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ORIGINAL ARTICLE

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Synergistic effects of co-administration of angiotensin 1–7 and Neupogen on hematopoietic recovery in mice

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Abstract *Purpose*: Angiotensin 1–7 [A(1–7)] is a seven amino acid peptide that has been shown to increase the proliferation of epidermal stem cells after dermal injury and the number of hematopoietic progenitors in the bone marrow of myelosuppressed mice. In this study, the effect of combining A(1–7) with Neupogen on hematopoietic recovery and bone marrow progenitors was evaluated. *Materials and methods*: Intravenous 5-fluorouracil (5FU) was administered to induce myelosuppression. Administration of A(1–7) and/or Neupogen was initiated 2 days after chemotherapy. Angiotensin II (AII) and A(1–7) binding were assessed by flow cytometric analysis. Hematopoietic progenitors were counted by colony forming assays. Recovery of formed elements in the blood was evaluated by hemocytometer. *Results*: Flow cytometric analysis indicated that the number of early hematopoietic progenitors ($Lin^-Sca1^+cKit^+$) that bind AII or A(1–7) increased 5–7 days after intravenous injection of 150 mg/kg 5FU. Further, administration of A(1–7) led to a slight increase in the number of circulating leukocytes and platelets after this chemotherapeutic regimen. When given in combination with a subclinical dose of Neupogen, a synergistic effect on the number of circulating leukocytes was observed, but there was no further effect on the number of circulating platelets. In myelosuppressed mice, A(1–7) had its most profound effect on the number of hematopoietic progenitors in the bone marrow. The progenitors evaluated in the study included BFU-E, CFU-Meg, CFU-GM and CFU-GEMM. There was an increase in the number of these progenitors in the bone marrow, indicating an effect on all hematopoietic lineages. When given in combination with Neupogen, these effects were synergistic for the numbers of BFU-E and CFU-Meg (Neupogen by itself had no effect) and for the myeloid progenitors at lower doses of A(1–7). *Conclusions*: These results suggest that these hematopoietic agents act at different sites within the hematopoietic cascade and that combining these two agents may be of benefit in the treatment of hematopoietic disorders.

Keywords Angiotensin 1–7 · Filgrastim Hematopoietic recovery · Synergistic effects

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Introduction

Angiotensin II (AII) is a peptide that has been traditionally recognized as a regulator of blood pressure, salt, and water homeostasis. However, recent studies have shown that AII and angiotensin (1–7) [A(1–7)], a non-hypertensive fragment of angiotensin I (AI), are potent regulators of tissue regeneration [13, 14, 16]. Studies over the past decade have shown that AII and A(1–7) also have regulatory effects on cellular proliferation and growth factor release, which may contribute to accelerated tissue regeneration. AII has been shown to act as a mitogen for smooth muscle cells, fibroblasts and endothelial cells [1, 4, 5, 6, 7, 11, 12, 18, 19]. AII also increases the protein content and size of vascular smooth muscle cells and cardiomyocytes [2, 3, 9]. A(1–7) is a peptide that shares some of the properties of AII, but can also oppose some of the actions of AII. For example, A(1–7) has been shown to inhibit the proliferation of smooth muscle cells, but to increase fibroblast and keratinocyte proliferation [6, 7, 8, 16].

A(1–7) is a member of the renin angiotensin system (RAS) that includes the first seven amino acids of AII

and, importantly, does not induce hypertension. While AII is derived from AI by angiotensin-converting enzyme, A(1–7) is the product of AI cleavage by neutral endopeptidases [10]. Recent studies have shown this fragment to be active in several systems including renal function and wound repair [10, 16].

In studies assessing the effect of AII on tissue healing after partial thickness burning, infiltration of mesenchymal stem cells into the burn site was observed. Further, histological studies have demonstrated that AII and A(1–7) increase the proliferation of epidermal stem cells in the base of the hair follicle [14, 16]. Recent studies in this laboratory have shown that AII stimulates the proliferation of human and murine hematopoietic progenitors in vitro [15]. Recovery of bone marrow and circulating white blood cells (WBC) after myelosuppression [17, 18] requires proliferation of progenitor cells which may be amenable to augmentation. Therefore, the ability of angiotensin peptides to act as hematopoietic stimulators after myelosuppression was evaluated and compared with that of filgrastim, a well-known stimulator of hematopoietic recovery which mobilizes hematopoietic precursors. The effect of systemic administration of AII and A(1–7) on the concentration of myeloid, erythroid and megakaryocytic progenitor cells in the bone marrow of myelosuppressed mice was also evaluated.

Recent studies have demonstrated a role for AII and A(1–7) in the regulation of tissue regeneration and the formation of hematopoietic progenitors in vitro. In these studies, the most potent effect of the peptides was an increase in the concentration of hematopoietic progenitors in the bone marrow. Based upon this observation, the effect of combining A(1–7) (as a means of increasing progenitor numbers) with a clinical dose of Neupogen (filgrastim) on recovery from intravenous chemotherapy was evaluated.

Materials and methods

Materials

A(1–7) was made following good manufacturing practice procedures by BACHEM (Torrance, Calif.). Neupogen was purchased from the Health Sciences Pharmacy at the University of Southern California. Reagents to evaluate progenitor numbers were purchased from Stem Cell Technologies (Vancouver, B.C.). Antibodies for flow cytometry were purchased from PharminGen (San Diego, Calif.). Fluoresceinated peptides were purchased from Phoenix Pharmaceuticals (Mountain View, Calif.).

Methods

Animals and treatment

All procedures were done in compliance with NIH guidance on animal welfare. Permission to conduct the studies was obtained from the USC Institutional Care and Use Committee prior to their initiation. Female C67Bl/6 mice (Harlan Laboratories, Indianapolis, Ind.), six per group, 6–8 weeks old, were treated with 1, 10, 100 and 200 μg/kg of A(1–7) with or without 50 μg/kg Neupogen starting 2 days after intravenous injection of 150 mg/kg 5FU. The dosage of Neupogen chosen was clinically subtherapeutic to determine whether A(1–7) could enhance hematopoietic recovery following chemotherapy. A control group of animals did not receive any chemotherapy and a placebo group received chemotherapy and saline (the A(1–7) vehicle). Administration of the treatments continued daily until the animals were killed. On days −10, 2, 7, 14 and 21 relative to 5FU administration, blood was taken under anesthesia from the retro-orbital sinus to assess WBC, hemoglobin and platelet numbers. The numbers of myeloid, erythroid and megakaryocytic progenitors in the bone marrow were determined on day 21.

