The Tetrapeptide AcSDKP Specifically Blocks the Cycling of Primitive Normal But Not Leukemic Progenitors in Long-Term Culture: Evidence for an Indirect Mechanism

By J.D. Cashman, A.C. Eaves, and C.J. Eaves

In the present study, we investigated the ability of the tetrapeptide NAc-Ser-Asp-Lys-Pro-OH (AcSDKP), a reported inhibitor of primitive hematopoietic cells, to influence the proliferative behavior of primitive normal and chronic myeloid leukemia (CML) progenitor cells in the adherent layer of long-term cultures (LTCs). Addition of 50-100 ng/mL of AcSDKP to LTCs of normal cells at the time of the regular weekly half-medium change selectively and reversibly decreased the proportion of high proliferative potential erythroid and granulopoietic progenitors in the adherent layer that were in S-phase without changing their numbers, but had no effect on either the cycling activity or number of analogous (neoplastic) cells in the adherent layer of CML LTCs. Specificity of the effect of AcSDKP on primitive normal progenitors was demonstrated by the finding that a similar addition of either the control peptide, AcSDKE, or 100 ng/mL of tumor necrosis factor-α (TNF-α, which contains the SDKP sequence), or SDKP itself (at 300 ng/mL) did not inhibit the proliferation of primitive normal progenitors in LTC adherent layers. Incorporation of 30 ng/mL of AcSDKP (but not the related control peptide, AcSDKE) directly into methylocellular—

THE PRODUCTION OF mature blood cells continues throughout adult life, and involves coordinated changes in cell phenotype that span many cell generations. Regulation of hematopoiesis is thus typically visualized in terms of the responses of a hierarchy of cells to extracellular factors that either directly or indirectly control their proliferation, differentiation, viability, and location. Many of these factors are believed to be produced by fixed cellular elements of the marrow where their actions are local. This may be due, in part, to the physiologic maintenance of levels of factor production that are of limited effectiveness, although additional mechanisms, including the production of membrane-bound forms of some growth factors (eg, colony-stimulating factor-1 (CSF-1) and Steel factor), and the ability of others to bind to extracellular matrix components (eg, granulocyte-macrophage-CSF [GM-CSF]) have also been described. Long-term marrow cultures (LTCs) have been particularly useful in the identification of candidate cytokines that may control the proliferation of primitive hematopoietic cells in vivo, because these cells are endogenously supported and regulated in the LTC system by mesenchymal fibroblast-like cells that appear ontologically related to the cells that constitute the marrow microenvironment. For example, both high proliferative potential erythropoietic and granulopoietic cells that are normally quiescent in vivo, are also maintained in a similar state in the fibroblast-containing adherent layer of LTCs, unless these are perturbed by the addition of any of a variety of indirect-acting, as well as direct-acting, cytokines following each weekly change of the medium. In contrast, the same types of progenitors located in the nonadherent fraction (or in cultures in which no fibroblasts are present) proliferate continuously.

Such observations suggest that the adherent layer of LTCs serve as a source of both inhibitors and stimulators of primitive hematopoietic cell cycle progression; the local balance of which can be altered by cytokine manipulation. We have previously identified transforming growth factor-β (TGF-β) as one of the endogenously produced inhibitors that contributes to the proliferation arrest of primitive normal hematopoietic cells in the adherent layer of unperturbed LTCs. More recent studies indicate that endogenously produced macrophage inflammatory protein-α (MIP-1α), or a related chemokine whose activity may likewise be blocked by MIP-1/3, may have a similar function and acts in LTCs in concert with TGF-β. Less is known about the identities of endogenous factors that mediate the supportive/stimulatory functions of the adherent layer. Multiple candidates have been suggested, including interleukin-6 (IL-6), granulocyte CSF (G-CSF), and GM-CSF alone, or in combination and additional possibilities exist, including factors like interleukin-11 (IL-11), Steel factor, and the ligand for flk-2/flt-3 that marrow fibroblasts are also known (or thought) to produce. Recent results demonstrating that human clonogenic progenitor production in vitro can be supported for

