The ectopeptidases dipeptidyl peptidase IV (DP IV) and aminopeptidase N (APN) and their related enzymes as possible targets in the treatment of skin diseases

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1. ABSTRACT

Skin cells express dipeptidvl peptidase IV (DP IV) and aminopeptidase N (APN) and their related molecules of the DP IV-like family DP2, DP6, DP8, DP9 and fibroblast activation protein (FAP), as well as the cytoplasmic alanyl aminopeptidase (cAAP). The inhibitors of DP IV-like activity, Lys[Z(NO₂)]-thiazolidide (LZNT) and Lys[Z(NO₂)]-pyrrolidide (LZNP), and the APN inhibitors actinonin and bestatin affect proliferation, differentiation and cytokine production in sebocytes and keratinocytes, which are involved in the initiation of acne. Furthermore. they suppress proliferation of Propionibacterium acnes-stimulated T cells ex vivo and induce an anti-inflammatory cytokine profile. In the mouse tail model of psoriasis they have a prodifferentiative effect. In addition, these inhibitors suppress skin fibroblast proliferation, whereas only inhibition of DP IV-like activity decreases TGF-beta₁ expression and abrogates the TGF-beta₁ mediated stimulatory effects on TGF-beta1 and fibronectin production, collagen synthesis and matrix deposition in these cells. Targeting enzyme activity of DP IV and APN and their related molecules might be a novel approach for the treatment of acne, psoriasis or keloids.

2. INTRODUCTION

The cell-surface peptidases DP IV (EC 3.4.14.5., CD26) and APN (EC 3.4.11.2, mAAP, CD13) are ubiquitously expressed and have pleiotropic functions (1, 2). DP IV is a 220-240 kDa type II transmembrane protein, which is expressed as a highly glycosylated homodimer and belongs to a family of proteins of DASH (DP IV activity and structural homologues), characterized by a similar substrate specificity and overlapping inhibitor profile (3). It is a serine exopeptidase catalyzing the release of Nterminal dipeptides from oligo- and polypeptides preferentially with proline in the penultimate position (4). Aminopeptidase N is a 150 kDa zinc-dependent metalloprotease cleaving neutral amino acids from the Nterminus of oligopeptides and is up-regulated in response to activation or malignant transformation on various leukocyte subpopulations including human T cells (5-7). Recent experimental work provided evidence that particularly CD4+CD25+ regulatory T cells express significant amounts of APN (8).

Both ectopeptidases play a role in the inactivation or activation of extracellular regulatory peptides, hormones, paracrine peptides, cytokines, and neuropeptides.

	SZ 95 (32)	HaCaT	PK-ROL	NHEK	NF	KF		
Gly-Pro-pNA hydrolysing activity [pkat/10 ⁶ cells]	37 ± 9	$30 \pm 5 (26)$	84 ± 7 (25)	61 ± 13 (25)	83 ± 24 (42)	48 ± 26 (42)		
Ala-pNA hydrolysing activity [pkat/10 ⁶ cells]	205 ± 54	$90 \pm 6 (34)$	$160 \pm 8 (34)$	141 ± 18	241 ± 33	189 ± 15		
CD26 surface expression [% positive cells]	98 ± 1	21 ± 4 (26)	$33 \pm 3 (25)$	$36 \pm 6 (25)$	73 ± 16 (42)	$46 \pm 25 (42)$		
CD13 surface expression [% positive cells]	100	$6 \pm 2 (34)$	26 ± 3 (34)	88 ± 1	97 ± 6	95 ± 6		

Table 1. DP IV/CD26 and APN/CD13 expression and enzyme activity on skin cells. References are in parentheses.

Interaction with agonistic antibodies or inhibitors revealed that ectoenzymes, beyond their proteolytic activity, influence fundamental biological processes such as growth, differentiation, adhesion, motility, invasion, cell-cell interaction, angiogenesis, transformation and apoptosis (6, 9, 10). This implies a possible role of these enzymes as targets to influence pathophysiologic conditions. Inhibitors of DP IV-like activity have potent immunosuppressive and anti-inflammatory effects in various disease models such as murine experimental autoimmune encephalomyelitis (EAE) (11), collagen- and alkyldiamone-induced arthritis (12) and rat cardiac transplantation (13); and DP IV has become a novel target for the treatment of type 2 diabetes (14). APN inhibitors have shown therapeutic efficacy in analgesia models and tumor neo-angiogenesis (15); bestatin is clinically used as an immunomodulator in cancer patients and has antiangiogenic properties (16, 17).

