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Dipeptidyl peptidase IV (DP IV, CD26) is involved in regulation of DNA synthesis in human keratinocytes

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Abstract Various studies have shown that the membrane ectoenzyme dipeptidyl peptidase IV (DP IV, CD26), expressed on T, NK, and B cells in the human immune system, is involved in the regulation of DNA synthesis and cytokine production. Here, we clearly demonstrate that this enzyme is highly expressed also on human epidermal foreskin and split-skin keratinocytes and that the specific DP IV inhibitors Lys[Z(NO_2)]-thiazolidide, Lys[Z(NO_2)]-pyrrolidide inhibit the enzymatic activity as well as the DNA synthesis of these cells. These data demonstrate that CD26 plays a role also in regulation of DNA synthesis of epidermal keratinocytes and that the enzymatic activity is required for mediating these effects.

Key words: Dipeptidyl peptidase IV; Keratinocyte; Synthetic inhibitor; Proliferation

1. Introduction

The dipeptidyl peptidase IV (DP IV, EC 3.4.14.5) is a transmembrane type II glycoprotein that is present on most mammalian cells [1,2].

In the immune system, this enzyme is found on T lymphocytes, but also on B lymphocytes and NK cells [1,3,4]. DP IV is a serine peptidase that catalyzes the release of N-terminal dipeptides from peptides and proteins preferentially with proline, hydroxyproline and alanine at the penultimate position [5]. In the plasma membrane, DP IV occurs as a dimer with a total molecular mass of 220–240 kDa. At the 4th Workshop on Leukocytes Differentiation Antigens a number of monoclonal antibodies recognizing DP IV was subsumed under the term CD26 [6–8].

DP IV/CD26 is also known to be an adenosine desaminase (ADA) binding protein [9] that interacts with collagen on the extracellular matrix [10–12] and to be involved in CD3/T cell receptor (TcR)-mediated signal transduction [13–18].

Using specific inhibitors of DP IV, it was demonstrated that DP IV is involved not only in the regulation of DNA synthesis, but also in the production of various cytokines by human $CD26^+$-immune cells [19–23].

In the present report, we demonstrate that DP IV/CD26 is highly expressed on human keratinocytes and that the synthetic reversible inhibitors of DP IV, Lys[Z(NO_2)]-thiazolidide and Lys[Z(NO_2)]-pyrrolidide, inhibit the specific enzymatic activity of human keratinocytes as well as the DNA synthesis in a dose-dependent manner.

2. Materials and methods

2.1. Cells

Commercially available human foreskin keratinocytes (NHEK, Clonetics) from adult and neonatal donors and primary human split-skin keratinocytes (RUM, ROL) from patients undergoing routine skin transplantation were cultured in serum-free keratinocyte growth medium (KGM, Promocell, Heidelberg). Pieces of split-skin obtained through routine skin transplantation were washed twice in Hank's balanced salt solution (HBSS, w/o Mg^{2+}, Ca^{2+}) with HEPES 10 mM (HBSS, Boehringer Ingelheim, Germany), minced, placed dermal side down in 0.25% trypsin solution and left overnight at 4°C. On the following day, dermis could be easily separated from epidermis using fine forceps and was placed in HBSS with 2% fetal calf serum (FCS, Gibco, Deisenhofen, Germany).

After 15 min of vortexing, epidermal cells were passed through a cell strainer (Falcon, Becton Dickinson, Heidelberg, Germany) to retain cornified cells and upper granular cells, then centrifuged at 900 rpm for 9 min. Finally, cells were seeded at a density of approximately 10 000 cells/cm^2 in equilibrated tissue culture flasks (Falcon) with KGM and stored at 37°C, 5% CO_2-saturated humidity. First medium change was on day 1, then every alternate day. Cells were passaged at 60–80% confluence and split in a 1:2–1:3 ratio using 0.025% Trypsin/0.01%EDTA (Boehringer Ingelheim). Cells from passages 1 to 6 were examined.

2.2. RNA isolation

In each experiment 5×10^6 cells were used for RNA preparation by means of an RNeasy isolation kit provided by Qiagen (Hilden, Germany) following the protocol recommended by the supplier. After the first round of purification, contaminating DNA was removed by DNase I digestion (Boehringer Mannheim, Mannheim, Germany; 20 U/50 μl reaction, 30 min at 37°C). The RNA was then subjected to a second round of purification by means of RNeasy, and the resulting RNA was quantified spectrophotometrically using a GeneQuant (Pharmacia LKB, Freiburg, Germany). RNA was aliquoted and stored ethanol-precipitated at −70°C until use.