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many weeks as effectively when cocultured with various types of murine fibroblasts, as with the complex array of indirect regulatory action by the Clinical Screening Committee for Research and Other Studies involving Human Subjects of the University of British Columbia. We have recently shown that the deregulated regulatory characteristics of the marrow microenvironment, and, if so, whether the primitive subsets of neoplastic progenitors in LTCs of CML cells would mimic the effects of MIP-1α and TGF-β on normal cells in a system that shows many of the regulatory characteristics of the marrow microenvironment, and, if so, whether the primitive subsets of neoplastic progenitors in LTCs of CML cells would prove responsive or unresponsive to AcSDKP addition. The present study was undertaken to investigate these possibilities.

MATERIALS AND METHODS

Reagents. The tetrapeptide AcSDKP, its unacetylated formSDKP, and a control peptide (AcSDKE) were synthesized and purified to homogeneity by high-performance liquid chromatography (HPLC) at the Microsequencing Centre of the University of Victoria, Victoria, Canada. The purity of all three peptides was established by amino acid analysis, as well as by mass spectrometry and HPLC. They were stored in concentrated form at 4°C until just before addition to either methylcellulose cultures or LTCs, as indicated. Highly purified (>95%) recombinant human MIP-1α expressed by transfected Chinese hamster ovary cells was obtained as a gift from S. Wolpe (Genetics Institute, Cambridge, MA). The final material was stored at -20°C in a proprietary buffer designed to minimize aggregation, and was diluted in methylcellulose LTC medium (StemCell Technologies, Vancouver, Canada) just before addition to the cultures. Recombinant human MIP-1β in the form of a cDNA transfected COS cell culture supernatant was also a gift from S. Wolpe. Purified recombinant human MIP-1β was purchased from R & D Systems, (Minneapolis, MN). Purified recombinant human tumor necrosis factor-α (TNF-α) was purchased from R & D Systems, and high specific activity 3H-thymidine (25 Ci/mmol) was purchased from Amersham (Oakville, Canada).

Cells. Normal bone marrow (BM) aspirate cells were obtained either as leftover cells from allogeneic transplants or as cadaveric material stored frozen at -153°C until use. Peripheral blood (PB) and BM cells from Philadelphia chromosome (Ph)-positive chronic-phase CML patients were obtained at diagnosis, or as part of routine follow-up procedures. All CML patients whose cells were used in these experiments had elevated white blood cell counts (>50 × 10⁹/L) at the time of study. Both normal and leukemic samples were obtained with informed consent according to guidelines approved by the Clinical Screening Committee for Research and Other Studies involving Human Subjects of the University of British Columbia.

Normal and CML BM cells were prepared for colony assays by ammonium chloride lysis of contaminating red blood cells and, after washing, were diluted in complete methylcellulose culture medium to give a final concentration of 2 × 10⁴ or 5 × 10⁴ cells/mL, respectively. Cell suspensions for use in colony assays of cultured marrow cells before and after adherent cell depletion were obtained from the nonadherent fraction of 10-day-old LTCs initiated with normal BM. For each experiment, the nonadherent cells from several such cultures established from a single inoculum of marrow were pooled, centrifuged on Ficoll-Paque (Pharmacia Biotech, Baie d’Urfe, Quebec, Canada) to isolate the light density (≤1.077 g/cm³) fraction, and the cells then washed and either cultured directly in methylcellulose at 10⁵ cells/mL, or first placed in tissue culture flasks (3 × 10⁶ cells/10 cm²) in Iscove’s medium with 20% fetal calf serum (FCS) and incubated overnight at 37°C to obtain an adherent cell-depleted fraction.

