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ORIGINAL ARTICLE

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Effect of angiotensin II and angiotensin(1–7) on hematopoietic recovery after intravenous chemotherapy

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Abstract *Purpose*: Previous studies have shown that angiotensin peptides stimulate the proliferation of hematopoietic progenitors in vitro, promote survival after exposure to lethal irradiation as well as accelerate the recovery of white blood cells (WBC), i.e., lymphocytes, monocytes and neutrophils, and platelets. These changes in the level of formed elements in the blood after irradiation was thought to be due to increases in the numbers of bone marrow progenitors including myeloid, erythroid and megakaryocyte progenitors by the action of angiotensin peptides. In view of these findings, the effect of angiotensin peptides on recovery after chemotherapy was assessed. *Materials and methods*: The effect of angiotensin II (AII) and angiotensin(1–7) (A1–7) on the recovery of WBC and platelets in the blood, as well as the number of myeloid, erythroid and megakaryocyte progenitors in the bone marrow and the number of myeloid progenitors in the blood after intravenous administration of chemotherapeutic drugs was assessed in a mouse model. *Results*: In initial studies, subcutaneous administration of 10 or 100 μg/kg per day of AII starting either 2 days before or 2 days after intravenous administration of 5-fluorouracil (5FU) accelerated WBC recovery (return to baseline between 7 and 14 days). Further, consistent with previous observations, the number of myeloid progenitors in the bone marrow and blood was increased after systemic administration of angiotensin peptides. The comparability of A(1–7) and AII in their effect on hematopoietic recovery after chemotherapy was shown in subsequent studies. Daily administration of both AII and A(1–7) increased platelet numbers in the peripheral blood and myeloid, erythroid and megakaryocyte progenitors in the bone marrow. As 5FU is not a stem cell toxin, these studies were repeated with administration of A(1–7) initiated before or after intravenous cyclophosphamide. Following treatment with A(1–7) before cyclophosphamide the numbers of circulating WBC initially increased and then decreased starting on day 14. Following treatment with A(1–7) 2 days after cyclophosphamide the numbers of WBC and the numbers of myeloid progenitors increased in the peripheral blood and bone marrow. *Conclusions*: These findings suggest that angiotensin peptides accelerate hematopoietic recovery in multiple cellular lineages after chemotherapy, perhaps through an increase in the number of early hematopoietic progenitors.

Keywords Angiotensin II · Angiotensin(1–7) · Hematopoietic recovery · Intravenous chemotherapy

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Introduction

Angiotensin II (AII) is a product of the renin angiotensin system that has recently been shown to have multiple effects beyond the usually recognized cardiovascular effects. However, AII is only one of the naturally occurring active peptides that are formed from angiotensin I (AI). Through angiotensin-converting enzyme, AI is converted to AII. However, another enzyme, termed neutral endopeptidase, catalyzes the cleavage of AI to the seven amino acid peptides A(1–7) [14]. A(1–7) shares the first seven amino acids of AII and lacks the eighth amino acid, phenylalanine, which is involved with the hypertensive actions of AII [27, 29]. Subsequent studies have shown that, although A(1–7) does not increase blood pressure, this peptide has biological actions in the cardiovascular and renal systems [3, 7, 8, 12, 14, 15].

Studies have shown that AII is also a potent regulator of tissue regeneration and angiogenesis [1, 4, 5, 9, 10, 11,

13, 19, 20, 30, 36, 37, 38, 39, 43, 44]. Further, studies have shown that AII increases the release of growth factors, growth factor gene expression, growth factor receptor expression, and expression of growth-related protooncogenes [2, 6, 21, 23, 24, 25, 26, 28, 33, 34, 45, 46, 47]. Some of the effects of AII on cell function are mediated through alterations in growth factor production and may be additive to the effects of growth factors per se. Recently, A(1–7) has been shown to be comparable to AII in their ability to accelerate repair dermal injuries [41].

During studies assessing the effect of AII on tissue healing it was noted that angiotensin peptides affect the infiltration of mesenchymal stem cells and the proliferation of epidermal stem cells [39, 41]. Based upon these observations, the effect of AII on the proliferation of hematopoietic progenitors was evaluated. These studies showed that AII stimulates the proliferation of human and murine hematopoietic progenitors in vitro in a concentration-dependent manner [40]. AII was shown to increase the number of colonies formed, under optimal culture conditions, by Lin^- and Lin^-Sca1^+ bone marrow cells from mice and $CD34^+$ and $CD34^+CD38^-$ cells from human cord blood.

As recovery of bone marrow and circulating white blood cells (WBC) after irradiation is a form of tissue regeneration, requires the proliferation of progenitor cells and may be augmented by growth factors, the ability of angiotensin peptides to act as a hematopoietic factor after irradiation was evaluated and compared with that of filgrastim. These studies showed that in vivo, systemic administration of AII and A(1–7) stimulates hematopoietic recovery as measured by circulating WBC and platelet concentrations as well as the number of myeloid, erythroid and megakaryocyte progenitors in the bone marrow of irradiated animals [42]. AII stimulates recovery of WBC from multiple lineages with an action more prolonged than filgrastim with cessation of therapy 7 days after irradiation. With administration of filgrastim, increases were observed in WBC numbers through day 13 after irradiation, whereas with AII, increases continued through day 21. Further increases in circulating myeloid progenitors and platelets were observed when angiotensin peptides were administered after myelosuppressive irradiation. If AII was given for 2 or 7 days after irradiation, with bone marrow evaluation occurring on day 35 (28–33 days after cessation of treatment), increased numbers of myeloid progenitors were observed. This is in contrast to the effects of filgrastim on the number of myeloid progenitors in the bone marrow.

The effect of AII may be as a stimulator of early hematopoietic progenitors as following administration of AII the numbers of granulocyte macrophage colony forming units (GM-CFU) in the bone marrow have been found to be increased for as long as 28 days after cessation of AII treatment. This is further supported by the stimulatory effects of angiotensin peptides on the multitude of hematopoietic lineages, including lymphocytes, monocytes, neutrophils and platelets in the blood and myeloid and megakaryocyte progenitors in the bone marrow, and types of hematopoietic progenitors, including GM-CFU, granulocyte erythroid, megakaryocyte, macrophage colony forming units (GEMM-CFU), burst-forming units erythroid (BFU-E) and megakaryocyte colony forming units (Meg-CFU) in the bone marrow after irradiation.

In the study reported here, the effects of administration of AII and A(1–7) on hematopoietic recovery after intravenous treatment with 5-fluorouracil (5FU) or cyclophosphamide were evaluated.

Material and methods

Animals

Female C57Bl/6 mice, 6–8 weeks old, were purchased from Simonsen Laboratories (Gilroy, Calif.) or Harlan and housed in the USC vivarium on a 12/12-h light/dark cycle with food and water available ad libitum. The mice were quarantined at least 1 week prior to use. After quarantine, the mice were placed in the study.

Study design

In the initial study, subcutaneous administration of AII (10 or 100 μg/kg per day) was initiated 2 days before, the day of, and 2 days after intravenous administration of 5FU (150 mg/kg), and the injections were continued until the animals were killed. On either day 7 or day 14 after administration of 5FU, the animals were killed and the peripheral blood and bone marrow were harvested. The peptide was administered for 9 or 16 days (starting 2 days before 5FU), for 7 or 14 days (starting the day of 5FU) or for 5 or 12 days (starting 2 days after 5FU). The numbers of WBC in the blood and the number of GM-CFU in the bone marrow and blood were determined.