Due to the similar expression pattern of DP IV and APN and their unique substrate specificities, both peptidases are supposed to cooperate in proteolysis and modulate cellular communication. Moreover, both, the inhibition of DP IV and APN, leads to increased TGF-beta1 production and secretion in immune cells (7, 18, 19). Their simultaneous inhibition acts additively in the induction of this immunosuppressive cytokine as well as the suppression of proliferation of T cells (20). Based on the increasing knowledge on the co-function of both extopeptidases in the regulation of lymphocyte functions and synergistic inhibitor effects, a novel therapeutic strategy was proposed named Peptidase-targeted immunoregulation and (PETIRTM), which aims at the restoration of immune balance by limiting T effector cells activation and the induction of endogenous immunosuppressive mechanisms, such as TGF-beta₁ and regulatory T cells (21) in parallel. This new therapeutic principle has recently proven efficacy in animal models of inflammatory bowel disease as well as in the EAE model for multiple sclerosis (21, 22).

The skin represents an interesting new target organ of ectopeptidase inhibitors, not only because skin diseases involve the activation of immune cells, but also because both DP IV and APN are expressed on skin cells and have been shown to be upregulated in pathologic conditions (23, 24). This review summarizes the recent data concerning inhibitor-related effects on skin cells with regard to potential therapeutic applications.

3. EXPRESSION OF DP IV AND APN AND THEIR RELATED ENZYMES ON SKIN CELLS

It has been shown that DP IV is expressed on the surface of various primary keratinocyte strains as well as the transformed hyperproliferative HaCaT keratinocyte cell

line (25, 26), with highest expression rates on freshly isolated primary keratinocytes (Table 1). The Gly-Pro-pNA hydrolysing activity of primary keratinocytes was up to fourteenfold higher than that of resting peripheral blood T cells (6 \pm 2 pkat/10⁶ cells (19)) and fivefold higher than that of T cells stimulated for 48 h with PHA, whereas APN activity exceeded that of PHA-activated T cells (31.4 ± 2.8) $pkat/10^6$ cells (7)) by fivefold. The inhibition of DP IV-like activity with LZNT and LZNP was shown to induce suppression of DNA synthesis in these cells. Recently, it was demonstrated that LZNT and LZNP are not specific for DP IV, but also inhibit some of the related family members of DASH proteins (3, 27, 28) DP 8, DP 9 and DP 2 (also named quiescent cell proline dipeptidase, OPP). Therefore, the published results have to be interpreted with regard to target-specific inhibitor effects. Other family members include fibroblast activation protein, which has poor Gly-Pro-pNA hydrolysing activity, Attractin (DPPT-L, Mahogany protein), whose enzymatic activity is controversially discussed (29-31), and DP 6 (DPL1), which is not enzymatically active.

DP IV expression has recently been demonstrated also in human sebocytes, the lipid-producing cells localized in the sebaceous glands (32, 33). However, to date, nothing is known about the expression and functional relevance of DASH-proteins in keratinocytes and sebocytes. Figure 1 summarizes the qualitative mRNA expression profile of DASH proteins in SZ 95 sebocytes (a human immortalized sebaceous gland cell line), in keratinocytes (HaCaT cell line, primary keratinocytes, ROL and commercially available human foreskin keratinocytes, NHEK) as well as in normal dermal fibroblasts (NF) and keloid-derived skin fibroblasts (KF).

DP IV is abundantly expressed in sebocytes, NHEKs and fibroblasts, whereas DP 8 and DP 9 are ubiquitously distributed in equal proportions in all skin cell types and have to be regarded as possible inhibitor targets. FAP expression is restricted to fibroblasts only, and marked DP 2 expression was found in one primary keratinocyte strain only. Although the expression profile of DP IV-like enzymes has not yet been confirmed on the protein level, it is obvious that DASH-proteins have to be considered in the design of new disease-specific inhibitors for the treatment of pathologic conditions of the skin.

The same holds true for APN and its intracellular counterpart, the cytosolic alanyl aminopeptidase (EC 3.4.11.14, cAAP, PSA) with substrate specifity nearly identical to that of APN. It has been shown that both enzymes are expressed on HaCaT and primary keratinocytes (34, 35); and studies using the cAAP-specific inhibitor PAQ-22 revealed that cAAP plays a role in the regulation of keratinocyte cell growth. Figure 1 completes



Figure 1. Qualitative mRNA expression of DP IV and APN and their related enzymes on skin cells (SZ = SZ95; HaC = HaCaT; PK = primary keratinocytes ROL; NHEK = human foreskin keratinocytes; NF = normal dermal fibroblasts; KF = keloid derived dermal fibroblasts). Enzymatic amplification was performed on cDNA derived from 70-80% confluent cell cultures.

the already published data by showing the mRNA expression APN/cAAP on the above mentioned skin cell strains. The data are further supplemented by table 1 which summarizes the data concerning surface expression of CD26 and CD13 on skin cells and their Gly-Pro-pNA hydrolysing activity (resulting from DP IV, DP 2, DP 8, DP 9) as well as Ala-pNA hydrolysing activity (resulting from APN and cAAP).

