0026-895X/99/040665-10$3.00/0

MOLECULAR PHARMACOLOGY, **56:**665–674 (1999).

The Hemoregulatory Peptide pEEDCK May Inhibit Stem Cell Proliferation via Hydropathic Binding to Antisense Sequence Motifs in Interleukin-11 and Other Growth Factors

JOHANNA B. PAUKOVITS, ROSALIA RUTTER, ERIKA GANGLBERGER, HEIDI I. KARLIC, BRIGITTE MARIAN, and WALTER R. PAUKOVITS

Institute of Tumorbiology-Cancer Research, University of Vienna, Vienna, Austria (W.R.P., J.B.P., E.G., B.M.) and Boltzmann-Institute of Leukemia Research, Hanuschspital, Vienna, Austria (H.I.K.)

Received July 27, 1998; accepted May 17, 1999

This paper is available online at http://www.molpharm.org

ABSTRACT

In undisturbed bone marrow, most hemopoietic stem cells are nonproliferating despite the presence of multiple growth factors. Endogenous inhibitory factors are responsible for maintenance of this quiescence. Previously we sequenced and synthesized the inhibitory pentapeptide pGlu-Glu-Asp-Cys-Lys (pEEDCK), which originally derives from granulocytes, and investigated the role of this peptide in stem cell quiescence. To provide some mechanistic insight, in the present work we studied the structural relationship of this peptide to specific growth-factor-derived sequence motifs. In the murine system in vivo as well as in long-term bone marrow, antiserum to pEEDCK produced a significant stimulation of formation of colony-forming units-granulocyte/macrophage. Binding of peptides to proteins often takes place at hydropathically complementary sites. Therefore, we searched for peptides corresponding to the complementary sequence to pEEDCK. We identified antisense sequences in the genes of various cytokines and cytokine receptors including interleukin-11. The corresponding peptide Val-Leu-Leu-Thre-Arg (VLLTR) and several other peptides hydropathically complementary to pEEDCK were synthesized. We found that pEEDCK binds specifically to these peptides as well as to complete interleukin-11. Dissociation constants were in the 10 μM range. The peptide hydropathically corresponding to pEEDCK (VLLTR) was found to stimulate colony-forming units-granulocyte/macrophage formation. Our data suggest that pEEDCK could exert a coordinating function in the hemopoietic cytokine network by binding to multiple regulatory proteins and modulating their activity.

The hemopoietic system is maintained by a population of stem cells capable of generating all functional blood cell types. In undisturbed hemopoiesis, most of these stem cells reside in a nonproliferating G_o state. Nevertheless, the multiplicative structure of the hemopoietic system ensures that this low rate of stem cell proliferation is sufficient to support an output of 4×10^{11} mature cells/day (Moore, 1995). It is known that the course of development from the multipotent stem cells to mature blood cells proceeds under the influence of hemopoietic growth factors (Schoefield et al., 1997), but most of the stem cells are quiescent despite the constitutive presence of growth factors in the bone marrow microenvironment (Cluitmans et al., 1995). Negative regulators are thought to be responsible, among them pyroGlu-Glu-Asp-Cys-Lys (pEEDCK; Paukovits et al., 1998), transforming growth factor-β (TGF-β; Keller et al., 1990), macrophage inflammatory protein-1α (MIP-1α; Maltman et al., 1993), tumor necrosis factor-α and -β (Sachs, 1994), and the peptides acetyl-Ser-Asp-Lys-Pro (acSDKP; Lenfant et al., 1989).

Our interest focused on the inhibitory pentapeptide pEEDCK and how it interferes with the action of stimulatory factors. pEEDCK is produced by mature granulocytes, is a normal constituent of the bone marrow milieu, and endogenous pEEDCK is present in the bone marrow and long-term bone marrow cultures (LTBMCs). Sequence homologies to effector binding motifs in G protein α-subunits (Laerum et al., 1990b, Amatruda et al., 1991) suggest an interference of pEEDCK in hematopoiesis-specific signal-transducing pathways. It reversibly inhibits proliferation of hemopoietic stem cells at a precommitment stage. An important in vivo target popula-

This work was supported by a grant from the Austrian Foundation for Scientific Research. We gratefully acknowledge the additional financial support by the Hertzfelder Foundation, the Hochschuljubiläums-Foundation, Vienna, and the Jubiläumsfond of the National Bank, Austria (Project No. 5921).

ABBREVIATIONS: pEEDCK, pyroGlu-Glu-Asp-Cys-Lys; VLLTR, Val-Leu-Leu-Thre-Arg, peptide complementary to pEEDCK; IL, interleukin; GM-CSF, granulocyte-macrophage-colony-stimulating-factor; LTBMCs, long-term-bone-marrow-cultures; CFU-GM, colony-forming units-granulocyte/macrophage; CFU-S, colony-forming unit-spleen; SCF, stem cell factor; FMOC, fluorenylmethyloxycarbonyl; tbs, *S*-tert-butyl-sulfenyl-group; FU, 5-fluoro-uracil; ara-C, 1-β-D-arabinofuranosylcytosine; TGF-β, transforming growth factor-β; MIP-1α, macrophage inflammatory protein-1α.

tion are pluripotent colony-forming units-spleen (CFU-S) from which all myeloid, erythroid, and megakaryocytic cells originate. Addition of exogenous pEEDCK to LTBMCs inhibits its primitive cells (preCFU-S). After differentiation and lineage commitment, only myeloid progenitors colony-forming units-granulocyte/macrophage (CFU-GM) remain sensitive to physiological concentrations of pEEDCK (Paukovits and Paukovits, 1995). When injected into mice treated with cytostatic drugs, pEEDCK inhibits the recruitment and cycling of quiescent CFU-S (Paukovits et al., 1990a), making them less sensitive to the toxic effects of repeated doses of these drugs. This results in attenuation of neutropenia and prevents the irreversible depletion of bone marrow reserve of primitive repopulating stem cells (Paukovits et al., 1993). The use of pEEDCK as a hemoprotector in cancer chemotherapy has been suggested (Gebran et al., 1992). It also reduces the duration of myelotoxicity associated with radioimmunotherapy (Alisauskas et al., 1997). The proliferative quiescence of primitive hemopoietic cells may thus reflect an intrinsic role of pEEDCK in hemopoietic regulation. To illustrate the role of pEEDCK in physiological stem cell quiescence we can show that hematopoietic activity is increased under pEEDCK-deficient conditions. This led us to the hypothesis that the mechanism of pEEDCK plays a role in the maintenance of stem cell quiescence.

Concerning the mechanism of action of pEEDCK, we raised the hypothesis of direct molecular interactions of the peptide with growth factors. The hypothesis was based on accumulating evidence that hydrophobic interactions are the dominant force in specific binding of growth factors to their receptors and of small peptides to proteins (Martin-Moe et al., 1995; Clackson and Wells, 1995; Bazan, 1995). Especially the ability of "antisense" peptides to specifically bind "sense" peptides has received much attention. Sequences of antisense peptides are derived by translating the complementary strand of the gene encoding a particular peptide (Blalock and Smith, 1995). Based on this knowledge, we show here that such antisense motifs complementary to pEEDCK are not only present in interleukin (IL)-11 (VLLTR) but also in other hemopoietic growth factors and receptors and that pEEDCK binds directly to these motifs. The aim of our work was to show that this direct binding of pEEDCK to some growth factors and receptors could be a result of hydrophobic interaction.

Materials and Methods

Animals. In vivo experiments described in this paper were performed in accordance with Austrian legislation on animal experiments under permit nos. GZ68 205/7–12/88, GZ68 205/259–12/88, and GZ68 205/181–12/89. Female BALB/c mice (8–12 weeks old) were kept under special pathogenic free-conditions with free access to autoclaved food and acidified water. New Zealand White rabbits were kept under conventional conditions. Immunized animals were checked daily for health status.

Chemicals. All chemicals and solvents were of reagent grade and were obtained from Merck (Darmstadt, Germany), Sigma (Vienna, Austria), or Boehringer Mannheim (Ingelheim, Germany). Culture media, sera, and other cell culture reagents were obtained from Life Technologies (Paisley, Scotland) or Boehringer Mannheim. Amino acid derivatives and other reagents required for peptide synthesis were purchased from Bachem (Bubendorf, Switzerland). Recombinant murine granulocyte-macrophage-colony-stimulating-factor (GM-CSF), IL-3, and IL-6 were obtained from Peprotech (London, UK). Recombinant human IL-11 was obtained from Becton Dickinson/Collaborative Biomedical Products (Bedford, MA) and recombinant murine stem cell factor (SCF) was purchased from R&D Systems Europe (Abington, UK).