In a second study, the female C57Bl/6 mice were injected with 150 mg/kg 5FU intravenously. Administration of AII and A(1–7) by subcutaneous injection was begun 2 days after administration of 5FU and continued daily until 10 days after 5FU administration at which time the mice were killed for determination of the numbers of WBC and platelets, and GM-CFU in the bone marrow and peripheral blood. On days 4 and 7 after 5FU administration, blood was taken under methoxyflurane anesthesia from the retro-orbital sinus for determination of WBC and platelet numbers. This study was repeated with determination of the numbers of platelets in peripheral blood on days 6, 10 and 14. These studies were performed with different times before the animals were killed to allow evaluation of the bone marrow at different stages of recovery.

In the final study, the female C57Bl/6 mice were injected with 200 mg/kg cyclophosphamide intravenously. Administration of A(1–7) by subcutaneous injection was begun 2 days before or 2 days after administration of cyclophosphamide and continued daily until the animals were killed (day 28) for evaluation of mature formed blood elements in the circulation and the number of GM-CFU in the bone marrow and peripheral blood. On days 5, 9, 14, 21 and 28 after cyclophosphamide administration, blood was taken under methoxyflurane anesthesia from the retro-orbital sinus for the determination of WBC numbers.

Evaluation of myeloid and erythroid progenitors in the blood and bone marrow

The blood was harvested by cardiac puncture at necropsy to assess mobilization of myeloid progenitors into the peripheral blood. The femurs and tibias were also collected and the bone marrow was

harvested by flushing with PBS containing 2% fetal calf serum. After collection of the blood and bone marrow, the red blood cells (RBC) were lysed with a hypotonic solution (0.83% NH_4C1, 10 m*M* EDTA, 0.5% $NaHC0_3$), mixed with 0.04% trypan blue and the nucleated cells counted by hematocytometer under light microscopy. Aliquots of cells were then resuspended at 1×10^5 cells/ml (bone marrow) or 1×10^6 cells/ml (blood). An aliquot of each suspension was added to semisolid medium (100 µl suspension to 900 µl medium) containing 0.9% methyl cellulose in Iscove's MDM, 15% fetal calf serum, 1% bovine serum albumin, 10 µg/ml bovine pancreatic insulin, 200 µg/ml human transferrin, 10^{-4} *M* 2-mercaptoethanol, 2 m*M* L-glutamine, 10 ng/ml recombinant murine interleukin 3 (IL-3), 10 ng/ml recombinant human IL-6, 50 ng/ml recombinant murine stem cell factor and 3 U/ml erythropoietin. This mixture was then added to duplicate wells of a 24-well plate. The cultures were then placed at 37ºC in a humidified atmosphere of 5% CO_2 in air. On day 12, the BFU-E formed were counted under phase-contrast microscopy. On day 14, the GM-CFU and GEMM-CFU formed were counted.

The BFU-E colonies contained predominantly erythroid cells with greater than 200 cells. GEMM-CFU colonies were large erythroid-containing colonies, granulocytes, monocyte/macrophage cells and megakaryocytes. GM-CFU colonies were large and contained cells from both the granulocyte and monocyte/macrophage lineage. The numbers of progenitors (CFU) present in bone marrow from untreated mice of this strain, sex and age are approximately 1×10^5 GM-CFU per femur, 5×10^3 GEMM-CFU per femur and 1.6×10^4 BFU-E per femur.

Evaluation of Meg-CFU in the bone marrow

Cells were isolated from the bone marrow as described above. After counting the viable nucleated cells isolated, an aliquot of the cells was resuspended to a concentration of 1×10^6 cells/ml. Of this suspension, 100 µl was mixed with 2 ml medium containing 1.1 mg/ml collagen, 1% BSA, 10 µg/ml bovine pancreatic insulin, 200 µg/ml human transferrin, 2 m*M* L-glutamine, 10 µg/ml 2-mercaptoethanol, 50 ng/ml recombinant human thrombopoietin, 20 ng/ml recombinant human IL-6, 50 ng/ml recombinant human IL-11, 100 ng/ml recombinant murine IL-3 in Iscove's MDM (MegaCult-C; Stem Cell Technologies, Vancouver, BC). The cells and medium were mixed and dispensed onto a tissue culture slide, spread evenly and allowed to gel. The culture slide was incubated at 37ºC in a humidified atmosphere of 5% CO_2 in air for 8 days.

At the end of the culture time, the formation of megakaryocytes from megakaryocyte precursors (Meg-CFU) was determined by staining for the expression of acetyl cholinesterase. The culture slides were opened and the collagen gel was fixed in an acetone solution and air-dried. The substrate solution (0.5 mg/ml acetylthiocholiniodide in 0.075 *M* sodium phosphate buffer, 0.01 *M* sodium citrate, 3 m*M* copper sulfate and 0.5 m*M* potassium ferricyanide solution) was added to the fixed slides and allowed to incubate in a humid chamber for 3.5 h. The slides were then fixed in a solution of 95% ethanol for 10 min, rinsed and air-dried. The slides were then counterstained with hematoxylin solution for 30 s, rinsed and dried. The colonies that stained positive for acetyl cholinesterase were counted under the microscope.

WBC and platelet numbers

At various times after irradiation, the mice were anesthetized with methoxyflurane and bled via the retro-orbital sinus. Approximately 100 µl blood was obtained from the retro-orbital sinus with a heparinized capillary tube. The blood was then placed in a 1.7-ml microfuge tube containing 10 m*M* EDTA and held on ice until further processing.

The blood (20 µl) was mixed with 200 µl of RBC lysing solution (0.83% NH_4Cl, 10 m*M* EDTA, 0.5% $NaHCO_3$). The mixture was then incubated for 10 min at 4°C. After this incubation, the supernatant was removed and the pellet was resuspended in 100 µl PBS. To this, 100 µl of 0.04% trypan blue was added. This mixture was vortexed and the number of WBC (baseline approximately 7–10×10^6 WBC/ml) was determined by hematocytometer under light microscopy and the number of platelets (baseline approximately 2.5×10^8 platelets/ml) was determined by hematocytometer under phase-contrast microscopy.

Statistics

The data were analyzed by comparing values from drug-treated mice with those from their respective saline-treated controls using Student's *t*-test. *P* values less than 0.05 were considered significant.

Results

Effect of angiotensin peptides on progenitor cell numbers after intravenous administration of 5FU

In the initial study, AII administration increased the numbers of progenitors in the bone marrow and peripheral blood of animals treated with 5FU. Administration of 5FU decreased the number of GM-CFU (myeloid progenitors) in the bone marrow of saline-treated mice. Subcutaneous administration of AII also had a profound effect on the number of myeloid progenitors in the bone marrow of mice treated with 5FU (Fig. 1). The greatest increase in myeloid progenitors in the bone marrow was observed if AII treatment was initiated 2 days after 5FU. The effects of AII on myeloid progenitors was dose-dependent and maximal in the 100 µg/kg dosage group.

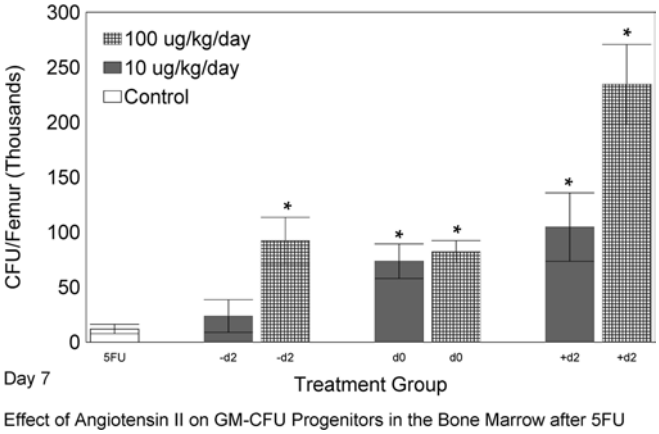


Fig. 1 Effect of AII on GM-CFU progenitors in the bone marrow after 5FU treatment. Female C57Bl/6 mice, 6–8 weeks old, were treated with 150 mg/kg 5FU by intravenous injection. Subcutaneous administration of AII was initiated 2 days before (*–d2*, 9 days of peptide), on the day of (*d0*, 7 days of peptide) or 2 days after (*+d2*, 5 days of peptide) 5FU injection. The animals were killed 7 days after 5FU injection, and the bone marrow harvested. The GM-CFU formed from cells isolated from bone marrow after RBC lysis by culturing in semisolid medium containing recombinant colony-stimulating factors were counted. The results presented are the means ± SE of the data from three animals per group (dose and time-point). $*P < 0.05$ vs saline-treated control

