIP-1 α 's role as a stem cell inhibitor. Indeed, they suggest that in the presence of active TGF- β *in vivo*, MIP-1 α is at best a weak contributor to the overall physiological inhibition of stem cells.

A number of inhibitors of hemopoietic stem cell proliferation have been identified recently (1), including TGF- β (2), a tetrapeptide inhibitor (3), and the hemoregulatory pentapeptide (4). We have identified and characterized another potent inhibitor of both murine and human hemopoietic stem cell proliferation, and subsequent work has revealed this molecule to be identical to a previously identified cytokine, macrophage inflammatory protein 1 α (MIP-1 α)¹ (5). We have also shown MIP-1 α to be functionally and antigenically indistinguishable from an inhibitory activity present in normal murine bone marrow extracts.

In vitro and *in vivo* results obtained thus far indicate that MIP-1 α has many functional similarities to another potent

inhibitory molecule, TGF- β . For example, it has been demonstrated that both of these molecules are capable of inhibiting primitive hemopoietic stem cell proliferation (5, 6), but appear to be stimulatory for less primitive progenitor cells (7–9). Both molecules have also been shown to be active *in vivo* in inhibiting the proliferation of primitive hemopoietic cells, and are thus currently being investigated as potential myelo-protective agents for use during chemotherapy (10–12). Furthermore, TGF- β is also functional as a potent inhibitor of clonogenic epidermal cells (13), and we have recently demonstrated MIP-1 α to be active in a similar manner in inhibiting this cellular population (14). Thus, the overlapping roles of TGF- β and MIP-1 α are not restricted to the hemopoietic system and may in fact be observed in a much wider range of embryologically distinct cell types. It is clear that both MIP-1 α and TGF- β are actively produced within resting bone marrow and skin (15–17), and it is therefore important to

¹ Abbreviations used in this paper: CM, conditioned medium; MIP-1 α , macrophage inflammatory protein 1 α .

define the relative roles, if any, of these two molecules in the overall control of stem cell proliferation.

There are no obvious structural similarities between MIP-1 α and TGF- β which might account for their similar functions. Indeed, whereas TGF- β is a member of a large family of related cytokines within which are three closely related mammalian TGF- β molecules (1, 2, and 3) (18), MIP-1 α is a member of a quite distinct family with very different primary, secondary, and tertiary structures to TGF- β and its related peptides (19, 20). Three major receptors for TGF- β (types 1, 2, and 3) (21) have been identified in mammalian tissues, one of which, β -glycan, has no signal transduction capacity (22), and the other two of which (types 1 and 2) are believed to be involved in forming a heterodimeric complex after TGF- β binding to cells (23). The type 2 receptor has been cloned recently and has been shown to be a member of the serine/threonine kinase family of receptors (24). A human receptor for MIP-1 α has recently been cloned (25, 26) and is a member of the G-protein linked family of seven membrane-spanning receptor molecules (27), which also contains the receptor for the MIP-1 α -related molecule, IL-8 (28, 29). We have shown that numerous cell types within the hemopoietic system have specific receptors for MIP-1 α including FDCP-MIX cells, a murine stem cell line, and K562 cells, a human myeloid leukemia cell line (30). Cross-competition analysis indicates that the receptor we are detecting on K562 cells may be identical to the recently cloned MIP-1 α /RANTES receptor, however the receptor on FDCP-MIX cells has a more restricted binding repertoire and may therefore represent a receptor specific for the stem cell inhibitory effects of MIP-1 α and related chemokines (30). MIP-1 α receptors have also been found on CTLL-R8, a T cell line, and on RAW 264.7, a macrophage cell line (31). Evidence is available implicating both Rb and *c-myc* (32–24) in the cellular response to TGF- β , although as yet, no information is available on the components of the MIP-1 α signal transduction system. Such studies await the availability of a suitable cell system.

The functional redundancy coupled with the knowledge of their distinct molecular nature suggest that there may be some form of interaction between MIP-1 α and TGF- β in the control of stem cell proliferation. It is conceivable that one or other of these molecules may exert its inhibitory effect by inducing synthesis of the other or by upregulating receptors or priming the necessary signal transduction systems. The aim of this study therefore was to look at any possible interactions between MIP-1 α and TGF- β , and to attempt to elucidate the possible role of each in the overall control of stem cell proliferation.

We now report that both MIP-1 α and TGF- β are capable of inhibiting the same population of hemopoietic stem cells (CFU-A/CFU-S cells) with similar potencies. We further demonstrate that TGF- β is capable of downregulating MIP-1 α gene expression in both murine bone marrow-derived macrophages, the presumed source of MIP-1 α in the bone marrow, and also in RAW cells, a murine macrophage cell line. TGF- β is extremely potent in this regard. We also demonstrate TGF- β to be capable of substantially downregulating

MIP-1 α receptor numbers on FDCP-MIX cells without affecting the affinity of the remaining receptors for ligand. These *in vitro* results suggest therefore that in the presence of TGF- β and in the absence of positive regulators, both arms of the MIP-1 α inhibitory response are downregulated and that *in vivo* in the presence of endogenous active TGF- β , MIP-1 α may be at best a weak contributor to the overall physiological control of hemopoietic stem cell proliferation in normal bone marrow.

Materials and Methods

Cytokines. Murine rMIP-1 α was obtained from R&D Systems, Inc. (Minneapolis, MN). Human rTGF- β 1 was obtained from British Biotechnology Ltd. (Oxon, UK) and Gibco BRL (Paisley, Scotland).

