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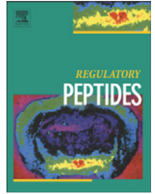

# Basic aminopeptidase activity is an emerging biomarker in collagen-induced rheumatoid arthritis

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ABSTRACT

The objective of this study was to investigate the catalytic activity of basic aminopeptidase (APB) and its association with periarticular edema and circulating tumor necrosis factor (TNF)-alpha and type II collagen (CII) antibodies (AACII) in a rat model of rheumatoid arthritis (RA) induced by CII (CIA). Edema does not occur in part of CII-treated, even when AACII is higher than in control. TNF-alpha is detectable only in edematous CII-treated. APB in synovial membrane is predominantly a membrane-bound activity also present in soluble form and with higher activity in edematous than in non-edematous CII-treated or control. Synovial fluid and blood plasma have lower APB in non-edematous than in edematous CII-treated or control. In peripheral blood mononuclear cells (PBMCs) the highest levels of APB are found in soluble form in control and in membrane-bound form in non-edematous CII-treated. CII treatment distinguishes two categories of rats: one with arthritic edema, high AACII, detectable TNF-alpha, high soluble and membrane-bound APB in synovial membrane and low APB in the soluble fraction of PBMCs, and another without edema and with high AACII, undetectable TNF-alpha, low APB in the synovial fluid and blood plasma and high APB in the membrane-bound fraction of PBMCs. Data suggest that APB and CIA are strongly related.

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## 1. Introduction

Rheumatoid arthritis (RA) is an inflammatory, chronic, systemic and autoimmune disease [1,2] of unknown etiology [1–4] that affects about 1% of the world's population [1,2,5]. It is associated with increased mortality and significant comorbidities and other factors that can specifically affect a patient's quality of life [6,7]. The prominent characteristic of RA is a symmetrical peripheral polyarthritis with bone erosions, mainly of cartilage, and resultant deformity and joint destruction [8]. This process is associated with inflammatory hyperplasia of the synovial membrane, also known as pannus [9], and pathological neovascularization, which occur after the infiltration of inflammatory cells into the synovial membranes [10], as well as an immune response against cartilage components, among them the type II collagen (CII) has been thought to be the most usual [8]. Tumor necrosis factor (TNF)-alpha has also been found in high concentrations in the serum and synovial fluid of RA patients [1,10].

The genetic predisposition linked to human leukocyte antigen system has been estimated to contribute with about 50% or less for the development of disease [11]. Thus, several epigenetic factors appear to be involved in the onset of RA, such as DNA methylation, alternative splicing, micro RNAs and histone modifications [11]. Moreover, factors such as infections, smoking, pregnancy, sex hormones and others may lead to autoimmunity [4]. CII is considered the primary antigen in RA, since it is the main constituent of cartilage [2] and because antibodies anti-CII (AACII) have been detected in synovial fluid [5,12], cartilage and B cells present in the synovial membrane of arthritic patients [12]. Furthermore, manifestations of RA have close similarity with those of the experimental model of CII-induced arthritis (CIA), that are: synovitis, progressive pannus formation, marginal erosion of bone and cartilage destruction [5,12,13]. The transfer of AACII is also able to induce RA, because these antibodies bind to normal cartilage and activate the complement system [5]. In the CIA model, symptoms of RA begin about 21 days after the injection of CII [13]. According to Stuart et al. [9], the initial lesion observed in this experimental model of RA is fibrin deposition in the synovial membrane 5 days after immunization. On the 12th day there is an intense deposition of fibrin and tissue hyperplasia. On the 19th day the infiltration of mononuclear and polymorphonuclear cells begins. Inflammation occurs so rapidly that the destruction of cartilage and the marginal erosion of the bone occur in a few days [9].