2.3. Reverse transcription

In a final volume of 20 μl in each case, 1 μg of total RNA was transcribed by 20 units of AMV reverse transcriptase (Boehringer Ingelheim, Heidelberg, Germany) in the supplied buffer with the addition of 0.5 mmol/l dNTP, 10 mmol/l DTT, 50 mmol/l random hexanucleotides (Boehringer Mannheim, Mannheim, Germany) and 50 units of placenta RNase inhibitor (Ambion, Austin, TX, USA) during a 1 h incubation at 37°C. The enzyme was inactivated by a 10 min incubation at 65°C and the reaction mixture was kept frozen at −70°C until enzymatic amplification.

2.4. Enzymatic amplification

One tenth of the reverse transcription reaction was used as the template for the amplification reaction. Twenty five cycles were performed in an Autogene II (CLF, Emmersacker, Germany) in 50 μl reaction buffer containing 0.5 units Goldstar Taq-polymerase (Euro-

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gentec, Brussels, Belgium), 0.5 mmol/l dNTP, and 1 μl of the corresponding RT primer set (Stratagene, Heidelberg, Germany) as recommended by the supplier. The initial denaturing step was for 1.5 min at 95°C. Each cycle consisted of annealing for 0.7 min at 60°C, elongation at 72°C for 1.0 min, and denaturing at 96°C for 0.3 min. The final elongation step was extended to 3.0 min. Ten microliter of each reaction mixture were loaded on a 1.9% agarose gel and electrophoresed at 5 V/cm in 1×TBE buffer and then stained with ethidium bromide.

2.5. Synthesis of DP IV substrates and inhibitors

$(Gly\text{-}Pro)_2$-Rhodamine 110 was prepared starting from Rhodamine 110 (Synthon AcMaRi Chemie, Wolfen, Germany) by coupling with t-butyloxycarbonyl (Boc)-proline and Boc-glycine using mixed anhydride method [24]. The Boc-group was removed by treatment with HCl in acetic acid. The substrate was purified by HPLC and characterized by mass spectrometry. The DP IV inhibitors Lys[Z(NO_2)]-thiazolidide and -pyrrolidide were synthesized as described previously [25,26].

2.6. Enzymatic assay

Enzymatic activity of DP IV was determined according to the method published by Schön [27] using 1.6 mM Gly-Pro-4-nitroanilide [28] and 4×10^4 cells in the reaction mixture.

2.7. Immunofluorescence CD26-staining of cells

Indirect immunofluorescence staining of keratinocytes was performed in ice-cold PBS using the monoclonal anti-DP IV (CD26) antibody EF5/A3 (IgG1) [29]. Labelled cells were analyzed by flow cytometry (FACScan, Becton Dickinson). For control of non-specific or Fc receptor-mediated labelling, we tested the binding of irrelevant IgG1 monoclonal antibodies (Coulter Electronics, Krefeld, Germany).

2.8. Enzymatic staining using the DP IV substrate $(Gly\text{-}Pro)_2$-Rhodamine 110, fluorescence microscopy

Keratinocytes were cultured for 72 h on chamber slides (Nunc, Wiesbaden, Germany) at 37°C, 5% CO_2 in serum-free KGM medium (CellSystems, Remagen, Germany). For enzymatic staining, 50–70% confluent cells were washed, incubated for 30 min at 37°C with the DP IV substrate $(Gly\text{-}Pro)_2$-Rhodamine 110 (0.02 mM) in presence and absence of the DP IV inhibitor Lys[Z(NO_2)]-thiazolidide (10^{-5} M) and analyzed under a fluorescence microscope (Axiovert, Zeiss, Oberkochen, Germany).

2.9. Proliferation assay

Keratinocytes (10^4 cells/100 μl) were incubated in serum-free KGM medium in the presence of different concentrations of the DP IV inhibitors Lys[Z(NO_2)]-thiazolidide, and Lys[Z(NO_2)]-pyrrolidide [22,26]. After 56 h the cultures were pulsed for an additional 16 h with ^{3}H-methyl-thymidine ([^{3}H]dThd, 0.2 μCi per well; Amersham, Braunschweig, Germany). Cells were harvested onto glass fiber filters, and the incorporated radioactivity was measured by scintillation counting [22].