The *In Vitro* CFU-A Assay. Bone marrow cells were obtained from female B6D2F₁ mice and prepared as suspensions by flushing femora with medium. For the detection of primitive progenitor cells, CFU-A assays were carried out as described previously (35). Briefly, 2 \times 10⁴ cells in 4 ml supplemented α -modified MEM containing 20% donor horse serum and 0.3% agar were seeded on top of an underlayer of the same medium containing 0.6% agar, 10% L929 cell conditioned medium ([CM] as a source of M-CSF), and 10% AF1-19 T cell CM (as a source of GM-CSF) in 45-mm petri dishes. Cultures were incubated at 37°C in a fully humidified atmosphere of 10% CO₂, 5% O₂, and 85% N₂ for 11 d. This gave rise to the formation of macroscopic colonies. Although colonies <2 mm in size do appear in the CFU-A assay, only colonies >2 mm in size were scored since it has been previously demonstrated that these larger colonies are derived from primitive hemopoietic cells (36). The inhibitory cytokines MIP-1 α and TGF- β were tested in this assay via direct addition to the bottom agar layer in various concentrations, as previously described (37).

Derivation of Bone Marrow Macrophages and Culture of RAW Cells and FDCP-MIX Cells. Bone marrow cells were obtained from female CD1 strain mice and prepared as suspensions by flushing femora with medium. These cells were suspended at 5 \times 10⁴/ml in α -modified MEM supplemented with 20% L929 CM as a source of M-CSF. These were incubated at 37°C for 7 d in a dry atmosphere of 5% CO₂ in air, and gave rise to a homogeneous population of bone marrow-derived macrophages as verified by immunohistochemical staining for macrophage-specific markers (data not shown). These cells were then used to investigate the effect of TGF- β on MIP-1 α expression.

The murine macrophage cell line RAW was maintained in special liquid medium (Gibco BRL)/10% FCS in a dry atmosphere of 5% CO₂ in air. These cells were also used to investigate the effect of TGF- β on MIP-1 α expression.

FDCP-MIX cells were a generous gift from Professor Mike Dexter and Dr. Elaine Spooner (Paterson Institute, Manchester, UK) and were cultured as described previously (38). Briefly, the cells were maintained in special liquid medium with 10% donor horse serum and made 10% with CM from the WEHI cell line, a source of IL-3.

Gene Expression Experiments. Bone marrow-derived macrophages were starved of L929 CM and thus M-CSF for 16 h to induce growth arrest. These cells were then refed with L929 CM \pm 250 pM TGF- β , and total RNA was made, either 0, 1, 2, 4, 6, or 24 h after refeeding for the time course experiment, or after 4 h for the TGF- β titration experiment, using a phenol-based extraction technique (RNAzol; Cinna/Biotech Laboratories, Inc., Houston, TX). 20 μ g of each RNA sample was run on a 1.4% (wt/vol) agarose gel. The RNA was transferred via Northern blot-

ting on to a GeneScreen membrane (Dupont NEN, Stevenage, Herts, UK) which was baked at 80°C for 2–3 h to fix the RNA. A MIP-1 α specific probe was generated as an in vitro transcription product of the coding sequence (279 bp) of MIP-1 α cDNA sequence cloned into the pSK⁻ Bluescript plasmid. Transcription was initiated from the T3 promoter to give an antisense RNA MIP-1 α specific probe. The probe was labeled using ³²P-labeled UTP and separated from unincorporated nucleotides by passage through a Nick column (Pharmacia, Piscataway, NJ). The membrane was pre-hybridized at 65°C for 3 h in prehybridization buffer (50% formamide, 5 \times SSPE, 5 \times Denhardt's, 0.1% SDS, 0.2% poly(A), 12.8% diethyl pyrocarbonate (DEPC)-treated H₂O and 0.1% salmon sperm DNA of a 10 mg/ml solution). Hybridization was then performed for 16 h at 65°C in prehybridization buffer containing the ³²P-labeled MIP-1 α probe. The filter was then washed twice in 2 \times SSPE/0.1% SDS for 30 min at room temperature and then twice in 0.2 \times SSPE/0.1% SDS for 30 min at 65°C. After washing, the membrane(s) were exposed to Kodak x-ray film for several hours at -70°C.

For the RAW cell experiment, the same protocol as above was used except that the cells were starved for FCS overnight, and then restimulated with fresh medium and FCS.

Western Blot Analysis. MIP-1 α protein was detected by Western blotting using a commercially available polyclonal antibody to MIP-1 α (R&D Systems, Inc.). Briefly, 15% SDS-PAGE gels were run according to the method of Laemli (39), and the gels blotted onto nitrocellulose filters (Schleicher & Schuell, Inc., Keene, NH) using a minigel electroblotter (Atto Instruments, Inc., Rockville, MD). Nonspecific binding sites on the blots were blocked with a 1-h wash in Blotto (5% powdered milk, 0.1% NP-40 in PBS), after which they were incubated, for 1 h, in 10 ml of a 1:1,000 dilution of the goat anti-MIP-1 α antibody in Blotto. The blot was removed from the antibody solution and washed extensively with multiple changes of Blotto over a period of 1 h after which it was placed in 10 ml of a 1:1,000 dilution of secondary antibody (horseradish peroxidase-labeled horse anti-goat IgG; Vector Laboratories, Inc., Burlingame, CA). The blot was then finally washed extensively with multiple changes of PBS-T (PBS/0.1% Tween 20) and the MIP-1 α bands visualized using the enhanced chemiluminescence detection system (Amersham International, Amersham, Buck, UK).

Receptor Binding Experiments. MIP-1 α was radiolabeled with

¹²⁵I as described previously (30). Briefly, labeling was by the iodogen method and resulted in labeling to high specific activity (2.5 \times 10⁷ cpm/ μ g) with full retention of biological activity.

FDCP-MIX cells were incubated overnight at a concentration of 10⁶ cells/ml with or without TGF- β (at a concentration of 100 pM, or as outlined in the figure legends). The cells were then washed twice in PBS, and 5 \times 10⁵ cells per point aliquoted. To each aliquot, varying concentrations of ¹²⁵I-labeled MIP-1 α were added in binding buffer (Special Liquid Medium/10% donor horse serum/0.2% azide) and either PBS or 100-fold excess unlabeled MIP-1 α competitor up to a final volume of 250 μ l. Cells were then incubated at 37°C in the above conditions for 90 min after which they were washed three times in PBS, and the incorporated radiation assessed in a gamma counter. Analysis of binding isotherms was performed using the LIGAND program (40).

