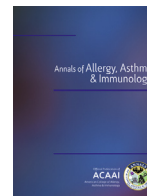




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Arginase I levels are decreased in the plasma of pediatric patients with atopic dermatitis

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ABSTRACT

Background: Serum arginase levels have been shown to be elevated in conditions, such as trauma, cancer, chronic wounds, pregnancy, and diabetes. This also has been found to be true in atopic diseases, such as asthma and allergic rhinitis.

Objective: To study arginase activity in patients with atopic dermatitis (AD).

Methods: In this pilot study, arginase activity levels in 15 pediatric patients with AD were compared with those in controls to determine whether arginase levels in AD are altered as in patients with other atopic diseases.

Results: In contrast to the other diseases studied, arginase activity was found to be decreased in granulocytes and in the plasma of patients with AD compared with controls. This finding was coupled with a trend toward higher L-arginine plasma levels.

Conclusion: In AD, a different mechanism of arginine metabolism seems to be stimulated, leading to the formation of nitric oxide pathway components causing suppression of the arginase pathway and impairment in skin hydration, collagen synthesis, and wound healing.

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Introduction

Arginase activity has been investigated in several conditions, including trauma states, cancer, chronic wounds, pregnancy, and diabetes. In atopic diseases, including asthma and allergic rhinitis, arginase research has been more limited and focused more toward another product of arginine metabolism, nitric oxide (NO).

L-arginine is a conditionally essential amino acid whose necessity is based on developmental age and health status. It is processed along 2 main pathways that may be coexpressed in the same cell: (1) through arginase (I or II) to create urea and L-ornithine and (2) through NO synthase (NOS; neuronal, inducible [iNOS], or endothelial) to form NO and L-citrulline. These catabolic pathways are differentially regulated by induction through different cytokine milieus: arginase I expression is induced by T-helper cell type (T_H)-2 cytokines (interleukin [IL]-4, IL-13, and transforming growth factor- β), whereas iNOS activity is induced by T_H1 cytokines (interferon- γ [IFN- γ], IL-1, and tumor necrosis factor).¹ In either

pathway, limitation in the availability of the L-arginine substrate will suppress enzymatic activity to some extent.

Although not previously investigated in atopic dermatitis (AD), the issue of arginine bioavailability has been studied in other atopic diseases, such as asthma and allergic rhinitis. Patients with asthma have been found to have transiently elevated serum arginase activity during acute asthma exacerbation,² and animal models of allergic asthma have shown a similar finding after allergen challenge.^{3,4} High arginase activity has been postulated to result in a low level of plasma L-arginine, which compromises the body's ability to synthesize NO, a potent vasodilator, and contributes to airway hypersensitivity.⁵ Moreover, high doses of L-arginine have been shown to decrease airway hyperresponsiveness and inflammation.⁶ Similarly to results seen in patients with asthma, in patients with allergic rhinitis, arginase I expression in nasal mucosa has been found to be elevated after allergen challenge,⁷ although serum arginase has not been found to change notably.⁸

Given these findings in other atopic diseases, this exploratory study was initiated to investigate the role of arginase in children with AD by measuring arginase activity and as serum arginine levels. Human arginase I is localized to the granules of polymorphonuclear (PMN) cells and found to be constitutively expressed.⁹ For this reason, this study looked into not only serum L-arginine levels but also arginase I activity within a patient's

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granulocytes to determine whether there was a difference in activity level at the source. Surprisingly, *decreased* levels of arginase I activity were found in the granulocytes of patients with AD coupled with low arginase I protein and some elevation of L-arginine within serum. These results, in contrast to the other atopic diseases investigated, imply a different involvement of arginase I in the inflammatory mechanism of AD.