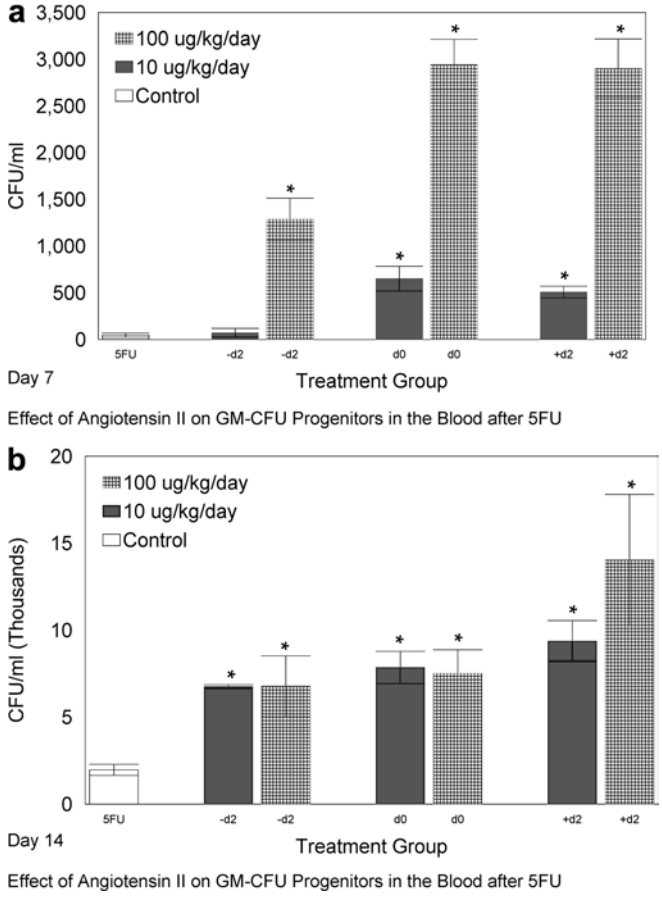


Fig. 2a, b Effect of AII on GM-CFU progenitors in the blood after 5FU treatment. Female C57Bl/6 mice, 6–8 weeks old, were treated with 150 mg/kg 5FU by intravenous injection. Subcutaneous administration of AII was initiated 2 days before (*–d2*, 9 days of peptide), on the day of (*d0*, 7 days of peptide) or 2 days after (*+d2*, 5 days of peptide) 5FU injection. The animals were killed 7 days (**a**) or 14 days (**b**) after 5FU injection, and peripheral blood harvested. The GM-CFU formed from nucleated peripheral blood cells isolated after RBC lysis by culturing in semisolid medium containing recombinant colony-stimulating factors were counted. The results presented are the means ± SE of the data from three animals per group (dose and time-point). $*P < 0.05$ vs saline-treated control

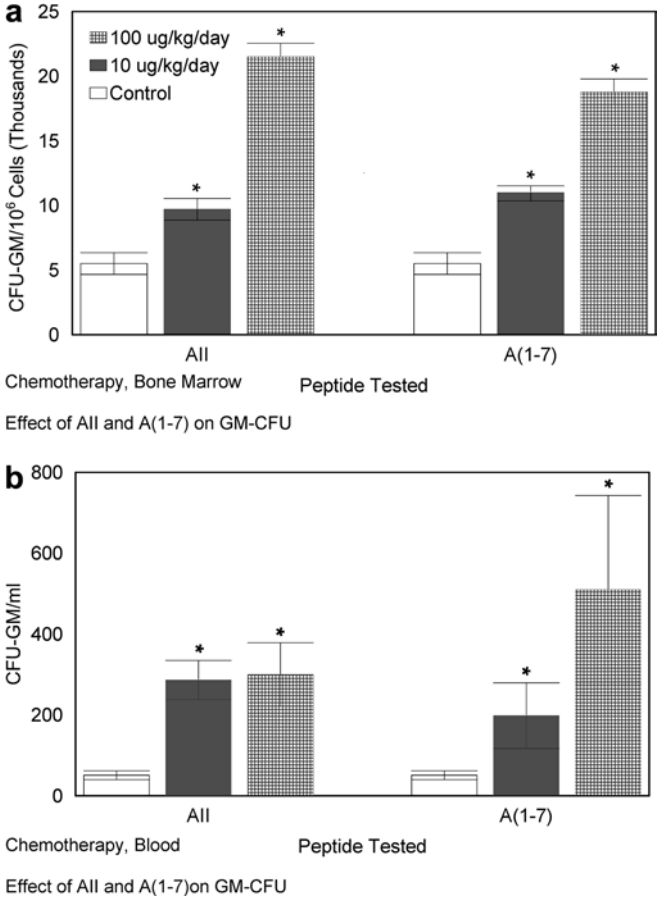


Fig. 3a, b Effects of AII and A(1–7) on GM-CFU. Female C57Bl/6 mice, 6–8 weeks old, were treated with 150 mg/kg 5FU by intravenous injection. Subcutaneous administration of AII or A(1–7) was initiated 2 days after 5FU injection (8 days of peptide). The animals were killed 10 days after 5FU injection, and bone marrow (**a**) or peripheral blood (**b**) harvested. The GM-CFU formed from cells isolated from bone marrow or peripheral blood after RBC lysis by culturing in semisolid medium containing recombinant colony-stimulating factors were counted. The results presented are the means ± SE of the data from up to four animals per group (dose and time-point). $*P < 0.05$ vs saline-treated control

The effect of AII administration in conjunction with 5FU on the mobilization of myeloid progenitors into the peripheral circulation was also assessed (Fig. 2a, day 7; Fig. 2b, day 14). Administration of 5FU alone increased GM-CFU in the blood over that expected in normal animals. Administration of AII on day 0 or day 2 relative to 5FU further increased in a dose-dependent fashion the mobilization of myeloid precursors by up to 50 times on day 7 over that observed with 5FU.

In a second study, the effects of AII and A(1–7) on the number of myeloid progenitors in the bone marrow and peripheral blood were investigated. Administration of 5FU significantly reduced the number of myeloid progenitors (GM-CFU) in the bone marrow. Administration of A(1–7) 2 days after 5FU increased the numbers of GM-CFU in the bone marrow 2–3.5 times and administration of AII increased the numbers of GM-CFU 2–4 times compared to the numbers in control animals (Fig. 3a). Administration of A(1–7) following 5FU increased the numbers of GM-CFU in the peripheral blood four to ten times and administration of AII increased the numbers of GM-CFU six times (Fig. 3b).