Results and Discussion

Direct Addition of TGF- β and MIP-1 α Can Inhibit CFU-A Colony Formation. The CFU-A assay is believed to be an in vitro correlate of the CFU-S hemopoietic stem cell assay (35, 36) and thus simplifies the testing of putative stem cell regulatory factors. We have previously reported that direct addition of MIP-1 α to the CFU-A assay results in inhibition of CFU-A colony formation (37). To determine whether direct addition of TGF- β was also active on this subset of stem cells, we performed CFU-A assays using both of these cytokines. The results show that both TGF- β and MIP-1 α are capable of preventing CFU-A colony formation (Fig. 1, A and B). TGF- β inhibits with an ED₅₀ of ~50 pM, whereas MIP-1 α inhibits with an ED₅₀ of around 150 pM. Previous work has shown that MIP-1 α is also capable of inhibiting CFU-A colony formation with an ED₅₀ of around 50 pM (37). Thus, it appears that both MIP-1 α and TGF- β are capable of inhibiting the same population of in vitro stem cells with similar potencies confirming the redundancy in the actions of these two cytokines on this cellular population. This system is well characterized in terms of inhibition by MIP-1 α , and thus offers an ideal experimental model for studying the in-

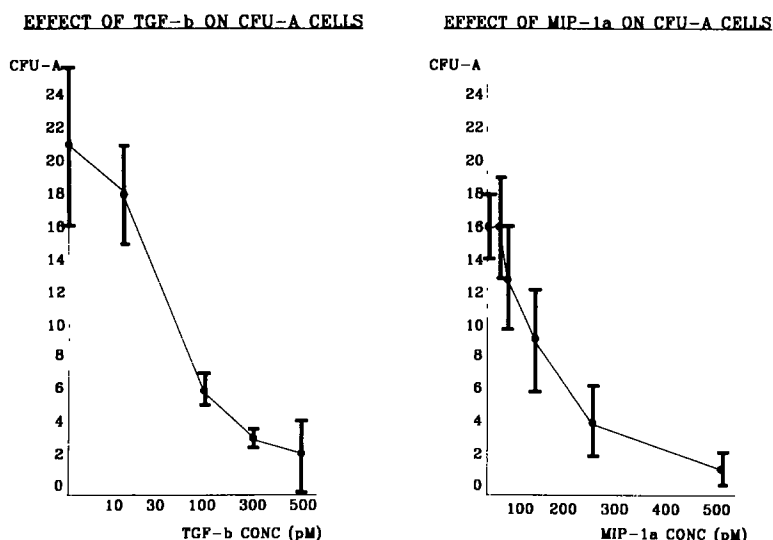


Figure 1. Inhibition of CFU-A colony formation by TGF- β and MIP-1 α . TGF- β (A) and MIP-1 α (B) were tested for inhibitory activity in the direct addition CFU-A assay (34). The cytokines were added directly to the feeder layers (0.6% agar in α -MEM with sources of M-CSF and GM-CSF) of the CFU-A assays and the target cells were normal bone marrow cells plated at 5 \times 10⁴/ml in the upper layer (0.3% agar in α -MEM). The assay plates were incubated at 37°C in a fully humidified atmosphere of 10% CO₂, 5% O₂, and 85% N₂ for 11 d. Colonies >2 mm were scored as being CFU-A type. The results represent inhibition of CFU-A colony formation compared with control values. The above results are representative of more than three separate experiments.