Methods

Patients

Fifteen pediatric patients with a history of AD requiring therapy of daily moisturizing, corticosteroids, and/or immunomodulators and current clinical manifestations of varying severity were enrolled in the study. AD was defined by a chronic, xerotic, excoriated, pruritic, and/or lichenified skin condition that was treated by the Division of Allergy/Immunology at the Louisiana State University Health Sciences Center (New Orleans). Most patients also had previously confirmed sensitizations to foods and/or inhalants. Each patient's dermatitis was documented, including location and percentage of body surface area involved, and a mild, moderate, or severe rating was assigned by the examiner for stratification purposes. Patients who showed greater than 15% body surface area involvement, no active infections, and no antibiotic use within 3 months of sample collection were enrolled in the study (Table 1). At the time of enrollment, 9 patients were receiving topical corticosteroids regularly, 5 patients were receiving topical calcineurin inhibitors regularly, 7 patients were on antihistamines, and 6 patients were on leukotriene inhibitors. The mean age of patients was 4.8 years (range 1–13 years). Nine patients were boys and 6 were girls; 8 patients were white, 5 were African American, and 2 were Hispanic.

Six age-matched control patients who had no personal or family history of atopy also were enrolled at the time of the study. Patients with a history of recurrent infections, skin disorders, or chronic medical conditions were excluded from participating as a control. Their mean age was 5.8 years (range 1–14 years). This group included 5 boys and 1 girl, with an equal distribution of white and African American patients.

At time of enrollment, blood was collected from each patient for determination of arginase activity, arginase protein quantitation, and amino acid levels.

Isolation of PMNs

Polymorphonuclear cells from patients and healthy donors were isolated by dextran sedimentation. Briefly, peripheral blood was centrifuged over Ficoll-Hypaque (GE Biosciences, Uppsala, Sweden),

after which peripheral blood mononuclear cells were collected and the remaining cells were resuspended in 3% dextran for 60 minutes at 37°C and 5% CO₂. The supernatants were collected and centrifuged for 5 minutes at 1,600 rpm at 4°C. Red blood cells were lysed by hypotonic lysis. PMN purity was determined by Giemsa staining and by flow cytometric staining of CD11b⁺/CD14⁺ and ranged from 97% to 100%.

Arginase I Activity Assay

Granulocyte cell extracts from patients and controls were tested for arginase activity by measuring the conversion of a known quantity of L-arginine to L-ornithine and urea. Briefly, 25 µg of protein from cell lysates was added to 25 µL of Tris-HCl (50 mmol/L, pH 7.5) containing 10 mmol/L of MnCl₂. This mixture was heated at 55°C to 60°C for 10 minutes to activate arginase. Then, a solution containing 150 µL of carbonate buffer (100 mmol/L; Sigma, St Louis, Missouri) and 50 µL of L-arginine (100 mmol/L) was added and incubated at 37°C for 20 minutes. The hydrolysis reaction from L-arginine to L-ornithine was identified by a colorimetric assay after the addition of Ninhydrin solution and incubation at 95°C for 1 hour. In addition, the hydrolysis reaction from L-arginine to urea was detected with diacetyl monoxime (Sigma) and incubation at 95°C for 10 minutes.

Myeloperoxidase and Metalloproteinase-9 Expression

Granule contents that colocalize with arginase I were identified to monitor for degranulation of storage granules. Cell extracts were suspended in lysis buffer (50 mmol/L of HEPES, 150 mmol/L of NaCl, 5 mmol/L of ethylenediaminetetraacetic acid, 1 mmol/L of NaVO₄, and 0.5% Triton) containing 50 µg/mL of aprotinin, 50 µg/mL of leupeptin, 100 µg/mL of trypsin-chymotrypsin inhibitor, and 2 mmol/L of phenyl methyl sulfonyl fluoride. Lysates were centrifuged at 3,000g for 10 minutes at 4°C. The expression of metalloproteinase-9 (MMP-9), myeloperoxidase (MPO), and glyceraldehyde-3-phosphate dehydrogenase was detected by immunoblot using 25 µg of cell extracts. Cytoplasmic extracts were electrophoresed in 10% Tris-glycine gels (Novex, San Diego, California), transferred to polyvinylidene difluoride membranes, and immunoblotted with the appropriate antibodies. The reactions were detected using an enhanced chemiluminescence kit (Amersham, Arlington Heights, Illinois).

Serum Arginase I Protein Levels

Arginase I levels were tested in the serum of healthy volunteer donors and patients with AD using an enzyme-linked immunosorbent assay kit (BioVendor, Candler, North Carolina).

