no difference in these variables could be seen between the groups of rats maintained on different sodium diets. Thus, sodium diet maintains an influence on gene expression of ANF after three weeks in rats with 1-K, 1-C renal hypertension but not in normotensive animals. At the present time, it is impossible to provide any clear reason for this different behavior of sodium-dependent ANF synthesis. It is nevertheless attractive to speculate that the concomitant hypertension may somehow contribute to the different secretory profile of the 1-K, 1-C renal hypertensive animals.

In summary, ANF gene transcription activity in the right and in the left atrium was, for a similar level of blood pressure, higher on a regular- than on a low-sodium diet in rats with established 1-K, 1-C renal hypertension, i.e. in animals known to have a suppressed and an activated renin-angiotensin system, respectively. The enhanced gene expression was associated with increased circulating levels of ANP only in some of the animals.

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Peptidylarginine deiminase in rat and mouse hemopoietic cells

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Summary. Peptidylarginine (protein-L-arginine) deiminase activities have been demonstrated in extracts of rat and mouse peritoneal macrophages, bone marrow cells, splenic adherent cells, neutrophils, and mouse monocyte/macrophage cell lines. The enzyme in these cells is indistinguishable from the skeletal muscle enzyme with respect to immunochemical properties.
Key words. Peptidylarginine deiminase; L-citrulline; hemopoietic cells.

Peptidylarginine (protein-L-arginine) deiminase (EC 3.5.3.15; PAD), which catalyzes the conversion of L-arginine residues in proteins to citrulline residues, has been described in various vertebrate tissues[1-5]. Although the distribution of PAD in functionally distinct mammalian tissues, and its unequivocal enzyme reaction, suggest the importance of this enzyme in the functional and metabolic modification of proteins, its role in cellular physiology is still largely unknown. Recently, it has been reported that murine bone marrow cells[6,7] and cytotoxic activated macrophages[8,9] can produce L-citrulline without the involvement of the urea cycle. The authors of these papers rule out a possible involvement of PAD in this citrulline-producing pathway, since they failed to demonstrate PAD activity in the lysates of these cells. But, in the study described here, we were able to demonstrate that rat and mouse hemopoietic cells, including macrophages and bone marrow cells, and some mouse leukemia cell lines do have PAD. Evidence was also obtained that the PAD in these cells is indistinguishable from the rat skeletal muscle enzyme.

Materials and methods

Female Wistar rats and C57BL/6 mice, 2–6 months old, were obtained from Shizuoka Experimental Animal Firm (Shizuoka) or from the animal facilities of Tokyo Metropolitan Institute of Gerontology. Animals were killed by cervical dislocation after ether anesthesia, and thymuses and spleens were dissected out and teased to make cell suspensions in phosphate buffered saline (PBS). Peritoneal exudate cells were harvested 4 days after the injection of 3% thioglycollate (Difco, Detroit), and macrophages were enriched by adherence to plastic dishes. To obtain neutrophils, peritoneal exudate cells were collected 20–24 h after copper rod transplantation[10] and centrifuged on the mouse lymphocyte separation medium M-SMF (P = 1.090; JIMRO, Tokyo). Neutrophils were recovered in the pellet with more than 95% purity. Tibial marrows were flushed with PBS containing 5 mM EDTA and the bone marrow cells were suspended by pipetting in centrifuge tubes. The cells were resuspended in Tris-buffered 150 mM NH_4Cl (pH 7.2) to lyse erythrocytes, washed and counted using a hemocytometer. Cells from 3–5 rats were pooled for each experiment. Mouse monocyte/macrophage cell lines, J774A.1, P388D1 and WEHI-3 were obtained from the Japanese Cancer Research Resources Bank, and mouse thymic lymphoma EL-4, plasmacytoma P3-X63Ag8.653 and mastocytoma P-815 from the stocks of our laboratory. The cells were cultured in RPMI-1640 or Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (all from GIBCO, Gland Island) in 5% CO_2 and 95% air at 37 °C.