Peptide Synthesis. Peptides were synthesized by standard fluorenylmethyloxycarbonyl (FMOC) solid-phase techniques with 2-(1-*H*-Benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium tetrafluoroborate activation. Trifluoroacetic acid-cleavable side-chain protection was used except where mentioned otherwise. The first (C-terminal) amino acid was purchased as NovySyn PA500 resin conjugate (Novabiochem AG, Laufelfingen, Switzerland). If necessary, crude peptides were purified by reversed phase chromatography and characterized by fast absorbance-mass spectrometry. pEEDCK was synthesized as the oxidation resistant, biologically inactive, mixed disulfide of pEEDCK and *tert.*-butyl mercaptane. The thiol-protective *S*-tert-butyl-sulfenyl-group (tbs) was removed immediately before use by reduction with dithioerythritol as described earlier (Paukovits et al., 1990a). Dilutions of fully deprotected pEEDCK were made in RPMI-1640 containing 10 μM $CaNa_2EDTA$ (Serva, Heidelberg, Germany). The other peptides were synthesized by analogous procedures. In some peptides C-terminal tyrosine extensions were included for diazo coupling with BSA or biotin. A 16-armed pEEDCK-dendrimer (Posnett et al., 1988) without tyrosine was synthesized for immunization to exclude tyrosine- and BSA-directed antibody specificity. α,ϵ-Bis-FMOC-Lys was coupled to resin-bound alanine. This was repeated four times, giving the branched core structure α,ϵ-$(FMOC)_{16}$-Lys_8-Lys_4-Lys_2-Lys-Ala-resin. To the 16 amino groups exposed after removal of FMOC protection, Nα-FMOC-Nϵ-BOC-Lys was coupled. The synthesis was then continued as described above, yielding $(pEEDCK_{tbs}K)_{16}$-Lys_8-Lys_4-Lys_2-Lys-Ala after cleavage and deprotection.

Preparation of Peptide Conjugates. Coupling of peptides to protein carriers usually involves reactive functional groups in the amino acid side chains. To keep all side chains of pEEDCK intact, we have extended the pentapeptide by a C-terminal tyrosine residue, which was used for conjugation. pEEDC(tbs)KY was coupled to tyrosine groups of the carrier protein (BSA, ovalbumin, and keyhole limpet hemocyanin) by bis-diazotized benzidine (Ratnam and Lindstrom, 1984). The yellow conjugate was purified by chromatography on a Pharmacia-Superose-12-column. A substitution ratio of 10 molecules of pEEDC(tbs)KY per molecule of BSA was achieved. Using similar procedures, conjugates of pEEDC(tbs)KY with ovalbumin and keyhole limpet hemocyanin were prepared. C-terminally biotinylated pEEDCK was prepared by reacting pEEDCK(tbs)KY with benzoic acid hydrazido-*N'*-(*N*-biotinyl-ϵ-aminocaproyl)-4-diazonium tetrafluoroborate (Diazobiotin, Boehringer). The thiol-protecting tbs group was removed immediately before use. N-terminally biotinylated EEDCK was prepared by reacting the free amino group of piperidine-treated resin-bound EEDCK-(*R*) with *N*-hydroxysuccinimidyl-biotin (Sigma) before cleavage from the resin.

Peptide Binding Assay. The peptides VLLTR-Y, ELDSTVLLTRSLLED-Y (P_{11}), AARNILLTHGRITK-Y (P_{kit}), and SLLTKVLLVRKFQNA-Y (P_{6R}) were coupled to BSA with bis-diazotized benzidine using the procedure of Ratnam and Lindstrom (1984). BSA conjugates and intact IL-11 were adsorbed (2 h, 37°C) to microwell plates (Nunc Maxisorb; Nunc, Naperville, IL) in sodium carbonate buffer (pH 9.6). After washing four times with PBS (pH 7.4), 0.1 to 5.0 μg C-terminally biotinylated pEEDCK was added to each well and incubation was continued (1 h, 37°C). Free biotinylated pEEDCK was removed by washing eight times with PBS containing 0.05% Tween 20. Bound biotinylated pEEDCK was determined with streptavidin-peroxidase conjugate (Boehringer, Vienna) using *O*-phenylene diamine and H_2O_2 as chromogenic substrates. In competition experiments, 0.1 to 5.0 μg free pEEDCK were added together with 0.5 μg biotinylated pEEDCK.

Antibodies against pEEDCK. Standard methods were used for the production of murine (BALB/c) or rabbit antibodies against

pEEDCKY-BSA or $(pEEDCK)_{16}$ dendrimer (Green and Manson, 1992). The thiol-protective tbs group was removed with dithioerythritol immediately before the antigen was suspended in complete Freund's adjuvant. Treatment was repeated twice in intervals of 2 weeks using incomplete Freund's adjuvant. Successfully immunized mice were anesthetized with ether and about 0.5 ml of blood per mouse was obtained from an incised tail artery. Then 10 mice were immediately sacrificed by cervical dislocation. Rabbit blood was drawn from ear veins. Serum aliquots were stored at −70°C. To avoid disturbances due to endogenous cytokines induced during the immunization procedure, we waited at least 1 month before bleeding the immunized mice and rabbits. Immune sera were checked for the presence of specific antibodies by dot-blotting assay using alkaline phosphate conjugates of second antibodies for detection. As a chromogenic substrate, a combination of 5-bromo-3-chloro-indolyl-phosphate and nitroblue tetrazolium was used. Specificity of antisera was checked against pEEDCKY bound to other carriers or against the $(pEEDCK)_{16}$-dendrimer to exclude carrier-specific reactions. Sera from mice and rabbits immunized against pEEDCKY-BSA were tested against pEEDCKY-ovalbumin, pEEDCKY-hemocyanin, or $(pEEDCK)_{16}$-dendrimer. Immune sera obtained against the $(pEEDCK)_{16}$- dendrimer were tested against the other three antigens, respectively. Sera taken before immunization, sera from animals immunized against unconjugated BSA, and sera from solvent-treated animals were used as controls. Control sera from normal individuals (50 human, 7 rabbit, and 30 mice) did not react with any of these antigens. In competition experiments, sera which had been preincubated with saturating amounts of antigen [pEDDCKY-ovalbumin, pEEDCKY-hemocyanin, or $(pEEDCK)_{16}$-dendrimer], were unable to detect any one of these antigens in dot-blot assays. Taken together, this demonstrates that our antisera specifically reacted with the pEEDCK epitope and that no antibodies against this peptide were present in normal sera. The Ig fraction was prepared from whole serum using affinity chromatography on protein G immobilized on sepharose (Pharmacia MAbTrap G II). IgG bound to the column was eluted and reconstituted following procedures given by the manufacturer.

LTBMCs. Stroma-supported LTBMCs were established using a modification of the methods of Eaves et al. (1991). A suspension of murine bone marrow cells (10^7 cells/2 ml of medium) was placed in 12.5 cm^2 cell culture flasks. The growth medium was minimum essential medium-α containing 10% horse serum, 10% fetal calf serum, 2 mM glutamine, 1% penicillin/streptomycin solution (10,000 U of penicillin/ml, 10,000 μg of streptomycin/ml), 0.5 mg/ml human transferrin, 10^{-5} M hydrocortisone sodium succinate, and 10^{-4} M β-mercaptoethanol. Cultures were incubated with 5% CO_2 at 37°C, which was lowered to 33°C after 1 week. They were fed in weekly intervals by replacing 50% of the supernatant with fresh medium. After 3 weeks, when a semiconfluent adherent cell layer had formed, nonadherent cells were removed by washing. The remaining adherent layer was covered with medium containing 1 μg/ml of 5-fluorouracil (5-FU; Sigma, St. Louis, MO). After 1 h at 37°C, the 5-FU-containing medium was replaced by fresh medium without 5-FU. This was sufficient to kill almost all CFU-GM. Few CFU-GM were present 24 h after 5-FU treatment (Fig. 1A), but "fresh" CFU-GM gradually reappeared due to the presence of 5-FU resistant long-term culture-initiating cells. Additions to the cultures started after removal of 5-FU. At selected time points the cultures were gently agitated to suspend nonadherent cells and a small aliquot of the supernatant was taken for determination of CFU-GM.

Stroma-Depleted Bone Marrow Cultures. Stromal elements and mature cells were removed from bone marrow by adherence to nylon wool columns (Nycomed, Oslo, Norway) using the procedure described by DiNicola et al. (1994). CFU-GM were enriched 3.4-fold from 86.0 ± 8.8 to 292 ± 42 per 10^5 plated cells. Nonadherent cells were cultured in medium containing recombinant growth factors in various combinations. After 7 to 14 days, the number of CFU-GM present in the culture was determined.

Determination of CFU-GM. CFU-GM were cultured as described previously (Paukovits and Paukovits, 1995) in minimum essential medium-α medium containing 20% horse serum and 0.8% methylcellulose-4000 (Colorcon, Königstein, Germany). Suitably diluted mouse lung conditioned medium was used as source of GM-CSF. Petridish cultures contained 50,000 bone marrow cells/ml or suitable aliquots of the nonadherent phase of LTBMCs. Aggregates of more than 50 cells were scored as colonies after 7 days at 37°C and 5%CO_2.