LTCs. Conventional LTCs were established by placing aliquots of 2.5 × 10⁵ fresh unprocessed normal BM aspirate cells into 60-mm tissue culture dishes in 8 mL of methylcellulose LTC medium (StemCell Technologies). This consists of an enriched α-medium supplemented with 12.5% FCS, 12.5% horse serum, and 10⁻⁴ mol/L 2-mercaptoethanol. Freshly dissolved hydrocortisone sodium succinate was added immediately before use to give a final concentration of 10⁻⁶ mol/L. LTCs were incubated in a humidified atmosphere of 5% CO₂ in air at 37°C for the first 3 to 4 days, and thereafter at 33°C. At the end of the first 10 days, after 14 days, and weekly thereafter, half of the medium and half of the nonadherent cells were removed, and an equal volume of fresh LTC medium was added as previously described.

For experiments in which LTCs were initiated on precultured feeders, the latter were obtained from conventional LTCs initiated with normal BM, and incubated for at least 2 to 3 weeks to generate confluent adherent layers. These cells were then trypsinized, irradiated in suspension with 15 Gy of 250 kVp X-rays, and subcultured into new tissue culture dishes at 10⁶ cells per 60-mm dish or 3 × 10⁶ cells per 35-mm dish in 8 or 2.5 mL of LTC medium, respectively. Normal or neoplastic hematopoiesis was then initiated by seeding 8 × 10⁴ light-density normal BM cells or 6,000 to 10,000 CD34+HLA-DRlow (FACS-sorted) light-density normal BM cells (also gated for low-forward and low-orthogonal light scatter properties as previously described) or 2.5 × 10⁴ light-density CML PB cells onto normal marrow feeder layers. These were then placed at 33°C and used for progenitor cycling studies at the time of the first (day 10), second (day 14), or third (day 21) half-medium change. The presence of the precultured feeders eliminated the 3 weeks otherwise required for the formation of an adherent layer able to inhibit the cycling of primitive normal clonogenic cells (eg, see Table 1).

Methylcellulose assays. Methylcellulose cultures containing 3 U/mL of highly purified (80,000 U/mg) human erythropoietin and 10% (vol/vol) agar-stimulated human leukocyte-conditioned medium (LCM) (StemCell Technologies) were used to assay for clonogenic cells in most experiments. The exceptions were those involving assays of cells from LTCs initiated with CD34+DRlow cells, where the LCM in the methylcellulose cultures was replaced with 50 ng/mL of Steel factor (Amgen), and 20 ng/mL each of IL-6 (Immunex, Seattle, WA) GM-CSF (Sandoz, Basel, Switzerland), G-CSF (Amgen, Thousand Oaks, CA), and IL-3 (Sandoz). This change in factors used to stimulate colony formation had no effect on the 3H-thymidine suicide values calculated for high and low proliferative potential clonogenic progenitors identified using the same scoring criteria originally developed for LCM-containing assays, and results have therefore been pooled (Table 1).

3H-thymidine suicide procedure. After trypsinization of the adherent layer, cells were washed twice in Iscove’s medium, diluted...
Table 1. Simultaneous Addition of MIP-1β and AcSDKP to Normal LTCs Allows the Activation of Primitive Progenitors in the Adherent Layer

<table>
<thead>
<tr>
<th>Addition</th>
<th>% Kill of Primitive Progenitors After Exposure to ³H-thymidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>62 ± 9</td>
</tr>
<tr>
<td>AcSDKP</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>50 ± 13</td>
</tr>
<tr>
<td>AcSDKP + MIP-1β</td>
<td>45 ± 15</td>
</tr>
</tbody>
</table>

Percent kill values for all primitive progenitors (ie, primitive BFU-E and primitive CFU-GM contained) in the adherent layer were determined 2 to 3 days after the first half-medium change, ie, 10 to 12 days after initiating the cultures, and are based on actual colony counts (in the control assays) of 18 to 113 colonies. In two experiments, these were initiated from light-density normal BM cells, and in two from FACS-sorted CD34+ HLA-DR++ light-density normal BM cells. None refers to a regular half-medium change. AcSDKP was added to give a final concentration of 200 ng/mL and MIP-1β to give a concentration of 300 ng/mL. Results from all four experiments were similar, and have therefore been combined. Values shown are the mean ± SEM of the individual percent kill values from these four experiments. The total number of primitive progenitors per LTC adherent layer relative to LTCs given a regular half-medium change only (=100%) was 230% ± 70%, 210% ± 36%, and 230% ± 70% for AcSDKP, MIP-1β, and both combined, respectively.

