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AMINOPEPTIDASE ACTIVITY IN NORMAL PRIMATE SKIN AND ITS CHANGES IN EXPERIMENTALLY PRODUCED PATHOLOGICAL LESIONS*

K. ADACHI, M.D., PH.D., S. YAMASAWA, B.S., F. ALLEGRA, M.D., M.J.C. IM, PH.D. AND S. KONDO, M.D.

Among the exopeptidases (1, 2), leucine aminopeptidase (LAP), which occurs in nearly all mammalian tissues (2), including skin (3), has been most frequently studied. The common substrate for LAP has been leucyl-β-naphthylamide, which provides a simplified and rapid procedure for both histochemical demonstration and biochemical assay of LAP in tissue. This use was suggested by the discovery that an enzyme that can split the peptide bond of the original substrate, leucylglycine (2), can also degrade leucyl-β-naphthylamide. As a result, the hydrolysis of amino acid-β-naphthylamides by tissues has been attributed to LAP action.

Recently, however, the validity of this assumption has been questioned, since arylamidase (or naphthylamidase) activity, which is distinctly different from that of LAP, has been shown to occur in serum (4), liver (5, 7), kidney (6), pancreas (7), and intestine (7). Behal *et al.* (8) apparently made the decisive finding when they chromatographically isolated two different enzymes, α and β, which can attack the same substrate. The former, like the classical LAP, can degrade both leucylglycine and leucyl-β-naphthylamide whereas the other, β, can split only leucyl-β-naphthylamide.

In the present paper, we are reporting: 1) the partial characterization and normal distribution of dipeptidases in rhesus monkey skin assayed with substrates like leucylalanine and alanylglycine, which arylamidase is unable to attack; 2) the changes in peptidase activities in pathological lesions experimentally produced in primate skin.

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* From the Oregon Regional Primate Research Center, Beaverton, Oregon 97005.

MATERIALS AND METHODS

All skin samples used in this study were freshly removed from normal adult rhesus monkeys without local anesthesia. Epidermis obtained from the abdominal and chest regions with a keratome (0.1 mm blade thickness) served as samples for the partial purification and characterization of dipeptidases. It was homogenized with a ground glass homogenizer at a concentration of 2 to 10% (wet weight/volume) in water. The crude enzyme (homogenate) was partially purified by vertical starch-gel electrophoresis in a manner identical with that described by Smithies (9). To study the normal distribution of dipeptidases in various parts of skin, we first prepared (10, 11) frozen-dried sections of scalp, lip, and sole skin from which we microdissected small samples (0.2 to 2.0 μg) of epidermis and skin appendages for subsequent micro procedures (11, 12).

Dipeptidase Assay.—The principle of the assay procedure is as follows. When the substrates (either L-leucyl-L-alanine or L-alanylglycine) have been hydrolyzed, alanine is converted first to pyruvate and then to lactate by the addition of the auxiliary enzymes, glutamate-pyruvate transaminase and lactate dehydrogenase, and cofactors. Since the conversion of pyruvate to lactate is a pyridine nucleotide-linked reaction, this series of reactions makes it possible to quantify dipeptidase activities in μg skin samples by the use of highly sensitive fluorometry (11, 12). The complete reagent mixture consisted of 10 mM alanylglycine (or 5 mM leucylalanine), 5 mM α-ketoglutarate, 0.5 mM nicotinamide adenine dinucleotide, reduced (NADH), 0.01% bovine plasma albumin, 10 μg/ml lactate dehydrogenase, 10 μg/ml glutamate-pyruvate transaminase, 0.1 M tris-HCl buffer pH 8.25, and 5 μl of epidermal homogenate in a total volume of 50 μl. After the reagent mixture had been incubated at 37°C for 30 minutes, the nicotinamide adenine dinucleotide, oxidized (NAD^+) formed was measured as described in the previous report (12). Blanks and internal standards with NAD^+ were run simultaneously. The internal standards with pyruvate or alanine gave the same values as the NAD^+ standards within the limits of linearity. Arylamidase assay was also carried out (8) in an experiment.