Determination of CFU-S Numbers In Vivo. CFU-S are analyzed (Till and McCulloch, 1961) by injecting bone marrow cells into lethally irradiated mice, where they seed in the spleen and form colonies. Six to ten mice per group were irradiated with 8.5 Gy (1 Gy/min) from a ^{60}C source and within 3 h received donor bone marrow cells (in 0.2 ml) into a lateral vein. After 11 days, the spleens were fixed in Carnoy's fixative and spleenic nodules were counted. The number of transplanted bone marrow cells was adjusted to give

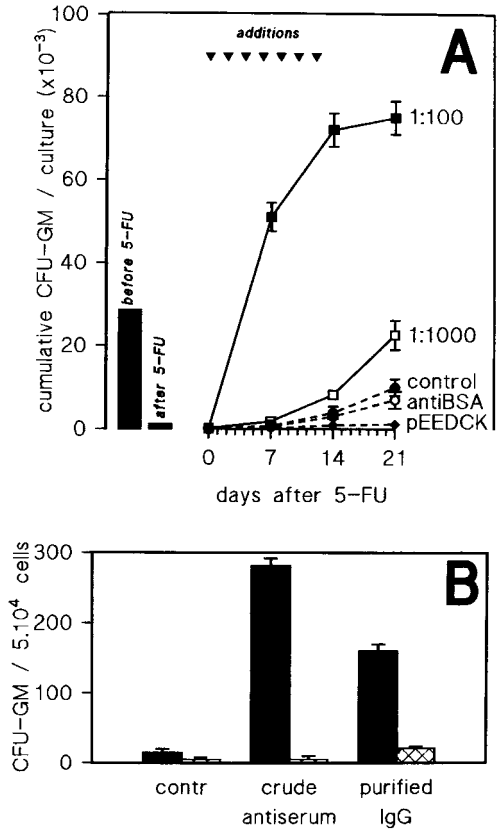

Fig. 1. Effects of pEEDCK and anti-pEEDCK-serum in LTBMCs. Murine bone marrow cells were cultured until the layer of adherent cells becomes semiconfluent. Then the cultures were treated with 5-FU to remove proliferating progenitors. Only a few CFU-GM are detectable 24 h after 5-FU treatment (solid bars in A). Under this conditions quiescent cells survive and continue to generate progenitors. Production of "fresh" CFU-GM is a measure of their activity. Crude antisera (A): on the indicated days (▼) the following additions were made to parallel cultures: RPMI-1640 as control, 1 ng/ml pEEDCK, rabbit antiserum against a pEEDCKY-BSA conjugate (dilution 1:100 and 1:1000, v/v), and rabbit antiserum against unconjugated BSA (dilution 1:100, v/v). On days 0, 7, 14, and 21, the number of nonadherent CFU-GM was determined. The values given are means ± S.E.M. of three parallel dishes. The experiment was repeated three times with similar results. The columns at the left give the CFU-GM content of the cultures before and 24 h after 5-FU treatment. Purified antibodies (B): RPMI-1640 or crude antiserum against pEEDCKY-BSA (1:100, v/v) or an equivalent amount of affinity-purified IgG were added to the cultures as above, either alone (dark columns) or together with 10 ng/ml pEEDCK (cross hatched columns). The CFU-GM content of the cultures was measured on day 20. Values given are means ± S.E. of three parallel determinations.

15 to 20 colonies per spleen. Proliferating CFU-S were assayed by the suicide method using 1-β-D-arabinofuranosylcytosine (ara-C) as S phase killing agent as described previously (Paukovits et al., 1993). In short, aliquots of the marrow cell suspension were incubated for 1 h at 37°C with or without 10^{-3} M ara-C. After washing, the cells were suspended in RPMI-1640 and transplanted. The percentage of CFU-S in S phase was calculated as $100 \times (n_o - n)/n_o$, where n and n_o are the numbers of spleenic nodules in the suicided and control group, respectively.

Statistical Procedures. Pairs of experimental groups were compared by Student's *t* test. Multiple comparisons were made by the Student-Newman-Keuls Test.

Results

Antiserum against pEEDCK Stimulates CFU-GM Production in LTBMCs. LTBMCs contain mature granulocytes, which secrete pEEDCK. To test the hypothesis that endogenous pEEDCK could reduce hemopoietic activity in LTBMCs, we measured the CFU-GM output in the presence of diluted antiserum against pEEDCK. On days 0, 2, 4, 6, 8, 10, and 12 after 5-FU, 1 ng/ml pf pEEDCK or diluted antiserum against pEEDCK-BSA (1:100 or 1:1000, v/v) was added to parallel cultures. Nonadherent CFU-GM were determined on days 7, 14, and 21. Figure 1 and Table 1 show that pEEDCK reduced the production rate of CFU-GM (per day and per cm^2) to 44% of the control value during the first week and to 8% during the third week, indicating that CFU-GM production in these cultures was sensitive to pEEDCK. Addition of 1:100, v/v, diluted antiserum resulted in a strong increase of progenitor output. During the first week the production rate (per day and per cm^2) had increased 136-fold (Table 1). It later declined below that in control cultures (third week). Thousand-fold diluted antiserum caused a more delayed reaction, resulting in a steady increase of the production rate (twice the control level after 2–3 weeks). Parallel produced antiserum against unconjugated BSA, obtained by an identical protocol, used as control, did not change CFU-GM production (Fig. 1A). A potential risk of contaminating endogenous cytokines was negligible. To provide further evidence to definitely exclude that the observed stimulations were caused by contaminating cytokines, we have repeated the experiments with IgGs purified from anti-pEEDCK serum by affinity chromatography on immobilized protein G (Fig. 1B). Purified IgGs at a concentration corresponding to 1:100 dilution or crude antiserum (1:100, v/v) were added to 5-FU-treated LTBMCs as above and the CFU-GM content was measured on day 20. Purified IgGs caused a similar stimulation of hemopoietic activity as crude antiserum against pEEDCK, strongly suggesting that antiserum stimulation was not caused by contaminating cytokines. Antibody-mediated stimulation could be abolished by addition of saturating amounts of synthetic pEEDCK (10 ng/ml, added together with the antibody).

TABLE 1

Mean CFU-GM production per day and per cm^2 in long-term bone marrow cultures treated with 5-FU, pEEDCK, and antiserum against pEEDCK

Cultures were established from unfractionated murine bone marrow as described in *Materials and Methods*. After 3 weeks, when a semiconfluent adherent layer had formed, nonadherent cells were removed by washing, and the adherent layer was treated with 1 μg/ml 5-FU (1 h, 37°C). The day of 5-FU treatment was counted as day 0. To some cultures 1 ng/ml pEEDCK was added on days 0, 2, 4, 6, 8, 10, and 12. Other cultures received diluted rabbit anti-pEEDCK antiserum on the same days. On days 7, 14, and 21, nonadherent cells were suspended by gentle shaking, small aliquots were removed, and the number of CFU-GM was determined by methylcellulose assay. The table gives average rates of CFU-GM production during the first and third week after 5-FU treatment. Values are means ± S.E.M. of triplicate cultures.

Treatment	CFU-GM Production per Day and per cm^2		
	First week	Second week	Third week
Controls	4.3 ± 1.3	44.3	69.8 ± 14.0
pEEDCK (1 ng/ml)	1.9 ± 1.9	8.5	5.5 ± 6.3
Antiserum 1:100	583.0 ± 51.0	244.1	33.6 ± 5.0
Antiserum 1:1000	19.1 ± 1.1	72.9	92.1 ± 16.9

Injection of Antiserum Increases Femoral CFU-GM Numbers. The question arose if antiserum injection in mice would enhance hemopoiesis in vivo. To avoid immune reactions against rabbit proteins, experiments were performed with syngeneic antisera. BALB/c mice were immunized against pEEDCKY-BSA, and controls were raised against unconjugated BSA. As above, antisera were obtained 1 month after immunization. Mice received a single i.v. injection of 200 μl antiserum. Three independent experiments were performed, each with another batch of antiserum. CFU-GM values were followed for up to 5 weeks. One experiment was terminated after 20 days. After antiserum injection, the femoral CFU-GM content (Fig. 2) increased strongly. Maximal values were reached after approximately 2 weeks and were dependent on the potency of the injected serum. The mean increase (over initial values) was 2.53 ± 0.33. After about 3 to 4 weeks, the femoral CFU-GM content returned to pretreatment levels. Serum from mice immunized against unconjugated BSA had no effect. CFU-GM were cultured as described above (Paukovits et al., 1990b).