to 10^6 cells/mL in Iscove’s medium (no FCS) and two 1-mL aliquots, incubated for 1 hour at 37°C in an environment of 5% CO2 in air to reestablish the cells at a fixed temperature and pH (~7.2). To one of these aliquots, a small volume containing 30 μCi of high-specific-activity ³H-thymidine was added and both aliquots of cells incubated at 37°C in an atmosphere of 5% CO2 in air for an additional 20 minutes. Excess cold thymidine was immediately added to both tubes, and the cells washed twice before being plated in methylcellulose medium at a final concentration equivalent to 10^5 original adherent cells/mL, assuming no losses during the entire procedure. Differences in the number of colonies obtained in assays of the ³H-thymidine–treated cells with the control cells are expressed as percent kill values. These represent the proportion of clonogenic cells (of the type designated) that were in S-phase at the time of harvesting the cells from the LTCs.

Colonies were evaluated according to their size and morphology to determine the stage of maturation of the different types of progenitor cells being assessed, as described previously.32 37 The terms primitive burst-forming unit—erythroid (BFU-E) and colony-forming unit—granulocyte macrophage (CFU-GM) refer to progenitors that are quiescent in normal adult marrow,24 and give rise to colonies containing more than eight clusters of hemoglobinized erythroid cells and more than 500 granulocytes and macrophages, respectively. The corresponding terms mature BFU-E and CFU-GM refer to the progenitors of all smaller colonies containing more than two clusters of erythroid cells or more than 20 granulocytes and macrophages, respectively.

RESULTS

AcSDKP addition to normal LTCs blocks the activation of primitive progenitors in the adherent layer. The ability of AcSDKP to influence the cycling status of primitive normal clonogenic cells was evaluated by adding varying concentrations of this tetrapeptide (from 20 to 300 ng/mL) at the time of the routine half-medium change to 3- or 4-week-old primary LTCs established from normal BM cells. As can be seen in Fig 1, addition of 100 ng/mL or more of AcSDKP was sufficient to consistently prevent the known ability of a fresh medium change to stimulate the primitive erythroid and granulopoietic progenitors in the adherent layer to enter S-phase.3 In contrast, at no concentration did AcSDKP have any effect on the proliferative activity of the more mature progenitors coexisting in the same adherent layers, and assessed at the same time. Similar concentrations of the control peptides, AcSDK and SDFK, added to parallel cultures in the same experiments had no effect on the proliferative status of any type of clonogenic cell. The total number of primitive progenitors detected in control cultures, given a routine half-medium change, was not significantly different from the number of progenitors detected in cultures treated with any of the tetrapeptides (Table 2). This latter result, together with the apparent specificity of the AcSDKP effect for primitive progenitors, and the demonstration that these could be reactivated on plating in methylcellulose, indicate that none of the tetrapeptide preparations were directly cytotoxic. Since the actions of AcSDKP appeared to be stage-specific, rather than lineage-specific, as previously shown also for the effects of the LTC adherent layer, itself, and for the two factors thus far identified as mediators of its inhibitory activity, ie, TGF-β and MIP-1α (or some other factor whose activity can be neutralized by MIP-1β),6,15 data for both types of primitive progenitors in subsequent experiments were pooled before calculation of percent kill values.