The microenzyme assays on the dissected frozen-dried samples were carried out with the same reagent mixture, but in a total volume of 10 μl and with an incubation time of 60 min. at 37° C. The NAD^+ formed was measured in an identical manner.

All chemicals except the enzymes used in this study were purchased from Sigma Co.. The crystalline glutamate-pyruvate transaminase, a product of Calbiochem, had a specific activity of 40 μmoles/min/mg protein at 30° C. The crystalline lactate dehydrogenase, a product of Sigma, had a specific activity of 500 μmoles/min/mg protein at 37° C.

Experimental production of pathological lesions. —Pseudoepitheliomatous hyperplasia was produced by simultaneous paintings of 0.5% 9,10-dimethyl-1,2-benzanthracene (DMBA) in acetone and the cocarcinogen dodecylbenzene 3 times weekly for 5 weeks on the backs of prosimians, *Perodicticus potto* and *Galago crassicaudatus*.

The skin lesions were resected under general anesthesia without any topical treatment, and the frozen-dried preparation was made as previously described (10, 11). Enzyme activities in the hyperplastic epidermis and in the adjacent dermis were compared with those in normal epidermis and upper dermis (Fig. 5).

Keratoacanthoma was experimentally produced on the backs of young rhesus monkeys (*Macaca mulatta*) with the same carcinogen and cocarcinogen paintings twice weekly for one year. The tumor was removed and processed in the same way as the hyperplasia. Samples for the enzyme assays were dissected from both the tumor tissue and the adjacent dermis (Fig. 6).

A homograft of whole thickness scalp skin was performed on an adult rhesus monkey. The graft, with underlying host tissue, was biopsied at the second, third, fourth, sixth, and tenth days after transplantation. Tissue samplings for the enzyme assays were always microdissected from the deeper dermis except for the sixth day sample, which included microsamplings from the upper dermis also (Fig. 7).

RESULTS

Partial purification of dipeptidases. Since the only source of enzymes was a limited amount of epidermis, the routine method for enzyme purification (column chromatography) was not practical. Therefore, we applied starch-gel electrophoresis for partial purification of the dipeptidases. One of the typical electrophoretic patterns is shown in Figure 1. Although only a 20-fold purification of the original crude homogenate was achieved, (cf. legends in Figure 1), 2 apparently different fractions were obtained, one containing alanylglycine dipeptidase and the other leucylalanine dipeptidase activity. The former fraction did not hydrolyze much leucyl-β-naphthylamide whereas the latter did (20 units vs. 86 units respectively).

Effect of pH on enzyme activities. The pH activity curves of the crude and partially purified enzymes are shown in Figure 2.

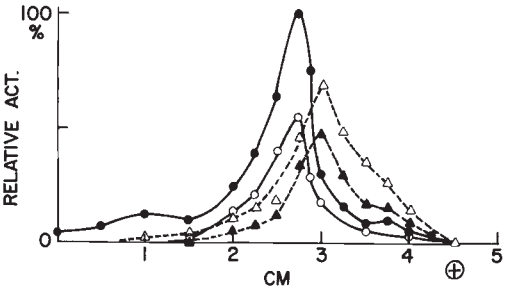

FIG. 1. Starch-gel electrophoresis of epidermal aminopeptidases. Alanylglycine dipeptidase (—●—) and leucylalanine dipeptidase (- - -▲- - -) activities. The peak alanylglycine dipeptidase activity is arbitrarily set for 100% in the ordinate. Note that the addition of manganese chloride (0.2 mM) inhibits alanylglycine dipeptidase activity (—○—) but activates leucylalanine dipeptidase (- - -△- - -); cobalt chloride (0.2 mM) causes almost identical results. This electrophoresis was run for 16 hours at 3° C at 150 volts. The buffer system was 0.1 M veronal-HCl buffer, pH 8.6. The crude enzyme had the specific activity of 17.5 mmoles/hr/mg protein and the peak fraction had 310 mmoles/hr/mg protein (alanylglycine).