Table 1
Clinical and laboratory profile of patients with atopic dermatitis

Patient number	Age (y)	Sex	Ethnicity	Sensitizations	Total IgE (IU/ml)	Absolute eosinophil count	Severity
1	6	F	W	inhalants	58	400	mild-mod
2	9	M	W	foods	1,838	ND	mild-mod
3	4	M	B	inhalants, foods	1,803	520	mild
4	5	M	H	foods	159	320	mild
5	7	M	W	inhalants	75.3	980	mod
6	1	M	B	inhalants	44.2	120	mod
7	2	F	B	foods	181	330	mild
8	1	F	H	foods	1,480	360	mod-severe
9	4	F	W	inhalants, foods	908	760	severe
10	13	M	B	ND	373	630	mild
11	7	F	W	inhalants, foods	104	110	mild
12	1	M	W	ND	137	210	mild
13	6	M	B	inhalants, foods	5,760	1,580	severe
14	4	F	W	inhalants, foods	4,106	300	mod
15	2	M	W	foods	214	320	mild

Abbreviations: B, black; F, female; H, Hispanic; M, male; mod, moderate; ND, not done; W, white.

Serum Levels of L-Arginine

L-arginine concentration in serum samples was measured by high-performance liquid chromatography and electrochemical detection using an ESA-CoulArray Model 540 (ESA, Inc, Chelmsford, Massachusetts) with an 80×3.2 column and 120A pore size. Briefly, serum samples were deproteinized in methanol. After centrifugation at 6,000g for 10 minutes at 4°C, the supernatant was derivatized with 0.2 mol/L of o-phthalaldehyde containing 7 mmol/L of β -mercaptoethanol. Fifty microliters of the sample was injected into the column. The retention time for L-arginine was 10.2 minutes.

Statistics

Statistical analysis was performed using GraphPad Prism 3.0 (GraphPad, San Diego, California). Differences between groups were determined by the Student *t* test and analysis of variance.

Results

Decreased Arginase I Activity in Granulocytes of Patients with AD

Arginase I activity found within PMN cells of patients with AD was significantly decreased compared with patients without atopic disease (361.8 ± 46.38 vs 530.9 ± 48.44 $\mu\text{M/L}$, $p < .01$; Fig 1).

Arginase I is thought to be stored in the gelatinase¹⁰ or azurophilic⁹ granules of PMNs, which also contain MPO and MMP-9, respectively. To assess degranulation of these 2 granule types, the intracellular protein expression of MMP-9 and MPO was determined by western blot. The enzymes were present in equal amounts in representative samples (3 patients and 2 controls), implying lack of degranulation of PMNs (Fig 1B).

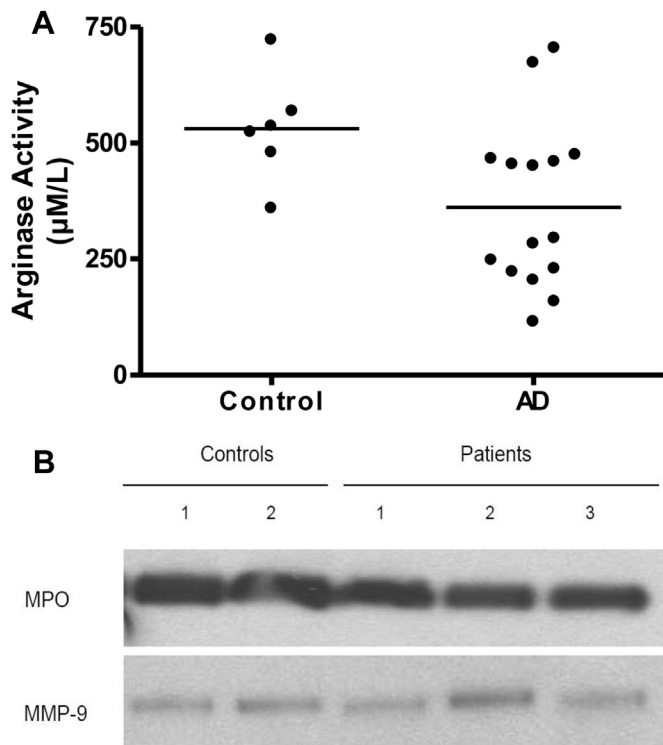


Figure 1. (A) Decreased arginase activity is found in granulocytes of patients with atopic dermatitis (AD). Granulocyte lysates were tested for arginase activity by measuring the production of L-ornithine using a colorimetric assay ($p < .01$, Student *t* test). (B) Decrease in arginase I activity in granulocytes is not due to degranulation and release. Western blot analysis shows comparable levels of myeloperoxidase (MPO) and metalloproteinase-9 (MMP-9) in the cells of controls and patients with AD, implying lack of degranulation. This suggests that the decrease of arginase I activity in the cells is not due to protein release into the extracellular space.

