

Overexpression of the Local Bone Marrow Renin-Angiotensin System in Acute Myeloid Leukemia

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Objectives: Local bone marrow renin-angiotensin system (RAS) is an autocrine-paracrine system affecting hematopoiesis. Angiotensin II stimulates the proliferation of bone marrow and umbilical cord blood hematopoietic progenitors. Angiotensin-converting enzyme (ACE) hyperfunction may lead to the acceleration of negative hematopoietic regulator peptide, AcSDKP, metabolism, which in turn lowers its level in the bone marrow microenvironment, finally removing the antiproliferative effect of AcSDKP on the hematopoietic cells and blasts. The aim of this study is therefore to search those major RAS components simultaneously in the leukemic blast cells taken from the bone marrow of patients with acute myeloid leukemia (AML).

Methods: Bone marrow aspiration materials were obtained from 10 patients with AML (8 males, 2 females; median age 48.5 years) and 8 patients with nonmalignant hematological disorders (6 males, 2 females; median age 45 years). EDTA-treated bone marrow samples were stored at -70°C until analysis. Total RNA was extracted from 200- μl bone marrow samples by High Pure RNA Isolation Kit.

Results: The medians of expression ratios of AML patient samples have been found 0.736 (IQR 1.359), 0.540 (IQR 0.725), and 0.075 (IQR 0.002) for ACE, ANG and REN genes, respectively. All three gene expressions were found to be significantly higher in the bone marrow samples of AML patients.

Conclusion: In this study, the expression of the mRNAs of the major RAS components—namely ACE, renin and angiotensinogen—in human bone marrow samples were quantified by reverse transcription-polymerase chain reaction (RT-PCR) to confirm the presence of the local bone marrow RAS. Elucidation of the pathological activity of the local RAS-mediated regulation of the leukemogenesis is both pathobiologically and clinically important, since the angiotensin peptides represent a molecular target in the disease management.

Key words: renin-angiotensin system ■ myeloid leukemia ■ angiotensin-converting enzyme ■ reverse transcription-polymerase chain reaction

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INTRODUCTION

The new frontiers of the renin-angiotensin system (RAS) include organ-, tissue- and even cell-based systems exhibiting autocrine, paracrine, endocrine and intracrine actions within the human body.^{1,2} The presence of a local bone marrow RAS affecting physiological and neoplastic blood cell production had been hypothesized in 1996.³ After a long literature debate,^{4,7} local hematopoietic bone marrow RAS has been demonstrated as an autocrine-paracrine system within the rat hematopoietic-lineage and marrow stromal cells.^{2,8} Angiotensin-II type 1a (AT1a) receptors are present on the CD34+ hematopoietic stem cells,⁹ in the context of the local bone marrow RAS.¹⁰ Likewise, angiotensin II stimulates the proliferation of bone marrow hematopoietic progenitors⁹ and umbilical cord blood cells.^{11,12} Major RAS components—namely renin, angiotensinogen and angiotensin-converting enzyme (ACE)—are present in human umbilical cord blood cells.¹³ Angiotensin II could serve in the erythroid and myeloid differentiation of stem cells.⁹ Likewise, the molecular mechanisms of angiotensin II-mediated hematopoiesis, such as arachidonic acid release from marrow stromal cells, have been searched.¹⁴