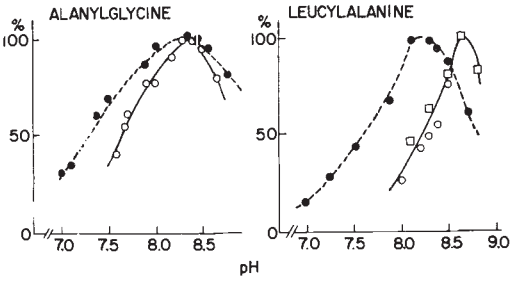

FIG. 2. pH activity curves. Relative enzyme activities at different pH's are expressed as percentages of maximal activity at optimal pH (as 100): partially purified enzyme (——), crude homogenate (- - -). The assay conditions are described in the text but with different pH values of buffers: 0.1 M Tris-HCl (● and ○) and 0.1 M 2-amino-2-methyl-1,3-propandiol-HCl (□).

Leucylalanine dipeptidase exhibited a shift in the optimal pH after purification whereas the optimal pH of alanylglycine dipeptidase remained about the same. These optimal pH values were used for the subsequent assays.

Effect of substrate concentration. The dipeptidase activities of crude and purified enzymes were tested on various concentrations of substrate (Fig. 3). Excessive substrate slightly inhibited the purified dipeptidase activity. In the absence of added substrate, no enzyme reaction occurred with either the crude or partially purified dipeptidase.

The Michaelis constant of the partially puri-

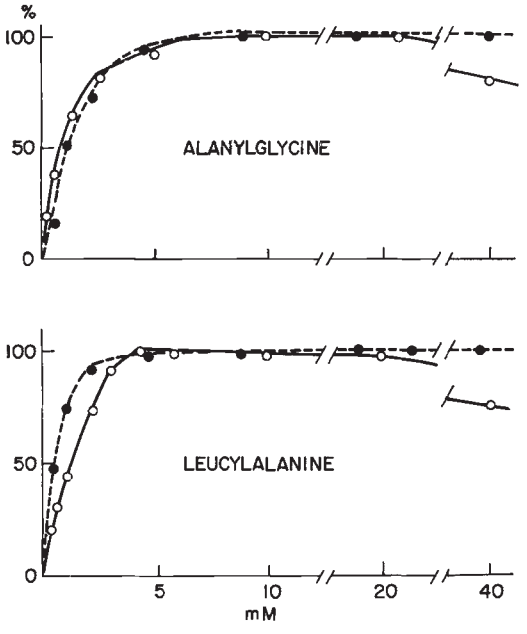

FIG. 3. Effect of substrate concentration. Assay system as in the text. The activities are expressed as percentages to the maximal (100%) activities at the optimal substrate levels.

TABLE I

Effect of activators and inhibitors on epidermal peptidases

Activator or Inhibitor	Peptidase	
	Alanylglycine (%)	Leucylalanine (%)
None	—	—
EDTA	−44	−21
Cobalt chloride	−25	+18
Manganese chloride	−40	+15

The compounds added were all 1 mM in final concentration. Source of enzymes were their respective peak fractions obtained by starch-gel electrophoresis (cf. Fig. 1).

fied alanylglycine dipeptidase was 8×10^{-3} (for alanylglycine) and 1×10^{-4} (for alanylglycylglycine) and that of leucylalanine dipeptidase 5.7×10^{-4} (for leucylalanine) as determined from Lineweaver-Burk plots.

Effect of activators and inhibitors. The results of adding the potential activators or inhibitors for LAP are summarized in Table I. Although the separation of peptidases was incomplete, they showed distinct differences in certain properties. The addition of magnesium ion (1 mM) caused no effect at all.

Effect of enzyme concentration and time course of the reaction. Under the assay condition described here, the reaction proceeded linearly with time and enzyme concentration (both crude and partially purified). A typical time course is shown in Figure 4.

Enzyme activities in various parts of the primate skin. The distribution of enzyme activities in epidermis and skin appendages is summarized in Table II. Generally, the distribution patterns of both alanylglycine and leucylalanine dipeptidase are similar among the different layers of sole epidermis and the various skin appendages, although the activities of alanylglycine dipeptidase are about 2 to 3 times higher than those of leucylalanine dipeptidase at each specific site. The most characteristic feature of these enzyme distributions is seen in the remarkably high activities in the hair follicles compared with those in the epidermis. The standard error of the mean of the activities is large in the hair follicle (also in the dermis). The possible significance of these characteristics will be discussed in the following section.