In vivo, a significant upregulation of CD26 and CD13 immunoreactivity was reported in the epidermis of patients with hyperproliferative and neoplastic skin diseases such as psoriasis, lichen planus or cutaneous T-cell lymphoma (23, 24), whereas they are only constitutively expressed in normal epidermis. CD13 expression has been especially detected at sites of epidermal-mesenchymal interactions (36). The downregulation or modulation of their epidermal expression *in vivo* has been associated with the success of therapeutic strategies (37-39).

In sebaceous glands of human scalp skin CD26 was shown to be mainly localized in basal lowdifferentiated sebocytes in the periphery of the glands, whereas CD13 presents a more diffuse staining pattern in the periphery as well as in clusters in the center of the glands (32), which represent highly proliferating undifferentiated cells sitting on the base of septa separating the lobules of the gland.

High expression of both DP IV and APN on human dermal fibroblasts has already been reported by Raynaud *et al.* (40), who also detected that DP IV expression is significantly higher in fibroblasts derived

from dermatological and rheumatic diseases (i.e. psoriasis, rheumatoid arthritis, and lichen planus) as compared to their normal counterparts, whereas APN expression was comparable. In contrast, DP IV expression was significantly downregulated in cultured cells derived from scleroderma species, which also was observed, to a lesser extent, in scleroderma biopsy specimens compared to normal skin (41). No difference could be detected concerning APN expression. A similar phenomenon has been observed in cultured keloid-derived skin fibroblasts, which showed a significantly lower DP IV surface and enzymatic expression as compared to normal dermal fibroblasts, whereas FAP expression was equal in both cell types (42). The pathophysiologic significance of this lower DP IV expression related especially to fibrotic conditions remains to be established.

4. TREATMENT OF ACNE: BROAD ATTACK WITH ECTOPEPTIDASE INHIBITORS

4.1. Pathogenesis of acne

Acne is an extremely common disease with a prevalence of 80–85% among adolescents. It can persist throughout adulthood in 12% of women older than 25 years and 3% of persons aged 35–44 years (43). To prevent physical or psychological scarring, as many as 15–30% of patients with acne need intense medical treatment, thus representing the largest patient group seen by dermatologists worldwide (44). Acne occurs in the pilosebaceous units localized on the face, chest and back, and is characterized by clinically noninflammatory (comedones) or inflammatory lesions (papules, pustules or nodular lesions).

Our knowledge about the pathogenetic steps leading to acne initiation is still incomplete and subject of controversial discussion (45-47). The most notable pathophysiological factors influencing the development of acne are sebaceous gland hyperplasia with hyperseborrhea, alterations in the growth and differentiation of follicular keratinocytes, *P. acnes* colonization of the follicle as well as inflammation and immune reactions involving both specific and non-specific immunity. Clinical experience and evidence of various studies has shown that the parallel targeting of these major pathogenetic factors, depending on clinical type and severity either by mono- or combination therapy, currently represents the most effective approach to treat acne (48).

Numerous factors have been identified to regulate sebaceous function and thereby contribute to the development of hyperseborrhea, among them androgens, neurohormones and their receptors, peroxisome proliferator activated receptors (PPARs) and neuropeptides, particularly alpha-MSH and substance P (49). The latter has been related to stress-induced exacerbation of the disease. The sequence of events initiating ductal hypercornification is not yet elucidated, and hypotheses implicate changes in sebum lipid composition, androgenic stimulation and immunologic stimulation in keratinocytes triggered by *P.acnes* and the proinflammatory cytokine interleukin-lalpha. The beginning of microcomedone (microscopically

visible precursor lesion of acne) formation is associated with aberrant differentiation of the follicular epithelium, vascular endothelial cell activation and inflammatory events which supports the hypothesis that acne may represent a genuine inflammatory disease. The perifollicular amounts of the proinflammatory cytokine ILlalpha and CD4+ memory T cells are significantly increased in early acne lesions and unaffected follicles of acne patients compared to non-acne subjects (50). The pathophysiological role of T cells found in uninvolved follicles prior to any detectable signs of keratinocyte hyperproliferation is yet unclear, but their further accumulation in acne lesion progress (51) in coincidence with other histological hallmarks of immunologic/vascular activation strongly suggests a functional contribution to disease aggravation. Within the increased perifollicular CD4+ T cell population, the majority consisted of memory/effector (CD45RO) cells, with a similar proportion exhibiting a skin-homing phenotype (50), suggesting a specific antigenic response.