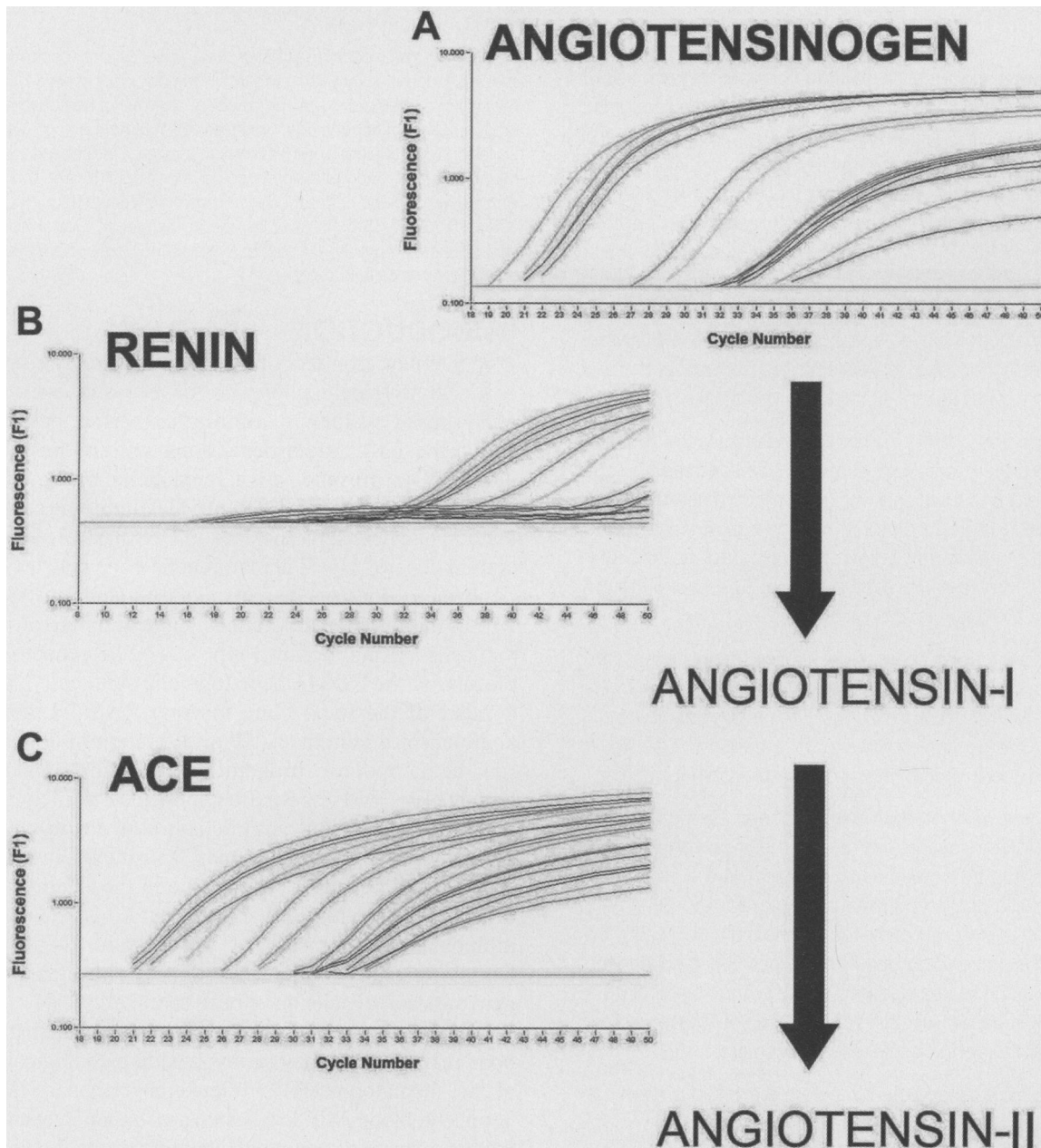
On the other hand, there are preliminary data that local bone marrow RAS may be involved in pathological neoplastic hematopoiesis and leukemogenesis.¹⁵⁻¹⁷ While peripheral blood ACE levels increase, blast percentages in the bone marrow accumulate and migrate to the circulation. Therefore, ACE hyperfunction may lead to the accel-

eration of AcSDKP metabolism, which in turn lowers its level in the bone marrow microenvironment, finally removing the antiproliferative effect of goralatide on the hematopoietic cells and blasts.¹⁵ Renin expression could have a role on the disease development and could be used as an aberrant marker of leukemia.¹⁸ Furthermore, angiotensin has been suggested to act as an autocrine growth factor for acute myeloid leukemia (AML) cells.¹⁹

The local synthesis of a particular molecule can be deduced from the presence of the corresponding messenger ribonucleic acid (mRNA) in a given local tissue.³

In this study, the expression of the mRNAs of the major RAS components—namely ACE, renin and angiotensinogen—in human bone marrow samples were quantified by reverse transcription-polymerase chain reaction (RT-PCR) to confirm the presence of the local bone marrow RAS. Furthermore, we assessed quantitative alterations of the ACE, renin and angiotensinogen mRNAs in leukemic bone marrow samples. The aim of this study is therefore to search major RAS components in the leukemic blast cells taken from the bone marrow of the patients with AML. Elucidation of the pathologi-

Figure 1. Real-time PCR amplifications and fluorescence curve graphics of the angiotensinogen (A), renin (B) and angiotensin-converting enzyme (ACE) (C) cDNA targets in bone marrow samples



cal activity of the local autocrine/paracrine RAS-mediated regulation of the leukemogenesis is both pathobiologically and clinically important, since the angiotensin peptides represent a molecular target in the management of disorders.^{1,2,20,21}