Retro-orbital bleeding of mice

The mice were bled from the retro-orbital sinus. The mice were anesthetized by placing the nose of the animal in a 50-ml conical tube containing cotton balls soaked with isoflurane. Approximately 100 μl of 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.

Hemoglobin assay

Blood (20 μl) was pipetted into a centrifuge tube to which 180 μl of distilled water was added. The blood and water were then mixed by inversion and allowed to incubate at 4°C for 20 min. The tube was then centrifuged at 12,000 rpm to precipitate the cellular debri for 20 to 30 min at room temperature. The supernatant from this centrifugation was then added in 20-μl aliquots into triplicate wells of a 96-well plate containing 180 μl of distilled water. The optical density was then read using a microplate reader at 570 nm.

WBC and platelet evaluation

Blood (20 μl) was mixed with 200 μl of red blood cell (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, the supernatant removed, and the pellet resuspended in 100 μl phosphate-buffered saline (PBS). To this was added 100 μl 0.04% trypan blue and the mixture was vortexed. WBC were counted using a hemocytometer under light microscopy and platelets were counted using a hemocytometer under phase contrast microscopy after further dilution in 0.04% trypan blue.

Evaluation of myeloid and erythroid progenitors in the bone marrow

The femurs and tibias were collected and the bone marrow was harvested by flushing with PBS containing 2% fetal calf serum. After collection of the bone marrow, the RBCs were lysed with a hypotonic solution, mixed with 0.04% trypan blue and the nucleated cells counted using hemocytometer under light microscopy. Aliquots of cells were then resuspended at 5×10^5 cells/ml, and 100 μl of each suspension was added to 900 μl of semisolid medium containing 0.9% methyl cellulose in Iscove's MDM, 15% fetal calf serum, 1% bovine serum albumin (BSA), 10 μg/ml bovine pancreatic insulin, 200 μg/ml human transferrin, 10^{-4} *M* 2-mercaptoethanol, 2 m*M* 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 37°C in a humidified atmosphere of air containing 5% CO_2. On day 14, the progenitor colonies formed were counted under phase contrast microscopy.

Evaluation of CFU-megakaryocyte in the bone marrow

Cells were isolated from the bone marrow as described above. After counting of viable nucleated cells, an aliquot of the cells was resuspended to a concentration of 1×10^7 cells/ml. A 100-µl aliquot of this suspension 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, B.C.). The cells and medium were mixed and dispensed onto a tissue culture slide, spread evenly and allowed to gel. The culture slides were placed in a humidified atmosphere and incubated at 37°C in an atmosphere of air containing 5% CO_2 for 8 days. At the end of the culture time, the formation of megakaryocytes from megakaryocyte precursors (CFU-Meg) 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 number of colonies expressing acetyl cholinesterase (as a measure of megakaryocyte formation) was evaluated.

Colony evaluation

BFU-E colonies contained predominantly erythroid cells with erythroid clusters containing eight or more erythroblasts. CFU-GEMM colonies were large and contained erythroid colonies, granulocytes, monocyte/macrophage cells and megakaryocytes. CFU-GM colonies were large and contained colonies from both the granulocyte and monocyte-macrophage lineage. The CFU-Meg colonies were counted using a ×10 objective. The cells considered positive by the enzymatic reaction contained brown granular deposits due to the staining described above. A CFU-Meg was a colony of at least 3 and up to 50 cells positive for brown staining clumped together.

Flow cytometry

Female C57Bl/6 mice were left untreated (control group) or treated with intravenous 5FU (150 mg/kg). Animals were killed on days 5 and 7 after 5FU treatment. To delineate the progenitor population and determine the subpopulation of $CD45^+$, $cKit^+/Sca1^+$ populations that express either AII or A(1–7), bone marrow cells were collected and labeled with PerCP-conjugated anti-murine CD45 (LCA-Ly-5) (PharminGen), APC-conjugated anti-murine cKit (CD117) (PharminGen), PE-conjugated anti-murine Sca1 (ly-8A/E) (PharminGen) and either FITC-conjugated AII or FITC-conjugated A(1–7) (Phoenix Pharmaceuticals).

The CD45 population was identified from the total forward vs side scatter (SSC) population. Gates which defined traditional areas for bone marrow progenitors (CD45 low/SSC low), lymphocytes (CD45 medium/SSC low), monocytes (CD45 high/SSC low), and granulocytes (CD45 high/SSC high) were set within a CD45 vs side scatter population. Two-parameter histograms representing the Sca1/cKit population were constructed from each of these gates. The $Sca1^+/cKit^+$ cells were gated on to determine the level of either AII or A(1–7) expressed within this population.

Statistics

The data were analyzed by one way analysis of variance followed by Tukey's analysis for subgroup comparisons.

Results

A(1–7) enhances Neupogen-mediated WBC recovery

The effect of administration of A(1–7) alone or in combination with Neupogen on the concentration of WBC was assessed at various times after treatment with 5FU. Subcutaneous administration of A(1–7) daily beginning on day 2 after chemotherapy increased WBC numbers starting on day 14 (Table 1, Fig. 1A, $P \leq 0.0001$, by ANOVA). The response peaked on day 14 and was dose-dependent. Administration of Neupogen increased WBC levels starting on day 7 with a peak on day 14 (Fig. 1B, by ANOVA). Coadministration of A(1–7) with a subclinical dose of Neupogen increased the concentrations of WBC in a dose-dependent manner. Importantly, this increase in WBC concentration was synergistic with Neupogen at lower concentrations of A(1–7). Groups that were significantly different by Tukey's analysis were: (1) on day 7, Neupogen with 1 or 10 µg/kg A(1–7) compared to placebo; (2) on day 14, Neupogen in combination with any dose of A(1–7) compared to placebo, and Neupogen plus 100 or 200 µg/kg A(1–7) compared to Neupogen; and (3) on day 21, all doses of A(1–7) in combination with Neupogen compared to either placebo or Neupogen.