In a final study, the effects of AII and A(1–7) on progenitors of multiple hematopoietic lineages were evaluated. Administration of 5FU significantly reduced the number of myeloid progenitors (GM-CFU), early mixed progenitors (GEMM-CFU) and erythroid progenitors (BFU-E) in the bone marrow. Administration of AII or A(1–7) 2 days after 5FU increased the number of these progenitor cells in the bone marrow (Fig. 4a–c). Administration of AII or A(1–7) after 5FU also resulted in an increase in the number of Meg-CFU in the bone

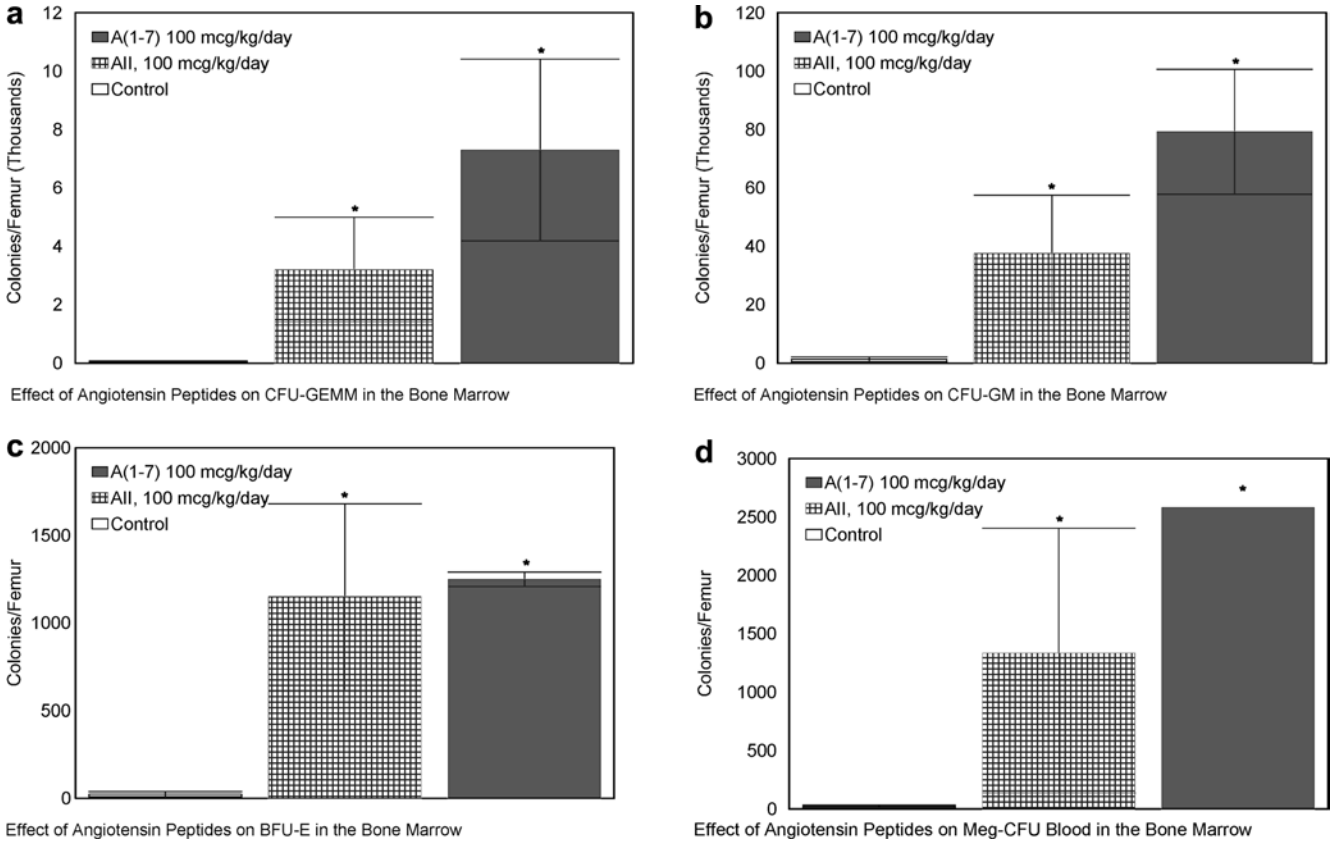


Fig. 4a–d Effects of angiotensin peptides on (**a**) GEMM-CFU, (**b**) GM-CFU, (**c**) BFU-E, and (**d**) Meg-CFU in the bone marrow. Female C57B;/6 mice, 6–8 weeks old, were treated with 150 mg/kg 5FU by intravenous injection. Subcutaneous administration of AII or A(1–7) was initiated 2 days after 5FU injection. The animals were killed 14 days after 5FU injection, and the bone marrow was harvested and cultured with the appropriate recombinant hematopoietic factors. The numbers of the hematopoietic progenitors were determined after culture. The results presented are the means ± SE of the data from up to five mice per group. $*P < 0.05$ vs saline-treated controls

marrow (Fig. 4d). Further, administration of A(1–7) or AII after 5FU increased the number of GM-CFU progenitors in the peripheral blood (Fig. 5).

Effect of angiotensin peptides on hematopoietic recovery

Given the effect of angiotensin peptides on the progenitors in the bone marrow and peripheral blood of animals treated with 5FU, the effect on hematopoietic recovery was also evaluated. An initial study involved determination of WBC numbers in the peripheral blood on days 7 and 14 after intravenous injection of 5FU. In this study, the nadir (approximately 83% reduction) in WBC numbers was observed on day 7 after 5FU administration (Fig. 6a). The WBC nadir was nearly two times higher in AII-treated animals than in control animals when the AII was begun on day 0. The WBC numbers were approximately 40% greater in AII-treated animals on day 14 than in control animals (Fig. 6b).

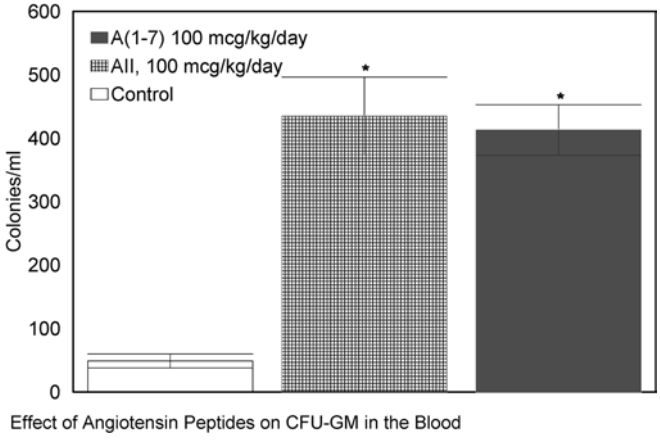
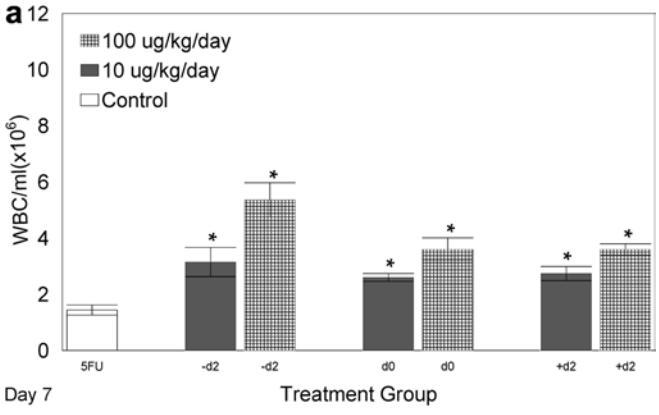
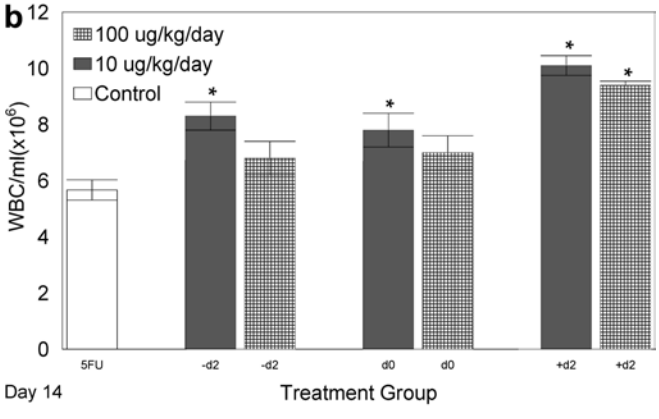


Fig. 5 Effects of angiotensin peptides on GM-CFU in the blood. Female C57Bl/6 mice, 6–8 weeks old, were treated with 150 mg/kg 5FU by intravenous injection. Subcutaneous administration of AII or A(1–7) was initiated 2 days after 5FU injection. Animals were killed 14 days after 5FU injection, and the blood harvested by cardiac puncture. After lysis of the RBCs in hypotonic solution, the nucleated cells were counted and adjusted, and the cells were cultured with the appropriate recombinant hematopoietic factors. The number of GM-CFU were determined after 14 days in culture. The results presented are the means ± SE of the data from up to five mice per group. $*P < 0.05$ vs saline-treated controls

With initiation of treatment on day 2 after 5FU, there was an approximately twofold increase in WBC numbers on day 7 and recovery was complete by day 14.