Design and Methods

Bone marrow aspiration materials and peripheral blood samples were obtained from 10 patients with AML [8 males, 2 females; median age 48.5 (range 22–80) years] and 8 patients with nonmalignant hematological disorders [6 males, 2 females; median age 45 (range 22–71) years]. The diagnosis of AML was based on the elevated bone marrow blast ratio (>20%) and flow cytometric analysis (the presence of ≥ 2 myeloid markers) as well as other standard diagnostic procedures of leukemia. The essential characteristics of the AML patients are given in Table 1. The study was conducted in accordance with the guidelines of the Helsinki Declaration. Hacettepe University Local Ethical Committee approved the study protocol. Written informed consents were provided by all participants.

Bone marrow samples were obtained with aspiration from the posterior iliac crest of all patients during the clinically indicated time point. The glass smears were prepared for wright staining, and 3.5 mL of aspirate were separated for RT-PCR analysis. Simultaneously, peripheral blood samples were also drawn from each patient for complete blood count analyses.

Isolation of RNA and Synthesis of cDNA

Ethylenediaminetetraacetic acid (EDTA)-treated bone marrow samples were stored at -70°C until analysis. Blood samples were collected and analyzed from

AML patients. Total RNA was extracted from 200- μL bone marrow samples by High Pure RNA Isolation Kit (Roche Diagnostics, Germany) according to manufacturer's instructions. RNA integrity was electrophoretically verified by ethidium bromide staining and by OD260/OD280-nm absorption ratio of >1.95 . One microgram of total RNA was used for cDNA synthesis using 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche Diagnostics, Germany) according to the manufacturer's protocol.

Quantitative Real-Time PCR Analysis

Real-time quantitative PCR analysis for ACE, renin and angiotensinogen gene expressions was done by using LightCycler[®] instrument (Roche Diagnostics, Germany), and results were analyzed by LC software 3.0. Amplifications were performed in 20- μL volume, including 2 μL cDNA, 4 nM of each primers, 2 nM of Taqman probe and LightCycler DNA master hybridization mix. The cycling parameters were 2 min at 95°C for denaturation, 40 cycles of 15 sec at 95°C , 30 sec at 60°C for amplification and quantification. The primer and probe sequences used in this study have been shown in Table 2.²² The beta-actin mRNA was quantified to adjust the amount of mRNA in each sample with beta-actin probe and primer set. The upstream and downstream primer sequences were 5' TCACCCACACTGTGCCCAT and 5' TCCTTAATGT-CACGCACGATTT 3', respectively, and the TaqMan probe selected between the primers was fluorescence labeled at the 5' end with 6-carboxyfluorescein (FAM) as the reporter dye and at the 3' end with 6-carboxytetramethylrhodamine (TAMRA) as the quencher; 5'-FAM-ATCCTGCGTCTGGACCTGGCT-TAMRA (Tibmolbiol, Germany). In every PCR reaction, the level of the

Table 1. Essential characteristics and laboratory findings of patients with acute myeloid leukemia (AML) and the control group

	AML Patients (n=10)	Control Patients (n=8)
Median age (years)	48.50 (27)*	45.00 (36.25)*
Gender	8 males/2 females	6 males/2 females
Hemoglobin (g/dl)	8.95 (3.87)*	10.00 (3.42)*
Leukocyte (/mm ³ x 10 ³)	50.5 (71.3)*	8.2 (3.975)*
Platelet (/mm ³ x 10 ³)	58.5 (101.75)*	245 (149)*

* median (IQR)

Table 2. The primer and probe sequences of angiotensin-converting enzyme (ACE), renin and angiotensinogen genes

ACE	Renin	Angiotensinogen
Forward 5' TCGGCCATGTTGAGCTACTTC 3'	5' GCCACCTTCATCCGAAAGTTC 3'	5' GCACCTCAGTGTCTGTCCCAT 3'
Reverse 5' TCCCATGCAGCTCGTTC 3'	5' GCCAAGGCGAAGCCAAT 3'	5' ACCGAGAAGTGTCTCTGGATGT 3'
Taqman 5' AGCCGCTGCTGGACTGGCTCC 3'	5' ACACAGAGTTTGATCGGCGTAACAACCG 3'	5' TGCCATGGGCACCTCCAGCA 3'
Probe		

housekeeping gene beta-actin was also quantified to normalize ACE, renin and angiotensinogen gene expression values of each sample with the same PCR conditions described above. Relative expressions were calculated according to mathematical model of M.W. Pfaffl²³ based on the PCR efficiencies and the crossing points.

