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Protein Deimination in Human Health and Disease

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On behalf of the editors, I wish to dedicate this book to the memory of Kerri Anne Mowen, who sadly passed away at age 41 on February 14, 2016 of a brain aneurysm. Although I had long been familiar with her work, I first met Kerri in 2008 at the FASEB Methylation meeting where we shared our equal passion for both the protein arginine deiminases and protein arginine methyltransferases. What impressed me most about our first meeting was that Kerri was not only whip smart but also a joy to be around. We quickly became collaborators,

and most importantly friends, leading us eventually to cofound Padlock Therapeutics. Kerri's contributions to the PAD field are indelible and include developing both PAD2 and PAD4 knockout mice, helping establish the key role of PAD4 in NETosis, and establishing the importance of PAD4 activity in the initiation versus effector phases of rheumatoid arthritis. Her imprint on the PAD field will long be felt, and her future contributions sadly missed.

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Chapter 1
A History of Deimination Research in Japan: The Founding Fathers

Hidenari Takahara

The research on protein deimination and the enzymes responsible for catalyzing this posttranslational modification started from investigations of the hair follicle and myelin in central nerve system. About 60 years ago, in 1958, the presence of protein-bound citrulline was first reported by Dr. George Rogers (Adelaide University, Australia) in the protein of the inner root sheath (IRS) of hair follicles (Rogers 1958). In order to obtain information about the protein composition of the IRS, he conducted a quantitative amino acid analysis of an acid hydrolysate on sufficient amounts of IRS that were dissected from the vibrissae follicle of rats. At that time, the common method for separating amino acids was paper chromatography, and when applied to the IRS hydrolysates, citrulline was discovered as a distinct ninhydrin-positive spot in an area adjacent to the basic amino acids. About 10 years after Rogers's discovery, Dr. Mario Moscarello (Toronto University, Canada) started an intensive investigation of myelin sheath proteins in the central nerve system. In 1971, he also found the presence of peptide-bound citrulline in myelin basic protein (MBP) using similar methods to Rogers (Finch et al. 1971). Moscarello continued the investigation of MBP until he passed away in 2013, publishing many papers concerning the hyper-deimination of MBP in the pathology of multiple sclerosis. His research career involving the deimination of MBP was described in a eulogy in the first volume of this book series (Nicholas and Bhattacharya 2014).

Although it was unclear as to how the citrulline was incorporated into proteins, the source was thought to be arginine. In 1977, Rogers and colleagues (1977) were the first to conclusively demonstrate that arginine residues were indeed converted to citrulline via a deimination reaction where they combined hair follicle extracts with calcium to promote this reaction. Following this report, research to identify the specific deiminating enzyme was energetically carried out in Japan. In 1979, Dr. Kiyoshi Sugawara (Fig. 1.1; Ibaraki University, Japan) reported the presence of protein-bound

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Fig. 1.1 The founding fathers: *left*, Professor Dr. George Rogers (Adelaide University, Australia). *Center*, Professor Dr. Hidenari Takahara (Ibaraki University, Japan). *Right*, Professor Dr. Kiyoshi Sugawara (Ibaraki University, Japan). Taken at the first International Symposium of Deimination and Skin Biology, April 2009 in Osaka, Japan

citrulline in the epidermal proteins of newborn rat (Sugawara 1979). Successively, he demonstrated the existence of the enzyme that converts arginyl to citrulline residues in the extracts from the newborn rat epidermis (Fujisaki and Sugawara 1981). To assay the enzyme activity, he introduced a colorimetric method using simple synthetic substrates of arginine blocked at the N- and C-terminals as a substrate. In this procedure, high temperatures (over 50 °C) and the presence of dithiothreitol (DTT) greatly enhanced enzyme activity. According to these procedures, he could overcome tedious and laborious work that was needed to measure enzyme activity using an amino acid analyzer. Dr. Sugawara then introduced the logical name peptidylarginine deiminase (PAD) for the enzyme, because it acts on arginine residues embedded in a peptide backbone and is distinct from deiminases that act on free arginine (Fujisaki and Sugawara 1981). In this year, PAD was registered as new enzyme to IUPAC Enzyme Committee and was classified into EC 3.5.3.15.