Tumor necrosis factor-α (TNF-α) contains the SDKP sequence, and TNF-α has been found to inhibit colony formation in semisolid culture systems containing certain accessory cells28,29 although stimulatory effects of TNF-α on primitive hematopoietic cells have also been reported.30,31 It was, therefore, of interest to investigate if the addition of TNF-α to normal LTCs might simulate the effects of AcSDKP. Table 3 shows the results of two such experiments. In both, TNF-α was added to 4-week-old primary LTCs, established with normal BM to give a final concentration in the medium of 100 ng/mL, using the same protocol as for the tetrapeptide experiments described earlier (Fig 1). However, in this case, the added cytokine had no effect on either the proliferative behavior or numbers of the primitive progenitors in the adherent layer (Table 3). Addition of TNF-α also did not affect the number or cycling status of the more mature progenitor cells present in these cultures (data not shown).

Evidence that the inhibitory action of AcSDKP on normal progenitors may involve accessory cells. The demonstration that adult rat hepatocytes are responsive to the inhibitory effects of AcSDKP in vivo,32 but not in vitro,33 suggested that at least some of the effects of this tetrapeptide could involve interactions with intermediate cells. To test this possibility, varying concentrations of AcSDKP were incorporated into methylcellulose assays of freshly isolated normal BM cells or normal BM cells that had been in LTC for 10 days, and the results compared with those obtained in assays of the same cells from which the adherent cells had been rigorously removed. As can be seen in Fig 2, a significant and reproducible dose-dependent inhibition of both erythroid...
INDIRECT REGULATORY ACTION OF AcSDKP

Fig 1. Effect of adding AcSDKP (circles), AcSDKE (squares), and SDKP (triangles) to normal LTC on the cycling status of the primitive (high proliferative potential) BFU-E (A) and CFU-GM (B) present in the adherent layer. Data for the response of mature CFU-GM (shaded circles, B) to AcSDKP for the same cell suspensions is also shown in (B). Conventional LTCs were established with normal BM. After 4 weeks, tetrapeptides were added with the weekly change of half of the medium. Adherent layer cells were harvested 2 to 3 days later, exposed to \(^{3}H\)-thymidine (or not), and plated in methylcellulose assays to determine percent kill values. Values shown are the mean ± SEM from a total of 16 experiments. The numbers of colonies scored in all of the control assays (no exposure to \(^{3}H\)-thymidine) of each group ranged from nine to 289 for primitive BFU-E, five to 175 for primitive CFU-GM, and 51 to 677 for mature CFU-GM.

Table 2. Addition of Tetrapeptides to LTCs Has No Effect on Primitive Hematopoietic Cell Numbers

<table>
<thead>
<tr>
<th>Additon (at time of the half-medium change)</th>
<th>No. of Primitive Progenitors per LTC Adherent Layer (% of controls)</th>
<th>Experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of Experiments</td>
<td>Mean ± SEM</td>
</tr>
<tr>
<td>AcSDKP</td>
<td>150 ± 30</td>
<td>12</td>
</tr>
<tr>
<td>AcSDKE</td>
<td>130 ± 30</td>
<td>3</td>
</tr>
<tr>
<td>SDKP</td>
<td>120 ± 30</td>
<td>7</td>
</tr>
</tbody>
</table>

Values shown are expressed as a percent of values measured in control LTCs given a regular half-medium change only. Data are from the same experiments shown in Fig 1 for LTCs to which 200 to 300 mg/mL of either AcSDKP or AcSDKE was added, or to which 300 mg/mL SDKP was added.

Table 3. TNF-α Does Not Block the Activation into S-Phase of Primitive Progenitors in the Adherent Layer of Normal LTCs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock feed</td>
<td>—</td>
<td>2 (449)</td>
</tr>
<tr>
<td>Regular feed</td>
<td>59 (620)</td>
<td>40 (294)</td>
</tr>
<tr>
<td>Regular feed + TNF-α (100 ng/mL)</td>
<td>41 (510)</td>
<td>51 (608)</td>
</tr>
</tbody>
</table>