Antigen Challenge Increases Hemopoietic Activity in Mice Previously Immunized against pEEDCK. Mice were immunized against pEEDCK-BSA or unconjugated BSA as above. Three months later, when all hematological parameters had returned to normal levels, they were challenged with the respective antigen (without adjuvant). Four independent experiments were performed. As can be seen in Fig. 3, femoral CFU-GM values increased after challenge, reaching maximum values after 1 week (7–11 days). Total bone marrow cellularity peaked on day 11 (ca. 2-fold elevation) and declined to control level after about 3 weeks. The number of myelopoietic cells in the bone marrow showed a

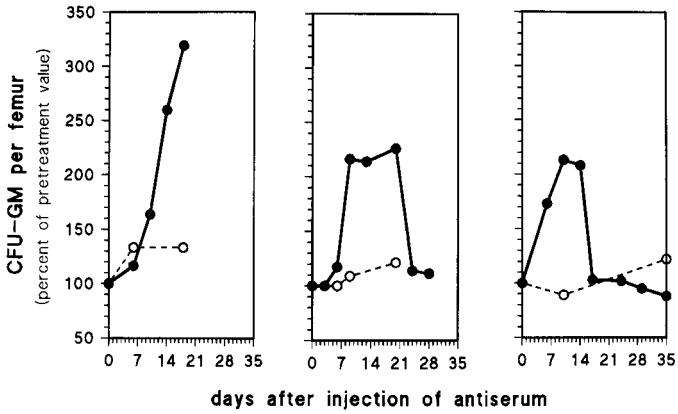

Fig. 2. Injection of anti-pEEDCK serum increases hemopoietic activity in BALB/c mice. Antisera against pEEDCKY-BSA conjugates were raised in syngeneic mice. Sera were taken at least 1 month after the last boost injection. Antiserum (200 μl) was injected through the tail vein and the femoral CFU-GM content was determined during 3 to 5 weeks after serum injection. Three experiments were performed, using three different batches of antiserum. Controls received anti-BSA serum.

maximum during the second week, with almost 80% of all bone marrow cells in the myeloid type. The increased content of myeloid cells occurred mostly at the expense of other lineages, the eosinophile/granulocyte ratio fell from values near 0.9 to 0.13 on day 12. After 5 weeks the overall cellularity had returned to normal but the percentage of myeloid cells was still elevated, the eosinophile/granulocyte ratio was still reduced. Leukocyte counts increased about 5-fold at the end of the second week. At the same time the percentage of granulocytes rose from a normal level of about 10% to almost 40%, but only occasionally immature cells were seen in blood smears.

Effects of pEEDCK in Suspension Cultures of Bone Marrow Depleted of Adherent Cells. Many hemopoietic growth factors originate in stromal elements and in mature blood cells. In our experiments we used a murine bone marrow cell suspension free of potential factor-producing cells (see *Materials and Methods*). These nonadherent cells were cultured in the presence of selected hemopoietic growth factors. The main attention of these experiments was directed to the interaction of pEEDCK with growth factors. We were aware to use suboptimal factor concentrations (1 ng/ml IL-3, IL-6, IL-11, GM-CSF, and 10 ng/ml SCF) to avoid a plateau effect. Factors and pEEDCK were added immediately after plating and then every other day. After 14 days, the number of CFU-GM was determined. The results of two independent experiments are summarized in Table 2. Cultures containing IL-11 or/and SCF (either with or without IL-3 or IL-6) produced significantly more CFU-GM than controls. Addition of pEEDCK inhibited IL-11-stimulated CFU-GM production but was not significantly effective in cultures that had received only SCF. Inhibition was strongest in cultures containing IL-11 and SCF together with IL-3 or IL-6. The output of CFU-GM in the bottom part of Table 2 is less than in the top part of Table 2. This is related to the fact that data are derived from two independent experiments. Cultures containing GM-CSF (either alone or in combination with other factors) were only sensitive to pEEDCK when IL-11 was present, but the degree of inhibition was not significant.

pEEDCK Binds Directly to IL-11. IL-11 and SCF, in synergy with other factors, are important regulators of stem cell activity. Our results (Table 2) suggest that pEEDCK antagonizes the effect of IL-11 alone and IL-11 combined with other factors. One possible mechanism for growth factor inactivation may be direct binding of pEEDCK to the respective protein molecule. This hypothesis was tested by studying the binding of biotinylated pEEDCK to IL-11. Microwell plates were coated with recombinant IL-11 (10 ng/well). Open sites were blocked with BSA. N- and C-terminally biotinylated pEEDCKY were added at various concentrations. and the plates were incubated for 1 h at 37°C. The C-terminally biotinylated pEEDCK bound to IL-11 in a concentration-dependent and -saturable manner (Fig. 4). N-terminally biotinylated pEEDCK did not bind to IL-11. Binding to plates coated with BSA was negligible. In competition experiments, biotinylated pEEDCK was displaced by unlabeled pEEDCK added to the incubation mixture at various concentrations, indicating the specificity of the interaction. As seen in Table 3 a dissociation constant of $K_d = 8.0 \times 10^{-6}$ was determined from the IC_{50} value.

pEEDCK Binds Hydropathically Complementary Sequence Motif in IL-11. We were further interested in possible binding motifs for pEEDCK in the IL-11 molecule. Binding of peptides to proteins often takes place at hydropathically complementary sites (Clackson and Wells, 1995; Bazan, 1995). Sequences complementary to pEEDCK corre-

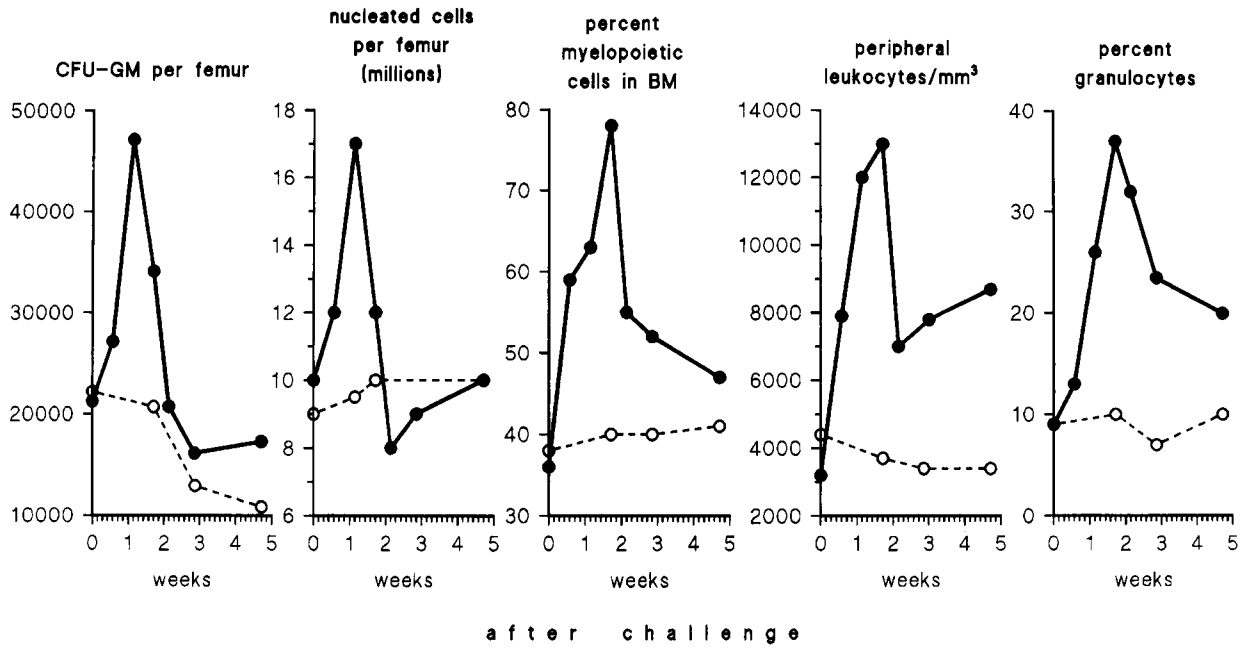

Fig. 3. Antigen challenge in mice previously immunized against pEEDCK leads to hemopoietic activity. Female BALB/c mice were immunized (see *Materials and Methods*) against pEEDCKY-BSA conjugate. Three months after the last boost injection the mice were challenged with the antigen (without adjuvant). At selected time points (day 0, 4, 8, 12, 15, 20, 33) up to 5 weeks, one mouse was sacrificed and the number of CFU-GM and other hematological parameters were determined. Controls were taken also at selected time points (day 0, 12, 20, 33, respectively, 0, 8, 12). The figure presents the data of one of four experiments, with similar results and gives detailed hematological analysis of the mice. Blood samples were drawn from caudal blood vessels. Femoral bone marrow was obtained after sacrifice. Blood and bone marrow smears were stained with May-Grünwald-Giemsa. control: ○——○ antigen: ●——●

spond to the general pattern AAABX (A. . . aliphatic hydrophobic side chain, B. . . polar side chain, X. . . large side chain-positively charged or uncharged). Such motifs are present in several hemopoietic growth factors, mostly notably IL-11 and c-kit, the receptor for SCF, and also in other growth factors, receptors, and inhibitors (Table 4). VLLTR corresponding to the relevant parts of IL-11, c-kit, and the IL-6 receptor was synthesized and extended C-terminally by tyrosine. It was conjugated to BSA with diazotized benzidine and used for coating microwell plates (10 ng conjugate/well). Control wells were coated with BSA. Binding of biotinylated pEEDCKY was determined as above. Figure 5 shows that C-terminally biotinylated pEEDCKY binds to VLLTR in a concentration-dependent and -saturable manner. Binding was negligible on plates coated with BSA or BSA conjugates of unrelated peptides. No binding was observed with N-terminally biotinylated pEEDCK(N). Competition experiments with free pEEDCK showed that binding is specific. Dissociation constants near 9×10^{-6} were optimal for binding to complementary peptides of the IL-11 and c-kit type (Table 3). P_{6R} showed a lower affinity for pEEDCK ($K_d = 31 \times 10^{-6}$).