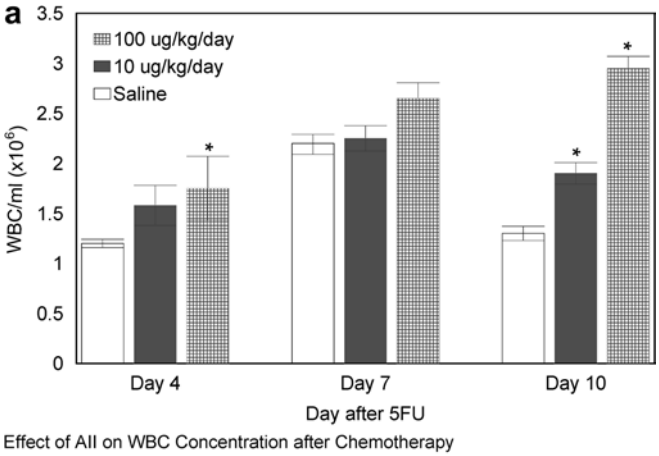


Effect of Angiotensin II on White Blood Cell Number in the Blood after 5FU

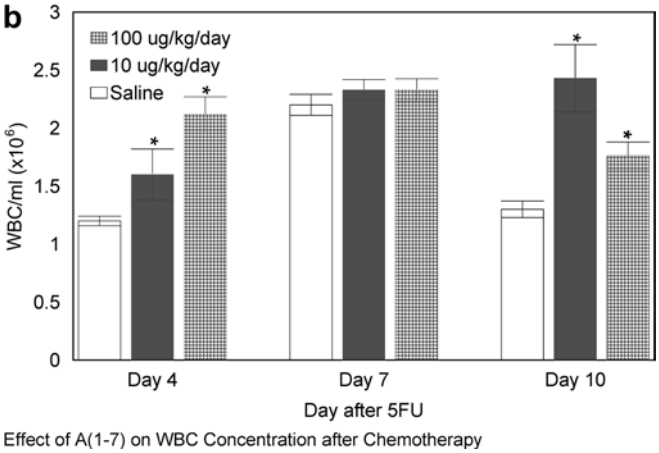


Effect of Angiotensin II on White Blood Cell Number in the Blood after 5FU

Fig. 6a, b Effects of AII on WBC numbers in the blood after 5FU treatment. Female C57Bl/6 mice, 6–8 weeks old, were treated with 150 mg/kg 5FU by intravenous injection. Subcutaneous administration of AII was initiated 2 days before (*–d2*), on the day of (*d0*) or 2 days after (*+d2*) 5FU injection. The animals were killed 7 days (**a**) or 14 days (**b**) after 5FU injection, and peripheral blood harvested. The WBCs were counted by hematocytometer after RBC lysis. The results presented are the mean ± SE of the data from three animals per group (dose and time-point). $*P < 0.05$ vs saline-treated controls



Effect of AII on WBC Concentration after Chemotherapy

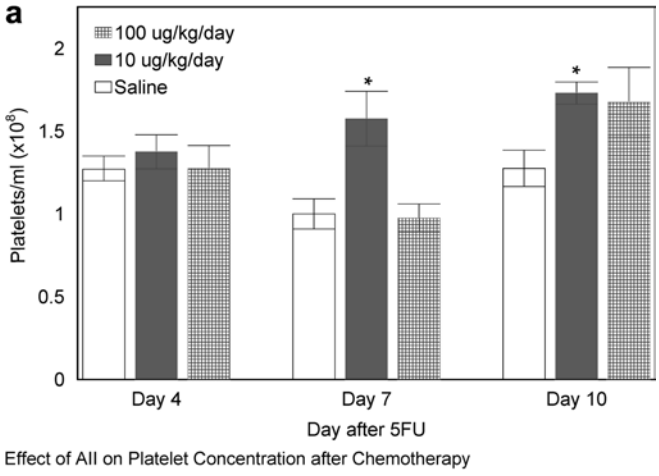


Effect of A(1-7) on WBC Concentration after Chemotherapy

Fig. 7a, b Effects of AII (**a**) and A(1–7) (**b**) on WBC numbers after 5FU treatment. Female C57Bl/6 mice, 6–8 weeks old, were treated with 150 mg/kg 5FU by intravenous injection. Subcutaneous administration of AII or A(1–7) was initiated 2 days after 5FU injection. On days 4, 7 and 10, peripheral blood was harvested. WBCs were counted microscopically using a hematocytometer after RBC lysis. The results presented are the means ± SE of the data from up to four animals per group

The second study showed that AII and A(1–7) accelerated the recovery of WBCs after intravenous administration of 5FU (Fig. 7). The decrease in WBC numbers as a result of administration of 5FU reached approximately 86% in saline-treated controls. In A (1–7)-treated animals, WBC levels were nearly twice as great as in control animals on day 7 and increased in the 100 μg/kg group to 64% of baseline. However, A(1–7) administration did not affect the time to nadir. In AII-treated animals, WBC levels were again nearly twice as high as in control animals by day 7 with further increases by day 10. A(1–7) and AII produced similar dose-dependent increases in WBC levels compared to control treatment. The increase in WBC continued throughout the experimental period with a maximum observed at the high dose of peptide on the day the animals were killed.

An increase in platelets after administration of both peptides (Fig. 8) was also observed. Intravenous administration 5FU resulted in a decrease of approximately 60% in platelet numbers that was maximal on day 7 in control animals. Animals treated with A(1–7) showed a consistent dose-dependent increase in platelet numbers, but the time to nadir did not vary. Animals treated with A(1–7) at the higher dose (100 μg/kg per day) showed a 50% higher platelet nadir compared to control animals on day 7. Animals treated with A(1–7) at 10 μg/kg per day did not show platelet numbers significantly different from control animals. Animals treated with AII at 10 μg/kg per day showed a 50% higher nadir than control animals, while those treated at higher doses showed similar levels to control animals on day 7. By day 10, AII-treated animals showed similar platelet numbers that were 40% higher than in control animals. These findings were reproduced in a second study in which the evaluation was continued through



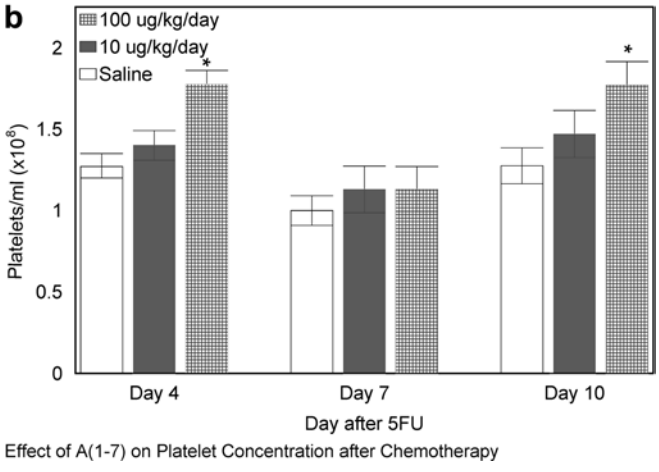


Fig. 8a, b Effects of AII (**a**) and A(1–7) (**b**) on platelet numbers after 5FU treatment. Female C57Bl/6 mice, 6–8 weeks old, were treated with 150 mg/kg 5FU by intravenous injection. Subcutaneous administration of AII or A(1–7) was initiated 2 days after 5FU injection. On days 4, 7 and 10 after 5FU injection, peripheral blood was harvested. The platelets were counted by hematocytometer after RBC lysis. The results presented are the means ± SE of the data from up to four animals per group (dose and time-point). $*P < 0.05$ vs saline-treated controls

day 14. In this second study, the nadir was seen in control animals on day 14. As expected, the effect of the peptide was more pronounced when the platelet numbers fell (Fig. 9).