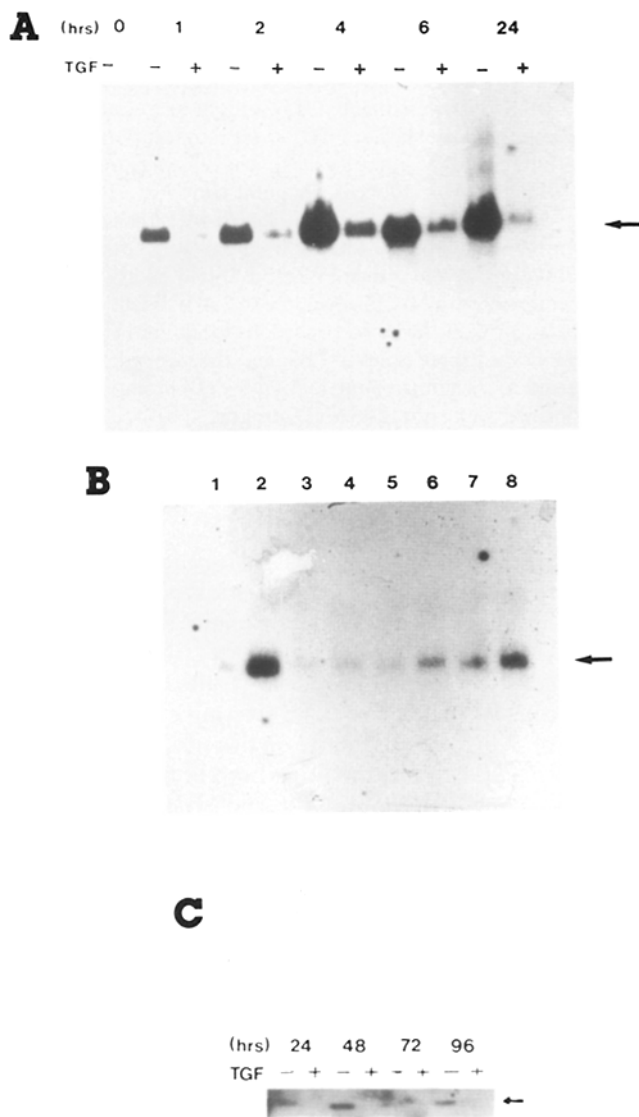


Figure 2. Inhibition of MIP-1 α gene expression and protein production in macrophages by TGF- β . (A) MIP-1 α gene expression induction from 0 to 24 h \pm TGF- β . Briefly, M-CSF starved bone marrow-derived macrophages were re-fed with M-CSF with or without TGF- β (250 pM). Total RNA was made 0, 1, 2, 4, 6, and 24 h after refeeding and 20 μ g of each RNA sample was run on a 1.4% (wt/vol) agarose gel. The RNA was transferred via Northern blotting, and the membrane probed using an MIP-1 α specific riboprobe. The autoradiograph was exposed for 18 h. (B) Titration of TGF- β downregulation of MIP-1 α gene expression. Lane 1, 0-h control; lane 2, 4-h control; lane 3, 300 pM TGF- β ; lane 4, 100 pM TGF- β ; lane 5, 30 pM TGF- β ; lane 6, 10 pM TGF- β ; lane 7, 3 pM TGF- β ; lane 8, 1 pM TGF- β . Briefly, M-CSF starved bone marrow-derived macrophages were re-fed with M-CSF plus varying concentrations of TGF- β . Total RNA was made 4 h after feeding, and blotted and probed as described above. (C) Downregulation of protein production by TGF- β . RAW cells were starved overnight and re-fed with fresh medium \pm 250 pM TGF- β . CM was collected and analyzed for the presence of MIP-1 α protein by Western blotting.

teraction, if any, between these two molecules in stem cell inhibition.

TGF- β Can Downregulate MIP-1 α Gene Expression in Bone Marrow-derived Macrophages. In functioning as stem cell inhibitors, it is possible that either MIP-1 α or TGF- β may act indirectly by inducing expression of the other in nontarget cells, many of which are present in the CFU-A assay. To investigate this possibility in more detail, we have looked at the effects of TGF- β on MIP-1 α gene expression in bone marrow-derived macrophages, the presumed source of MIP-1 α in the marrow.

It has been shown previously that bone marrow-derived macrophages starved of M-CSF for 16 h enter growth arrest (41). Upon refeeding with M-CSF, MIP-1 α expression is rapidly induced to an optimum at around 4 h. As it is difficult to determine exactly what level of MIP-1 α expression will be observed in normal steady state bone marrow macrophages, and given the low level of gene expression detected in quiescent cultured macrophages, it was decided to use this technique of MIP-1 α gene expression induction to obtain a readily measurable level of expression.

The effect of TGF- β on the induction of MIP-1 α was investigated as described in Materials and Methods. Fig. 2 A shows a time course of MIP-1 α gene expression in cells after refeeding with M-CSF, with or without TGF- β as measured by Northern blot analysis. As previously demonstrated, in the absence of TGF- β , MIP-1 α is induced to an optimum at around 4 h after feeding, with this level beginning to decline to resting levels within 24 h. In the presence of TGF- β however, the induction of MIP-1 α expression is essentially inhibited throughout the time course investigated. Even after 24 h, densitometric analysis shows that expression of MIP-1 α in TGF- β treated cells remains inhibited by 87% compared with the control cells. Reprobing of blots for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA revealed similar loadings in all tracks (data not shown). It appears therefore that TGF- β is capable of inhibiting the induction of MIP-1 α gene expression in bone marrow-derived macrophages, the presumed source of MIP-1 α in the bone marrow, and whereas for practical reasons we have observed this effect during induction of MIP-1 α gene expression, it is evident from the varying levels of MIP-1 α induction seen in Fig. 2 A that such an effect is observed at a range of levels of MIP-1 α gene expression, even as low as those detected in normal bone marrow.

To assess the potency of this effect, we performed a titration of the effects of TGF- β on MIP-1 α gene expression using the methodology outlined above. Cells were studied at 4 h after feeding as this leads to the optimum induction of MIP-1 α gene expression, therefore allowing any inhibition by TGF- β to be easily measured. The results show MIP-1 α gene expression to be inhibited in a dose-dependent manner (Fig. 2 B), and even at concentrations of TGF- β as low as 1 pM, densitometric analysis shows that MIP-1 α gene expression is reduced by \sim 70% compared with control values. TGF- β is therefore very potent in the inhibition of MIP-1 α gene expression, and appears to be capable of mediating this effect at femtomolar concentrations.

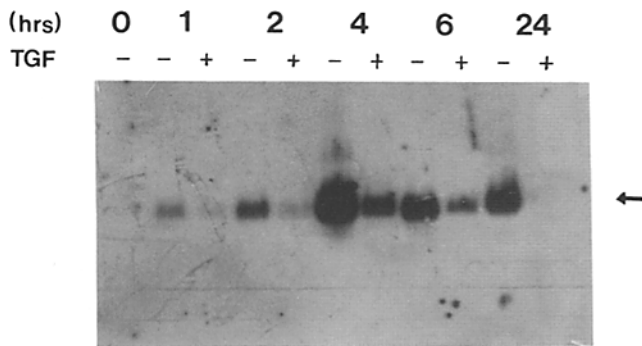


Figure 3. Inhibition of MIP-1 β gene expression in macrophages by TGF- β . A time course of MIP-1 β gene expression induction from 0 to 24 h \pm TGF- β . Briefly, the membrane shown in Fig. 2 was stripped of MIP-1 α specific probe by boiling in several hundred milliliters of 0.05 \times SSC, 0.01 M EDTA (pH, 8.0), and 0.1% SDS for 10 min. The filter was then rinsed in 0.01 \times SSC at room temperature and probed with a MIP-1 β specific riboprobe. The autoradiograph was exposed for 18 h.