A(1–7) enhances platelet recovery after 5FU-mediated thrombocytopenia

The effects of administration of A(1–7) alone, Neupogen alone, or a combination of A(1–7) and Neupogen on numbers of platelets were assessed at various times after treatment with 5FU. Following A(1–7) administration, there was approximately a 50% increase in platelet numbers (Fig. 2).

Lack of erythropoietic effects of A(1–7) and Neupogen

Peripheral blood hemoglobin levels declined in all groups treated with 5FU beginning on day 2. Neither A(1–7) nor Neupogen affected hemoglobin recovery (Fig. 3).

Table 1 WBC counts on day 14. Values are means ± SE

	WBC count ($\times10^6$/ml)		
		A(1–7)	
		Alone	Plus Neupogen
A(1–7) dose (µg/kg)			
0		4.23 ± 0.22	8.36 ± 0.87
1		5.6 ± 0.49	12.0 ± 0.87
10		5.72 ± 0.43	12.9 ± 1.14
100		6.24 ± 0.49	14.6 ± 2.1
200		6.8 ± 0.49	18.3 ± 3.06
Control[a]	7.73 ± 0.28		

[a]No chemotherapy

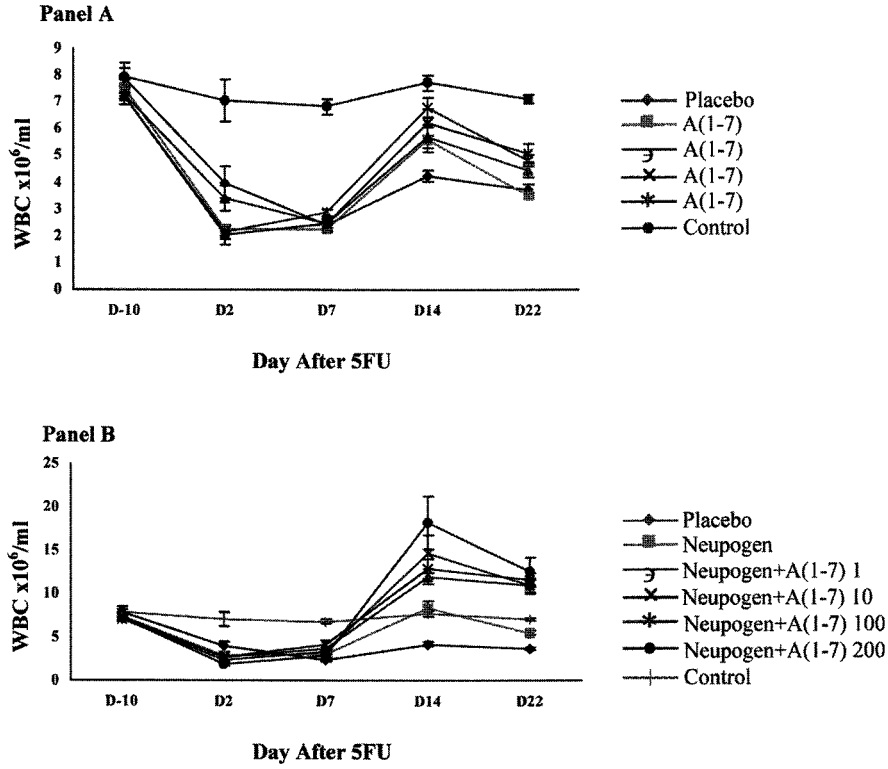

Fig. 1A, B Effects of administration of A(1–7) and Neupogen on WBC count after 5FU treatment. The effects of administration of various concentrations of A(1–7) alone (**A**) or in combination with Neupogen (**B**) are shown. The data are presented as the means ± SE from four to six animals per group. Administration of A(1–7) daily beginning on day 2 after chemotherapy increased WBC levels starting on day 14. Further, administration of Neupogen increased WBC numbers starting on day 7 with a peak on day 14. Groups that were significantly different by Tukey's test were: (1) on day 7 Neupogen with 1 or 10 μg/kg A(1–7) compared to placebo; (2) on day 14:: Neupogen in combination with any dose of A(1–7) compared to placebo, and Neupogen plus 100 or 200 μg/kg A(1–7) compared to Neupogen; and (3) on day 21 all doses of A(1–7) in combination with Neupogen compared to either placebo or Neupogen

Synergistic effects of coadministration of A(1–7) and Neupogen

A(1–7) enhanced Neupogen-mediated formation of nucleated cells in the bone marrow following 5FU treatment. The concentration of nucleated cells in the bone marrow was increased by administration of A(1–7) alone (Fig. 4A). Neupogen alone also increased the number of nucleated cells. Coadministration of A(1–7) and Neupogen increased the number of nucleated cells in the bone marrow in excess of that observed with administration of A(1-7) or Neupogen alone (Fig. 4B).

A(1–7) increases the amount of BFU-E in the bone marrow

A(1–7) increased the concentration of BFU-E in the bone marrow following 5FU treatment. The effect was dose-dependent with 200 μg/kg eliciting the greatest increase (Fig. 5A). Neupogen alone did not affect BFU-E formation. Coadministration of A(1–7) with Neupogen increased the concentration of BFU-E in the bone marrow (Fig. 5B).

Neupogen and A(1–7) increased the number of myeloid progenitors in the bone marrow

Treatment with A(1–7) increased the concentration of CFU-GM in the bone marrow after treatment with 5FU. The response was dose-dependent (Fig. 6A). Coadministration of A(1–7) and Neupogen synergistically increased the number of CFU-GM per femur (Fig. 6B). Coadministration of A(1–7) with Neupogen synergistically increased the level of myeloid progenitors in the bone marrow at lower concentrations of A(1–7).