TABLE 2

Inhibition of progenitor production in suspension cultures of bone marrow

In two independent experiments, adherent cells were removed from murine bone marrow by incubation in nylon wool columns. Nonadherent cells were cultured in the presence of suboptimal concentrations of growth factors. Recombinant mGM-CSF and interleukins mIL-3, mIL-6, and hIL-11 were used at 1 ng/ml; mSCF was used at 10 ng/ml. pEEDCK was added at 1 ng/ml. Factors and peptide were added every other day. On day 14 the numbers of CFU-GM were determined in corresponding aliquots of the cultures. Values given are means of three cultures ± S.E.M. GM-CSF is abbreviated by GM, the interleukins by their numbers. Significances were calculated by unpaired, two-sided t test, N.S. indicates $p > .1$.

Factors	CFU-GM per Culture		Inhibition	t Test
	−pEEDCK	+pEEDCK		
			%	p
None	12.0 ± 2.1	5.3 ± 3.0	56	.05
SCF	120.0 ± 7.8	108.5 ± 4.9	10	N.S.
IL-3	47.3 ± 9.8	67.7 ± 2.5		
IL-6	14.7 ± 11.6	13.7 ± 10.1		
IL-11	50.7 ± 8.1	19.3 ± 4.0	62	.025
IL-11+SCF	137.3 ± 11.3	83.0 ± 7.8	40	.017
IL-11+SCF+IL-3	118.0 ± 2.0	32.2 ± 20.1	73	.013
IL-11+SCF+IL-6	96.0 ± 3.5	18.4 ± 3.6	81	.0001
None	5.3 ± 0.6	2.3 ± 0.6	57	.003
GM	9.8 ± 0.5	10.5 ± 2.2		
GM+IL-3	12.8 ± 1.5	12.4 ± 1.7		
GM+IL-6	8.4 ± 2.2	9.1 ± 1.9		
GM+IL-11	16.9 ± 2.4	12.5 ± 1.0	26	N.S.
IL-11+GM+SCF+IL-3	11.0 ± 1.0	9.5 ± 0.5	14	N.S.
IL-11+GM+SCF+IL-6	5.5 ± 0.5	3.5 ± 1.0	36	N.S.

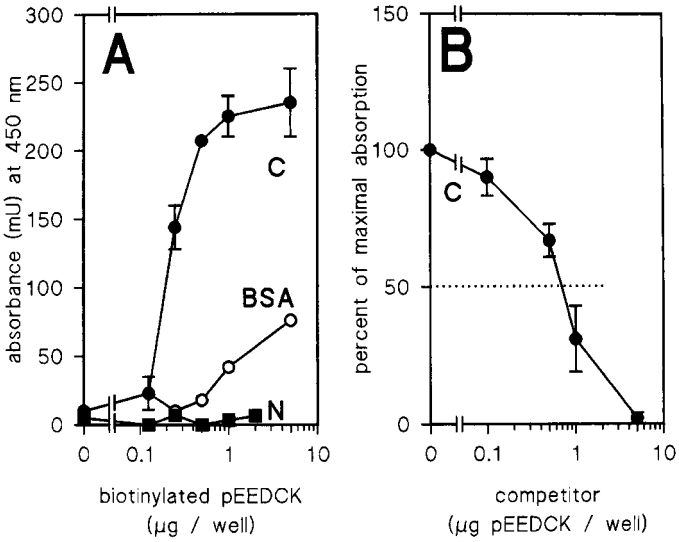

Fig. 4. Binding of pEEDCK C-terminal and N-terminal to IL-11. A, recombinant hIL-11 was adsorbed to microtiter plates (10 ng/well) and unoccupied binding sites were blocked with BSA. The plates were incubated for 1 h at 37°C with the indicated amounts of C-terminally or N-terminally biotinylated pEEDCK (pEEDCKY or YEEDCK). After washing, bound biotin was determined by streptavidin-peroxidase. ●, binding of pEEDCKY-biotin to IL-11; ○, binding of pEEDCKY-biotin to BSA; ■, binding of biotin-YEEDCK to IL-11. B, in competition experiments, 0.5 μg C-terminally biotinylated pEEDCK was added together with the indicated amounts of free pEEDCK and the extent of binding of biotinylated pEEDCK to IL-11 was determined.

Peptides Complementary to pEEDCK Stimulate Hemopoiesis In Vitro and In Vivo. Taken together, these results suggest that pEEDCK may exert its inhibitory effect by directly interacting with complementary recognition sequences in growth factors. The possibility arose that such complementary peptides, when added to cultures or injected in vivo might stimulate hemopoiesis, either by sequestering pEEDCK or by directly mimicking the respective growth factor. We have investigated effects of VLLTR (contained in IL-11) in vitro and of VLLTF, which is the directly corresponding antisense peptide to pEEDCK in vivo (Bost and Blalock, 1989).

In vitro, combinations of IL-11, SCF, and VLLTR (1 ng/ml) were added daily to progenitor-depleted stroma-supported LTBMCs. On day 9, the number of nonadherent CFU-GM was determined. Figure 6A shows that VLLTR stimulated progenitor production about 2-fold. The combination of IL-11 with VLLTR resulted in a 7-fold stimulation. This was also evident in SCF-containing cultures. The combination IL-11 + SCF caused a 4-fold stimulation, whereas substitution of IL-11 with VLLTR resulted in a 16-fold increase of progenitor production.

For in vivo experiments, VLLTF was diluted in PBS containing 1% BSA. Mice were injected i.p. with 1 μg VLLTF. Controls received 1% BSA/saline. Ten hours after peptide application the mice were sacrificed and their bone marrow subjected to suicide and CFU-S determination as described in *Materials and Methods*. Figure 6B shows that VLLTF injection doubled the number of CFU-S that seeded to the spleens of the recipient mice. About 50% were sacrificed in S phase by a 1-h pulse of ara-C, indicating that most of them had entered the cell cycle.

Discussion

The physiological quiescence of hemopoietic stem cells is thought to result from the action of inhibitory factors. There is experimental evidence that addition of TGF-β, MIP-1α, pEEDCK, and acSDKP to bone marrow cultures or injection in vivo reduces stem cell proliferation and/or the output of downstream cells. All four factors are present in the bone marrow and may be involved in inhibition of early hemopoietic cells. Increased hemopoietic activity under inhibitor-deficient conditions has been interpreted (Baserga et al., 1992; Waegell et al., 1994) as indicating that the respective inhibitor is involved in keeping primitive hemopoietic cells in G_o (Lenfant et al., 1989). Following this strategy, we show here that antibody-mediated sequestration of pEEDCK can increase the production of progenitors from more immature

pluripotent cells in vitro and in vivo. Similar to other authors we conclude that stem cells are under inhibitory control by pEEDCK under undisturbed conditions. Our experiments have followed three lines: 1) addition of antibodies against pEEDCK-BSA conjugates to LTBMCs, 2) injection of these antibodies into mice, and 3) active immunization of mice against pEEDCK followed later by challenge with the antigen. Each of these approaches induced a strong stimulation of hemopoietic activity, suggesting a role of pEEDCK in physiological stem cell quiescence. First, antiserum addition to LTBMCs raised the rate of progenitor production more than 100-fold (Fig. 1). A similar rise was observed when affinity-purified IgGs were used instead of antiserum. This excludes the possibility that the stimulation by crude antiserum might have been caused by adjuvant-induced cytokines. As a further precaution against cytokine contamination, blood samples for antiserum preparation were taken 4 weeks after immunization when hematopoiesis was back to normal.

The second approach involved creating pEEDCK-deficiency in mice by injecting antisera against pEEDCK-BSA. For these experiments antisera were raised in syngeneic mice to avoid reactions against foreign (rabbit) proteins. As a precaution against cytokine contamination, antisera were obtained at least several weeks after the last boost injection. Anti-pEEDCK serum injection was followed after a few days by a strong increase of progenitor output (Fig. 2), suggesting that primitive hemopoietic cells (preCFU-GM) were released from inhibition. Anti-pEEDCK serum obtained under identical conditions had no effect. This corresponds to our previous findings that pluripotent CFU-S and committed CFU-GM are differentially regulated by pEEDCK in such a way that after a single peptide injection, CFU-S are inhibited for at least 24 h whereas CFU-GM inhibition lasted only 2 to 3 h (Paukovits and Paukovits, 1995).