TGF- β Downregulates MIP-1 α Protein Synthesis. We have demonstrated similar inhibition of MIP-1 α gene expression in RAW cells (data not shown), the murine macrophage cell line from which MIP-1 α was initially isolated (19). This cell line produces detectable levels of MIP-1 α protein and thus has allowed us to investigate the effects of the observed TGF- β downregulation of MIP-1 α gene expression on MIP-1 α protein levels. Such analysis is difficult in cultured macrophages because of the very low and often undetectable levels of MIP-1 α protein production (data not shown).

Results from an analysis of CM from control and TGF- β -treated RAW cells for presence of MIP-1 α protein using Western blotting (Fig. 2 C) indicate that in the presence of TGF- β , MIP-1 α protein levels are substantially reduced, and that this effect is evident even after only 24 h. This inhibition was maintained over the 96-h time course of this experiment.

These results suggest that the inhibition of gene expression by TGF- β is also observed as an inhibition of protein synthesis and that the potent blocking of MIP-1 α production by TGF- β may have important physiological and functional implications.

TGF- β Downregulates MIP-1 β Gene Expression in Bone Marrow-derived Macrophages. To investigate whether the TGF- β inhibition of MIP-1 α expression was a specific effect, we have examined the effect of TGF- β on MIP-1 β gene expression in bone marrow-derived macrophages. MIP-1 β is a related molecule to MIP-1 α , but, with the exception of recombinant material produced in prokaryotic systems, it does not appear to exhibit stem cell inhibitory properties (30). A time course was performed as outlined in the previous experiments and the blot probed with a MIP-1 β specific probe. The results (Fig. 3) show that TGF- β can also inhibit MIP-1 β gene expression, and therefore it appears that TGF- β is not specific in inhibiting MIP-1 α gene expression in bone marrow-derived macrophages.

Much evidence is now available pointing to a role for TGF- β

as a modulator of inflammatory cytokine production (42, 43), and it has been shown previously that TGF- β is a potent deactivator of macrophages (44). It is likely that the downregulation of MIP-1 α and MIP-1 β cytokine production falls into this category of cellular control and may not be specifically related to stem cell inhibition. It is interesting to note in this context that we have also demonstrated TGF- β to be active in inhibiting induction of MIP-1 α after treatment of bone marrow-derived macrophages with LPS (data not shown). The more general nature of the TGF- β downregulation of MIP-1 α is, however, to a large extent irrelevant as the demonstration of the ability of TGF- β to downregulate MIP-1 α protein synthesis suggests that it will interfere with all of the MIP-1 α -mediated functions, and not simply those relating to immunological phenomena.

Recently, targeted disruption studies on the TGF- β 1 gene have been reported (45), and analysis of the cytokine profile of these animals indicate MIP-1 α gene expression to be up-regulated in the TGF- β 1-deficient mice compared with control mice. This is interpreted as being a secondary product of the tissue inflammation observed in the TGF- β 1-deficient mice. The present results indicate that this may, at least in the case of MIP-1 α , be a more specific result of removal of a TGF- β block on cytokine gene expression. Such a conclusion would predict that TGF- β 2 and TGF- β 3 would not be potent downregulators of MIP-1 α gene expression, as these genes are intact in the transgenic mice. We are currently investigating the effects of these isoforms of TGF- β in this regard.

TGF- β Can Downregulate MIP-1 α Receptors on FDCCP-MIX Cells. Given the observed response of macrophages (the likely in vivo source of MIP-1 α) to TGF- β , we have also investigated what, if any, response is observed in the MIP-1 α target cells. To address this question, we have studied the effects of TGF- β on the levels of receptor for MIP-1 α on hemopoietic stem cell like lines. The specific cell line used in the current study was the murine FDCCP-MIX cell line which, as initially isolated, displays considerable similarity to the CFU-S stem cell (38). We have previously demonstrated these cells to have numerous cell surface receptors for MIP-1 α and have also shown these receptors to be specific in recognizing only those members of the MIP-1 α peptide family that are capable of stem cell inhibition (30). Receptor binding assays were performed as described in Materials and Methods. Initially, binding was performed using a titration of radiolabeled MIP-1 α to investigate the effects of TGF- β on MIP-1 α receptor levels. MIP-1 α receptor levels are typically saturated at around 1 nM, however, when cells are incubated with TGF- β , the MIP-1 α receptor levels on the cell surface appear to be reduced by \sim 50% (Fig. 4). We have shown that TGF- β does not compete with MIP-1 α for binding to its receptor and thus these results do not simply reflect TGF- β blocking of MIP-1 α receptor binding. More comprehensive binding curves were carried out (Fig. 5) and Scatchard analysis of the data performed to investigate whether the observed downregulation results from reduction in receptor numbers or receptor affinity. The results indicate that whereas the control cells

EFFECT OF TGF- β ON MIP-1 α RECEPTORS ON FDCP MIX CELLS

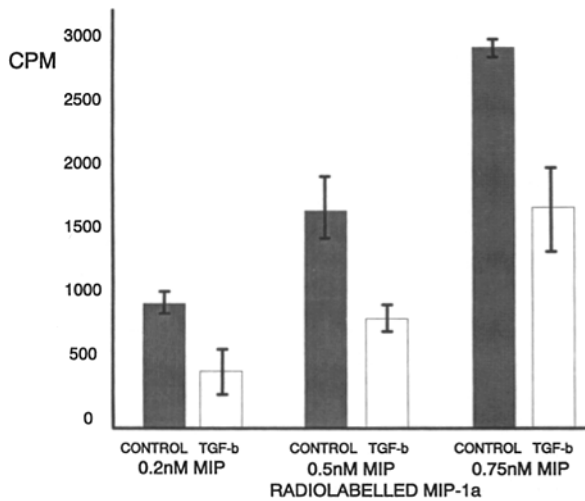


Figure 4. TGF- β downregulates MIP-1 α receptors on FDCP-MIX cells. Titration of specific binding of 125 I-labeled MIP-1 α to FDCP-MIX cells after treatment with or without TGF- β (100 pM). FDCP-MIX cells were incubated overnight at a concentration of 10^6 cells/ml \pm TGF- β after which the cells were washed twice in PBS and 5×10^5 cells per point aliquoted. To each aliquot, varying concentrations of 125 I-labeled MIP-1 α were added in binding buffer and either PBS or 100-fold excess unlabeled MIP-1 α competitor. Cells were then incubated at 37°C for 90 min after which they were washed 3 times in PBS and the incorporated radiation assessed in a gamma counter. Data are representative of three experiments and are plotted as the mean of duplicate determinations.