Decreased Arginase I Protein in Serum of Patients with AD

Because the decrease in granulocyte arginase might be a result of degranulation of the PMNs with release of arginase into the local environment, patients' serum levels of arginase I were compared with those of controls. In congruence with the decreased intracellular activity level, there was a notable decrease in the level of arginase I protein in the serum of patients with AD (39.7 ± 14.35 vs 121.1 ± 20.33 $\mu\text{M/L}$, $P < .01$; Fig 2).

Increase in L-Arginine in Serum of Patients with AD

Consistent with the findings of decreased arginase protein in patients with AD, there was a trend toward higher levels of L-arginine compared with controls (Fig 3).

Discussion

This is the first report of arginase activity in the setting of AD. In contrast to other atopic diseases studied, the present patients with AD showed lower levels of arginase activity in their granulocytes and lower arginase I protein expression in their serum. This was not due to degranulation and dilution in the serum, because the other PMN granule proteins were notably intact, but likely was due to decreased production or increased degradation. These results are supported further by the slightly higher levels of plasma L-arginine compared with controls, although these levels did not reach statistical significance.

In the complex pathophysiology of acute AD, the T_H2 cytokine milieu predominates in the early phase of disease; as the process becomes chronic, the cytokines become skewed toward the T_H1 balance, with IFN- γ and IL-12 being the predominant effectors.¹¹ This change has importance for arginine metabolism because these T_H1 cytokines found in chronic AD, specifically IFN- γ , could skew processing toward the NOS pathway, causing increased production of NO. In mechanisms of wound repair, NO in large quantities has been shown to have deleterious effects on wound healing.¹² In patients with AD, this could contribute to impaired skin barrier healing.

In addition, the seeming disparity between the present results and those of previously investigated atopic diseases may be due to this same T_H1 cytokine skewing. Although T_H2 cytokines dominate in asthma and allergic rhinitis, presumably causing an increase in arginase I expression and activity, the T_H1 -dominated chronic AD milieu may have the opposite effect. Therefore, there may be a differential of expression based on the chronicity of the disease.

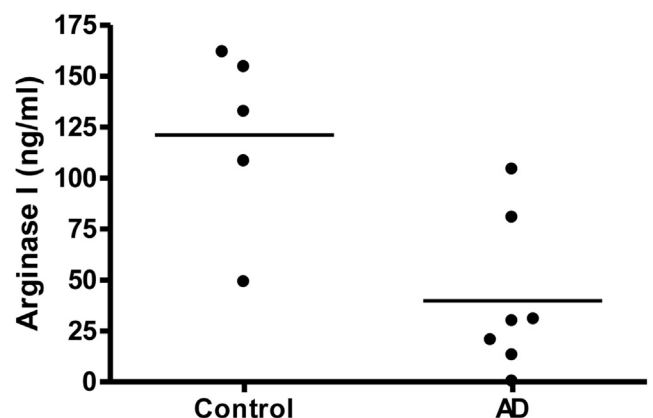


Figure 2. Decreased arginase I protein found in the plasma of patients with atopic dermatitis (AD). Plasma samples of 7 patients with AD and 5 controls were analyzed for arginase I protein using an enzyme-linked immunosorbent assay ($p < .01$).

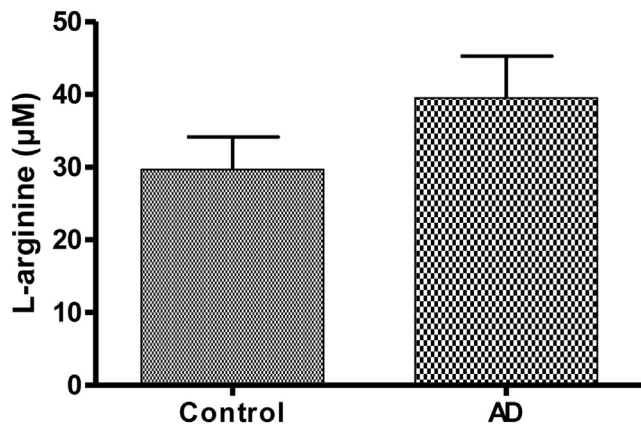


Figure 3. Increased levels of L-arginine are found in plasma of patients with atopic dermatitis (AD). Assessment of L-arginine levels of 7 patients and 6 controls using high-performance liquid chromatography shows a trend toward lower levels of arginine in controls compared with patients with AD.