Thirdly, we have immunized mice against pEEDCK-BSA (or unconjugated BSA). Three months after the last boost injection they were challenged with the antigen without using adjuvant. This long interval between immunization and challenge was considered as precaution against possible interference from cytokines produced during immunization (see above). Cytokine effects were also excluded by the fact that at this time all measured hematological parameters had normal values and that immunization and challenge with unconjugated BSA had no effect. Challenge with pEEDCK was followed by increased CFU-GM numbers in the bone marrow and increased production of mature blood cells with a pronounced shift toward myeloid cells. Channeling of hemopoiesis into the myeloid lineage may be a consequence of the fact that, in addition to pluripotent stem cells, committed myeloid progenitors and precursors are also inhibited by pEEDCK, whereas cells belonging to other lineages, e.g., immature erythropoietic cells, are not (Laerum et al., 1990a). Blood and bone marrow changes were accompanied by multilineage extramedullary hematopoiesis in several organs. No signs of malignancy according to the criteria of Frith et al. (1993) were detected at the end of the observation period (5 weeks). All short-term changes were reversible. This is in contrast to the effects of constitutive expression of hemopoietic growth factors (GM-CSF) in transgenic mice. Lang et al. (1987) have observed that these mice showed little change in hemopoietic activity, but rapidly developed massive organ infiltrations of mature macrophages, blindness, fatal tissue damage, and early death.

We conclude from these results that under steady-state conditions, stem cell quiescence is maintained in vitro and in vivo by endogenous pEEDCK. Two aspects of this require careful consideration. Firstly, similar conclusions have been reached by others (Baserga et al., 1992; Waegell et al., 1994) working with inactivation of other endogenous inhibitors (TGF-β, MIP-1α, acSDKP). Secondly, the in vivo situation may be complex, but in LTBMCs it is difficult to see how the addition of purified antibodies against one particular inhibitor could render the whole set of other stem cell inhibitors ineffective.

An aspect requiring consideration is that pEEDCK keeps stem cells quiescent despite the constitutive presence of sev-

TABLE 3

Dissociation constants for binding pEEDCK to IL-11 and to complementary peptides in IL-11, c-kit, and IL-6 receptor

The table gives also dissociation constants of peptides with altered side chain patterns (Glu, Asp, and Cys replaced by Gly or α-aminobutyric acid = Abu). Values were obtained from the concentration of competing peptide yielding 50% inhibition of biotinyl-pEEDCK binding.

Complementary Peptide	pEEDCK	K_d ($\times 10^{-6}$)	pE**X**DCK	pEE**X**CK	pEED**X**K
Full length IL-11	8.0				
VLLTR	9.3	X = Gly	16.7	16.7	no binding
P_{11} = ELDST**VLLTR**SLLED	9.0	X = Abu	22.9	14.4	no binding
P_{kit} = AARN**ILLTH**GRITK	10.1	X = Gly	8.6	17.2	no binding
P_{6R} = SLLTK**VLLVR**KFQNA	31.0	X = Abu	22.6	14.3	no binding

TABLE 4

Sequence motifs in growth factors, inhibitors, and receptors, which are similar to the hydropathically complementary sequence of pEEDCK

Hydropathy indices of aminoacid residues are from Kyte and Doolittle (1982): A, 1.8 to 4.5 (hydrophobic); B, −0.7 to −0.8 (polar); X, −3.2 to −4.5 (pos. charged). Extracellular and cytoplasmic domains of receptors are designated by ex or cy, resp.

Sequence	Protein	Receptor for	Domain	Hydropathy Pattern	Reference
VLLTR	IL-11			AAABX	Paul et al., 1990
VLLTH	TNFα			AAABX	Banner et al., 1993
ILLTH	c-kit	SCF	cy	AAABX	Yarden et al., 1987
VLLTN	c-fms	CSF-1	cy	AAABX	Yarden et al., 1987
LLVLR	IL-3Rα	IL-3	cy	AAAAX	Doshi and DiPersio, 1994
LLVLQ	IL-3Rβ	IL-3	cy	AAAAX	Doshi and DiPersio, 1994
VLLVR	IL-6R	IL-6	ex	AAAAX	Bazan, 1989
LLVLQ	GM-CSF-Rβ	GM-CSF	cy	AAAAX	Doshi and DiPersio, 1994

eral growth factors (Cluitmans et al., 1995). Full stem cell activity requires the simultaneous synergistic action of multiple growth factors causing better growth in media. However, they still show limited growth when a suboptimal cocktail is provided, but IL-11, SCF (c-kit ligand), IL-3, and IL-6 seem to be essential (Peters et al., 1995). Keeping stem cells quiescent would thus require "deactivation" of several growth factors at the same time, especially those mentioned above. It is intriguing that a single pentapeptide (or, in fact, any one of the above-mentioned inhibitors) seems to render the whole network of synergistic growth factors ineffective. Taken together, these observations suggest the existence of a network of interdependent inhibitors (Jacobsen et al., 1994), analogous to the better known stimulatory network of cytokines and ILs. Because several growth factors share common signal transduction pathways, it is possible that pEEDCK works at a common step in signal transduction.

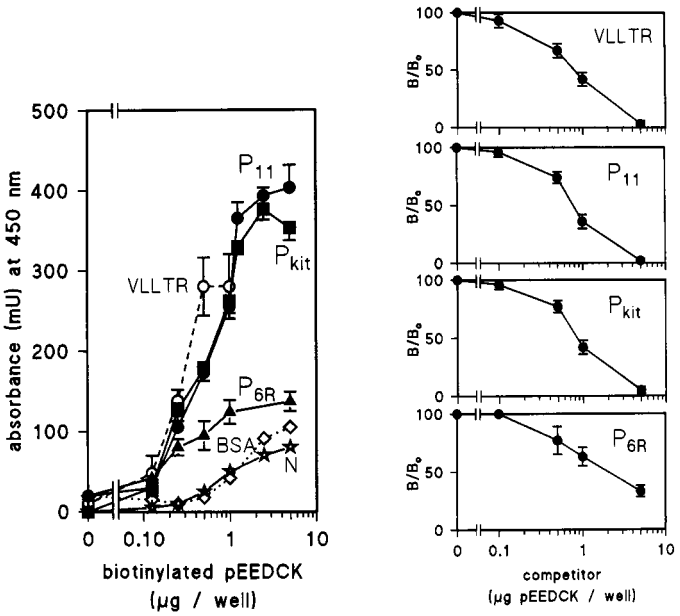

Fig. 5. Binding of pEEDCK to VLLTR (hydropathically complementary sequence in IL-11), c-kit, and IL-6 receptor. Synthetic V**LLTR**Y and the hexadecapeptides P_{11} = ELDST**VLLTR**SLLEDY, P_{kit} = AARN**ILLTH**GRITKY, P_{6R} = SLLTK**VLLVR**KFQNAY from IL-11, c-kit, and IL-6R, C-terminally extended by a tyrosine residue, were coupled to BSA with diazotized benzidine. Microtiter plates were coated with 10 ng conjugate/well. Binding of C-terminally biotinylated pEEDCK was determined as in Fig. 4. Left, concentration dependence of binding of C-terminally biotinylated pEEDCK to the indicated complementary peptides. N designates an experiment in which N-terminally biotinylated EDDCK was used with P_{11}. Right, in competition experiments, 0.5 μg C-terminally biotinylated pEEDCK was added together with the indicated amounts of free pEEDCK. B/B_o = percent maximal absorption.

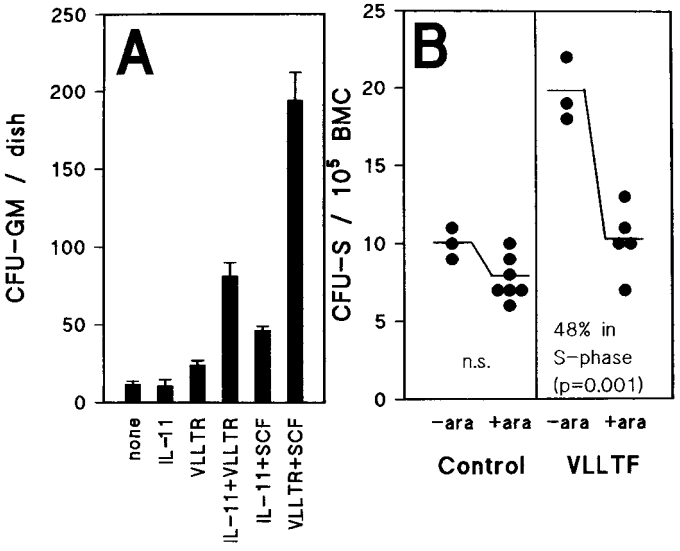

Fig. 6. Peptides hydropathically complementary to pEEDCK stimulate hemopoiesis in vitro and in vivo. A, CFU-GM-depleted LTBMCs were established as in Fig. 1. One nanogram per milliliter of IL-11, SCF, and VLLTR (= $IL\text{-}11_{21-25}$), were added every other day. On day 9 the number of newly formed CFU-GM was determined. B, 1 μg of VLLTF (complementary consensus sequence of pEEDCK) was injected into 10 mice. Ten hours later, the bone marrow was obtained and the percentage of CFU-S in S phase and spleen colony assay were determined as described in *Material and Methods*.