An ideal substance for acne treatment would be capable to reduce sebocyte proliferation and consecutive hyperseborrhea, suppress keratinocyte proliferation and restore disturbed desquamation, and have direct antiinflammatory activity. Additional antibiotic activity agains *P. acnes* would be desirable but also bears the risk of inducing bacterial resistance.

4.2. Inhibitor effects on three major pathogenetic factors of acne **4.2.1.** Hyperseborrhea

4.2.1.1. SZ95 sebocytes

One of the most frequently used models for studying the regulation of sebaceous function in vitro is the immortalized SZ95 sebaceous gland cell line (52). In these cells, the inhibitors of DP IV LZNT and LZNP and the APN inhibitors actinonin and bestatin dosedependently suppressed proliferation in a range of IC50 values between 36 and 92 μ M and IC25 values between 2.5 and 5 μ M (32). Comparable to synergistic effects observed in T cells (20), the simultaneous application of DP IV and APN inhibitors showed additive efficacy in the suppression of DNA synthesis in SZ95 cells. Notably, comparable antiproliferative effects were obtained with 13-cis-retinoic acid (isotretinoin), the most powerful sebosuppressive substance in clinical use, only at high concentrations of 10⁻⁶ M or higher, and with a delayed onset of action as compared to ectopeptidase inhibitors. The strong and rapid suppression of proliferation in the presence of inhibitors was accompanied by an increase in sebocyte differentiation, characterized by size, granularity and lipid production. Despite the increase of single-cell differentiation seen in the Nile Red (a fluorescent dve binding to neutral lipids) assay, the total neutral lipid content isolated from cultured cells showed a trend for a decrease already after 48 h of incubation, which means that the balance between antiproliferative and prodifferentiative effects will probably not induce increased sebum production. However, the in vivo effects of peptidase inhibitors on sebaceous function in humans remain to be established.

Sebocytes are able to produce pro-inflammatory cytokines such as TNF-alpha, IL-1alpha, IL-1beta (53). The pro-inflammatory cytokine IL-1alpha, most probably produced by keratinocytes and sebocytes, represents another hallmark in early acne pathogenesis. Increased levels can be found in uninvolved follicles, early acne lesions (50) and abundantly in extracted mature comedones. Its functional relevance was studied in an in vitro organ model, where it induced a follicular hyperkeratosis in isolated sebaceous infundibula (54). This process could be blocked or reversed by the application of interleukin 1receptor antagonist (IL-1RA), the anti-inflammatory opponent which abrogates IL-1 induced inflammation. Therefore the induction of IL-1RA would be advantageous in the prevention of early inflammatory pathogenetic events of acne. It was shown that both, inhibitors of DP IV and APN as well as their combination, significantly induce IL-1RA in SZ95 sebocytes in a time-dependent manner and thereby exert direct antiinflammatory activity in these cells (32).

4.2.1.2. Syrian Hamster

Acne research is hampered by the fact that the disease is exclusively limited to humans and that animal models are unable to model the whole disease complexicity. The existing models refer to single pathogenetic factors and, even within these limitations, have shown to be of restricted predictive value for the human situation. The Syrian hamster flank organ is a widely used model for the control of sebaceous gland activity by androgens and anti-androgens, because the paired hamster flank organs, one on each side of the costovertebral angle, are highly sensitive to androgen stimulation. The inhibitors of DP IV activity LZNT and LZNP and the inhibitors of APN activity, actinonin and bestatin, were tested in this model and topically applied to the flank organs at concentrations of 10⁻³ M for 30 days. Flutamide, a topically used antiandrogen already tested in this model (55), was used as a control substance. Figure 2 summarizes the reduction in flank organ size measured by the multiplication of the greatest longitudinal and transversal diameter (LD x TD) of the visible bulk of the treated flank organs. In the groups treated with ectopeptidase inhibitors, a 20-25% reduction was observed after one month compared to 32% in the presence of corroborates flutamide. which the observed sebosuppressive inhibitor effects obtained in the SZ95 cell line.