Enzyme activities in pathological lesions. Since the data on normal skin suggested that the high peptidase activities were associated with mesenchymal tissue, we attempted to

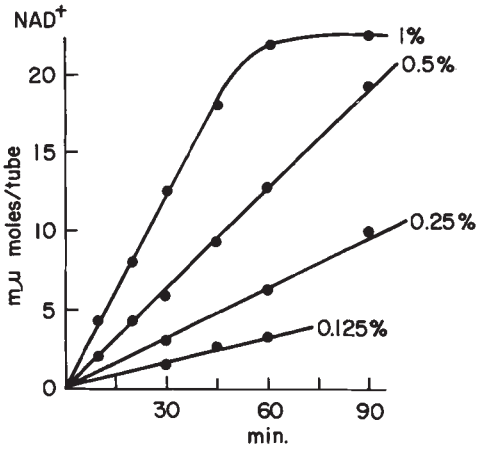

FIG. 4. Effect of enzyme concentration and the time course. The linearity was assessed up to 18–20 mμmoles (alanylglycine) per reaction tube (50 μl in total volume). The crude homogenate was used as enzyme source. A similar range of linearity was obtained with partially purified enzyme for alanylglycine and leucylalanine dipeptidase activities.

assay enzyme activities in experimentally produced pathological lesions in sites where we anticipated a heightening of peptidase activities.

1. Pseudoepitheliomatous hyperplasia. Changes in both alanylglycine and leucylalanine dipeptidase activities are summarized in Table III. In all cases, the enzyme activities in the epidermis increased 2 to 3 times in the hyperplasia whereas those in the dermis increased 10 to 15 times at comparable sites in the hyperplasia.

2. Keratocanthoma. Changes in leucylalanine dipeptidase activities at various sites (Fig. 6) of the pathological lesions are summarized in Table IV. Again it is clear that the enzyme activities in keratoacanthoma (epidermal cells) were increased up to 3 times whereas those in the upper dermis (adjacent to the keratoacanthoma) showed remarkable increase. Changes in enzyme activities in the deeper dermis also increased but less significantly.

3. Skin homograft. Both alanylglycine and

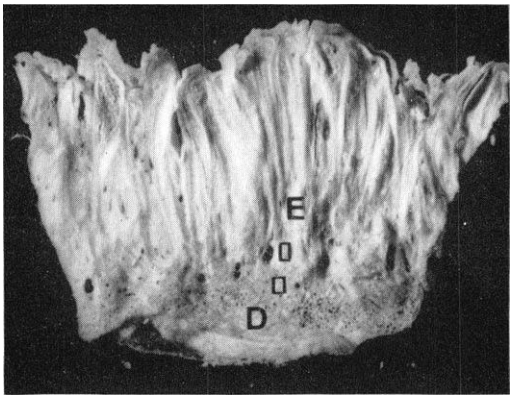

FIG. 5. Pseudoepitheliomatous hyperplasia. Frozen-dried preparation (unstained) shows considerable thickening and downward proliferation of the epidermis. The rectangular areas indicate the specific sites where the samples of both the epidermal hyperplasia and the adjacent dermis were microdissected.

TABLE II

Alanylglycine and leucylalanine peptidase activities in the various parts of rhesus monkey skin*

Region	Substrate	
	Alanylglycine	Leucylalanine
Scalp		
Epidermis	1.4 ± .13	0.60 ± .05
Hair follicle**	11.8 ± 1.5	5.6 ± .46
Eccrine gland	5.7 ± .62	2.8 ± .26
Sebaceous gland	2.6 ± .14	1.3 ± .09
Dermis	0.15 ± .05	0.16 ± .02
Sole		
Keratin layer	≦0.1	≦0.1
Granular layer	0.58 ± .08	0.36 ± .03
Prickle layer	1.5 ± .09	0.59 ± .04
Basal layer	2.2 ± .27	0.89 ± .06
Eccrine gland	5.5 ± .58	1.3 ± .25
Dermis	0.16 ± .05	0.17 ± .03
Lip		
Mucous, upper	7.9 ± .35	3.4 ± .45
Mucous, lower	4.9 ± .54	2.5 ± .48
Sebaceous gland	3.9 ± .57	1.7 ± .16

* The enzyme activities are expressed as moles of substrate converted per hour per kg of dry weight tissue (± standard error of mean). Each figure is the mean of 5 determinations.