Effect of A(1–7) on hematopoietic recovery after cyclosphosphamide

A study was then conducted that showed that A(1–7) accelerated the recovery of WBC after intravenous administration of cyclophosphamide when A(1–7) treatment was initiated 2 days after cyclophosphamide (Fig. 10a). The increase in WBC numbers was observed within 9 days after cyclophosphamide treatment. However, if A(1–7) treatment was initiated 2 days prior to administration of cyclophosphamide, there was a decrease in WBC numbers compared to that in control

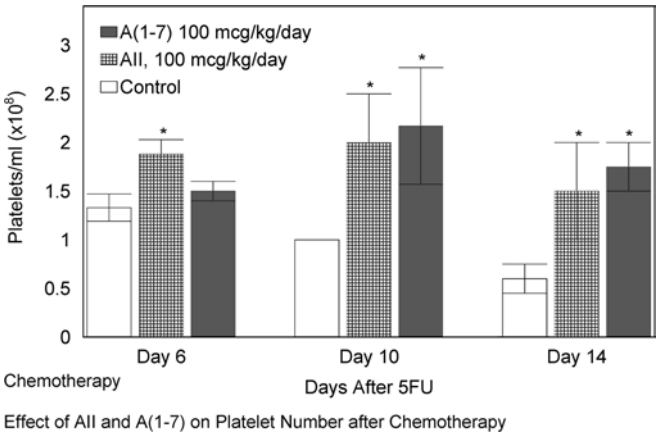


Fig. 9 Effects of AII and A(1–7) on platelet numbers after 5FU treatment. Female C57Bl/6 mice, 6–8 weeks old, were treated with 150 mg/kg 5FU by intravenous injection. Subcutaneous administration of AII or A(1–7) was initiated 2 days after 5FU injection. On days 6, 10 and 14 after 5FU injection, peripheral blood was harvested. The platelets was counted microscopically using a hematocytometer after RBC lysis. The results presented are the means ± SE of the data from up to five mice per group. $*P < 0.05$ vs saline-treated controls

animals starting on day 14 after cyclophosphamide treatment (Fig. 10b). This suggests that, as with irradiation, pretreatment with an angiotensin peptide prior to and continuing during administration of a stem cell toxin may reduce hematopoietic recovery.

Administration of cyclophosphamide also significantly reduced the number of myeloid progenitors (GM-CFU) in the bone marrow. Treatment with A(1–7) (100 μg/kg per day) 2 days prior to treatment with cyclophosphamide slightly reduced the number of GM-CFU in the bone marrow of treated animals (Fig. 11a). However, A(1–7) administration 2 days after cyclophosphamide increased the number of myeloid progenitor cells in the bone marrow (Fig. 11a). This may have been due to the ability of cyclosphosphamide to injure cells that are proliferating at the time of treatment. Further, administration of A(1–7) after cyclophosphamide increased the number of myeloid progenitors in the peripheral blood (Fig. 11b).

Discussion

Evidence is increasing that angiotensin peptides are potent modulators of cellular proliferation and function. Numerous studies have shown that AII can stimulate growth factor release and increase the expression of growth factor receptors. More recent studies have shown that exposure to AII can increase the production of IL-6, a cytokine that can contribute to hematopoiesis, by mesangial cells, vascular smooth muscle cells and macrophages [22, 31, 35]. Further, the levels of extrarenal erythropoietin production in animals and the levels of circulating erythropoietin in patients made anemic by induced hemorrhage are increased by administration of

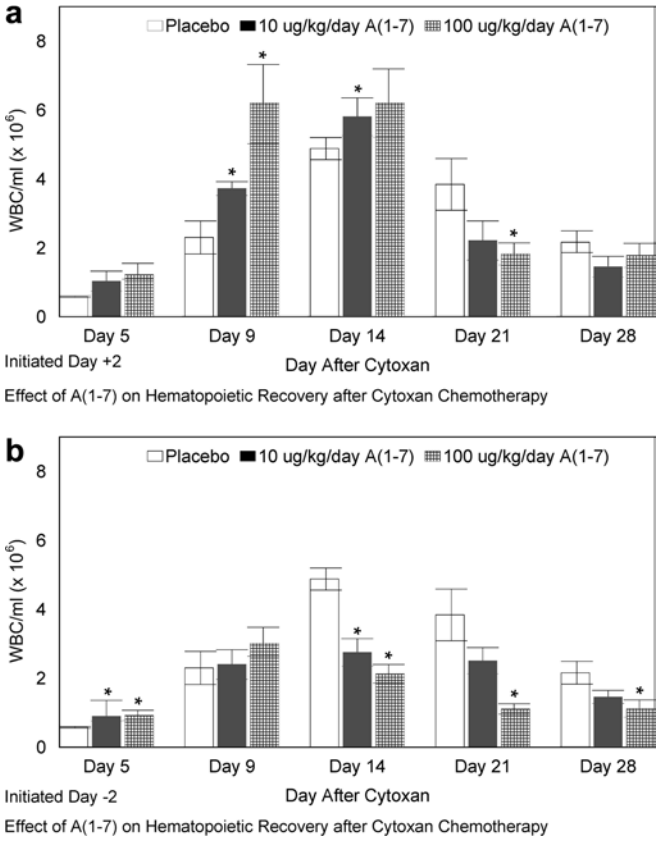


Fig. 10a, b Effects of A(1–7) on hematopoietic recovery after cyclophosphamide treatment. Female C57Bl/6 mice, 6–8 weeks old, were treated with 200 mg/kg cyclophosphamide by intravenous injection. Subcutaneous administration of A(1–7) was initiated 2 days after (**a**) and 2 days before (**b**) cyclophosphamide injection. The animals were killed 5, 9, 14, 21 and 28 days after cyclophosphamide injection, and peripheral blood harvested. The WBCs were counted by hematocytometer after RBC lysis. The results presented are the means ± SE of the data from three animals per group (dose and time-point). $*P < 0.05$ vs saline-treated controls