A(1–7) increased the number of CFU-GEMM in the bone marrow

Treatment with A(1–7) significantly increased the concentration of CFU-GEMM in the bone marrow after administration of 5FU (Fig. 7A). Treatment with Neupogen alone had no effect on the concentration of CFU-GEMM. However, coadministration of A(1–7) with Neupogen compensated for the lack of an effect on early progenitors observed in the group treated with Neupogen alone.

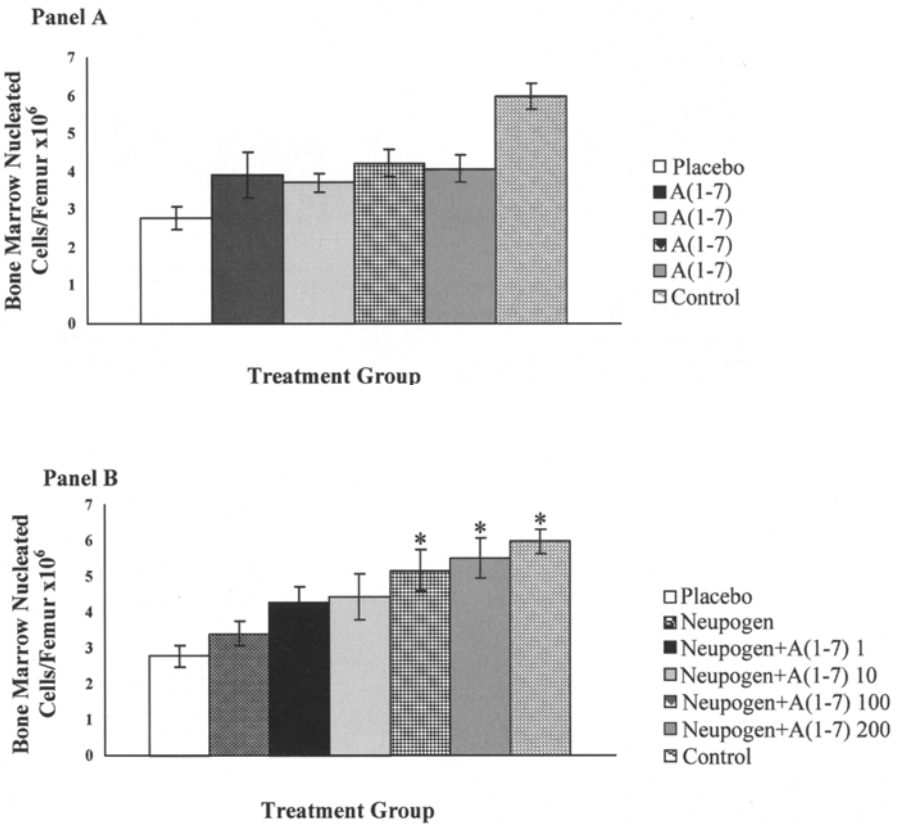

Fig. 4A, B Effects of A(1–7) and Neupogen on formation of nucleated cells in the bone marrow after 5FU treatment. The effects of administration of various concentrations of A(1–7) alone (**A**) and Neupogen alone or in combination with various concentrations of A(1–7) (**B**) are shown. The data are presented as the means ± SE from four to six animals per group. *Significantly increased compared with placebo control by Tukey's analysis. The groups are significantly different by ANOVA ($P \leq 0.0001$)

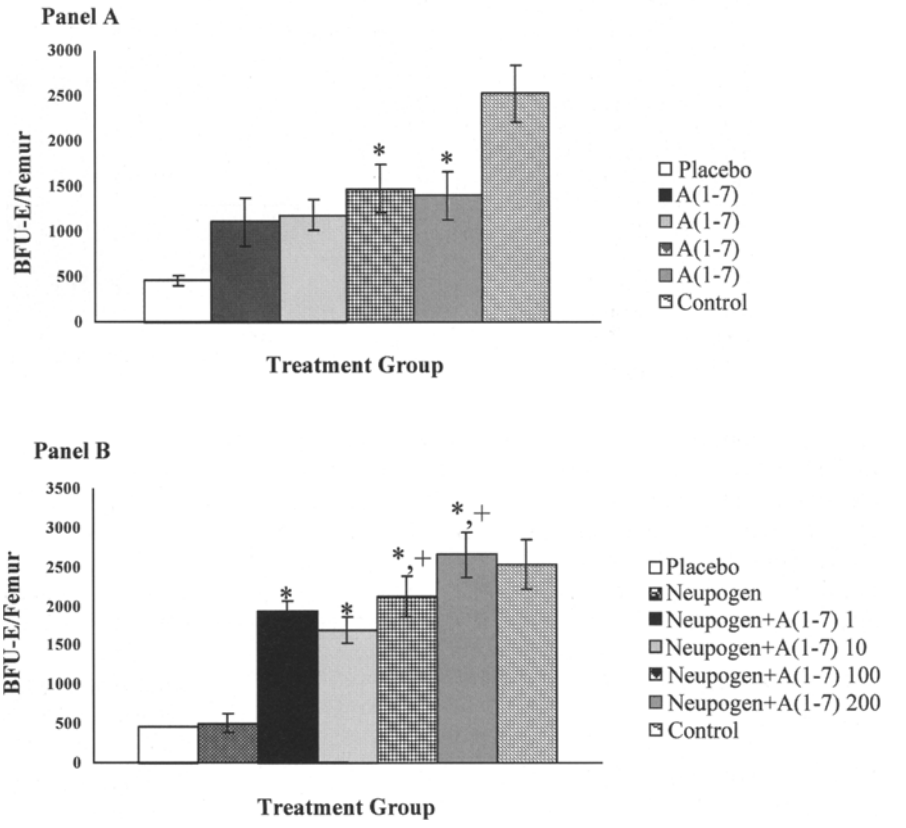

Fig. 5A, B Effects of A(1–7) and Neupogen on BFU-E formation. The effects of administration of various concentrations of A(1–7) alone (**A**) and Neupogen alone or in combination with various concentrations of A(1–7) (**B**) are shown. The data are presented as the means ± SE from four to six animals per group. *Significantly increased compared with placebo control; $^{+}$significantly different from Neupogen (Tukey's analysis). The groups are significantly different by ANOVA ($P \leq 0.0001$)