4.2.2. Follicular Hyperkeratosis (HaCaT and Primary Keratinocytes)

Obstruction of the pilosebaceous follicle occurs in the infrainfundibulum due to abnormalities of proliferation, adhesion and differentiation of keratinocytes and leads to the development of the microcomedo as the initial acne lesion. Inhibitors of DP IV and APN/cAAP enzymatic activity were shown to dose-dependently suppress proliferation of primary keratinocytes (25, 34) as well as the hyperproliferative HaCaT keratinocyte cell line (26) *in vitro*. The additive effects of combined inhibition of DP IV and APN have also been demonstrated in keratinocytes (32). A similar synergistic effect was shown



p < 0.05; p < 0.01

Figure 2. Effect of topical application of peptidase inhibitors $(10^{-3}M)$ or flutamide $(300 \ \mu g)$ or vehicle (ethanol) on the left flank organ size, measured by the multiplication of the greatest longitudinal and transversal diameter (LD x TD) of the visible bulk of the treated flank organs. The animals were treated for 30 consecutive days. The data are presented as mean \pm SD.

for the induction of the anti-inflammatory and differentiation-restoring cytokine IL-1RA in HaCaT keratinocytes. *In vitro*, addition of IL-1alpha to follicular infundibula maintained *ex vivo* led to the formation of a microcomedo, which was inhibited or reversed by the application of IL-1RA (54), thus underlining the particular pathophysiological relevance of this cytokine in acne.

4.2.3. Inflammation (P.acnes stimulated T cells)

The perilesional cell infiltrate precursor and early acne lesions is essentially composed of CD4+ lymphocytes. In vitro, P.acnes stimulates proliferation of T lymphocytes by both specific antigenic and mitogenic stimulation. Human T cell clones isolated from lesional acne skin showed a significant increase in proliferation and interferon (IFN)-gamma production in response to P.acnes stimulation, which could not be achieved with psoriatic T cell lines or in presence of other skin commensals (56). Inhibitors of DP IV and APN activity, as well as their combination, were shown to suppress proliferation and IL-2 production of *P.acnes*-stimulated T cells ex vivo and to induce the expression of the immunosuppressive cytokine TGF-beta₁ (32). In conclusion, these inhibitors exert direct anti-inflammatory activity in the specific immune response branch of acne pathogenesis. The main factors of acne pathogenesis and their interactions with ectopeptidase inhibitors (PETIRTM) are summarized in Figure 3.

5. ROLE OF ECTOPEPTIDASE INHIBITORS IN THE TREATMENT OF PSORIASIS

5.1. Pathogenesis of psoriasis

Psoriasis is probably the most prevalent immunemediated skin disease in adults and affects about 25 million people in North America and Europe. It is a T-cellmediated chronic inflammatory skin disease believed to be of autoimmune nature that is considered to have key genetic underpinnings and can be triggered or worsened by streptococcal throat infections (57, 58). In addition to conventional chronic inflammatory changes, psoriasis is characterized by complex and striking alterations in epidermal growth and differentiation. The trigger of the keratinocyte response is thought to be activation of the cellular immune system, with T cells, dendritic cells, factors of innate immunity and various immune-related cytokines and chemokines implicated in pathogenesis. Basically, the genetic background predisposes for alterations in antigen presentation, T cell activation and epidermal differentiation, whereas exogenous factors such as infections and stress aggravate and promote T cell infiltration and antigen-specific activation in the skin. The increased production of Th₁ cytokines as well as exogenous factors induce increased irritative keratinocvte proliferation. The "stressed" keratinocytes produce proinflammatory cytokines which, being part of a vicious circle, in turn re-stimulate T cell activation in the skin. Recently, it was found that dysfunctional regulatory T cells are also involved the pathogenesis of this complex disease (59). The newest therapies for psoriasis target its immune components and may predict potential treatments for other inflammatory human diseases.

5.2. In vitro effects of ectopeptidase inhibitors

CD26 and CD13 have been identified as activation markers in immune cells (6, 7, 9) and in keratinocytes (23, 24, 37). Their epidermal expression was especially up-regulated in psoriatic plaques, and successful therapies in psoriasis were associated with a reduction in their expression on keratinocytes (37-39). The inhibitorrelated effects on keratinocytes in vitro are summarized in chapter 4.2.2.. In peripheral blood T cells of psoriatic donors, a significant reduction of CD26 expression on CD8+ cells was observed compared to control persons (60) and was presumed to contribute to pathogenesis of guttate psoriasis by decreased chemokine truncation mediated by DP IV, but no difference was found in the CD4+ (predominant in the psoriatic plaque) and CD3+ populations (61). The proliferation of peripheral blood mononuclear cells isolated from psoriatic patients was suppressed by the DP IV inhibitors LZNT and LZNP in a range comparable to that of normal healthy donors (61). It has been previously shown that these inhibitors suppress various proinflammatory cytokines (IFN-gamma, TNFalpha, IL-2, IL-10, IL-12) in different (mitogen and antigen-stimulated) T cell populations in vitro (62) and exert potent effects in animal models of autoimmune diseases. As some of these cytokines are considered to be of crucial relevance in the complex cytokine network of psoriasis, it is conceivable that these inhibitors might have immunosuppressive effects in psoriasis. However, the proof of concept in T cell-mediated animal models of psoriasis is vet lacking.