expressed $\sim 11,000$ receptors per cell, the TGF- β -treated cells expressed only 3,400 receptors per cell, indicating a 70% reduction in receptor numbers. MIP-1 α receptors on control cells exhibited a K_d of 1.3×10^{-9} compared with 1.21×10^{-9} for the TGF- β -treated cells. Thus, TGF- β inhibition of MIP-1 α receptors is due to a decrease in receptor number and not to a decrease in receptor affinity. The K_d of the MIP-1 α

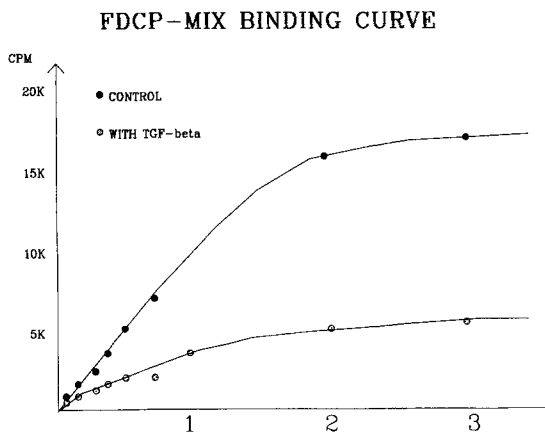


Figure 5. Analysis of TGF- β -mediated downregulation of MIP-1 α receptor levels. Equilibrium binding of 125 I-labeled MIP-1 α to FDCP-MIX cells. Cells were preincubated in medium with or without TGF- β (100 pM) for 18 h and binding of 125 I-labeled MIP-1 α assessed as outlined in Fig. 4.

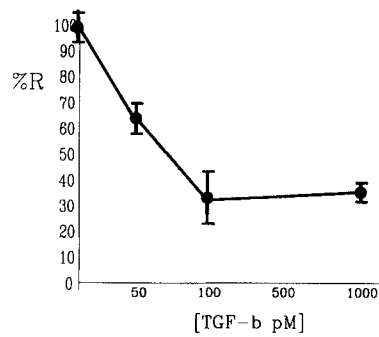


Figure 6. Titration of TGF- β -mediated downregulation of MIP-1 α receptors. FDCP-MIX cells treated for 24 h with varying concentrations of TGF- β were treated as outlined in Fig. 4 and specific binding of 125 I-labeled MIP-1 α measured. The results are expressed as percent incorporation of 125 I MIP-1 α compared with control cells.

receptors measured in the present study is approximately three times that measured in previous studies (30). The reasons for this are unclear but may reflect variations in the molecular weight of the MIP-1 α used in the binding studies (for a discussion see reference 30).

To fully characterize the kinetics of this receptor downregulation, and to ascertain the maximum downregulation that could be obtained, a titration from 50 pM to 1 nM TGF- β was performed. Fig. 6 shows that after 24 h, the maximum downregulation appears to be $\sim 50\%$, and this is achieved using TGF- β at a concentration of 100 pM. The maximum downregulation achievable varied from experiment to experiment between 50 and 70%. Increasing the concentration of TGF- β does not however cause a further decrease in MIP-1 α receptor numbers.

In vivo, it is possible that stem cells are continually exposed to active TGF- β and preliminary evidence from studies on long-term exposure of FDCP-MIX cells to TGF- β indicate that such continued exposure maintains the reduced levels of MIP-1 α receptors on FDCP-MIX cells. If a single treatment of TGF- β is applied to the cells, the MIP-1 α receptor levels are seen to return to normal within 72 h (data not shown). This suggests that the TGF- β -mediated inhibition of MIP-1 α receptor levels is fully reversible but that on prolonged exposure, such as may be encountered in vivo, MIP-1 α receptor levels will be downregulated on cells for the duration of their exposure to TGF- β .

Demonstration of receptor downregulation by TGF- β is not peculiar to MIP-1 α receptors and has been observed for a range of receptor molecules, most notably those for IL-3, G-CSF, and GM-CSF (46). Indeed, it is postulated that one of the modes of inhibitory action of TGF- β on hemopoietic stem cells involves downregulation of receptors for mitogenic stimuli, thus blocking response to these factors. In contrast to the three factors mentioned above, the MIP-1 α receptor is likely not to be a member of the hemopoietin receptor family, and thus the ability of TGF- β to downregulate cytokine receptor levels may be a very general phenomenon affecting receptors for both mitogenic and inhibitory cytokines. Again, although these results have been obtained using in vitro cel-

lular systems, they indicate the ability of TGF- β to down-regulate MIP-1 α receptor levels on any cells, including stem cells, that are exposed to active TGF- β . The functional significance of a 50–70% reduction in MIP-1 α levels on FDC-MIX cells in terms of ability to respond to MIP-1 α is unclear. Certainly it is apparent from studies with other receptor/ligand systems that only partial receptor occupancy is often sufficient for generation of full biological effect (47), and it remains a formal possibility that the remaining 30–50% of MIP-1 α receptor may be sufficient to achieve this end. This possibility is currently under investigation.