Conventional normal BM LTCs were established and, after 4 weeks, treated as indicated. Two or 3 days later, percent kill values for total primitive progenitors (primitive BFU-E and primitive CFU-GM) in the adherent layer were determined based on actual counts (in the control assays) of 54 to 187 colonies. Values shown in parentheses are the numbers of primitive progenitors per LTC adherent layer in each group at the time of assessment.
primitive target cell specificity of TGF-β in LTCs of normal cells, but that would be unable to regulate primitive CML cells. Because AcSDKP appeared to have such an effect on normal cells, it was of interest to determine if this tetrapeptide would have a similar action on primitive CML targets. This was initially examined in a series of experiments with LTCs of CML cells using the same protocol as described above for LTCs of normal cells (Fig 1), in which AcSDKP was added at the time of a fresh medium change. To approximate the conditions operating in the adherent layer of these CML cultures as closely as possible to those present in the 3- to 4-week-old LTCs of normal cells used in Fig 1, and at the same time to ensure that all clonogenic progenitors in the CML LTCs would be of neoplastic origin, the latter were established by seeding PB cells from patients with high white blood cell counts onto preestablished, irradiated, normal marrow feeder layers.

As expected, both the primitive erythroid and granulopoietic progenitors in the adherent layer of routinely maintained CML LTCs exhibited a consistently deregulated cycling behavior. The data for both types of progenitors in each experiment were therefore pooled to derive 3H-thymidine suicide values for total primitive progenitors. These are shown in Table 4. In three of the 10 experiments performed (each with cells from a different CML patient), 100 ng/mL of TNF-α was added with the half-medium change to one of the cultures to investigate if this cytokine might also have an inhibitory effect on primitive neoplastic progenitors in the LTC setting. It can be seen that neither AcSDKP nor TNF-α addition blocked the cycling or altered the numbers of primitive or mature (data not shown) CML progenitors in any of these experiments. In each of these, 5 ng/mL of TGF-β was added with the regular half-medium change to one of the cultures to serve as a positive control. Previous experiments of this type had shown that TGF-β can transiently arrest the cycling of primitive CML progenitors without affecting their viability. As a result, they can subsequently be removed from treated LTCs, washed free of TGF-β, and plated in colony assays where they are then restimulated to divide and express their clonogenic potential at an undiminished frequency. Similar results were obtained in the present series of experiments (Table 4).

Since primitive CML progenitors remain in cycle in LTCs that have not been perturbed for 7 days under conditions
where there is not an adequate positive stimulus to activate
their normal counterparts, we undertook a further experiment
to test if AcSDKP (and/or TNF-α) might be able to inhibit
the cycling of primitive CML progenitors under this condi-
tion of minimal stimulation. Accordingly, another set of
CML LTCs were initiated. After 10 days, either 300 ng/mL
of AcSDKP or 100 ng/mL of TNF-α, but no new medium
(or nothing), was added. The results were not different from
those shown in Table 4, i.e., all categories of progenitors in
the adherent layer of all of these cultures were found to be
proliferating (with percent kill values ranging from a mini-
mum of 41% to a maximum of 59%) when their cycling
status was assessed 2 days later.

Since the inhibitory action of AcSDKP on normal progeni-
tors could be demonstrated in colony assays of unseparated
BM, the responsiveness of CML progenitors to AcSDKP
was also tested under these conditions. As shown in Fig 4,
fresh unseparated CML BM showed no decrease in either
erthyroid or granulopoietic colony formation when AcSDKP
was included in the methylcellulose medium over a wide
range of concentrations (up to 30 times those that signifi-
cantly decreased colony formation by normal progenitors in
assays of unseparated normal BM; Fig 2).