Statistical Analyses

Statistical analyses were performed via SPSS* 11.5 statistical software. Descriptives were depicted as the median and IQR (interquartile range). Nonparametric tests have been used, since the sample sizes of the groups were small. The differences between two groups were assessed by the Mann-Whitney U test. A p value of ≤0.05 was considered statistically significant.

RESULTS

Specific mRNAs for all three components of the RAS could be detected in bone marrow samples by real-time PCR analysis (Figure 1). Relative expressions of each target were calculated according to housekeeping gene of beta-actin expression. All calculations were made according to median test results of duplicate

patient samples. Relative gene expression of quantitative real-time PCR of AML patients and the control group are shown in Table 3. The median expression ratio was found to be 0.033 (IQR 0.025) for ACE and 0.029 (IQR 0.06) for angiotensinogen in control samples. Renin gene expression has not been found in 4 out of 8 control samples with an median expression of 0.002 (IQR 0.001). Relatively higher expression of all three genes has been observed in 7 of 10 patients. The medians of expression ratios of AML patient samples have been found to be 0.736 (IQR 1.359), 0.540 (IQR 0.725) and 0.075 (IQR 0.002) for ACE, angiotensinogen and renin genes, respectively. All three gene expressions were found to be significantly higher in the bone marrow samples of AML patients compared to the bone marrow samples of the control group (Table 4).

DISCUSSION

In this study, local syntheses of the major RAS components—namely ACE, renin and angiotensinogen—have been identified via demonstrating their corresponding mRNAs in human bone marrow samples. These findings support the existence of a local RAS in human

Table 3. Relative gene expression results of angiotensin converting enzyme (ACE), renin and angiotensinogen genes

Patients	No	ACE Ratio	Renin Ratio	Angiotensinogen Ratio
AML	1	1.260	0.009	0.703
	2	1.774	0.016	0.806
	3	1.002	0.007	0.269
	4	0.029	ND*	0.039
	5	0.019	ND*	0.012
	6	0.439	0.007	0.800
	7	0.028	0.003	0.047
	8	2.188	0.008	0.761
	9	0.470	0.006	0.410
	10	1.102	0.008	0.671
Control	1	0.02	0.002	0.028
	2	0.036	ND*	0.029
	3	0.054	0.003	0.036
	4	0.026	ND*	ND*
	5	0.031	ND*	0.022
	6	0.071	0.002	0.029
	7	0.024	0.002	0.029
	8	0.035	ND*	0.023

Beta-actin gene expression of the same samples was used for calculations; * ND: not determined; AML: acute myeloid leukemia

Table 4. Renin, angiotensinogen and angiotensin-converting enzyme (ACE) expressions in the bone marrow samples of AML patients compared to the bone marrow samples of the control group

Expression Ratio	AML Patients			Controls			p
	Median	IQR	n	Median	IQR	n	
Renin	0.075	0.002	8	0.002	0.001	4	0.018
Angiotensinogen	0.540	0.725	10	0.029	0.06	7	0.005
ACE	0.736	1.359	10	0.033	0.025	8	0.05

AML: acute myeloid leukemia

bone marrow. Quantitative mRNA expression levels of the individual marrow RAS components disclosed variations between the samples. That variability may be due to the dynamic interactions during the ongoing biological events of the normal and neoplastic hematopoiesis in the bone marrow. Furthermore, ACE, renin and angiotensinogen gene expressions were significantly higher in the bone marrow samples of AML patients compared to the bone marrow samples of the control group. Recent investigations revealed several RAS elements and receptors on normal and neoplastic hematopoietic cells.^{2,8,18,19,24} Strawn and coworkers⁸ detected the presence of ACE, angiotensin II, angiotensin AT(1) and AT(2) receptors in rat unfractionated bone marrow cells, hematopoietic-lineage bone marrow cells and cultured marrow stromal cells. The mRNAs for angiotensinogen, renin, ACE, and AT(1a) and AT(2) receptors were found to be present in bone marrow cells and in cultured marrow stromal cells, whereas ACE2 mRNA was detected only in bone marrow cells in their study. Flow fluorocytometry analyses by Strawn et al. also showed immunodetectable angiotensinogen, ACE, AT(1) and AT(2) receptors, and angiotensin II as well as binding of angiotensin II to AT(1) and AT(2) receptors, in CD4(+), CD11b/c(+), CD45R(+) and CD90(+) bone marrow cells and cultured marrow stromal cells. Renin was found to be present in almost all cell types, excluding CD4(+) bone marrow cells, and angiotensin II was detected in marrow stromal cells homogenates. Strawn et al.⁸ concluded that the presence of angiotensin-II receptors in both hematopoietic-lineage bone marrow cells and marrow stromal cells, and the de novo synthesis of angiotensin II by marrow stromal cells suggests a potential autocrine-paracrine mechanism for local RAS-mediated regulation of hematopoiesis.