Another possibility would be direct molecular interaction of pEEDCK with growth factors and/or receptors. Binding of peptides may alter the secondary structure of proteins (Martin-Moe et al., 1995), which may have profound effects on the function of the respective protein. We asked if pEEDCK could directly interact with IL-11, SCF, IL-3, and IL-6 or with their receptors, and if so, which part of the protein would bind the peptide.

To answer the first question, we have experimentally measured the interaction of biotinylated pEEDCK with IL-11. The results show that pEEDCK is capable of binding directly to recombinant full-length IL-11. Binding is concentration dependent, saturable, and specific for pEEDCK with a dissociation constant of $K_d = 8 \times 10^{-6}$ corresponding to rather weak binding. Low-affinity binding ensures that the concentration of free IL-11 is strongly dependent on the concentration of pEEDCK, allowing demand-related up- and down-modulation of stem cell activity (see below).

The second question was then to identify possible binding sites for pEEDCK in IL-11 and other growth factors. Protein sites responsible for binding of other proteins or peptides have been identified in many systems by searching for sequences characterized by hydropathic complementarity. Complementary peptides may be a dominant factor in the binding of ligands and receptors (Martin-Moe et al., 1995). Among others, complementary binding sequences have been identified in IL-1 and IL-2 and their receptors, in G-protein coupled receptors and their hormone ligands, and in tyrosine kinase growth factor receptors and the respective growth factors. Peptides encoded by complementary DNA strands are characterized by the opposition of hydrophilic and hydrophobic residues and are capable of specifically binding to each other with dissociation constants in the 10^{-5} to 10^{-6} M range (Martin-Moe et al., 1995; Blalock and Smith, 1995). Binding constants between pEEDCK and IL-11 are in the same range. The molecular basis of binding between hydrophobic and polar (or charged) amino acid residues is still disputed, but X-ray crystallographic investigations of hormone receptor complexes (Clackson and Wells, 1995; Bazan, 1995) indicate stereospecific interactions of the hydrophobic portions of the involved amino acid side chains. Polar or charged end-groups play a minor role. Clackson and Wells (1995) have shown that protein-protein binding is mediated by small functional patches often containing a hydrophobic core of 3 to 10 amino acid residues, which can account for more than 75% of the total binding free energy ΔG. Surrounding charged or hydrophilic residues are less important energetically, and contribute mainly to the specificity of the

interaction. Promiscuous binding of cytokines and receptors in the hemopoietic system often involves multiple epitopes consistent with these structural rules guiding one ligand to different targets (Bazan, 1995).

The nucleotide sequence of the gene encoding pEEDCK or a putative precursor protein is not known. We have thus used other ways to define possible antisense sequences. Because pEEDCK-like sequences are present in several G protein α subunits, we have used their nucleotide sequences (Karlic et al., 1995) for obtaining alternative antisense peptides. All peptides deduced in this way follow the structural pattern outlined by Clackson and Wells (1995) and Bazan (1995): an N-terminal cluster of three or four aliphatic hydrophobic residues (e.g., Leu, Ile, Val) and a polar C-terminal group of one or two residues (e.g., Ser, Thr, His, Lys, Arg). Searching the amino acid sequence of IL-11 for such sequences resulted in the identification of VLLTR as a putative binding site for pEEDCK. Interestingly, sequence motifs corresponding to this general pattern are present also in other hemopoietic growth factors and receptors, such as IL-3R and IL-6R, which may be involved in stem cell proliferation.

Our interest concentrated on IL-11 and SCF because these seem to be of prime importance in stem cell regulation, and on IL-3 and IL-6 because they synergistically support IL-11 and SCF-mediated stem cell stimulation. IL-11 contains the sequence VLLTR, SCF does not contain a relevant sequence, but its receptor, the c-kit proto-oncogene, contains the motif ILLTH in the kinase domain, midway between two major phosphorylation sites (Blume-Jensen et al., 1995). The receptor for IL-6 contains an antisense site (VLLVR) in one of its conserved factor-binding domains (Doshi and DiPersio, 1994) and may thus have different pEEDCK-binding characteristics. Other sequences follow the same general hydropathic pattern and constitute a set of topologically related potential binding epitopes for pEEDCK.

We show here that pEEDCK binds to such antisense peptides. For our binding studies we have used 15-amino acid stretches from IL-11, c-kit, and IL-6R, centered around the sequence motifs mentioned above. We found that pEEDCK binds specifically to VLLTR, P_{11} (ELDST**VLLTR**SLLEDY), and P_{kit} (AARN**ILLTH**GRITKY). That pEEDCK binds to plain VLLTR suggests that binding of pEEDCK to IL-11 (and possibly also to c-kit and other proteins) occurs at the hydropathically complementary recognition site. Dissociation constants range between 8 and 10 μM (Table 4) for IL-11 and c-kit-related AAABX-type complementary peptides. Binding to AAAAX-type P_{6R} (SLLTK**VLLVR**KFQNA) is weaker with K_d = 31 μM. It has to be kept in mind that these dissociation constants were determined with one partner bound to a solid phase. Solution values may be different although the use of BSA as "spacer" may reduce the effect of the plastic surface.

The pGlu and the SH group are essential for inhibitory activity of pEEDCK (Paukovits et al., 1990b). This is also reflected by the binding properties of pEEDCK. N-terminally biotinylated pEEDCK, which lacks the pyroglutamyl motif, did not bind to IL-11. For investigating the role of side chains in binding to complementary partners we have synthesized a set of "mutant" peptides in which Glu^2, Asp^3, or Cys^4 of pEEDCK were replaced either by a glycyl residue (no side chain) or by an α-aminobutyryl residue (C_2H_5 side chain). These peptides were used as competitors for binding of biotinylated pEEDCK to P_{11} and P_{kit}. Peptides with Gly or Abu in position 2 or 3 are good competitors. With the exception of pEGDCK-binding to P_{kit}, their binding affinities were lower than that of pEEDCK, resulting in elevated dissociation constants (Table 4). Substitution of Cys by Gly or Abu completely abolished the ability to bind to P_{11} and P_{kit}. The presence of intact pyroGlu and SH-groups seems thus to be as important for binding to IL-11 and c-kit as it is for the biological activity of pEEDCK. Interestingly, peptides complementary to pEEDCK are themselves strong stem cell stimulators.

Our results show that pEEDCK can bind to the antisense motifs in IL-11 and c-kit, and less strongly to the motifs present in the receptors for IL-6 (and possibly IL-3). It is known that the binding of peptides to proteins can cause conformational changes and functional alterations. Interestingly, the pEEDCK-binding VLLTR-motif in IL-11 is located at the N-terminal end of helix A, a region identified as important for structural stabilization of IL-11 (Czupryn et al., 1995). Simultaneous binding to specific recognition sites in several factors and receptors essential for stem cell regulation may thus constitute the structural basis for the ability of pEEDCK to keep stem cells quiescent in the presence of stimulatory factors. The role of potential pEEDCK-binding sites in stem cell inhibitors is unknown. Because the actions of stem cell inhibitors seem to be interdependent, the presence of pEEDCK binding sites may have an integrative function.

Conclusion

Taken together, our results indicate that endogenous pEEDCK may be involved in the physiological regulation of stem cells. Hematopoiesis in a pEEDCK-deficient environment proceeds at a greatly accelerated rate in vitro and in vivo, involving widespread and multilineage extramedullary hemopoiesis. Because pEEDCK is a product of mature leukocytes, this is reminiscent of earlier suggestions that in some organs, cell proliferation may be controlled by negative feedback (Weiss and Kavanau, 1957; Iversen, 1968). The presence of pEEDCK keeps stem cells quiescent in the presence of growth factors, and the absence of pEEDCK allows stem cells to proliferate in the presence of other inhibitors. We show here that the inhibitory effect of pEEDCK is most pronounced in the presence of growth factors known to be essential for stem cell functioning (especially IL-11, SCF, IL-6, and IL-3). We also show that IL-11, c-kit (the receptor of SCF), and the receptors for IL-6 and IL-3 contain complementary recognition sites to which pEEDCK binds specifically by hydrophobic interaction. Our data indicate that structural interactions of endogenous pEEDCK with complementary recognition sites in cytokines such as IL-11 may be involved in the physiological regulation of stem cells. Interestingly, such pEEDCK-specific recognition sites are also present in inhibitory factors thought to be involved in stem cell quiescence. At least in some proteins (e.g., IL-11) pEEDCK-binding motifs are located at sites involved in stabilization of secondary structure, and binding of pEEDCK may cause structural and functional changes. pEEDCK binding to multiple growth factors and/or receptors may thus simultaneously influence the functional properties of several members of the stem cell regulatory cytokine network. Is this the first glimpse on a superimposed regulatory system, acting as a network-coordinator?

Acknowledgments

We thank Ch. Balcarek for his excellent and reliable technical assistance. R. Rutter contributed to this work as part of her Ph.D. thesis, S. Kneissl as part of her M.D.vet. thesis. Work done by E. Ganglberger was in fulfillment of requirements for a M.Sc. degree.

References

Alisauskas RM, Goldenberg DM, Sharkey RM and Blumenthal RD (1997) Reduction in the duration of myelotoxicity associated with radioimmunotherapy with infusions of the hemoregulatory peptide, HP5b in mice. *Int J Cancer* **70:**323–329.