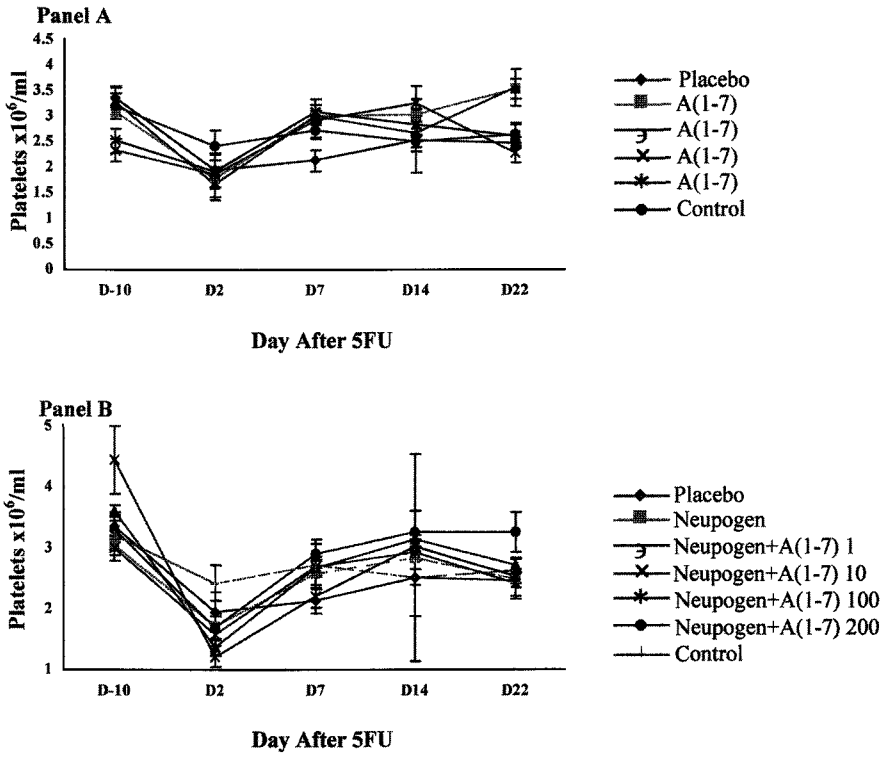

Fig. 2A, B Effects of A(1–7) and Neupogen on platelet formation after 5FU treatment. Changes in platelet concentrations following administration of A(1–7) alone (**A**) or in combination with Neupogen (**B**) are shown. The data are presented as the means ± SE from four to six animals per group. Treatment with A(1–7) increased the platelet concentration by 50% after 5FU. Administration of A(1–7) in combination with Neupogen augmented platelet recovery starting on day 7

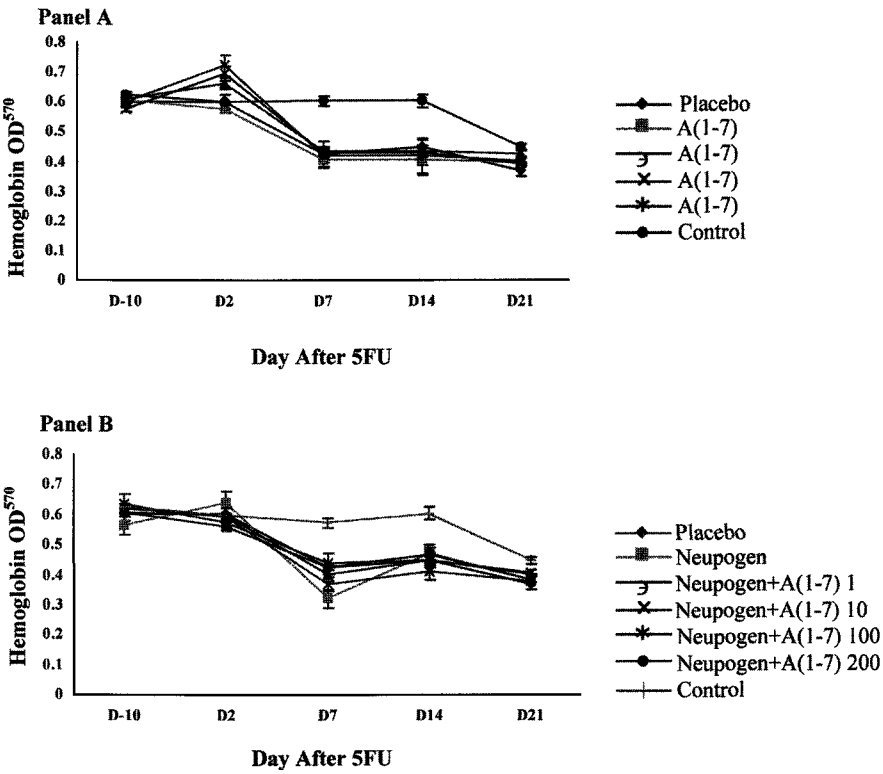

Fig. 3A, B Effects of A(1–7) and Neupogen on hemoglobin levels after 5FU treatment. The effects of administration of various concentrations of A(1–7) alone (**A**) and Neupogen in combination with various concentrations of A(1–7) (**B**) are shown. The data are presented as the means ± SE from four to six animals per group

A(1–7) increases the number of CFU-Meg in the bone marrow

Treatment with A(1–7) significantly increased the concentration of CFU-Meg in the bone marrow (Fig. 8). The highest concentration of CFU-Meg was generated at a dose of 10 μg/kg of A(1–7). Neupogen alone increased the levels of CFU-Meg to levels similar to those following placebo treatment. Coadministration of A(1–7) with Neupogen caused a rapid increase in the