** The samples were obtained generally from bulb portions. The middle portions of the external hair sheath contain approximately 20% less activities than the bulb portions do (3 determinations only).

TABLE III

Aminopeptidase activities in the experimental pseudoepitheliomatous hyperplasia***

		Substrate	
		Alanylglycine	Leucylalanine
Perodicticus potto			
normal	epidermis	1.59 ± .20	.85 ± .09
	dermis	.18 ± .05	.16 ± .05
hyper.	epidermis	4.51 ± .43	1.87 ± .16
	dermis	4.00 ± .68	2.21 ± .31
Galago crassicaudatus			
normal	epidermis	1.33 ± .13	.52 ± .05
	dermis	.19 ± .03	.25 ± .05
hyper.	epidermis	4.02 ± .73	1.76 ± .33
	dermis	3.39 ± .41	2.24 ± .20

* Enzyme activities are expressed as moles per kg dry weight sample per hour (± standard error of mean). Each figure represents an average of 10 determinations on 2 animals (2 *P. potto* and 2 *G. crassicaudatus* were used).

** The microdissection of the pathological lesions are illustrated in Figure 1.

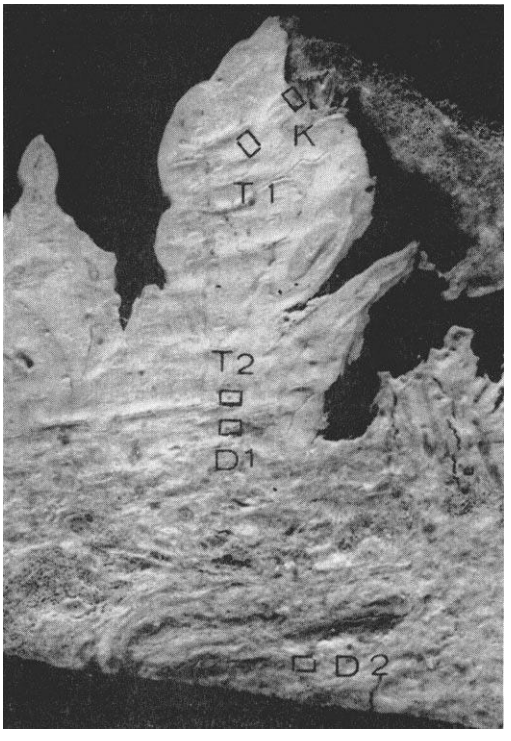

FIG. 6. Keratoacanthoma. Frozen-dried sections (unstained). The rectangular sites (1–5) were microdissected for the enzyme assays: (1) keratin, (2) tumor cells from the protruding portion near the surface (sample tumor I in Table II), (3) tumor cells at the base of the keratoacanthoma (sample tumor II in Table II), (4) upper dermis adjacent to the base of the tumor (dermis I in Table II) and (5) lower dermis (dermis II in Table II).

leucylalanine peptidases were assayed after a whole thickness homograft. Here we focused on the enzyme changes in the deeper dermis and the granulation tissue at the junction of the grafted skin. Generally, the enzyme activities decreased at the outset (2nd day), then increased concomitantly as the granulation tissue developed. The fully developed granulation tissue at day 6 showed 10 to 20 times more peptidase activity than normal dermis, whereas the upper dermis showed normal peptidase activities (Table V).

DISCUSSION

Although a variety of enzyme systems in skin have been quantified biochemically in recent years, practically no one has attempted to purify a skin enzyme except Liss and Lever, who purified ribonuclease from psoriatic scales (13) and Zaruba *et al.*, who isolated phosphodiesterase from mouse skin (14). Obviously, however, purification is one of the most important steps in characterizing an enzyme in a tissue, particularly when it has a broad spectrum of specificity. This neglect is chiefly due to the complexity of skin structures and hence to the limited supply of "pure" samples.