Continued stimulation of the iNOS pathway also leads to downregulation of arginase I. Primary cultured keratinocytes, when exposed to T_H1 cytokines IFN- γ , tumor growth factor- α , and IL-1 β , showed an increased expression of iNOS and a decreased arginase I mRNA expression of approximately 45%.¹³ In mouse models of AD-like lesions, skin showed increased iNOS expression and serum samples were noted to have increased nitrites compared with controls.¹⁴ In addition, even after epicutaneous sensitization, iNOS knockout mice did not show the normally induced symptoms of swelling/scratching or increased nitrite levels as found in the control mice.¹⁵ More specifically to AD in humans, serum nitrate levels were found to be elevated in pediatric patients with AD and were related to severity of disease and eosinophil count.¹⁶ Elevated urinary nitrite levels also were correlated with an AD diagnosis,¹⁷ although severity was not consistently correlated by other groups. In the present patients, arginase activity did not show a correlation in severity or IgE level, although there was some consistency with absolute eosinophil count (data not shown). However, the continued stimulation of the iNOS system, which is found in patients with AD, could account for the decreased levels of arginase I protein seen in the present patients and be a factor in the pathophysiology of AD, because the nitrites produced would induce further breakdown and inability to heal. These findings are corroborated by the successful use of AD treatments, such as topical hydrocortisone¹⁸ and FK506,¹⁹ which inhibit iNOS mRNA expression. In addition, a small study showed an 80% decrease in itching and 60% decrease in erythema with daily application of the NOS inhibitor N ω -nitro-L-arginine in 1% petrolatum.²⁰

Suppression of the arginase I pathway also can have direct effects on wound-healing pathways. Less processing of arginine into L-ornithine, which is metabolized further to proline by ornithine aminotransferase and to polyamines by ornithine decarboxylase, could result in impaired collagen production and cell proliferation, respectively.²¹ Recently, researchers have shown that local arginase I deficiency induces delay in wound healing through an altered inflammatory response and abnormal matrix deposition.²² This issue also is important for patients with AD who often have barrier function defects associated with impaired healing.²³

Recent research has focused on the filaggrin protein, which is a structural component of the epidermis. Mutations in filaggrin also have been associated with AD.²⁴ At degradation, filaggrin releases free amino acids, including arginine, which are processed into natural moisturizing factors in the skin.²⁵ Then, arginase I hydrolyzes this arginine into ornithine and urea. Urea is known to enhance hydration in the stratum corneum and has been used in

topical form to improve skin barrier function and enhance antimicrobial peptides.²⁶ Recent proteomic profiling of patients with AD has found a significant decrease in the expression of arginase I in skin samples.²⁷ The investigators posited that decreased expression of arginase I also might decrease urea generation. This finding, in congruence with the present findings, also might provide insight into another problem with the epidermal barrier in AD: low arginase I expression would decrease urea production, thereby causing barrier hydration dysfunction.

Although this early research has begun to link the iNOS-arginase pathway to AD, many issues remain to be elucidated. First, expansion of patient numbers will clarify differences among patients based on severity stratification. Second, the mechanism of action for the decreased arginase I enzyme expression found in these patients will need to be investigated further based on pre- and post-transcriptional analyses. Third, direct measurement of iNOS and arginase I activity in the AD skin microenvironment (acute and chronic) may help to clarify the local interactions between these 2 enzymes.

In summary, this exploratory study has identified a role for arginase I in AD. In this disease state, overproduction of iNOS may contribute to the suppression of the arginase system, causing impaired wound healing and barrier dysfunction. Further research into the causes of iNOS overexpression and resultant arginase I suppression may lead to therapeutic interventions in the future.

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