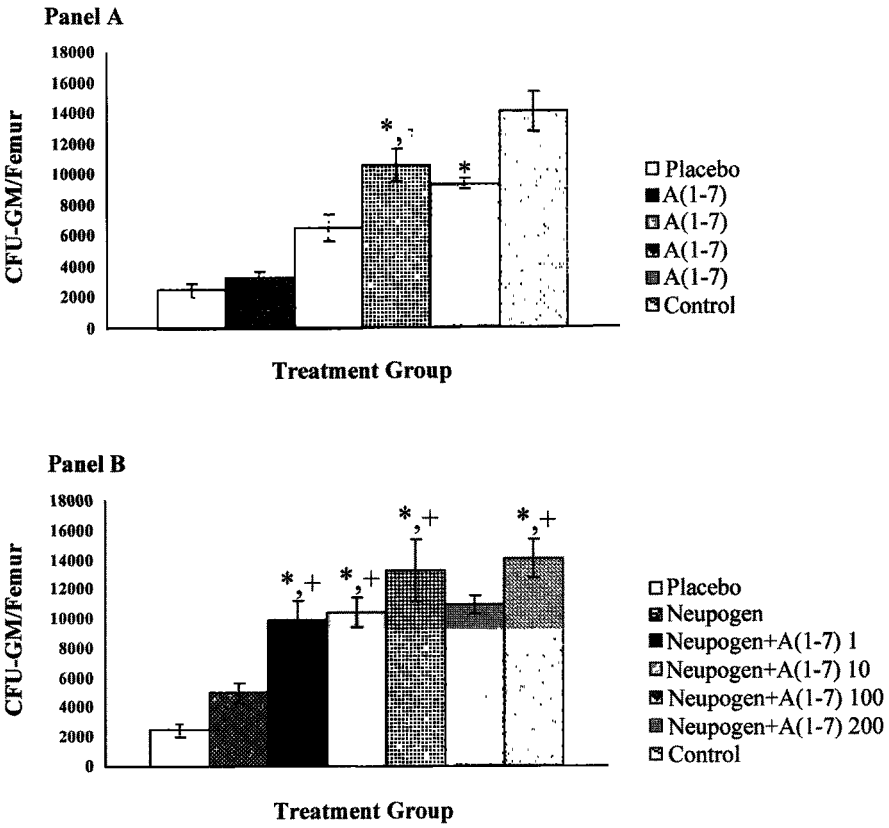

Fig. 6A, B Effects of A(1–7) alone or in combination with Neupogen on CFU-GM formation. The effects of administration of various concentrations of A(1–7) alone (**A**) and Neupogen alone or in combination with various concentrations of A(1–7) (**B**) are shown. The data are presented as the means ± SE from four to six animals per group. *Significantly increased compared with placebo control; $^{+}$significantly different from Neupogen (Tukey's analysis). The groups are significantly different by ANOVA ($P \leq 0.0001$)

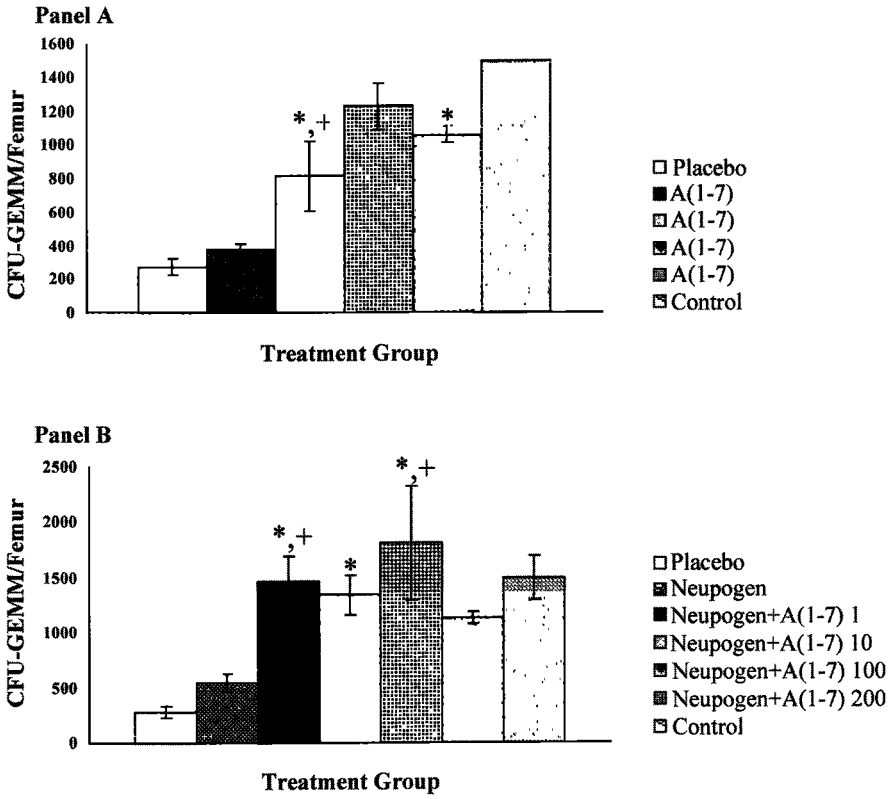

Fig. 7A, B Effects of A(1–7) alone or in combination with Neupogen on CFU-GEMM formation. The effects of administration of various concentrations of A(1–7) alone (**A**) and Neupogen alone or in combination with various concentrations of A(1–7) (**B**) are shown. The data are presented as the means ± SE from four to six animals per group. *Significantly increased compared with placebo control, $^{+}$significantly different from Neupogen (Tukey's analysis). The groups are significantly different by ANOVA ($P \leq 0.0001$)