Amatruda TT, Steele DA, Slepak VZ and Simon MI (1991) G-alpha16, a G-protein alpha subunit specifically expressed in hematopoietic cells. *Proc Natl Acad Sci USA* **88:**5587–5591.

Baserga R, Reiss K, Alder H, Pietrzkowski Z and Surmacz E (1992) Inhibition of cell cycle progression by antisense oligodeoxynucleotides. *Ann NY Acad Sci* **660:**64–69.

Bazan JF (1995) Protein-protein interactions. Postmodern complexes. *Nature (Lond)* **376:**217–218.

Blalock JE and Smith EM (1995) Hydropathic anti-complementarity of amino acids based on the genetic code. *Biochem Biophys Res Comm* **121:**203–207.

Blume-Jensen P, Wernstedt C, Heldin CH and Rönnstrand L (1995) Identification of the major phosphorylation sites for protein kinase C in kit/stem cell factor receptor in vitro and in intact cells. *J Biol Chem* **270:**14192–14200.

Bost KL and Blalock JL (1989) Preparation and use of complementary peptides. *Methods Enzymol* **168:**16–28.

Clackson T and Wells JA (1995) A hot spot of binding energy in a hormone-receptor interface. *Science (Wash DC)* **267:**383–386.

Cluitmans FHM, Esendam BHJ, Landegent JE, Willemze R and Falkenburg JHF (1995) Constitutive in vivo cytokine and hemopoietic growth factor gene expression in the bone marrow and peripheral blood of healthy individuals. *Blood* **85:**2038–2044.

Czupryn MJ, McCoy JM and Scoble HA (1995) Structure-function relationships in human interleukin-11. *J Biol Chem* **270:**978–985.

DiNicola MD, Siena S, Bregni M, Ravagnani F, Vitello F and Belli N (1994) Large-scale enrichment of mobilized CD34(+) peripheral blood hematopoietic progenitors by removal of nylon wool adherent mature cells. *Bone Marrow Transplant* **14:**863–869.

Doshi PD and DiPersio JF (1994) Three conserved motifs in the extracellular domain of the human granulocyte-macrophage colony-stimulating factor receptor subunit are essential for ligand binding and surface expression. *Blood* **84:**2539–2553.

Eaves CJ, Cashman JD, Kay RJ, Dougherty GJ, Otsuka T, Gaboury LA, Hogge DE, Landsdorp PM and Eaves AC (1991) Mechanisms that regulate the cell cycle status of very primitive hematopoietic cells in long-term human marrow cultures. Analysis of positive and negative regulators produced by stromal cells within the adherent layer. *Blood* **78:**110–117.

Frith CH, Ward JM and Chandra M (1993) The morphology, immunohistochemistry, and incidence of hematopoietic neoplasms in mice and rats. *Toxicol Pathol* **21:**206–218.

Gebran S, Romano E and Soyano A (1992) Biomolecules suppressing myelopoiesis. *Acta Cient Venez* **43:**255–265.

Green JA and Manson MM (1992) Production of polyclonal antisera, in *Immunochemical Protocols* (Manson MM ed) pp 1–5, Humana Press, Totowa, NJ.

Iversen OH (1968) Effect of epidermal chalone on human epidermal mitotic activity in vitro. *Nature (Lond)* **219:**75.

Jacobsen SEW, Ruscetti FW, Ortiz M, Gooya JM and Keller JR (1994) The growth response of Lin(−)Thy-1(+) hematopoietic progenitors to cytokines is determined by the balance between synergy of multiple stimulators and negative cooperation of multiple inhibitors. *Exp Hematol* **22:**985–989.

Karlic HI, Mühlberger H, Fritsch G, Paukovits WR, Pavlova B, Pfeilstöcker M, Salamon J and Heinz R (1995) Expression of the hematopoiesis-specific G-protein α-subunit Gα16 correlates with CD34 surface antigen expression and CFU-GEMM. *(Meeting Abstract). Acta Haematol* **93:**258.

Keller JR, McNiece IK, Sill KT, Ellingsworth LR, Quesenberry PJ and Sing GK (1990) Transforming growth factor-beta directly regulates primitive murine hematopoietic cell proliferation. *Blood* **75:**596–602.

Laerum OD, Aakvag A, Frostad S, Kalland T, Langen P and Maurer HR (1990a) Selectivity of the hemoregulatory peptide (HP5b) action in culture. *Int J Cell Cloning* **8:**431–444.

Laerum OD, Frostad S, Ton HI and Kamp D (1990b) The sequence of the hemoregulatory peptide is present in G-i-alpha proteins. *FEBS Lett* **269:**11–14.

Lang RA, Metcalf D, Cuthbertson RA, Lyons I, Standley E, Kelso A, Kannourakis G, Williamson DJ, Klintworth GK, Gonda TJ and Dunn AR (1987) Transgenic mice expressing a hemopoietic growth factor gene (GM-CSF) develop accumulations of macrophages, blindness, and a fatal syndrome of tissue damage. *Cell* **51:**675–686.

Lenfant M, Wdzieczak-Bakala J, Guittet E, Prome J, Sotty D and Frindel E (1989) Inhibitor of hematopoietic pluripotent stem cell proliferation: Purification and determination of its structure. *Proc Natl Acad Sci USA* **86:**779–782.

Maltman J, Pragnell IB and Graham GJ (1993) Transforming growth factor-beta– Is it a downregulator of stem cell inhibition by macrophage inflammatory protein 1-alpha. *J Exp Med* **178:**925–932.

Martin-Moe SA, Lehr R, Cauley D and Moe GR (1995) Hydrophobic interactions and the design of receptor mimetic peptides. *Pept Res* **8:**70–76.

Moore MAS (1995) Hematopoietic reconstruction: New approaches. *Clin Cancer Res* **1:**3–9.

Paukovits JB and Paukovits WR (1995) Stem cell stimulation in vitro by the dekapeptide (pEEDCK)$_2$: A single-factor alternative for multifactor cocktail. *Leukemia* **9(Suppl 1):**48–52.

Paukovits WR, Guigon M, Binder KA, Hergl A, Laerum OD and Schulte-Hermann R (1990a) Prevention of hematotoxic side effects of cytostatic drugs in mice by a synthetic hemoregulatory peptide. *Cancer Res* **50:**328–332.

Paukovits WR, Hergl A and Schulte-Hermann, R (1990b) Hemoregulatory peptide pGlu-Glu-Asp- Cys-Lys: A new synthetic derivative for avoiding dimerization and loss of inhibitory activity. *Mol Pharmacol* **38:**401–409.

Paukovits WR, Moser MH and Paukovits JB (1993) Pre-CFU-S quiescence and stem cell exhaustion after cytostatic drug treatment: Protective effects of the inhibitory peptide pGlu-Glu-Asp-Cys-Lys (pEEDCK). *Blood* **81:**1755–1761.

Paukovits WR, Paukovits JB, Moser MH, Konstantinov S and Schulte-Hermann R (1998) Activated granulocytes oxidize the endogenous stem cell inhibitory peptide pGlu-Glu-Asp-Cy-Lys (pEEDCK) to the stimulatory dimer: A redox-mediated mechanism for demand-induced hemopoietic regulation. *Exp Hematol* **26:**1521–1529.

Peters SO, Kittler ELW, Ramshaw HS and Quesenberry PJ (1995) Murine marrow cells expanded in culture with IL-3, IL-6, IL-11, and SCF acquire an engraftment defect in normal hosts. *Exp Hematol* **23:**461–469.

Posnett DN, McGrath H and Tam JP (1988) A novel method for producing anti-peptide antibodies: Production of site specific antibodies to the t cell antigen receptor beta-chain. *J Biol Chem* **263:**1719–1725.

Ratnam M and Lindstrom J (1984) Structural features of the nicotinic acetylcholine receptor revealed by antibodies to synthetic peptides. *Biochem Biophys Res Comm* **122:**1225–1233.

Sachs L (1994) The molecular control of haemopoiesis and leukaemia. *Eur J Cancer.* **30A:**852–860.

Schoefield KP, Rushton G, Humphries MJ, Dexter TM and Gallagher JT (1997) Influence of interleukin-3 and other growth factors on alpha(4) beta(1) integrin mediated adhesion and migration of human hematopoietic progenitor cells. *Blood* **90:**1858–1866.

Till JE and McCulloch EA (1961) A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat Res* **14:**213–222.

Waegell WO, Higley HR, Kincade PW and Dasch JR (1994) Growth acceleration and stem cell expansion in Dexter-type cultures by neutralization of TGF-β. *Exp Hematol* **22:**1051–1057.

Weiss P and Kavanau JL (1957) A model of growth and growth control in mathematical terms. *J Gen Physiol* **41:**1–47.

Send reprint requests to: Dr. Johanna B. Paukovits, Institute for Tumorbiology-Cancer Research, University of Vienna, Borschkegasse 8a, A-1090 Vienna, Austria. E-mail: walter.paukovits@univie.ac.at