*Abbreviations:* AACII, antibody anti-type II collagen; Ang, angiotensin; APB, basic aminopeptidase; BSA, bovine serum albumin; CII, type II collagen; CIA, collagen-induced arthritis; LDH, lactate dehydrogenase; LTA4, leukotriene-A4; LTA4H, leukotriene A4-hydrolase; MF, membrane-bound fraction; NADH, nicotinamide adenine dinucleotide, reduced form; PBMCs, peripheral blood mononuclear cells; PGP, Pro-Gly-Pro; RA, rheumatoid arthritis; SF, soluble fraction.

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Regarding the evidences that altered matrix metalloproteinase and other proteins implicated in matrix degradation, cell activation, inflammation and bone collagen degradation products accompany early rheumatoid and osteoarthritis development and can precede the canonical diagnostic detection by several years [14,15], as well as that CD13, an aminopeptidase type enzyme, was histologically expressed strongly in RA synovial tissue lining cells [16], it can be hypothesized that changes of other aminopeptidase hydrolytic activities, such as the basic aminopeptidase (APB) activity, could be important in RA, mainly because APB activity was reported to be increased in the soluble fraction of elicited macrophages [17] and because kallidin [18], leukotriene(LT)-A4 [19,20] and angiotensin (Ang) III [21], three of the known natural substrates susceptible to hydrolysis by isozymes with APB activity, have recognized actions in regulating angiogenesis [22] and inflammation [23].

To prospect new markers and contribute to elucidate the etiology of RA, this study aims to assess changes in the APB activity of blood plasma, synovial fluid and soluble (SF) and solubilized membrane-bound (MF) fractions from the synovial membrane of the knee and from peripheral blood mononuclear cells (PBMCs), associating this enzyme activity with hind paw edema and with the AACII titer in blood plasma and TNF-alpha levels in blood serum of control and CII-treated rats.

## 2. Materials and methods

### 2.1. Animals, treatments and samples

Adult male Wistar rats, weighing 150–160 g, provided by the Animal Facility of the Instituto Butantan, were maintained in polyethylene cages (inside length × width × height = 56 × 35 × 19 cm) with food and tap water *ad libitum*, in a container with controlled temperature of 25 °C, relative humidity of 65.3 ± 0.9% and 12 h:12 h photoperiod light:dark (lights on at 6:00 am). The animals and research protocols used in this study are in agreement with the COBEA (Brazilian College of Animal Experimentation) and were approved by the Ethics Committee of the Instituto Butantan (682/09).

The animals were anesthetized with a solution of ketamine (Ketamine, Syntec, Cotia, São Paulo, Brazil) (3.75%) and xylazine (Calmiun, Agener Union, Planalto Paulista, São Paulo, Brazil) (0.5%) at a dose of 0.2 mL/100 g body weight, via intraperitoneal, and then subjected to induction of arthritis by administration of CII from chicken (Sigma, St. Louis, MO, USA) dissolved in 0.01 M acetic acid and emulsified in an equal volume of Freund's incomplete adjuvant (Sigma, St. Louis, MO, USA) (prepared at 4 °C just before use), via a single intradermal dose of 0.4 mg/0.2 mL/animal, into the proximal one-third of the tail (induced animals), or with 0.9% saline at the same scheme of administration (sham induction). On day 41 after treatment, the animals were anesthetized using the same scheme specified above and the thickness of the hind paws were measured in order to evaluate edema. Based on this measurement the following experimental groups were formed: *Control* (all animals submitted to sham induction); *Arthritic* (induced animals with hind paw thickness >7 mm); *Resistant* (induced animals with hind paw thickness similar to control). Thus, blood was withdrawn from the left ventricle with heparinized and not heparinized syringes in order to obtain plasma and serum, respectively. Heparinized blood was centrifuged (at 200 × *g* for 10 min at room temperature) to separate plasma (supernatant) from the pellet containing PBMCs and other blood cells. The non-heparinized blood was left at room temperature for 10 min and then centrifuged under the same conditions to obtain the serum. The synovial fluid and synovial membrane were subsequently removed from both knees of each animal. The withdrawal of synovial fluid and synovial membrane was performed as follows: 200 μL of 0.9% NaCl was injected intraarticularly into each knee and aspirated with a syringe and, after such washing, the synovial membrane was excised together with the connective tissue of the joint capsule.