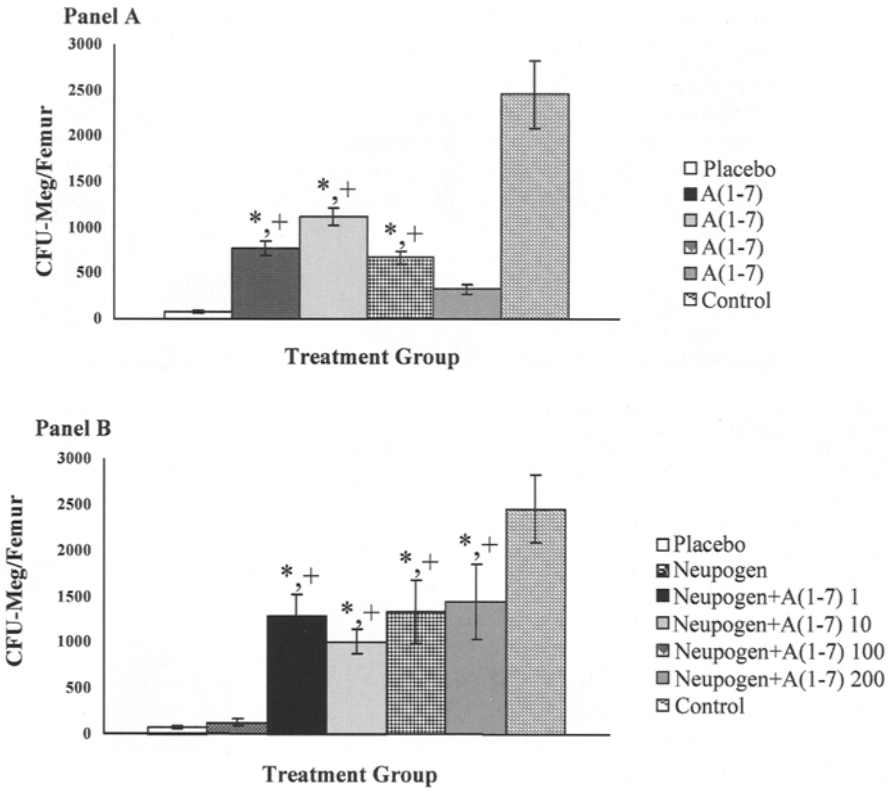

Fig. 8A, B Effects of A(1–7) alone or in combination with Neupogen on CFU-megakaryocyte formation. The effects of administration of various concentrations of A(1–7) alone (**A**) and Neupogen alone or in combination with various concentrations of A(1–7) (**B**) are shown. *Significantly increased compared with placebo control, $^{+}$significantly different from Neupogen (Tukey's analysis). The groups are significantly different by ANOVA ($P \leq 0.0001$)

concentration of CFU-Meg in the bone marrow. Peak CFU-Meg production started at 1 μg/kg of A(1–7) and reached a plateau thereafter.

AII and A(1–7) receptors are expressed in early progenitor populations in 5FU-treated mice

To determine which bone marrow progenitor populations expressed AII and A(1–7) receptors, mice were treated with 5FU and the bone marrow cells harvested 5 and 7 days later (Table 2). Cells isolated from the bone marrow of treated and nontreated mice were labeled for CD45, Sca1, c-Kit, and either FITC-conjugated AII or FITC-conjugated A(1–7). Treatment with 5FU induced an increase in levels of both AII and A(1–7) markers in bone marrow progenitors. A(1–7) receptor expression peaked at 5 days. AII appeared to peak at 7 days (estimation only). The interaction of FITC-conjugated AII and A(1–7) was specific since it competed with unlabeled AII or A(1–7).

Discussion

Neupogen is a recombinant G-CSF growth factor extensively used to treat neutropenia following chemotherapy. Neupogen efficiently assists hematopoietic recovery by stimulating bone marrow progenitors to produce neutrophils. The use of Neupogen to augment

Table 2 Percentage of early progenitors binding angiotensin peptides following treatment with 5FU (150 mg/kg) or with no chemotherapy (*control*)

	Markers	Percent positive
Control	CD45 PerCP, cKit APC, ScaI PE, AII-FITC	1.53
	CD45 PerCP, cKit APC, ScaI PE, AII-FITC + cold AII	0.93
	CD45 PerCP, cKit APC, ScaI PE, A(1–7)-FITC	2.55
	CD45 PerCP, cKit APC, ScaI PE, A(1–7)-FITC + cold A(1–7)	3.03
5 days after 5FU treatment	CD45 PerCP, cKit APC, ScaI PE, AII-FITC	3.0
	CD45 PerCP, cKit APC, ScaI PE, AII-FITC + cold AII	3.7
	CD45 PerCP, cKit APC, ScaI PE, A(1–7)-FITC	8.89
	CD45 PerCP, cKit APC, ScaI PE, A(1–7)-FITC + cold A(1–7)	4.78
7 days after 5FU treatment	CD45 PerCP, cKit APC, ScaI PE, AII-FITC	4.55
	CD45 PerCP, cKit APC, ScaI PE, AII-FITC + cold AII	1.74
	CD45 PerCP, cKit APC, ScaI PE, A(1–7)-FITC	3.5
	CD45 PerCP, cKit APC, ScaI PE, A(1–7)-FITC + cold A(1–7)	4.83

neutrophil recovery has enabled the development of high-dose chemotherapy regimens for the treatment of particularly aggressive and non-responsive cancers.

However, the spectrum of cellular end-products elicited by Neupogen is narrow, primarily enhancing neutrophil recovery. Reconstitution of other circulatory components is delayed, awaiting hematopoietic recovery from the bone marrow. Despite enhancing recovery of neutrophils, Neupogen does not stimulate the production of other essential peripheral factors and cellular components. For instance, thrombocytopenia remains potentially life-threatening in those patients myelosuppressed as a consequence of chemotherapy. However, therapeutic infusions of platelets are frequently required as a supplement since Neupogen treatment does not stimulate megakaryocyte progenitors to produce platelets.

We have previously demonstrated that administration of AII or A(1–7) stimulates recovery of circulating WBCs in a murine model of hematopoietic depletion. Daily administration of AII following irradiation enhances both WBC recovery and diminishes mortality [17]. AII stimulates multiple hematopoietic lineages in the peripheral blood. The non-hypertensive A(1–7), a fragment of AI, also stimulates peripheral WBC recovery. AII and its analogs stimulate hematopoietic recovery in irradiated mice.

In this study, we extended these observations by using a 5FU model of hematopoietic depletion and tested the effects of A(1–7) alone or coadministered with Neupogen on recovery of circulating WBCs, hemoglobin, platelets, and progenitor populations in the bone marrow. Previous studies in our laboratory have clearly demonstrated that A(1–7) stimulates bone marrow progenitors in 5FU-treated mice. In light of this observation, we chose to examine the effect of A(1–7) given in combination with subtherapeutic levels of Neupogen to determine whether A(1–7) could enhance Neupogen-induced WBC recovery. That is, could augmented progenitor populations enable Neupogen to be utilized at lower and safer doses? We did not evaluate Neupogen at clinically therapeutic levels. Treatment of mice with A(1–7) enhanced hematopoietic recovery following 5FU treatment, an observation consistent with previously described leukocyte recovery after irradiation. Neupogen enhanced WBC recovery after 5FU treatment. However, coadministration of A(1–7) with Neupogen enhanced WBC recovery in a dose-dependent manner, allowing Neupogen to be used at clinically subtherapeutic levels.