After washing with PBS, cells were incubated for 30 min on ice in a lysis buffer containing 0.25% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl (pH7.4), 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. The lysates were centrifuged for 10 min at 10 000 g, and the supernatants were kept at − 70 °C until enzyme assay. PAD activity was determined using N-benzoyl-L-arginine-o-ethylester (BAEE; Nakarai, Tokyo) as a substrate according to the previously described procedure[4]. The reaction mixtures were treated for 1 h at 37 °C with urease (2.3 U/ml), before the quantitation of L-citrulline by a colorimetric method[11]. One unit of enzyme activity was defined as the activity required to produce 1 µmole of L-citrulline derivatives in 1 h at 50 °C. To examine possible interference with the PAD assay by arginine deiminase or arginase, the extracts were incubated with the substrate buffer containing L-arginine in place of BAEE. L-citrulline produced in the reaction mixtures was quantitated as described above, and urea and ammonia by using the F-kit urea/ammonia from Boehringer (Mannheim).

For immunoprecipitation, 200 µl samples of lysates were incubated for 3 h at 4 °C, with continuous rocking, with protein A-Sepharose CL-4B (Pharmacia, Uppsala) coated with either rabbit anti-rat muscle PAD antibody[4] or normal rabbit IgG (20 µg IgG). The mixtures were centrifuged and the supernatant was saved for the enzyme assay. The precipitated beads were washed and resuspended in 200 µl of extraction buffer for the enzyme assay (our anti-PAD antibody does not block the enzyme activity).

Western immunoblot analyses were performed as described previously[4].

Results and discussion

When the rat macrophage extract was incubated with BAEE, a ureido compound was produced in the reaction mixture that was detected by the colorimetric reaction. This compound was resistant to urease treatment and its absorption spectrum was identical to that of L-citrulline (maximum absorption at 528 nm), identifying it as a L-citrulline derivative. The production of an approximately equal amount of ammonia was also detected in the reaction mixture (table 1). When the extracts were incubated similarly with L-arginine, production of urea but only of trace amounts of citrulline and ammonia was detected, demonstrating arginase but not arginine deiminase activities (table 1).

The macrophage extracts were immunoprecipitated with anti-rat skeletal muscle PAD antibody, and the supernatant and precipitates were incubated with BAEE or L-arginine. The results summarized in table 1 showed that the activity which produced L-citrulline from BAEE was specifically immunoprecipitated (table 1). Arginase activity remained in the supernatant. These results, and those of Western immunoblot analyses (see below) demonstrate that the activity in the macrophage extracts which produces L-citrulline from BAEE is that of PAD. The results of PAD assays on cell extracts are summarized in table 2. In the rat cell extracts, the activity was

Table 1. Determination of citrulline, urea and ammonia production after incubation of rat macrophage extracts with BAEE or L-arginine, before and after immunoprecipitation with anti-rat skeletal muscle PAD antibody

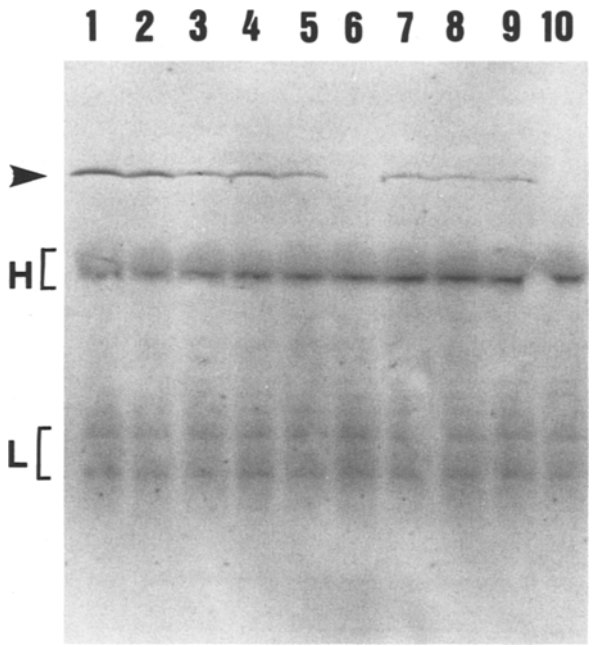
Sample	Substrate	L-Citrulline	Urea	Ammonia
		(µmoles/ml reaction mixture)		
Extraction buffer	BAEE	< 0.01	< 0.01	< 0.01
Macrophage extract	None	< 0.01	0.09	0.06
	BAEE	0.38	0.07	0.42
	L-arginine	0.02	0.82	0.03
Macrophage extract after immunoprecipitation				
Supernatant	BAEE	0.01	0.04	0.03
	L-arginine	0.02	0.63	0.01
Precipitate	BAEE	0.31	< 0.01	0.29
	L-arginine	< 0.01	< 0.01	0.02

After enzyme samples had been incubated with same volume of substrate solution for 1 h at 50 °C, amount of citrulline, urea and ammonia in reaction mixtures was determined as described in materials and methods. Each value indicates the increase of each substance during incubation. In control immunoprecipitation using normal rabbit IgG, 87% of the citrulline-producing activity was recovered in supernatant. Similar results were obtained in two separate experiments performed under the same conditions.