Evidence that the inhibitory action of AcSDKP in LTCs
may be indirectly mediated. In a recent series of experi-
ments, we found that the addition of MIP-1α to LTCs of
normal cells could, like the addition of TGF-β, block the
activation of both primitive erythroid and primitive granulo-
poietic progenitors in the adherent layer.15 However, unlike
TGF-β, addition of MIP-1α to LTCs of CML cells was
unable to influence the cycling activity of the primitive neo-
plastic progenitors present in the adherent layer of these
cultures.15 Thus, the effects of AcSDKP, seen here in terms
of activity and target cell specificity, appear to closely paral-
lel those previously documented for MIP-1α. Because of the
findings that the effects of AcSDKP in colony assays of
normal progenitors involved accessory cells (Fig 2), and
because of the known ability of other cytokines to alter
MIP-1α expression,16 it seemed possible that the inhibitory
activity attributed to AcSDKP might reflect its ability to
stimulate the release or availability of MIP-1α (or a factor
with related activities). To test this hypothesis, we used the
strategy of adding exogenous MIP-1β (a known antagonist
of MIP-1α and several related chemokines17) with AcSDKP.
These experiments followed the same general protocol used
initially to detect the ability of AcSDKP addition to block

\[ % \text{ Kill of Primitive Progenitors After Exposure to } ^{3} \text{H}-\text{Thymidine} \]

\[ \begin{array}{cccccccccccc}
\text{Addition} & \text{Exp 1} & \text{Exp 2} & \text{Exp 3} & \text{Exp 4} & \text{Exp 5} & \text{Exp 6} & \text{Exp 7} & \text{Exp 8} & \text{Exp 9} & \text{Exp 10} & \text{Mean ± SEM} \\
\hline
\text{None} & 34 & 61 & 54 & 59 & 61 & 5 & 37 & 63 & 63 & 57 & 54 ± 3 \\
\text{TNF-α} & 0 & 7 & 9 & 0 & 0 & 3 & 4 & 4 & 4 & 0 & 1 ± 2 \\
\text{AcSDKP} & 49 & 62 & 42 & 36 & 52 & 49 & 61 & 41 & 71 & 71 & 51 ± 4 \\
\end{array} \]

Percent kill values shown for all primitive progenitors (i.e., primitive BFU-E and primitive CFU-GM combined) in the adherent layer of LTCs
were determined 2 to 3 days after various cytokine additions, and are based on actual counts (in the control assays) of 24 to 256 colonies.
These were made at the time of the first, second, or third weekly half-medium change as described in Materials and Methods. None refers to
a regular half-medium change. TGF-β was added to give a concentration of 5 ng/mL AcSDKP to give a concentration of 20 or 300 ng/mL, and
TNF-α to give a concentration of 100 ng/mL. The total number of primitive progenitors per LTC adherent layer relative to LTCs given a regular
half-medium change only (≈100%) was 120% ± 20%, 140% ± 30%, and 190% ± 30% for addition of TGF-β, AcSDKP, and TNF-α, respectively.
Abbreviation: Exp, experiment.
primitive progenitor activation in normal BM LTCs (Fig 1). However, in this case, additional groups of LTCs were included, and to these, 300 ng/mL of MIP-1β both with and without AcSDKP (at 200 ng/mL) were added to the new medium. Results from the four experiments performed are shown in Table 1. It can be seen that 300 ng/mL of MIP-1β alone (without AcSDKP) had no effect on the activation of primitive progenitor cycling induced by the addition of fresh medium, but was sufficient to completely overcome the ability of simultaneously added AcSDKP to block the activation of these cells.

**DISCUSSION**

During steady-state hematopoiesis in vivo, the proportion of cells in the most primitive progenitor compartments that are in S-phase is low, often undetectable. This has been attributed to the presence of a large G₀ population. Recently, a large number of intracellular intermediates that control the cell-cycle progression of mammalian cells have been identified, and their role and mechanisms of action are beginning to be delineated in a variety of cell types, including hematopoietic cells. Because of the evolutionary conservation of many aspects of these mechanisms, it is likely that some of the unique features of hematopoietic stem-cell control will be found to be determined, at least in part, by the spectrum of extrinsic cytokines to which these cells are responsive. In fact, current evidence indicates that early hematopoietic cell differentiation involves changes, not only in responsiveness to specific factors or factor combinations, but also in the subsequent biologic response(s) they elicit. Thus, an important aspect of studies of the physiologic regulation of primitive hematopoietic cells lies in the specificity of the assays used to measure responsive target cells, as well as the use of an experimental system that, at least, appears to mimic the regulatory mechanisms operative in the marrow in vivo.