In this study, we have attempted to purify the epidermal peptidases with starch-gel elec-

TABLE IV

Aminopeptidase activities in the experimental keratoacanthoma***

	Alanylglycine	Leucylalanine
Normal		
epidermis	1.47 ± .15	.57 ± .02
dermis	.13 ± .04	.11 ± .04
Acanthoma**		
keratin	.18 ± .03	.09 ± .03
tumor I	1.96 ± .31	1.12 ± .14
tumor II	3.76 ± .70	1.62 ± .07
dermis I	1.74 ± .44	.96 ± .27
dermis II	.75 ± .21	.30 ± .05

* Expressed as moles per kg dry weight per hour (± standard error of mean). Each figure is an average of 5 determinations. All samples were obtained from the same rhesus monkey (the normal skin was biopsied from the upper neck region since the entire back region had been painted with carcinogen).

** For the sampling sites cf. Figure 2.

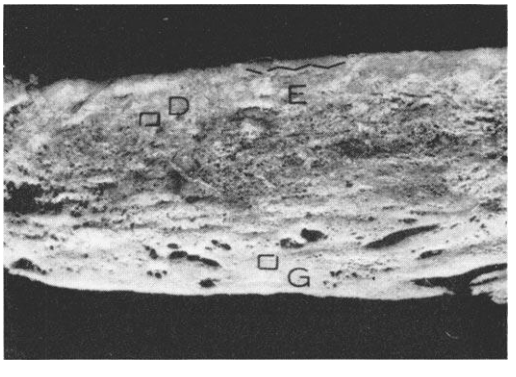

FIG. 7. Day 6 after homotransplantation. Frozen-dried preparation. Without staining, the epidermis (E), dermis (D), appendages, and granulation tissue (G) are easily identified. The peptidase activities in the upper dermis (rectangle) were also assayed on this day 6 preparation.

trophoresis, which initially requires only 0.1 ml of the crude enzyme (homogenate) and seems to be the only practical method. The rather low degree of purification was sufficient, however, to distinguish two different types of dipeptidases in the epidermis. Three facts emerge and appear to lend significance to these data. 1) Primate epidermis contains at least 2 different types of peptidases. 2) For the first time, alanylglycine dipeptidase has been shown to occur in skin. 3) Leucylalanine dipeptidase of skin can be regarded as the "classical" LAP since it acts on both leucyl-naphthylamide and leucylalanine (possibly also on leucylglycine).

So far, we have not been able to separate the LAP and arylamidase activities in skin. Therefore we are still unable to interpret the histochemical data on LAP and are uncertain whether the positive LAP staining means the occurrence of LAP, arylamidase, or both. Certain discrepancies in the degree of stainability in the histochemical reaction of LAP, such as a different intensity of epidermal coloration with different types of substrate, have been attributed by Decker and Dicken (3) to the different ratios and distributions of lyo and desmo forms of LAP. We propose another factor to explain the discrepancy—the possible occurrence of skin arylamidase, which, if present, should be purified and characterized in the future.

It should be pointed out that the activities of these peptidases were extremely high in the bulb portion of the hair follicle. This fact may concur with the histochemical data, which showed an extremely intense reaction in the dermal papilla of the hair follicle (15). We also noticed that the dermis occasionally contained a relatively high activity (up to 0.5 moles/hr/μg dry wt), and this variation was reflected by the high values of the standard errors. This may also concur with the previous histochemical finding that the LAP reaction is strong in the young fibroblasts of the dermis (15). From these combined biochemical and histochemical data, it appears that the dipeptidase activities in skin may serve as a useful indicator of the mesenchymal tissue activity.