Table 2. Peptidylarginine deiminase (PAD) activities in rat and mouse hemopoietic cells, and mouse monocyte/macrophage cell lines

Cells	PAD activity (U/10^9 cells)
Rat peritoneal macrophages	14.22 ± 2.21
Rat neutrophils	3.10 ± 1.00
Rat bone marrow cells	2.83 ± 0.75
Rat splenic adherent cells	1.14 ± 0.47
Rat thymocytes	< 0.10
Rat erythrocytes	< 0.10
Mouse peritoneal macrophages	9.08 ± 2.91
Mouse cell lines	
J774A.1	1.36 ± 0.14
P388D1	1.07 ± 0.19
WEHI-3	0.81 ± 0.10

PAD activity was determined for each extract prepared from 3–5 animals or cultured cells as described in materials and methods, and the results were presented as mean ± SD of 3–5 separate assays. No PAD activity was detected in the extracts of mouse thymic lymphoma EL-4, plasmacytoma P3-X63Ag8.653 and mastocytoma P-815.



Immunochemical detection of PAD on Western blots using anti-rat muscle PAD antibody. Extracts prepared from 10^7 cells were immunoprecipitated with anti-rat muscle PAD antibody (lane 1–9) or normal rabbit serum (lane 10), followed by protein A-Sepharose CL-4B. Immunoprecipitated samples from peritoneal macrophages (lane 2), neutrophils (lane 3), bone marrow cells (lane 4), splenic adherent cells (lane 5), thymocytes (lane 6), J774A.1 (lane 7), P388D1 (lane 8), WEHI-3 (lane 9) cells and 10 ng purified rat muscle PAD (lane 1 and 10) were electrophoresed on 10% polyacrylamide gels containing 0.1% SDS. After the transfer to nitrocellulose membrane, PAD on blots was detected using anti-rat muscle PAD rabbit serum and peroxidase-labeled anti-rabbit IgG antibody (Bio Rad, Richmond). Specific bands for PAD (apparent molecular weight of about 80 k daltons) are indicated by arrowhead. H, immunoglobulin heavy chain; L, immunoglobulin light chain.

highest in peritoneal macrophage extracts; activity in extracts of bone marrow cells, splenic adherent cells, and neutrophils was lower but still at significant levels. No activity was detectable in the lysates of rat thymocytes and erythrocytes. PAD activities were also detected in mouse peritoneal macrophages, bone marrow cells and monocyte/macrophage cell lines, J774A.1, P388D1 and WEHI-3, but not in thymic lymphoma EL-4, plasmacytoma P3-X63Ag8.653, and mastocytoma P-815 cells.

On Western immunoblot analysis, purified rat muscle PAD gave a single peptide band of apparent molecular weight of about 80 k daltons. This band was also seen on the blots of the extracts from macrophages, neutrophils, bone marrow cells and splenic adherent cells, but it was hardly detected on the blots of rat thymocyte and mouse leukemia cell extracts. However, when the immunoprecipitates from the larger amount of extracts were analyzed by immunoblotting, the band was clearly visualized in the extracts of all these cells except thymocytes (fig.).

The above results present a sharp contrast to the previous reports[6–9] that describe the failure to detect PAD activity in murine bone marrow cells and cytotoxic activated macrophages. This discrepancy may be, at least in part, due to differences in the sensitivity of the enzyme assays used. The muscle-type PAD shows only limited (less than 20%) activity towards N-benzoyl-L-arginine, which was used as the substrate in the previous study, as compared to that towards BAEE, which was used here[3,4]. Previous papers have described at least 3 types of PAD in mammalian tissues, designated from their localization as muscle, hair root and epidermal types, which differ in substrate specificity and immunological properties[2,4]. Since the anti-rat skeletal muscle PAD antibody used here specifically recognizes muscle-type enzyme[4], the PAD found in the rat and mouse hemopoietic cells is probably of the muscle type.

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