In the present study, we have attempted to meet the first requirement through the use of rigorously standardized colony assay culture components and colony sizing criteria. These allow a reproducible distinction to be made between classes of normal progenitors that differ dramatically in their turnover rates both in vivo and in LTCs. Because these progenitors are not only characterized by marked differences in the proliferative potential they manifest under defined conditions in vitro, but have also been shown to represent sequential stages of development, they are referred to as primitive and mature. There is thus a strong rationale for interpreting the cycling changes found to characterize Ph-positive progenitors, defined by similar criteria, as indicative of the deregulation of a control mechanism normally operative in analogously defined normal cells. Consistent with this is the well-documented expansion of the neoplastic clone in patients with CML at all levels of clonogenic cell maturity, as defined by their different proliferative potentials.

The observed parallels between the cycling behavior of both normal and neoplastic hematopoietic cells in the adherent layer of LTCs, and in vivo provide support for the validity of the LTC system as an experimental model for investigating physiologically relevant control mechanisms. In this report, we have confirmed (Fig 2) the ability of AcSDKP to inhibit the generation of colonies of erythroblasts or granulocytes and macrophages from normal progenitors stimulated to proliferate in semisolid media, and have demonstrated the reversibility of this inhibition following removal of normal progenitors from the adherent layer of LTCs to which AcSDKP had been added 2 to 3 days previously (Fig 1). Moreover, the activity of AcSDKP in the LTC system showed considerable structural specificity, in that neither TNF-α, which contains the SDKP sequence, nor a slightly different tetrapeptide, AcSDK, had similar effects. Our present results further show that removal of adherent cells can eliminate the inhibitory effect of AcSDKP in colony assays of normal progenitors (Fig 3), and that addition of excess MIP-1α, an antagonist of MIP-1α and certain functionally related chemokines, can eliminate the effect we have observed of AcSDKP in the LTC system (Table 1). Given the similarities in the target-cell specificity of MIP-1α and AcSDKP (inhibitory to primitive normal, but not to either mature normal or primitive neoplastic clonogenic cells), these findings suggest that at least one of the mechanisms by which AcSDKP can elicit its effects is by stimulating adherent cells to produce or release MIP-1α (or some other molecule antagonized by MIP-1β). However, preliminary
studies have failed to show detectable changes in MIP-1α mRNA levels in the adherent layers of LTCs to which AcSDKP was added, nor was immunoreactive MIP-1α detectable in the supernatants of these cultures (Humphries RK, Wolpe S, Cashman JD, et al, unpublished observations).

In summary, these findings support and extend a model of primitive hematopoietic cell regulation in which the control of the proliferation of these cells is dependent on the relative concentrations of stimulators and inhibitors to which they are exposed. The involvement of secondary mediators of inhibition shown here, as well as secondary mediators of stimulation demonstrated previously, 8 highlights the complexity of the mechanisms that need to be considered. In addition, they reinforce the concept that reversible inhibition of proliferation may be a major mechanism controlling primitive hematopoietic cell behavior in vivo, and is, itself, subject to independent regulation. Finally, these studies raise the interesting possibility of investigating AcSDKP for its clinical potential to optimize the therapeutic benefit achievable with cell cycle-specific chemotherapeutic agents by eliciting localized in vivo production of inhibitors of primitive hematopoietic progenitor proliferation; one example of which (MIP-1α) has already been shown to be effective in this regard in various animal models. 40,41

ACKNOWLEDGMENT

The authors thank Dr S. Wolpe of the Genetics Institute (Cambridge, MA) for generous gifts of recombinant human MIP-1α and MIP-1β. The excellent technical assistance of Dianne Reid and the secretarial expertise of Heather Calladine are also gratefully acknowledged.

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The tetrapeptide AcSDKP specifically blocks the cycling of primitive normal but not leukemic progenitors in long-term culture: evidence for an indirect mechanism

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