5.3. Inhibitor effects in the mouse tail model of psoriasis

Over the past decades, several animal models of psoriasis have been developed, among them transgenic, knockout, and reconstituted models (63). Xenograft models, in which involved and uninvolved psoriatic skin are transplanted onto immunodeficient mice, are the only models that come close to incorporating the complete genetic, immunologic, and phenotypic changes of the



 $PETIR^{TM} = Peptidase-targeted immunoregulation$

Figure 3. Summary of main pathogenetic factors contributing to the development of acne lesions and their interaction with inhibitors of DP IV/APN activity or their combination

disease. However, due to several limitations, they are not suitable for (especially topical) drug testing, at least not when different substance groups and dose-dependent effects are to be evaluated.

The mouse tail test is a morphometry-based, relatively sensitive, fast and well reproducible method which allows the quantitative evaluation of the effects of antipsoriatic drugs on epidermal differentiation, related to the fact that mouse tail skin shows histologic epidermal alterations that are similar to those observed in the psoriatic plaque (64). Despite its definite limitations regarding the disease complexicity, it provides the advantage that almost all available topical antipsoriatic drugs have been tested in this model. The specimens are histometrically analyzed for the relative orthokeratosis (OK), i.e. the length of the granular layer per scale related to the total scale length. Higher values in OK correspond to a greater capacity of a substance to restore disturbed differentiation.

Inhibitors of both DP IV and APN (34) and their combination have been tested in this model and dose-depently restored disturbed differentiation in a comparable range to effects seen with topical vitamin D_3 preparations (65). Figure 4 summarizes the degree of orthokeratosis obtained after a 2-week topical treatment with ectopeptidase inhibitors compared to tacalcitol at 4 µg/g.

6. TARGETING KELOIDS AND SKIN FIBROSIS BY INHIBITORS OF DP IV-LIKE ACTIVITY

6.1. The cytokine TGF-beta₁ in the pathogenesis of keloids and fibrosis

A major concern for the systemic use of inhibitors of DP IV-like activity was the possible induction of fibrosis via the temporary induction of TGF-beta₁ in T

cells (18). However, despite the fact that TGF-beta₁ plasma levels were increased and discussed to be responsible for various immunosuppressive effects, no fibrotic conditions have been observed in treated animals (11) and, to our knowledge, in contrast to the related FAP (27), DP IV has never been shown to be associated with the development of fibrosis. To date, only few investigations are available on the effects of DP IV inhibitors on fibroblast activity (66). Most importantly, it is not known whether inhibition of DP IV-like activity induces pathological changes in fibroblast function leading to the generation of fibrosis.

Fibrosis and sclerosis of the skin and subcutaneous tissue can be seen in a variety of disorders such as keloids, hypertrophic scars, morphea, or systemic sclerosis. The major pathogenetic feature of fibrosis is represented by excessive accumulation of collagen and extracellular matrix leading to progressive organ dysfunction. TGF-beta₁ has been identified as a central player in the induction and aggravation of fibrosis (67, 68) with pleiotropic effects (Figure 5), among them the increase of collagens type I, III, VI, VII, X, fibronectin, proteoglycans, fibrogenic growth factors such as connective tissue growth factor (CTGF), or the inhibition of matrix degradation by regulating the synthesis of proteases and their inhibitors.

In keloid-derived fibroblasts, TGF-beta₁ expression and the response to TGF-beta₁-induced stimulation of proliferation, collagen and fibronectin production are increased as compared to normal fibroblasts (69-71), suggesting a crucial role of TGFbeta₁ in the development of keloids and scarring. Furthermore, alterations in the expression and signalling of this cytokine have been implicated in scleroderma pathogenesis (72).



p < 0.05 vs. vehicle

Figure 4. Inhibitors of DP IV and APN activity and their combination dose-dependently induce relative orthokeratosis in the mouse tail test. The proximal tales of male NMRI mice aged 4-6 weeks were treated for 2h daily 5 days a week for a treatment period of 2 weeks. The specimens were histometrically analyzed for the relative orthokeratosis (OK), i.e. the length of the granular layer per scale related to the total scale length Data are presented as mean \pm SEM of 60 measurements. Tacalcitol was used in a concentration of 4 µg/g.