Conclusions. The results presented in this manuscript show

TGF- β to be a potent downregulator of MIP-1 α production by bone marrow macrophages and also of MIP-1 α receptor levels on primitive hemopoietic cells *in vitro*. These results suggest that *in vivo*, in the presence of active TGF- β which has been demonstrated to be present in normal bone marrow and in the absence of additional factors which may act to upregulate MIP-1 α , MIP-1 α may only be a weak contributor to inhibition of hemopoietic stem cells. It may be that MIP-1 α is relatively more important in inhibiting stem cells during or after stress, or in situations in which TGF- β is present in low levels.

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References

- Graham, G.J., and I.B. Pragnell. 1990. Negative regulators of haemopoiesis-current advances. *Prog. Growth Factor Res.* 2:181.
- Ohta, M., J.S. Greenberger, P. Anklesaria, A. Bassols, and J. Massague. 1987. Two forms of transforming growth factor- β distinguished by multipotential haemopoietic progenitor cells. *Nature (Lond.)* 329:539.
- Lenfant, M., J. Wdzieczak-Bakala, E. Guittet, J. Prome, D. Sotty, and E. Frindel. 1989. Inhibitor of hematopoietic pluripotent stem cell proliferation: purification and determination of its structure. *Proc. Natl. Acad. Sci. USA.* 86:779.
- Paukovits, W.R., and O.D. Laerum. 1982. Isolation and synthesis of a hemoregulatory peptide. *Z. Naturforsch.* 37c:1279.
- Graham, G.J., E.G. Wright, R. Hewick, S.D. Wolpe, N.M. Wilkie, D. Donaldson, S. Lorimore, and I.B. Pragnell. 1990. Identification and characterisation of an inhibitor of haemopoietic stem cell proliferation. *Nature (Lond.)* 344:442.
- Migdalska, A., G. Molineux, H. Demuyneck, G.S. Evans, F. Ruscetti, and T.M. Dexter. 1991. Growth inhibitory effects of transforming growth factor- β 1 *in vivo*. *Growth Factors.* 4:239.
- Jacobsen, E.W.S., R. Keller, F.W. Ruscetti, P. Kondaiah, A.B. Roberts, and L.A. Falk. 1991. Bidirectional effects of transforming growth factor-induced human myelopoiesis *in vitro*: differential effects of distinct TGF- β isoforms. *Blood.* 78:2239.
- Broxmeyer, H.E., B. Sherry, L. Lu, S. Cooper, K. Oh, P. Tekamp-Olson, B.S. Kwon, and A. Cerami. 1990. Enhancing and suppressing effects of recombinant murine macrophage inflammatory proteins on colony formation *in vitro* by bone marrow myeloid progenitor cells. *Blood.* 76:1110.
- Clements, J.M., S. Craig, A.J.H. Gearing, M.G. Hunter, C.M. Heyworth, T.M. Dexter, and B.I. Lord. 1992. Biological and structural properties of MIP-1 α expressed in yeast. *Cytokine.* 4:76.
- Bonewald, L.F. 1992. Can transforming growth factor beta be useful as a protective agent for pluripotent haematopoietic progenitor cells. *Exp. Hematol.* 20:1249.
- Dunlop, D.J., E.G. Wright, S. Lorimore, G.J. Graham, T. Holyoake, D.J. Kerr, S.D. Wolpe, and I.B. Pragnell. 1992. Demonstration of stem cell inhibition and myeloprotective effects of SCI/rhMIP-1 α *in vivo*. *Blood.* 79:2221.
- Lord, B.I., T.M. Dexter, J.M. Clements, M.A. Hunter, and A.J.H. Gearing. 1992. Macrophage inflammatory protein protects multipotent haemopoietic cells from the cytotoxic effects of hydroxyurea *in vivo*. *Blood.* 79:2605.
- Parkinson, K., and A. Balmain. 1990. Chalcones revisited—a possible role for transforming growth factor β in tumour promotion. *Carcinogenesis.* 11:195.
- Parkinson, E.K., G.J. Graham, P. Daubersies, J.E. Burns, C. Heuffer, M. Plumb, G. Schuler, and I.B. Pragnell. 1993. A haemopoietic stem cell inhibitor (SCI/MIP-1 α) also inhibits clonogenic epidermal keratinocyte proliferation. *J. Invest. Dermatol.* In press.
- Akhurst, R.J., F. Lee, and A. Balmain. 1988. Localized production of TGF- β mRNA in tumour promoter-stimulated mouse epidermis. *Nature (Lond.)* 331:363.
- Thompson, N.L., K.C. Flanders, J.M. Smith, L.R. Ellingsworth, A.B. Roberts, and M.B. Sporn. 1989. Expression of transforming growth factor-1 β in specific cells and tissues of adult and neonatal mice. *J. Cell Biol.* 108:661.
- Heuffer, C., G. Topar, F. Koch, B. Trokenbacher, E. Kampgen, N. Romani, and G. Schuler. 1992. Cytokine gene expression in murine epidermal cell suspensions: interleukin 1 beta and macrophage inflammatory protein 1 alpha are selectively expressed in Langerhans cells but are differentially regulated in culture. *J. Exp. Med.* 176:1221.
- Massague, J. 1990. The transforming growth factor- β family. *Annu. Rev. Cell Biol.* 6:597.