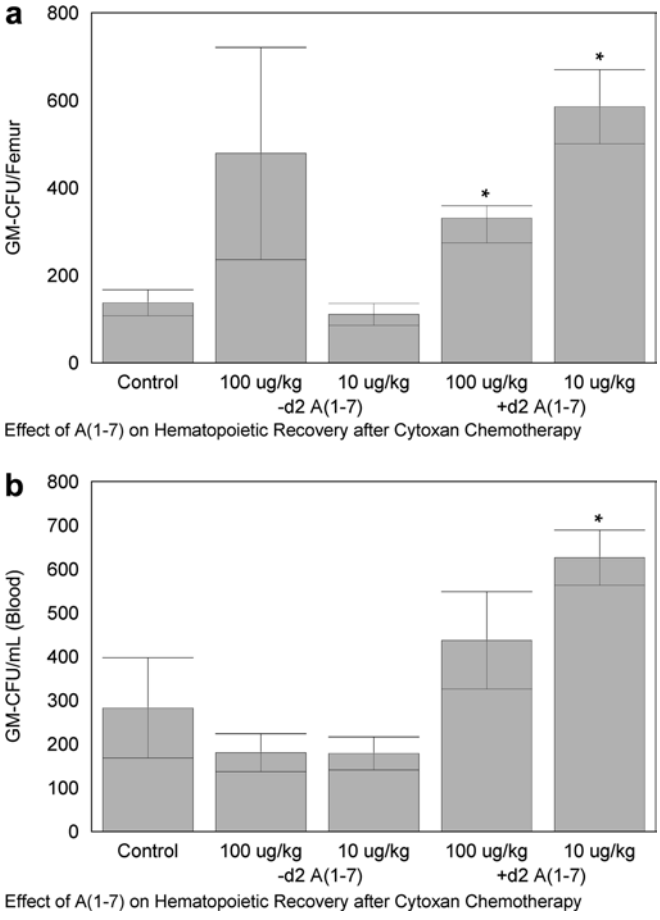


Fig. 11a, b Effects of A(1–7) on hematopoietic recovery after cyclophosphamide treatment. Female C57Bl/6 mice, 6–8 weeks old, were treated with 200 mg/kg cyclophosphamide by intravenous injection. Subcutaneous administration of A(1–7) was initiated 2 days after and 2 days before cyclophosphamide injection. The animals were killed 28 days after cyclophosphamide injection, and bone marrow (**a**) and peripheral blood (**b**) harvested. The numbers of GM-CFU formed from cells isolated from bone marrow and peripheral blood after RBC lysis by culturing in semisolid medium containing recombinant colony-stimulating factors were determined. The results presented are the means ± SE of the data from three animals per group (dose and time-point). $*P < 0.05$ vs saline-treated controls

both pressor and subpressor concentrations of AII [16, 17, 18].

While AII affects factors known to affect hematopoiesis, there is also an effect on progenitor proliferation. Studies from this laboratory have shown that angiotensin peptides have a profound effect on the proliferation of less mature cells, including epidermal stem cells [38, 39, 41] and hematopoietic progenitors [40, 42]. As both early hematopoietic progenitors and bone marrow stromal cells express receptors for AII, and proliferation and function of these cells are necessary for hematopoietic recovery, the effect of angiotensin peptides after myelosuppressive therapy has been evaluated [40, 42]. After myelosuppressive irradiation, administration of AII promotes survival and accelerates recovery of WBC, lymphocytes, monocytes, neutrophils, and platelets. Further, angiotensin peptides increase the number of myeloid, erythroid and megakaryocyte progenitors in the bone marrow of irradiated mice. In the studies reported here the effect of AII and A(1–7), an angiotensin peptide that does not increase blood pressure, on hematopoietic recovery after intravenous chemotherapy were evaluated.

In an initial study, it was demonstrated that administration of AII accelerated, in a dose-dependent manner, the recovery of WBC after intravenous 5FU. The increase in WBC appeared to be the result of an increase in the number of myeloid (GM-CFU) progenitor cells in the bone marrow. Further, administration of AII increased the number of GM-CFU progenitors in the peripheral blood induced by 5FU administration. Increased platelet concentrations were also observed following A(1–7) and AII treatment at a dose of 100 μg/kg after 5FU. Increased concentrations of GM-CFU progenitors in the bone marrow and mobilized GEMM-CFU, GM-CFU, BFU-E, and Meg-CFU progenitors from the bone marrow into the peripheral blood were

observed in a dose-dependent manner following AII and A(1–7) treatment.

Finally, the results of the administration of A(1–7) in conjunction with cyclophosphamide were similar to those observed after irradiation. This correspondence between irradiation and cyclophosphamide treatment and the disparity between cyclophosphamide and 5FU may have been due to the site of action of these myelosuppressive agents. Cyclosphosphamide, like radiation, causes damage to DNA, whereas 5FU is an antimetabolic agent. Pretreatment with A(1–7) resulted in a decreased WBC recovery compared with saline-treated controls starting on day 14 after chemotherapy. As with irradiation, there was an initial increase that may have reflected an effect on later committed progenitors and a late effect on WBC which might have resulted from an effect on early hematopoietic progenitors.

This is consistent with the results of a comparative study between AII and filgrastim after myelosuppressive irradiation [42]. Along with a more prolonged effect on WBC recovery after cessation of therapy (6 days with filgrastim, 14 days with AII), an increase was observed in the number of myeloid progenitors in the bone marrow 28 days after cessation of AII administration, whereas no increase was observed at this time after filgrastim treatment. As filgrastim and AII or A(1–7) might act at distinct sites of the hematopoietic pathway, these drugs might have increased benefit in terms of WBC recovery when used in combination. Further, A (1–7) and AII, in contrast to filgrastim, increased circulating platelets, perhaps through an effect on megakaryocyte precursors and megakaryocytes. These findings, together with previously reported effects of AII on erythrogenesis [32, 40], suggest that AII and A(1–7) have the potential to stimulate the recovery of multiple hematopoietic lineages after myeloblative therapy. This is confirmed by the results of a phase I clinical study in which A(1–7) was administered daily starting 2 days after Adriamycin/cyclophosphamide chemotherapy for breast cancer and continuing for 12 to 15 days. In this trial, a reductions in anemia, thrombocytopenia and lymphopenia were observed confirming the multilineage effect of this peptide in hematopoietic recovery after chemotherapy (Blachly et al., personal communication).