6.2. The effects of DP IV inhibitors LZNT and LZNP on skin fibroblast biology *in vitro*

The inhibitors of DP IV-like activity LZNT and LZNP moderately suppressed proliferation of normal dermal (NF) and keloid-derived skin fibroblasts (KF) by 30-40% at concentrations of 50 µM (42). An additive dosedependent antiproliferative effect was seen when the APN inhibitors actinonin and bestatin were applied in combination with DP IV inhibitors. Surprisingly, a significant time-dependent suppression of TGF-beta₁ expression in fibroblast supernatants was observed in the presence of DP IV inhibitors, whereas APN inhibitors were ineffective in this assay. This unexpected result with regard to previously described stimulatory effects on TGF-beta₁ production in immune cells prompted us to deeper investigate interactions of DP IV inhibitors with fibroblast functions. Using a [³H]-proline incorporation assay, we found that collagen and matrix deposition, stimulated in this system by addition of the profibrotic cytokine TGFbeta₁ were significantly suppressed in the presence of inhibitors (42). Furthermore, these inhibitors abrogated the stimulatory effects of TGF-beta₁ on procollagen type I Cterminal peptide expression, a marker for collagen synthesis, as well as on fibronectin production, an important extracellular matrix protein. Using a commercially available matrix metalloprotease (MMP) array, we additionally found that the stimulatory effect of TGF-beta₁ on MMP-2 and MMP-13 expression was diminished in the presence of inhibitors. To further clarify the underlying mechanisms of the relationship between DP IV-like activity and fibroblast functions, we next investigated whether the phosphorylation status of the mitogen activated protein kinases p38 and ERK1/2 was influenced in the presence of inhibitors. The rationale for these experiments was that inhibitors of DP IV-like activity were previously shown to affect ERK1/2 and p38 activation in lymphocytes (19), and that both kinases were implicated in the regulation of collagen and matrix metabolism (73). We found that that inhibition of DP IV-like activity downregulates pp38 and pERK1/2 compared to unstimulated control cells and abrogates the TGF-beta₁induced stimulation of the phosphorylation of both kinases, whereas the SMAD3 pathway was completely unaffected.

6.3. In vivo effects in a mouse model of skin fibrosis

To examine the *in vivo* relevance of our findings, we used an animal model in which cutaneous fibrosis is induced by repetitive intracutaneous injections of high doses of TGF-beta₁ (74, 75). In this model, newborn mice are used because of their lower collagen content in the skin compared to adult mice, thus rendering them more sensitive for fibrogenic stimuli. When compared with PBS alone, injections with TGF-beta1-induced dermal thickening as well as increased numbers of collagen fibers and α -smooth muscle actin (a-SMA) expression. Simultaneous injection with TGF-beta₁ plus LZNP or LZNP resulted in a significant reduction in dermal thickening and a-SMA expression as compared with mice injected with TGF-beta₁ alone (42). Injections with inhibitors alone did not produce significant changes as compared with mice treated with PBS. These results confirm the anti-fibrogenic activity of inhibitors of DP IV-like activity in vivo.

7. ROLE OF DP IV AND APN IN SKIN CANCER

7.1. Melanoma

Several reports indicate that the loss of expression and enzymatic activity of DP IV occurs during malignant transformation of melanocytes into melanoma (76, 77). Another author reported that DP IV expression was almost absent in benign pigment-cell lesions, whereas 34% of melanomas in the *in situ* or invasive radial growth phase and 12% in the vertical growth phase expressed CD26, thus suggesting a role of this enzyme in the early invasion of melanoma (78). Reexpressing DP IV in melanoma cells in vitro profoundly changed their phenotype, leading to a loss of tumorigenicity and anchorage-independent growth, a reversal of a block in differentiation and an aquired dependence on exogenous growth factors for cell survival, the latter being independent on proteolytic activity (79, 80). Interestingly, upon transfection of DP IV also FAP expression was rescued, suggesting an interaction of both ectopeptidases in malignant transformation. FAP, which has been reported to be co-expressed with CD26 in invadopodia of fibroblasts as a prerequisite for cell invasion (81), might also be responsible for the observation that DP IV retransfection in melanomas leads to a significantly suppressed invasive potential, independent of the extracellular serine protease activity of the transfected enzyme. It has been extensively speculated about mechanisms how DP IV expression mediates anti-tumor effects, and hypotheses include proteolytic modification of chemokines, growth factors, adenosine or effects related to the function of DP IV as binding protein for adenosindeaminase, collagen or fibronectin (82). Only few data are available about inhibitor-related effects with regard to treatment of skin malignancies. Val-boro-Pro, a small molecule DP IV



Figure 5. Pleitropic effects of TGF-beta₁ in the aggravation of fibrosis.

inhibitor, was shown to exert anti-tumor effects in mice injected with tumor cells from fibrosarcoma, melanoma and lymphoma cell lines (83). These effects were related to a complex immunostimulatory of Val-boro-Pro activity in the host organism, but not to a direct cytotoxic or suppressive effect on tumor cells, which did not express CD26 and FAP. Interestingly, similar anti-tumor effects were obtained in CD26 knockout mice, suggesting that other targets than DP IV are responsible for these effects.