In 1982, I joined Dr. Sugawara's laboratory. This was just after he obtained a new finding that the extract from rabbit skeletal muscle contains very high deiminase activity, about 120-fold compared to that of the newborn rat epidermis. Since the available amounts of the newborn rat epidermis were very low and the tissue preparations were burdensome, the high abundance of a PAD in rabbit skeletal muscle was an exciting research finding that gave me a tremendous head start to further characterize this enzyme. My first research project at Ibaraki University was to purify the PAD from rabbit skeletal muscle. Very fortunately, I quickly purified the enzyme to homogeneity and determined the chemical, physiochemical, and kinetic properties toward several synthetic arginine derivatives including natural proteins (Takahara et al. 1983). This was the first and most definitive report demonstrating that the

enzyme could catalyze the conversion of arginyl to citulline residues in native protein substrates in vitro. Among the protein substrates examined using this purified PAD, the reaction toward the Kunitz soybean trypsin inhibitor (STI) attracted our attention (Takahara et al. 1985). The effect of the enzyme on STI activity was remarkable as treatment with this PAD rapidly abolished the inhibitory activity of STI without altering its overall conformation; complete inactivation of STI was attained within several minutes at 37 °C. Surprisingly, only the modified arginine residue was the reactive site (or primary contact site) despite the fact that all of the remaining nine arginine residues in STI are exposed on the protein surface (Takahara et al. 1985). This study was first an indication of a biological function for the deimination and biochemical application of PAD. Furthermore, the observation that skeletal muscle PAD showed a high affinity for only the functional arginine residue in STI inspired the idea of an effective affinity adsorbent composed of immobilized STI for PAD purification. Our expectation was fully realized, as a 1800-fold purification with 50% yield was achieved by this affinity column (Takahara et al. 1986). Thereafter, we could supply a sufficient amount of purified rabbit skeletal muscle PAD to other researchers. Although recombinant enzymes from various sources superseded the rabbit skeletal muscle PAD since the latter half of the 1990s, the natural enzyme is still under requisition today. Several earlier experiments conducted with rabbit skeletal muscle PAD elicited important insights into the physiology and pathophysiology of protein deimination. For instance, our collaborative work with Dr. Masaki Inagaki (Aichi Cancer Center, Japan) provided very interesting results. In general, vimentin, an intermediated filament protein, is expressed by various cells and forms a stable, less dynamic molecular network. In 1989, we found that there was a complete loss of filament-forming ability of vimentin after PAD treatment. The enzyme could also deiminate the filaments that had been polymerized and induced filament disassembly. The deimination reduced the isoelectric point of the head domain, in which the positive charge of arginine residues are essential for maintaining the ability to form filaments, resulting in the complete loss of their intermediate filament constructs. Similar results were obtained with other intermediate filaments such as desmin and glial fibrillary acidic protein (GFAP) (Inagaki et al. 1989). Thus, we presumed that deimination of intermediated filaments controls the cytoskeletal network. This hypothesis was verified by several subsequent reports by others in the field. In particular, deiminated vimentin was found in vivo, and this modification triggers structural collapse and promotes apoptosis (Asaga et al. 1998; Hsu et al. 2014). There is also some evidence that deimination of GFAP is a characteristic feature of neurodegenerative diseases (Ishigami et al. 2005).

In parallel with research on rabbit skeletal muscle PAD, we attempted to develop a model system using the mouse/rat for investigation of the physiological function of PAD. From 1988, Dr. Tatsuo Senshu (Tokyo Metropolitan Institute of Gerontology, Japan) started his investigations into the PADs. Together, by 1995, our findings, coupled with those of Senshu's laboratory, established that PAD is widely distributed in many tissues with the notable exception of serum and the location of the enzyme was essentially in cytoplasm. Among the tissues tested thus far, the activity of PAD in the salivary glands, pancreas, and uterus far exceeded those of any other tissues.

Immunohistochemical analyses indicated that the enzyme is preferentially located in acinal cells of the salivary glands and pancreas and in the luminal and glandular epithelia of the uterus (Takahara et al. 1989; Watanabe et al. 1988). Additionally, we noted estrous cycle-dependent changes in enzyme expression in the uterus, with the level being highest and lowest at diestrus (Takahara et al. 1989). Senshu's group also found estrous cycle-dependent change of this enzyme in the rat pituitary gland (Senshu et al. 1989). The expression of PAD in the pituitary and uterus responds adequately to administration with 17β-estradiol (Senshu et al. 1989; Takahara et al. 1992). In the uterus, a remarkable series of events takes place during the estrous cycle. The luminal and glandular epithelia of the uterus at the estrous stage show hyperplasia and vigorously secrete fluid into the lumen. Therefore, PAD may be important for exocrine events, but the physiological roles of PAD in the uterus and pituitary are still unknown.