These results indicate that both alanylglycine and leucylalanine dipeptidase activities respond

TABLE V

Aminopeptidase activities in the granulation tissue in the homograft*

Days after homograft	Substrate	
	Alanylglycine	Leucylalanine
0	.24 ± .07	.20 ± .03
2	.15 ± .04	.09 ± .01
3	.21 ± .05	.35 ± .03
4 A	.33 ± .15	.43 ± .05
4 B	.91 ± .36	1.46 ± .13
6 G	4.29 ± .81	2.40 ± .35
6 UD	.18 ± .04	.14 ± .13
10	6.11 ± 1.7	1.83 ± .10

* Expressed as moles/kg dry wt./hr (± S.E.). Each figure is an average of quintuplicate determinations. For specific sampling sites, cf. Figure 3. All samples were obtained from the same animal. There were 2 biopsies on day 4 (4A and 4B). From the 6th day sample, the upper dermis (6 UD) was also microdissected in order to compare its activities with those in the granulation tissue (6 G).

in a similar way to the proliferation of the mesenchymal tissue in pathological skin conditions. That is, a dramatic increase in aminopeptidase activities is associated with specific sites at the periphery of keratoacanthoma and hyperplastic epidermis and is also seen in the granulation tissue at the site of the homograft. It is noteworthy that different sites in the dermis show different enzyme activities. For example, in keratoacanthoma, the enzyme activity in the upper dermis adjacent to the tumor is much higher than that in the deep dermis, whereas in the homograft the enzyme activity in the granulation tissue in the subcutaneous region is much higher than that in the upper dermis of the grafted skin. These findings may further support the assumption that the aminopeptidase activities are due to a proliferation of the mesenchymal tissue, such as fibroblasts and endothelial cells. However, in spite of Burstone's finding (16) of an intense histochemical reaction in polymorphonuclear leukocytes and macrophages, we are still uncertain whether the increase represents another kind of mesenchyme, such as hemocytoblasts, macrophages, or mast cells.

It is thought that one of the pathophysiological functions of aminopeptidase is to control

the chemical mechanisms involved in the infiltrative and destructive growth of tumors. That is, that some proteinase(s) (possibly derived from tumor cells) digest the normal structural proteins, which thus become transformed into a "gelatinous matrix" that is conducive to invasion by growing tumor cells (quoted in Ref. 17). However, this hypothesis, particularly the assumption that a digestive enzyme derives from a tumor into the dermis, is naive since the dermis itself can produce the mesenchymal tissue containing high aminopeptidase activities.

SUMMARY

Two dipeptidases from adult rhesus monkey epidermis—alanylglycine dipeptidase and leucylalanine dipeptidase—were partially purified and characterized by starch-gel electrophoresis. Leucylalanine dipeptidase also degraded leucyl-β-naphthylamide and hence was considered to be the so-called leucine aminopeptidase.

In normal rhesus monkey skin, alanylglycine dipeptidase activities averaged about 1.5 (moles/kg dry wt/hr, 37° C) in the epidermis, 2.5 to 5.5 in sebaceous and sweat glands, 5 to 8 in mucous membrane, and 12 in the bulb portion of the hair follicle. Dermis and keratin contained minute activity. Leucylalanine dipeptidase (LAP) showed a similar distribution pattern, with ⅓ to ½ as much activity as that of the alanylglycine dipeptidase.

The most striking characteristic of these 2 enzyme activities was their extremely high values in the bulb portion of the hair follicle, the activity being 8 to 9 times greater in the follicle than in the epidermis. The dermal papilla of the hair follicle is composed of active mesenchymal tissue; and our quantitative data, together with previous histochemical data, suggest the close association of aminopeptidase activity with mesenchymal tissue activity.

Topographical variations of alanylglycine and leucylalanine dipeptidase activities were quantified in 3 pathological skin conditions produced experimentally in primates—pseudoepitheliomatous hyperplasia, keratoacanthoma, and homograft. Aminopeptidase activities increased 2 to 3 times in the hyperplastic epidermis, whereas they were 10 to 20 times higher in specific sites at the periphery of the hyperplasia and in the epidermal tumor than in normal dermis. Marked increases in enzyme activities were also observed in the newly developed granulation tissue at the sites of the homograft. Not all dermal tissue in these pathological conditions showed high enzyme activity; only those sites in the dermis where the mesenchymal tissue response to the pathological stress was significant showed high aminopeptidase activities. It appears, therefore, that the aminopeptidases play a significant role in the pathophysiological response of the dermis and that their activities are one indication of mesenchymal tissue response.

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