APN/CD13 is present on human melanoma cells (84) and has recently been described to be (i) a distinctive marker for spindle cell melanomas (85) and (ii) to be upregulated during hypoxia (86). High expression has been related to enhanced invasive potential and metastasis (87, 88); and invasion and angiogenesis of melanoma cells have been significantly inhibited in the presence of APN inhibitors and CD13 specific antibodies *in vitro* (89-91). *In vivo*, bestatin was found to significantly inhibit the melanoma cell-induced angiogenesis in a mouse dorsal air sac assay (90).

7.2. Other skin cancers

Only few data are available concerning DP IV and APN expression or activity in epithelial skin tumors. Strong APN activity was detected in the stroma or peritumorous connective tissue of basal cell carcinoma (92, 93), which reflects the particular tumor-stroma interaction of this semimalignant tumor, whereas it was not detectable in either healthy epidermis or the tumor parenchyma. DP IV showed a highly positive reaction in both tumor epithelium and surrounding connective tissue. Precancerous dermatoses and basal cell carcinomas had higher levels of DP IV-activity than normal skin or benign seborrheic keratosis. Poorly differentiated malignant squamous cell carcinomas, however, showed no histochemically detectable DP IV-activity at all, which corresponds to reports of decreased activity of this enzyme in cases of malignancy.

Keratinocytes show a strong upregulation of CD26 expression in malignant skin diseases associated with the presence of a neoplastic or reactive lymphocytic infiltrate, i.e. cutaneous T cell lymphoma (CTCL) (23). Furthermore, CD26 has been identified as a distinctive marker in Sézary syndrome, where, in contrast to other cutaneous lymphoma, both the cutaneous infiltrate and skin-homing CLA+ CD4+ T lymphocytes in peripheral blood show an absence of CD26 expression (94-96).

CD13 expression was shown to be upregulated in basal and suprabasal epidermal layers in patients with mycosis fungoides (24) and has been recently proposed as a marker for distinction between MF and parapsoriasis (97), whereas is was absent in basal keratinocytes of cutaneous T cell lymphoma specimens. The modulation of CD13 expression has been associated with response to therapeutic strategies in CTCL (39, 98), but to date no direct inhibitorrelated therapeutic application has been suggested in any form of skin cancer.

8. SUMMARY AND PERSPECTIVE

A growing body of evidence suggests that DP IV and APN and their related enzymes are differentially expressed on different types of skin cells and, due to their profound effects on proliferation, differentiation and inflammation (Table 2), represent promising targets to modulate pathologic inflammatory and hyperproliferative skin diseases.

	Proliferation	Cytokines	Other functions	In vivo
SZ 95 sebocytes	$\downarrow\downarrow$	IL-1RA↑ (protein/mRNA)	Lipid Production, Differentiation	Hamster Flank Organ size ↓
Keratinocytes (HaCaT, PKC)	$\downarrow\downarrow$	IL-1RA↑ (protein/mRNA)		Mouse Tail test: Relative
				Orthokeratosis↑
P.acnes stimulated T cells	$\downarrow\downarrow\downarrow\downarrow$	IL-2↓TGF-β1↑		
Skin fibroblasts (normal and keloid)	\downarrow	TGF-β₁↓↓	Collagen (PICP) and Matrix	Mouse Model of skin fibrosis: dermal
			(fibronectin) production, MMP	thickness↓, α-SMA expression↓
			expression, MAPK signalling	

Table 2. Effects of inhibitors of DP IV/CD26 and APN/CD13 enzymatic activity on skin cells

The presented data suggest that especially the treatment of acne, psoriasis and fibrotic skin conditions or keloids might be enriched in the future by new substance groups of ectopeptidase inhibitors.

Further investigations will have to clarify the differential contribution of DP IV- or APN-related enzymes to the observed biological effects and the detailed mechanisms to provide a rationale for improved moleculeand disease-targeted inhibitor design.

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Abbreviations: APN = Aminopeptidase N, alpha–SMA = alpha-smooth muscle actin, DP = dipeptidyl peptidase, FAP = fibroblast activation protein, IL = interleukin; IL-1RA = interleukin-1 receptor antagonist, KF = keloid fibroblasts, LZNP = Lys[Z(NO₂)]-pyrrolidide, LZNT = Lys[Z(NO₂)]thiazolidide, MMP = matrix metalloproteinase, NF = normal skin fibroblasts, PICP = procollagen type I Cterminal peptide, *P.acnes* = Propionibacterium acnes, PK = primary keratinocytes, TGF- β_1 = transforming growth factor beta 1

Key words: dipeptidyl peptidase IV, aminopeptidase N, acne, psoriasis, mouse tail model, fibrosis, keloid, transforming growth factor beta, collagen

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