In addition, A(1–7) stimulated platelet production in 5FU-treated mice. Coadministration of A(1–7) and Neupogen resulted in a rapid increase in the concentration of platelets in the peripheral circulation. Interestingly, A(1–7) facilitated platelet recovery most efficiently at doses of 1–10 μg/kg. A(1–7) appeared to enhance hematopoiesis by acting synergistically with Neupogen to broaden the spectrum of responsive hematopoietic constituents.

Neither A(1–7) nor Neupogen stimulated hemoglobin production after treatment with 5FU. However, A(1–7) alone and synergistically with Neupogen stimulated production of BFU-E, the bone marrow progenitors responsible for production of RBCs. Erythropoietin (EPO), a growth factor used clinically to stimulate RBC production in anemic patients, typically exhibits a 3-month lag time before restoration of RBCs in the periphery, far beyond the time-course of this study. Tubular and juxtatubular endothelial/interstitial cells and, to a lesser extent, hepatocyte and Kupffer cells of the liver are the predominant source of EPO in mammals. While it is apparent that in spite of a robust stimulation in the production of BFU-E, a delay exists between A(1–7)-mediated induction of progenitor cells and the manifestation of peripheral RBCs. Additional experiments will determine whether A(1–7) accelerates RBC production by stimulating the proliferation of an RBC progenitor population in the bone marrow receptive to endogenous or supplemented EPO.

The bone marrow is a site responsive to A(1–7)-mediated hematopoiesis. Examination of A(1–7) receptors following 5FU treatment clearly demonstrates that bone marrow progenitors express A(1–7) receptors. This observation is significant since it reveals that cells from the bone marrow are receptive to A(1–7). Administration of A(1–7) alone increased the number of nucleated cells in the bone marrow following 5FU treatment. Coadministration of A(1–7) with Neupogen synergistically increased the number of nucleated cells in the bone marrow to levels significantly greater than those observed with A(1–7) or Neupogen alone. Neupogen alone poorly stimulated CFU-GM production. However, CFU-GM production was stimulated by A(1–7) alone, and A(1–7) again acted synergistically with Neupogen to accelerate CFU-GM production in the bone marrow.

Prior to stem cell harvest for autologous bone marrow transplantation, donor bone marrow is mobilized with Neupogen to increase the resident progenitor population. Mobilization increases the chances of successful autologous transplant following high-dose chemotherapy. Neupogen, frequently administered post-transplant to treat neutropenia, stimulates CFU-GM in the bone marrow to produce neutrophils. A(1–7) alone increased the number of CFU-GMs in the bone marrow of treated mice. A(1–7) coadministered with Neupogen synergistically increased CFU-GM to higher levels than those resulting from treatment with each compound alone. In addition, A(1–7) allowed Neupogen to be administered at subtherapeutic doses, with the possible consequence of diminishing deleterious side effects. Thrombocytopenia is a persistent problem following high-dose chemotherapy/autologous stem cell transplantation. While Neupogen successfully stimulates the production of neutrophils, it does not stimulate platelet formation. A(1–7) induced an increase in CFU-Meg, the progenitor population responsible for the production of platelets.

References

1. Bell L, Madri JA (1990) Influence of the angiotensin system on endothelial and smooth muscle cell migration. Am J Pathol 127:7
2. Berk BC, Rao GN (1993) Angiotensin II-induced vascular smooth muscle cell hypertrophy: PDGF A chain mediates the increase in size. J Cell Physiol 154:368
3. Berk BC, Vekshtein V, Gordon HM, Tusuda T (1989) Angiotensin II stimulated protein synthesis in cultured vascular smooth muscle cells. Hypertension 13:305
4. Campbell-Boswell M, Robertson AL (1981) Effects of angiotensin II and vasopressin on human smooth muscle cells in vitro. Exp Mol Pathol 35:265
5. 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
6. 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
7. Freeman EJ, Chisolm GM, Ferrario CM, Tallent EA (1996) Angiotensin(1–7) inhibits smooth muscle cell proliferation induced by vascular injury. Hypertension 28:104
8. 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
9. Geisterfer AAT, Peach MJ, Owens GK (1988) Angiotensin II induces hypertrophy not hyperplasia of cultured rat aortic smooth muscle cells. Circ Res 62:749
10. Jackson EK, Garrison JC (1996) Renin and Angiotensin. In: Hardman JG, Limbird LE, Molinoff PB, Ruddon RW, Gilman AG (eds) Goodman and Gilman's the pharmacological basis of therapeutics, 9th edn. McGraw-Hill, New York, p 733
11. 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
12. 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
13. Rodgers KE, Abiko M, Girgis W, St. Amand K, Campeau J, diZerega GS (1997) Acceleration of dermal tissue repair by angiotensin II. Wound Repair Regen 5:175
14. 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 Rehabil 18:381
15. Rodgers KE, Xiong S, Steer R, diZerega GS (2000) Effect of angiotensin II on hematopoietic progenitor cell proliferation. Stem Cells 18:287
16. 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
17. Rodgers KE, Xiong S, diZerega GS (2002) Accelerated recovery from irradiation injury by angiotensin II. Cancer Chemother Pharmacol 49:403–411
18. Rodgers KE, Xiong S, diZerega GS (2003) Effect of angiotensin II and angiotensin (1–7) on hematopoietic recovery after intravenous chemotherapy. Cancer Chemother Pharmacol 51:97–106
19. Schelling P, Ganten D, Speck G, Fischer H (1979) Effects of angiotensin II and angiotensin II antagonist saralysin on cell growth and renin in 3T3 and SV3T3 cells. J Cell Physiol 98:503