### 2.2. Separation of PBMCs

According to the method of Maldonado and Curi [24] with modifications as follow: the pellet resulting from the obtainment of blood plasma was reconstituted to the original volume with 0.9% NaCl and then carefully layered on equal volume of Percoll ($\rho = 1.077$ mg/mL) (GE-Healthcare) and subsequently centrifuged (200 × *g* for 20 min at room temperature). The layer containing the PBMCs was then removed from the tube and washed twice with 0.9% NaCl (1:3 v/v diluted blood cells) and centrifuged again at the same conditions to discard the supernatant.

### 2.3. Cell counting and viability

PBMCs were resuspended in 0.5 mL of 0.9% NaCl. The total number of PBMCs was assessed in 20 μL aliquots of this suspension diluted with Turk's fluid (1:20, v/v). The cell viability was assessed using 40 μL aliquots of this suspension diluted in equal volume of Trypan. Cell counting was performed in a Neubauer chamber under optical microscopy.

### 2.4. Evaluation of the canonical signs of RA

#### 2.4.1. Paw edema

Hind paw swelling was quantified by macroscopic measurement of the dorsal–plantar thickness of the hind paws in the region of the metatarsus with a paquimeter (Mitutoyo, Aurora, IL, USA). Both paws were measured and the average thickness was calculated for each animal.

#### 2.4.2. Molecular markers

AACII was measured in blood plasma and TNF-alpha was measured in blood serum with Enzyme-Linked ImmunoSorbent Assay (ELISA) kits purchased from Chondrex (Redmond, WA, USA) and Biosource (Carlsbad, CA, USA), respectively, according to the manufacturer's recommendations.

### 2.5. Obtaining SF and MF from the synovial membranes and PBMCs

The synovial membranes from both knees of each animal were homogenized in 10 mM Tris–HCl buffer, pH 7.4 (0.1 g tissue/3.0 mL) for 3 min at 15,000 rpm (homogenizer Polytron-Aggregate, Kinematica, Lucerne, Switzerland). PBMCs, resuspended in 0.9% NaCl, were sonicated in 10 mM Tris–HCl, pH 7.4 ($3.0 \times 10^6$ cells/mL), for 10 s at an amplitude level of 40 microm at a constant frequency of 20 KHz. These samples were then ultracentrifuged at 100,000 × *g* for 35 min (ultracentrifuge Hitachi model CP60E). The resulting supernatants correspond to SF. The resulting pellets were washed twice with the same buffer and ultracentrifuged at 100,000 × *g* for 35 min, to assure the complete removal of SF. Subsequently, the pellet was homogenized for 3 min at 800 rpm (homogenizer Tecnal TE 099, Tecnal, Piracicaba, SP, Brazil) with the same volume of the same buffer plus Triton X-100 (0.1%) and ultracentrifuged again (100,000 × *g* for 35 min). The resulting supernatants correspond to MF. All procedures were carried out at 4 °C.

As a marker for the fractionation procedure, lactate dehydrogenase (LDH) activity was determined, photometrically, at 340 nm, in SF and MF, as previously described [25]. Briefly, samples of 3 μL of SF and MF, in triplicates, were incubated with 297 μL of 100 mM phosphate buffer, pH 7.4, containing 200 mM NaCl and 1.6 mM sodium pyruvate solution (Sigma, St. Louis, MO, USA) and 0.2 mM nicotinamide adenine dinucleotide, reduced form (NADH) disodium salt (Sigma, St. Louis, MO, USA). Two measurements of absorbance at 340 nm were performed: the first at

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