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Research paper

Peptidylarginine deiminase expression and activity in PAD2 knock-out and PAD4-low mice

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ABSTRACT

Citrullination, the conversion of peptidylarginine to peptidylcitrulline is catalyzed by peptidylarginine deiminases (PAD). The expression of PAD isoforms displays great variation among different tissues as demonstrated by PAD mRNA analyses. Here we have analyzed the differential expression of PAD2, PAD4 and PAD6 in mouse tissues at the protein level and by enzymatic activity assays using PAD2 and PAD4 knock-out strains. As expected, no PAD2 expression was detected in the PAD2−/− mice. In contrast, the PAD4 protein was observed in several tissues of the PAD4 knock-out mice, albeit at reduced levels in most tissues, and are therefore referred to as PAD4-low mice. In material from PAD2−/− mice, except for leukocyte lysates, hardly any PAD activity was found and no citrullinated proteins were detected after incubation in the presence of calcium. PAD activity in the PAD4-low mice was similar to that in wild-type mice. In both PAD knock-out strains the expression of PAD6 appeared to be up-regulated in all tissues analyzed, with the exception of spleen and testis. Our data demonstrate that the PAD2 protein is expressed in brain, spinal cord, spleen, skeletal muscle and leukocytes, but not detectably in liver, lung, kidney and testis. PAD4 was detected in each of these tissues, although the expression levels varied. In all tissues where PAD2 was detected, except for blood cells, this PAD isoform appeared to be responsible for virtually all peptidylarginine deiminase activity.

1. Introduction

Peptidylarginine deiminases (PAD; EC 3.5.3.15) represent a family of calcium-dependent enzymes that catalyze the conversion of peptidylarginine into peptidylcitrulline, which is generally indicated with (protein) citrullination. This post-translational modification is associated with the loss of a positive charge of the protein involved, which can have important structural and functional consequences [1]. Several family members have been identified, including PAD1, PAD2, PAD3, PAD4 and PAD6, the genes of which are clustered in the human and mouse genomes and share sequence similarities. Several studies have addressed PAD expression in different tissues on the mRNA [2,3] as well as on the protein [1,4,5] level. Each isotype showed a tissue-specific expression pattern. It was observed that PAD1 was mainly expressed in epidermis, hair follicles, sweat glands and arrector pili (small muscle that connects hair follicle to dermis) [6,7]. In some studies PAD1 was detected in uterus, pancreas, colon and thymus [5,8]. PAD2 appeared to be ubiquitously expressed, since it was detected in many tissues, including white blood cells [4,9]. PAD3 was reported to be expressed in (the upper layers of the) epidermis, hair follicles, brain and colon [5,7,10]. PAD4 has been found in white blood cells (including lymphocytes), brain, colon, kidney, pancreas, spleen, as well as in several tumours [4,5,9,11,12]. Finally, PAD6 expression so far has only been observed in oocytes, ovary, embryonic stem cells and (peripheral) white blood cells [2,5]. The different mRNA as well as protein expression patterns of the mammalian PAD isotypes are listed in Supporting Table 1.

For their activity PAD enzymes are dependent on high calcium concentrations. Under normal circumstances, the intracellular

Abbreviations: ABAP, antibody-based assay for peptidylarginine deiminase activity; NET, neutrophil extracellular trap; PAD, peptidylargine deiminase; PBMC, peripheral blood mononuclear cell; PMN, polymorphonuclear leukocytes; RA, rheumatoid arthritis; RBC, red blood cell.

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Table 1
PAD protein expression in mouse and human tissues.

	WT mice			PAD2−/− mice			PAD4-low mice			Human		
	PAD2[a]	PAD4[a]	PAD6[a]	PAD2	PAD4	PAD6	PAD2	PAD4	PAD6	PAD2	PAD4	PAD6
Brain	76	74 + 94[b]	–	–	74 + 94[b]	77	76	–	77	76	–	77
Liver	–	74 + 94[b]	–	–	74 + 94[b]	77	–	74	77	76	74	77
Lung	–	74 + 94[b]	–	–	74 + 94[b]	77	–	74[c]	77	–	74	–
Spleen	76	74	–	–	74	–	76	74	–	60[b]	74	–
Kidney	–	74	–	–	74	77	–	74[c]	–	–	74	–
Muscle	76	74 + 94[b]	–	–	74 + 94[b]	77	76	–	77	76	–	–
Spinal cord	76	74 + 94[b]	–	–	74 + 94[b]	77	76	–	77	n.d.	n.d.	n.d.
Testis	–	74 + 94[b]	60	–	74 + 94[b]	60	–	–	60	n.d.	n.d.	n.d.
Skin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	–	–	–
Ovary	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	–	–	85[b]
Placenta	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	–	74	–
Thymus	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	60[b]	74 + 77	77

[a] The calculated mass of human PAD2, PAD4 and PAD6 is 76, 74 and 77 kDa, respectively. The numbers in the table refer to the relative molecular masses estimated by the migration in SDS-PAGE.
[b] Putative alternative or post-translationally modified PAD isoforms.
[c] Reduced expression compared to wild-type mice. n.d.: not determined.

calcium levels in the cell are too low for PAD enzymes to be active. PAD activation especially occurs in dying cells as a result of elevated calcium concentrations. PAD (probably PAD4) has recently been found to be involved in neutrophil extracellular trap (NET) formation [13,14]. NETs represent an extracellular fibrillar matrix, which is produced as a result of various stimuli by neutrophils (through a cell death mechanism other than apoptosis and necrosis called NETosis) and which can bind pathogens [15,16]. NETs are composed of chromatin and granular proteins [16,17]. Peptidylarginine deiminase 4 (PAD4)-mediated deimination of histone H3 and H4 is required for NET formation [14].