Our findings provide further valuable indication to previous investigations focusing on different RAS elements in leukemic patients. Renin mRNA is found in leukemic blast cells.^{2,18} Angiotensin gene is expressed in leukemic cell lines as well as primary AML patient samples. Angiotensin may act as an autocrine growth factor for AML cells.¹⁹ ACE is produced at higher quantities in the leukemic bone marrow. ACE degrades a tetrapeptide called AcSDKP (goralptide), a negative hematopoietic regulator. Furthermore, while peripheral blood ACE levels increase, blast percentages in the bone marrow accumulate and migrate to the circulation. Therefore, ACE hyperfunction may lead to the acceleration of AcSDKP metabolism, which in turn lowers its level in the bone marrow microenvironment, finally removing the antiproliferative effect of goralptide on the hematopoietic cells and blasts.¹⁵ In vitro incubation of AML cells with an ACE inhibitor decreased the growth and colony-forming ability of AML cells in a dose-dependent manner. The addition of angiotensin-II peptide to AML cells in reduced serum partially rescued their colony-forming ability at the same study.¹⁹ ACE may also be physiologi-

cally important for the functioning of dendritic cells.²⁵ ACE is involved not only in intracellular volume regulation but also in the control of cellular proliferation. Since both ACE gene polymorphism (I/D ACE) and ABO blood group determine ACE level in peripheral blood and probably also in bone marrow, the hypothesis to the interindividual differences in survival of leukemic patients was suggested by Hajek et al.²⁶

The findings of the present study together with the previous investigations shed further light on the presence and (patho)biological functions of the local hematopoietic bone marrow RAS.^{2,27} RAS regulates cellular growth in a variety of tissues, including the bone marrow,^{2,13,21,28-30} with numerous molecular signal mechanisms.^{31,32} RAS can exert significant effects on erythropoietic progenitors and primitive pluripotential hematopoietic stem cell populations. Angiotensin II, the effector peptide of RAS, acts as a systemic and "locally active" hematopoietic growth factor or cytokine through activation of CD 34+ cell AT1 receptors.^{2,27} Angiotensin II can alter the differentiation of erythroid and myeloid precursors from hematopoietic stem cells. ACE inhibitors inhibit the proliferation of hematopoietic stem and progenitor cells.³³ Angiotensin-signaling pathways, such as the JAK-STAT pathway, represent the point of crosstalk between RAS and hematopoiesis.³⁴ Angiotensin II can affect the proliferation and differentiation of CD34(+) cells of umbilical cord blood and bone marrow origin.¹³ Angiotensin peptides act to stimulate the formation of bone marrow progenitors, thereby facilitating recovery after myelosuppressive irradiation in experimental studies.^{27,35,36}

Our findings indicate that locally produced angiotensin peptides, in the context of local hematopoietic bone marrow RAS, could be active in the pathobiological basis of leukemogenesis. This issue is not just academic,¹ since the pharmacological modification of RAS affects the course of diseases.^{2,37-39} Possible inhibitory effects of AcSDKP at high doses on human leukemia cells have been shown.⁴⁰ Therefore the negative hematopoietic regulator peptide, AcSDKP, may be an ideal starting point for the alteration of local RAS kinetics in the bone marrow microenvironment.^{20,40-51} A new class of organometallic compound, the Biphosphinic Palladacycle Complex [Pd (C2, N-S (-) (dmpa) (dppf)] Cl (BPC), as an ACE inhibitor, which resulted in increased levels of its substrate AcSDKP, has already been shown to act in hematological regulation.⁵² Further experimental and clinical studies should focus on the elucidation of the critical leukemogenic mechanisms in relation to the molecular and cellular basis of the local bone marrow RAS for the better management of leukemic patients.

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