During investigations of PAD activity in the skin, we had a question: why are the substrate specificities of the skeletal muscle PAD toward several arginine derivatives different from those of the epidermal PAD reported previously (Fujisaki and Sugawara 1981)? Both PADs showed high activity toward the synthetic arginine derivatives blocked at the N- and C-termini (i.e., benzoyl-L-arginine ethyl ester), whereas the skeletal muscle PAD showed very low activity to C-terminal free arginine derivatives such as benzoyl-L-arginine and acetyl-L-arginine. On the other hand, these C-terminal free substrates were comparably processed by epidermal PAD. This question was resolved by our comprehensive work published in 1991 (Terakawa et al. 1991). We compared the elution profiles of the PAD activities of the extracts from several tissues of mouse using anion-exchange chromatography, in which PAD activity was simultaneously measured with the different substrates. As shown in Fig. 1.2, three peaks were eluted upon chromatography of the skin extract. Since each peak showed different substrate specificities, we proposed designating them as peptidylarginine deiminase type I (PAD1), II (PAD2), and III (PAD3) according to the order of elution. The extracts of the skeletal muscle, pancreas, salivary gland, and brain (spinal cord) showed a single peak that corresponded to type II enzyme. Type I enzyme is specifically located in the uterus and epidermal cells, and type III enzyme is present in the hair follicle. These three types of enzyme were not significantly different in catalytic properties, including absolute dependence on calcium ions for activity and the stimulation with DTT. Senshu's group also described the presence of three isozymes in rat tissues and called them "epidermal type, skeletal muscle type, and hair follicle type," which correspond to PAD1, PAD2, and PAD3, respectively (Watanabe et al. 1988). Thereafter, by innovative techniques such as molecular genetics and proteomics, two new PAD isozymes were found in rat epidermis (Yamakoshi et al. 1998)/a keratinocyte cell line (Ishigami et al. 1998) and in the mouse ovary (Wright et al. 2003), and they were named PAD4 and ePAD, respectively. In 1999, Dr. Michiyuki Yamada (Yokohama City University, Japan) and colleagues identified a novel PAD in human myeloid leukemia HL-60 cells, which can induce to differentiate into granulocytes by retinoic acid (Nakashima et al. 1999). By comparison of the amino acid sequence and substrate specificity of HL-60 PAD with those of the four known rat PADs, they