Furthermore, PAD enzymes are involved in cornification of the skin (citrullination of filaggrin and keratin), in maintaining the hydration of the stratum corneum and the barrier function of the epidermis, in hair follicle differentiation, in hair growth (citrullination of trichohyalin), myelin formation (citrullination of myelin basic protein) and in the regulation of gene expression (citrullination of histones) [7,10,11,18]. Alterations in citrullination have been reported to occur in several diseases, including multiple sclerosis, Alzheimer's disease, psoriasis and rheumatoid arthritis (RA) [19–22]. Moreover, antibodies against citrullinated proteins are produced in rheumatoid arthritis (RA) patients and are employed as a biomarker for early RA [23,24]. Furthermore, in East Asian populations specific PADI4 (the PAD4 gene) genotypes appeared to be associated with RA. In addition, autoantibodies against PAD4 have been detected in RA patients and can predict disease outcome [25–27].

To study the potential role of citrullination in pathological conditions, the availability of PAD knock-out animals might be very helpful. Indeed, using PAD2 knock-out mice we have previously demonstrated that PAD2 and citrullination are not essential to the development of experimental autoimmune encephalomyelitis [28].

The PADs that seem to be most relevant from a pathological point of view are PAD2 and PAD4. Here, we systematically addressed the expression and activity of PADs in murine PAD2 knock-out and PAD4-low strains.

2. Materials and methods

2.1. Mice strains

PAD2−/− mice were generated as described previously by Raijmakers and colleagues [28]. PAD4-low mice were generated via gene trap mutagenesis (Lexicon). Control B6/129 mice (wild-type) were purchased from Jackson Laboratories (Bar Harbor, ME).

2.2. Genomic DNA isolation from PAD2−/− and PAD4-low mice

The toes, routinely used for identification of siblings, of PAD2−/− and PAD4-low mice were incubated in 250 ul lysis buffer (100 mM Tris–HCl, pH 7.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl and 100 ug/ml proteinase K) at 50 °C overnight. The next day, insoluble material was removed by centrifugation for 5 min at 7000*g*. The supernatant was transferred to a new tube and isopropanol (250 ul) was added. The resulting pellet after centrifugation for 10 min at 18,000*g* was air-dried and dissolved in 100 ul TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA).

2.3. Polymerase chain reaction

Genomic DNA (150 ng; isolated from the toes) was subjected to PCR using the following primers: $mPAD2_{Exon1}$-fwd, 5′-GCCGCCTATACGGGAAAATATG-3′; $mPAD2_{Intron1}$-rev, 5′-GGTGAAAGGTCAGCCAAGAAG-3′; $mPAD4_{LTR}$-fwd, 5′-GGCGTTACTTAAGCTAGCTTGC-3′; $mPAD4_{Intron1}$-fwd, 5′-CTTTGTTTCATCCGGACCTC-3′; $mPAD4_{Intron1}$-rev, 5′-GAAATGTCAGCTCGGTCCTC-3′.

To determine the amount of PAD4 mRNA present in the PAD4-low mice the following primers were used: mP4-E5-Fo, 5′-ATGGACTTTGAGGATGAC-3′; mP4-E8-Re, 5′-TGTCTTGGAACACCAGGG-3′; mP4-E1-Fo, 5′-GTGTGTGTGGTGGGCACAG-3′; mP4-E2-Re, 5′-ACTGGAGGACCATGGATGAC-3′;

As a DNA quality control and/or reference beta-actin primers and GAPDH primers were used: beta-actin-fwd, 5′-ACTCCATCATGAAGTGTGACG-3′ and beta-actin-rev, 5′-CATACTCCTGCTTGCTGATCC-3′. GAPDH-fwd, 5′-TGTGTCCGTCGTGGATCTGA-3′ GAPDH-rev, 5′-TTGCTGTTGAAGTCGCAGGAG-3’.

PCR reactions were performed with Red hot polymerase (ABgene) according to the manufacturer's protocol. Annealing temperature and elongation time were dependent on primer combinations (Supporting Table 2) used and the length of the amplicon. A total of 38 cycles were used for amplification.

Quantitative RT-PCR (qRT-PCR) was performed using POWER SYBR Green PCR master mix (Applied Biosytems) with a total of 40 cycles for amplification. The relative PAD4 mRNA expression was analyzed with the $2^{-\Delta\Delta Ct}$ method as previously described [29].

2.4. Tissue isolation and preparation

Mouse tissue samples were obtained from the animal facility at the Radboud University Nijmegen. Freshly dissected tissue samples (pooled material from two mice per group), kept on ice, were