References

1. Bell L, Madri JA (1990) Influence of the angiotensin system on endothelial and smooth muscle cell migration. Am J Pathol 127:7–12
2. Bell L, Luthringer DJ, Madri JA, Warren SL (1992) Autocrine angiotensin system regulation of bovine aortic endothelial cell migration and plasminogen activator involves modulation of proto-oncogene pp60c-src expression. J Clin Invest 89:315–320
3. Benter IF, Diz DI, Ferrario CM (1993) Cardiovascular actions of angiotensin(1–7). Peptides 14:679–684
4. Berk BC, Vekshtein V, Gordon HM, Tusuda T (1989) Angiotensin II stimulated protein synthesis in cultured vascular smooth muscle cells. Hypertension 13:305–314
5. Campbell-Boswell M, Robertson AL (1981) Effects of angiotensin II and vasopressin on human smooth muscle cells in vitro. Exp Mol Pathol 35:265–276
6. Chua BH, Krebs CJ, Chua CC, Diglio CA (1992) Induction of endothelin-1 transcripts by angiotensin II in rat heart endothelial cells. FASEB J 6:A1491
7. DelliPizzi AM, Hilchey SD, Bell-Quilley CP (1994) Natriuretic action of angiotensin (1–7). Br J Pharmacol 111:1–3
8. Diz KI, Pirro NT (1992) Differential actions of angiotensin II and angiotensin-(1–7) on transmitter release. Hypertension 19 [Suppl II]:41–48
9. Dzau VE, Pratt R, Gibbons G, Schunkert H, Lorell B, Ingelfinger J (1989) Molecular mechanism of angiotensin in the regulation of vascular and cardiac growth. J Mol Cell Cardiol 21 [Suppl III]:S7
10. Emmett N, Archibald E, Harris-Hooker S, Dukes L (1986) Effect of saralasin (angiotensin II antagonist) on 3T3 cell growth and proliferation. J Cell Biol 103:171
11. Fan TPD, Hu DE (1992) Losartan (DuP753) blocks the angiogenic effect of angiotensin II in rats. FASEB J 6:A937
12. Felix D, Khosla MC, Barnes KL, Imboden H, Montani B, Ferrario CM (1991) Neurophysiological responses to angiotensin (1–7). Hypertension 17:1111–1114
13. Fernandez LA, Twickler J, Mead A (1985) Neovascularization produced by angiotensin II. J Lab Clin Med 105:141–145
14. Ferrario CM, Barnes KL, Block CH, Brosnihan KB, Diz KI, Khosla MC, Santos RAS (1990) Pathways of angiotensin formation and function in the brain. Hypertension 15:13–19
15. Ferrario CM, Brosnihan KB, Diz KI, Jaiswal N, Khosla MC, Milsted A, Tallant EA (1991) Angiotensin-(1–7): a new hormone of the angiotensin system. Hypertension 18 [Suppl III]:126–133
16. Freudenthaler SM, Schreeb K, Korner T, Gleiter CH (1999) Angiotensin II increases erythropoietin production in healthy human volunteers. Eur J Clin Invest 29:816–823
17. Freudenthaler SM, Lucht I, Schenk T, Brink M, Gleiter CH (2000) Dose-dependent effect of angiotensin II on human erythropoietin production. Pflugers Arch 439:838–844
18. Fried W, Barone-Varelas J, Barone T, Anagnostou A (1982) Effect of angiotensin infusion on extrarenal erythropoietin production. J Lab Clin Med 99:520–525
19. Ganten D, Schelling P, Flugel RM, Fischer H (1975) Effect of angiotensin and angiotensin antagonist P113 on iso-renin and cell growth in mouse 3T3 cells. IRCS Med Sci Biochem 3:327
20. Geisterfer AAT, Peach MJ, Owens GK (1988) Angiotensin II induces hypertrophy, not hyperplasia, of cultured rat aortic smooth muscle cells. Circ Res 62:749–756
21. Gibbons GH, Pratt RE, Dzau VJ (1992) Vascular smooth muscle cell hypertrophy vs hyperplasia. Autocrine transforming growth factor-beta 1 expression determines growth response to angiotensin II. J Clin Invest 90:456–461
22. Han Y, Runge MS, Brasier AR (1999) Angiotensin II induces interleukin-6 transcription in vascular smooth muscle cells through pleiotropic activation of nuclear factor-kappa β transcription factors. Circ Res 84:695–703
23. Hsueh WA, Do YS, Anderson PW, Law RE (1995) Angiotensin II in cell growth and matrix production. Adv Exp Med Biol 377:217–223
24. Itoh H, Mukoyama M, Pratt RE, Gibbons GH, Dzau VJ (1993) Multiple autocrine growth factors modulate vascular smooth muscle cell growth response to angiotensin II. J Clin Invest 91:2268–2274
25. Iwami K, Ashizawa N, Do YS, Graf K, Hsueh WA (1996) Comparison of ANG II with other growth factors on Egr-1 and matrix gene expression in cardiac fibroblasts. Am J Physiol. 270:H2100–2107
26. Kawahara Y, Sunako M, Tsuda T, Fukuzaki H, Fukumoto Y, Takai Y (1988) Angiotensin II induces expression of the c-fos gene through protein kinase C activation and calcium ion mobilization in cultured vascular smooth muscle cells. Biochem Biophys Res Commun 150:52–59

27. Khosla MC, Smeby RR, Bumpus FM (1974) Structure-activity relationship in angiotensin II analogs. In: Page IA, Bumpus FM (eds) Angiotensin. Springer, New York Berlin Heidelberg, p 126
28. Koibuchi Y, Lee WS, Gibbons GH, Pratt RE (1993) Role of transforming growth factor β-1 in the cellular growth response to angiotensin II. Hypertension 21:1046–1050
29. Kono T, Taniguckh A, Imura H, Oseko F, Khosla MC (1986) Biological activities of angiotensin II-(1–6)-hexapeptide and angiotensin II-(1–7)-heptapeptide in man. Life Sci 38:1515–1519
30. LeNoble FAC, Hekking JWM, Van Staaten HWM, Slaaf DW, Struyker Bouder HAJ (1991) Angiotensin II stimulates angiogenesis in the chorio-allantoic membrane of the chick embryo. Eur J Pharmacol 195:305–306
31. Moriyama T, Fujibayashi M, Fujiwara Y, Kaneko T, Xia C, Imai E, Kamada T, Ando A, Ueda N (1995) Angiotensin II stimulates interleukin-6 release from cultured mouse mesangial cells. J Am Soc Nephrol 6:95–101
32. Mrug M, Stopka T, Julian BA, Prchal JF, Prchal JT (1997) Angiotensin II stimulates proliferation of normal early erythroid progenitors. J Clin Invest 100:2310–2314
33. Naftilan AJ, Pratt RE, Dzau VJ (1989) Induction of platelet-derived growth factor A-chain and c-myc gene expression by angiotensin II in culture rat vascular smooth muscle cells. J Clin Invest 83:1419–1424
34. Nakahara K, Nishimura H, Kuro-o M, Takewaki S, Iwase M, Ohkubo A, Yazaki Y, Nagai R (1992) Identification of three types of PDGF-A chain gene transcripts in rabbit vascular smooth muscle and their regulated expression during development and by angiotensin II. Biochem Biophys Res Commun 184:811–818
35. Nakamura A, Johns EJ, Imaizumi A, Yanagawa Y, Kohsaka T (1999) Effect of beta(2)-adrenoceptor activation and angiotensin II on tumour necrosis factor and interleukin 6 gene transcription in the rat renal resident macrophage cells. Cytokine 11:759–765
36. Owens GK (1985) Differential effects of antihypertensive drug therapy on vascular smooth muscle hypertrophy, hyperploidy and hyperplasia in the spontaneous hypertensive rat. Circ Res 56:525–536
37. Paquet JL, Baudouin-Legros M, Brunelle G, Meyer P (1990) Angiotensin II-induced proliferation of aortic myocytes in spontaneously hypertensive rats. J Hypertens 8:565–572
38. Rodgers KE, Abiko M, Girgis W, StAmand K, Campeau J, diZerega GS (1997) Acceleration of dermal tissue repair by angiotensin II. Wound Repair Regen 5:175–183
39. Rodgers KE, DeCherney AH, St. Amand KM, Dougherty WR, Felix JC, Girgis WW, diZerega GS (1997) Histologic alterations in dermal repair after thermal injury. Effects of topical angiotensin II. Burn Care Rehabilitation 18:381–388
40. Rodgers KE, Xiong S, Steer R, diZerega GS (2000) Effect of Angiotensin II on the proliferation of hematopoietic progenitors. Stem Cells 18:287–294
41. Rodgers KE, Xiong S, Felix JC, Roda R, Espinoza T, Maldonado S, diZerega GS (2001) Development of angiotensin (1–7) as an agent to accelerate dermal regeneration. Wound Repair Regen 9:238–247
42. Rodgers KE, Xiong S, diZerega GS (2002) Accelerated recovery from irradiation injury by angiotensin II. Cancer Chemother Pharmacol 49:403–411
43. Schelling P, Ganten D, Speck G, Fischer H (1979) Effects of angiotensin II and angiotensin II antagonist saralasin on cell growth and renin in 3T3 and SV3T3 cells. J Cell Physiol 98:503–513
44. Schorb W, Conrad KM, Singer HA, Dostal DE, Baker KM (1995) Angiotensin II is a potent stimulator of MAP-kinase activity in neonatal rat cardiac fibroblasts. J Mol Cell Cardiol 27:1151–1160
45. Stouffer GA, Owens GK (1992) Angiotensin II induced mitogenesis of spontaneously hypertensive rat derived cultured smooth muscle cells is dependent on autocrine production of transforming growth factor-β. Circ Res 70:820
46. Taubman MB, Berk BC, Izumo S, Tsuda T, Alexander RW, Nadal-Ginard B (1989) Angiotensin II induces c-fos mRNA in aortic smooth muscle. Role of Ca^{2+} mobilization and protein kinase C activation. J Biol Chem 264:526–530
47. Temizer DH, Yoshizumi M, Perrella MA, Susanni EE, Quertermous T, Lee ME (1992) Induction of heparin-binding epidermal growth factor-like growth factor by phorbol ester and angiotensin II in rat aortic smooth muscle cells. J Biol Chem 267:24892–24896