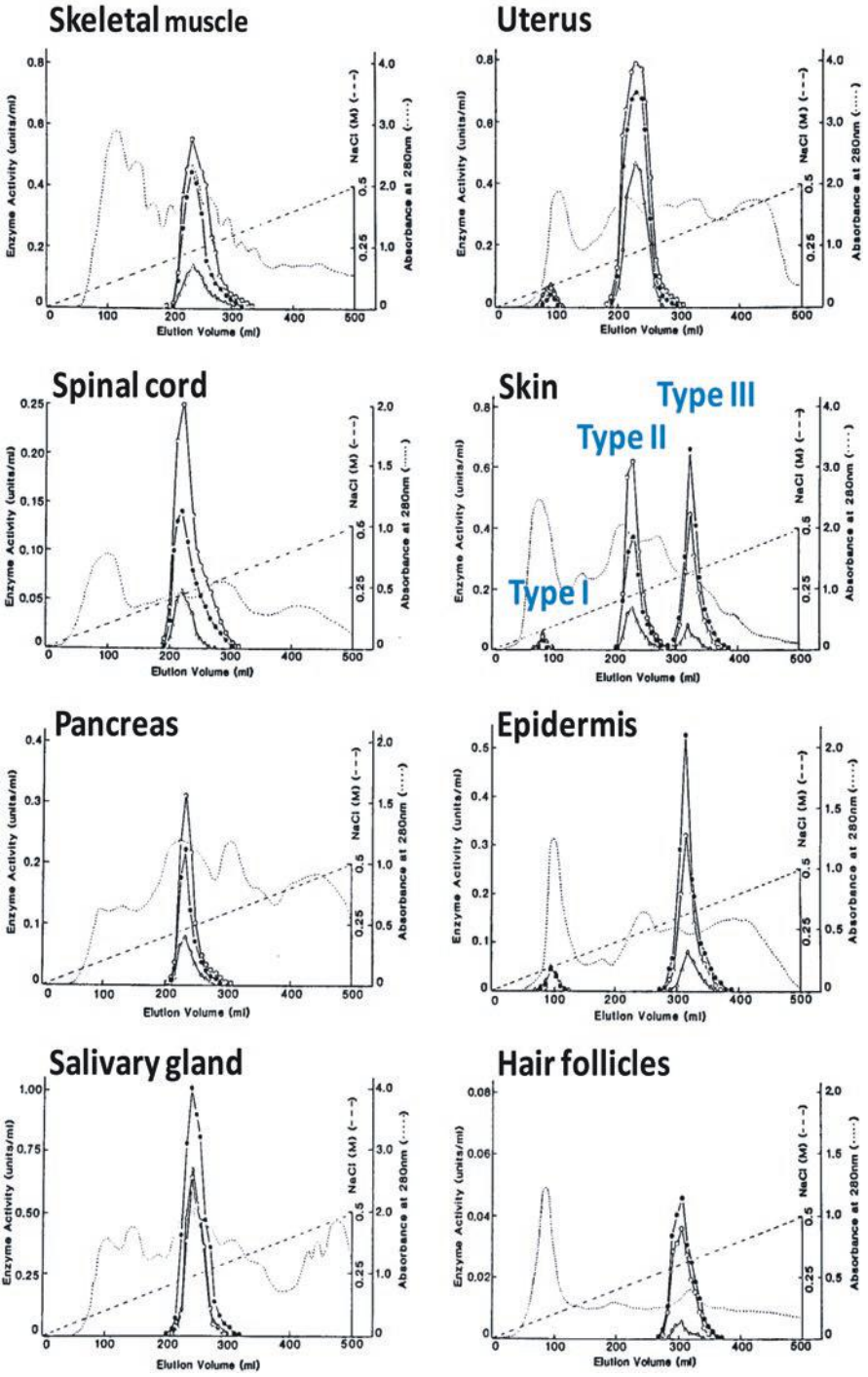

Fig. 1.2 DEAE-Sephacel ion-exchange column chromatography of the PAD activity from various tissues from mouse. The substrates used for measuring PAD activity were Benzoyl-L-Arg-*O*-ethyl ester (*open circle*), Benzoyl-L-Arg (*open triangle*), and protamine (*filled circle*)

concluded that HL-60 PAD did not belong to any PADs and named it PAD5 (Nakashima et al. 1999). However, human PAD5 proved to be the human orthologue of mouse PAD4 (Chavanas et al. 2004), and it was subsequently named PAD4 by the HUGO Gene Nomenclature committee (HGNC). In addition, to avoid confusion, the HGNC recommended that PAD5 remains unused and ePAD be renamed PAD6. In total, it is now recognized that there are five PAD isozymes, i.e., PAD1, PAD2, PAD3, PAD4, and PAD6.

Cloning of the cDNAs for the five PAD isozymes was a historic struggle that stretched from 1989 to 2004. In 1989, Watanabe and Senshu first reported on the cDNA of rat PAD2 and deduced its amino acid sequence (Watanabe and Senshu 1989). Four years later, we revealed at long last in the full nucleotide sequence of mouse the PAD2 cDNA (Tsuchida et al. 1993). Looking back on that time, we had to overcome several obstacles to reach our goal. The N-terminal amino acid sequence of mouse PAD2 was N^{α}-acetyl-Met-Gln-, a sequence which has never previously been reported among N^{α}-acetyl-Met protein. As such, it was difficult to assign the methionine codon (ATG) at the translational start of the cDNA. In 1997, we purified a small amount of PAD3 from newborn rat epidermis by a procedure that included STI-affinity chromatography and carried out peptide mapping by the in situ protease digestion method (Nishijyo et al. 1997). Subsequently, we succeeded in cloning the cDNA and sequenced the full-length cDNA encoding rat PAD3 by RT-PCR and 3′−/5′-RACE methods using synthesized nucleotide primers designed from the internal amino acid sequences (Nishijyo et al. 1997). This was the first report exhibiting the entire amino acid sequence of the isozyme, and by alignment of the PAD2 and PAD3 sequences, we found that a half of C-terminal region was highly conserved, and we predicted that the conserved region was likely responsible for the catalytic activity of this enzyme. This notion was ultimately proved by the excellent works of Dr. Mamoru Sato (Yokohama City University, Japan) and his coworkers in 2004, who determined the X-ray crystal structure of human PAD4 (Arita et al. 2004). These leading researches ultimately resulted in the cloning of the cDNAs for other PAD isozymes. Most cDNA isozymes from the rodent (Yamakoshi et al. 1998; Ishigami et al. 1998; Wright et al. 2003; Watanabe and Senshu 1989; Tsuchida et al. 1993; Nishijyo et al. 1997; Rus'd et al. 1999) and human PAD (Nakashima et al. 1999; Chavanas et al. 2004; Kanno et al. 2000; Ishigami et al. 2002; Guerrin et al. 2003) were established in laboratories in Japan. In addition, we produced the bacterial recombinant PADs of mouse and human in run-up to other laboratories (Ohsugi et al. 1995). The constructed plasmids had a unique DNA linker containing a pair of Shine-Dalgarno sequences and a short preceding cistron inserted into the adjacent 5′-region of the coding region, so that we could obtain a large quantity of the PADs without a sequence tag in bacteria. These recombinant PADs were also easily purified by STI-affinity chromatography and helped a great deal for many investigations (Ohsugi et al. 1995).

In 1992, Senshu and coworkers developed an excellent procedure for the detection of deiminated proteins on membranes or fixed tissues (Senshu et al. 1992). This method involves a three-step process. In the first step, citrulline-containing proteins immobilized on the membrane or fixed tissues are chemically modified. In the