19. Wolpe, S.D., and A. Cerami. 1989. Macrophage inflammatory proteins 1 and 2: members of a novel superfamily of cytokines. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 3:2656.
20. Schall, T.J. 1991. Biology of the RANTES/SIS cytokine family. *Cytokine.* 3:165.
21. Massague, J. 1992. Receptors for the TGF- β family. *Cell.* 69:1067.
22. Wang, X.F., H.Y. Lin, E. Ng-Eaton, J. Downward, H.F. Lodish, and R.A. Weinberg. 1991. Expression cloning and characterization of the TGF- β type III receptor. *Cell.* 67:797.
23. Wrana, J.L., L. Attisano, J. Carcamo, A. Zentella, J. Doody, M. Laiho, X.F. Wang, and J. Massague. 1992. TGF- β signals through a heteromeric protein kinase receptor complex. *Cell.* 71:1003.
24. Lin, H.Y., X.F. Wang, E. Ng-Eaton, R.A. Weinberg, and H.F. Lodish. 1992. Expression cloning of the TGF- β type II receptor, a functional transmembrane serine/threonine kinase. *Cell.* 68:775.
25. Neote, K., D. DiGregorio, J.Y. Mak, R. Horuk, and T.J. Schall. 1993. Molecular cloning, functional expression and signaling characteristics of a C-C chemokine receptor. *Cell.* 72:415.
26. Gao, J.-L., D.B. Kuhns, H.L. Tiffany, D. McDermott, X. Li, U. Franke, and P.M. Murphy. 1993. Structure and functional expression of the human macrophage inflammatory protein 1 α /RANTES receptor. *J. Exp. Med.* 177:1421.
27. Sweeney, K., and J. Pouyssegur. 1992. G-protein-controlled signal transduction pathways and the regulation of cell proliferation. *Adv. Cancer. Res.* 58:75.
28. Murphy, P.M., and H.L. Tiffany. 1991. Cloning of the complementary DNA encoding a functional human interleukin-8 receptor. *Science (Wash. DC).* 253:1280.
29. Holmes, W.E., J. Lee, W.-J. Kuang, G.C. Rice, and W.I. Wood. 1991. Structure and functional expression of a human interleukin-8 receptor. *Science (Wash. DC).* 253:1278.
30. Graham, G.J., L. Zhou, J.A. Weatherbee, M.L.-S. Tsang, M. Napolitano, W.J. Leonard, and I.B. Pragnell. 1993. Characterisation of a receptor for MIP-1 α and related proteins on human and murine cells. *Cell Growth. & Differ.* 4:137.
31. Oh, K.O., Z. Zhou, K.-K. Kim, H. Samanta, M. Fraser, Y.-J. Kim, H.E. Broxmeyer, and B.S. Kwon. 1991. Identification of cell surface receptors for murine macrophage inflammatory protein-1 α . *J. Immunol.* 147:2978.
32. Laiho, M., J.A. DeCaprio, J.W. Ludlow, D.M. Livingston, and J. Massague. 1990. Growth inhibition by TGF- β linked to suppression of retinoblastoma protein phosphorylation. *Cell.* 62:175.
33. Yan, Z., S. Hsu, S. Winawer, and E. Friedman. 1992. Transforming growth factor β 1 (TGF- β 1) inhibits retinoblastoma gene expression but not pRB phosphorylation in TGF- β 1-growth stimulated colon carcinoma cells. *Oncogene.* 7:801.
34. Pietenpol, J.A., R.W. Stein, E. Moran, P. Yaculik, R. Schlegel, R.M. Lyons, M.R. Pittelkow, K. Munger, P.M. Howley, and H.L. Moses. 1990. TGF- β 1 inhibition of c-myc transcription and growth in keratinocytes is abrogated by viral transforming proteins with pRB binding domains. *Cell.* 61:777.
35. Pragnell, I.B., E.G. Wright, S.A. Lorimore, J. Adam, M. Rosendaal, J.F. DeLamarter, M. Freshney, L. Eckmann, A. Sproul, N. Wilkie. 1988. The effect of stem cell proliferation regulators demonstrated with an *in vitro* assay. *Blood.* 72:196.
36. Lorimore, S., I.B. Pragnell, L. Eckmann, and E.G. Wright. 1990. Synergistic interactions allow colony formation *in vitro* by murine haemopoietic stem cells. *Leuk. Res.* 14:481.
37. Graham, G.J., M.F. Freshney, D. Donaldson, and I.B. Pragnell. 1992. Purification and biochemical characterisation of human and murine stem cell inhibitors (SCI). *Growth Factors.* 7:151.
38. Spooncer, E., C.M. Heyworth, A. Dunn, and T.M. Dexter. 1984. Self-renewal and differentiation of interleukin-3 dependent multipotent stem cells are modulated by stromal cells and serum factors. *Differentiation.* 32:111.
39. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680.
40. Munson, P.J., and D. Robard. 1980. A versatile computerised approach for the characterisation of ligand binding systems. *Anal. Biochem.* 107:220.
41. Tushinski, R.J., and E.R. Stanley. 1985. The regulation of mononuclear phagocyte entry into S Phase by the colony stimulating factor CSF-1. *J. Cell. Physiol.* 122, 221.
42. McCartney-Francis, N., D. Mizel, H. Wong, L. Wahl, and S. Wahl. 1990. TGF- β regulates production of growth factors and TGF- β by human peripheral blood monocytes. *Growth Factors.* 4:27.
43. Chantry, D., M. Turner, E. Abney, and M. Feldmann. 1989. Modulation of cytokine production by transforming growth factor- β 1. *J. Immunol.* 142:4295.
44. Tsunawaki, S., M. Sporn, A. Ding, and C. Nathan. 1988. Deactivation of macrophages by transforming growth factor- β . *Nature (Lond.).* 334:260.
45. Schull, M.M., I. Ormsby, A.B. Kier, S. Pawlowski, R.J. Diebold, M. Yin, R. Allen, C. Sidman, G. Proetzel, D. Calvin, et al. 1992. Targeted disruption of the mouse transforming growth factor- β 1 gene results in multifocal inflammatory disease. *Nature (Lond.).* 359:693.
46. Jacobsen, S.E.W., F.W. Ruscetti, C.M. Dubois, J. Lee, T.C. Boone, and J.R. Keller. 1991. Transforming growth factor- β transmodulates the expression of colony stimulating factor receptors on murine hematopoietic progenitor cell lines. *Blood.* 77:1706.
47. Dinarello, C.A., and R.C. Thompson. 1991. Blocking IL-1: interleukin 1 receptor antagonist *in vivo* and *in vitro*. *Immunology.*