2.10. Cell viability assay

Cell viability was determined using the EZ4U cell proliferation and cytotoxicity assay (Biomedica, Wien, Austria).

3. Results

3.1. DP IV RT-PCR, enzymatic DP IV activity and CD26 antigen expression

To clarify whether DP IV/CD26 is expressed on human keratinocytes, first we studied DP IV mRNA levels of the four keratinocyte preparations NHEK Ad, NHEK Neo, RUM, and ROL. Enzymatic amplification was performed on cDNA derived from 70–80% confluent cell cultures. Total RNA was isolated, reverse transcribed, and polymerase chain reaction (PCR) amplified using primers specific for DP IV. DP IV mRNA was found to be expressed on all four cell populations (Fig. 1).

Viable keratinocytes were further analyzed for enzymatic DP IV activity. Using Gly-Pro-4-nitroanilide as a substrate, high DP IV activity was detected on viable cells of all four preparations (Table 1). The enzymatic activity of these cells was found to be much higher (three- to ten-fold) than on resting peripheral blood T cells and close to that of T cells 48 h after PHA stimulation (data not shown). These results were supported by flow cytometric studies obtained with the monoclonal anti-CD26 antibody EF5/A3 (Table 1). All four keratinocyte preparations expressed the CD26 antigen on the cell surface.

Furthermore, the enzymatic activity of DP IV was studied on keratinocytes preincubated for a period of 30 min with different concentrations of the DP IV inhibitors Lys[Z(NO_2)]-thiazolidide and Lys[Z(NO_2)]-pyrrolidide. As shown in Fig. 2, the DP IV inhibitors were capable of suppressing the enzymatic activity of these cell populations in a dose-dependent manner.

Fig. 1. DP IV mRNA levels of human keratinocytes. Enzymatic amplification was performed on cDNA derived from 70–80% confluent cell cultures of the four keratinocyte preparations NHEK Ad (1), NHEK Neo (2), RUM (3), and ROL (4). Total RNA was isolated, reverse transcribed, and polymerase chain reaction (PCR) amplified using primers specific for DP IV.

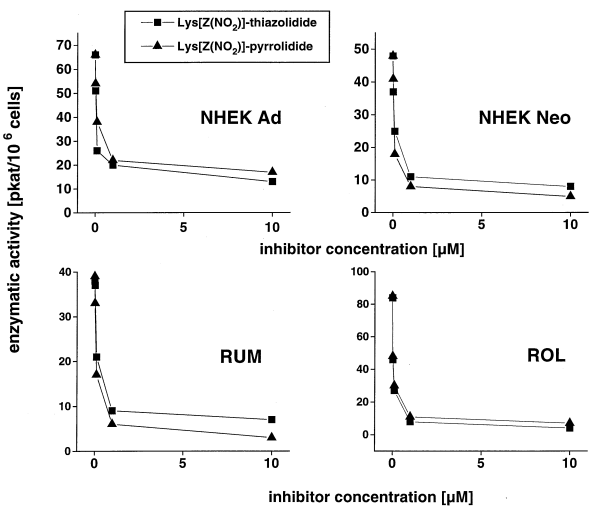

Fig. 2. Influence of DP IV inhibitors on the enzymatic DP IV activity of human keratinocytes. Enzymatic DP IV activity was measured on human keratinocytes using the DP IV-specific substrate Gly-Pro-4-nitroanilide in absence and presence (preincubation 30 min) of different concentrations of the DP IV inhibitors Lys[Z(NO_2)]-thiazolidide, and Lys[Z(NO_2)]-pyrrolidide. The standard deviation from triplicates was less than 15%.

Table 1
CD26 expression and enzymatic DP IV activity

	CD26 positive cells %	Enzymatic DP IV activity (pkat/10^6 cells)
NHEK Ad	36 ± 6	61 ± 13
NHEK Neo	25 ± 4	48 ± 10
RUM	34 ± 3	39 ± 5
ROL	33 ± 3	84 ± 7

Additionally, NHEK Ad keratinocytes were cultured on chamber slides in serum-free KGM medium. For enzymatic staining, 50–70% confluent cells were washed, incubated for 30 min at 37°C with the DP IV substrate (Gly-Pro)$_2$-Rhodamine 110 (0.02 mM) in presence and absence of the DP IV inhibitor Lys[Z(NO$_2$)]-thiazolidide (10^{-5} M). As demonstrated in Fig. 3, the DP IV, expressed on NHEK Ad keratinocytes, catalyzed the hydrolysis of these DP IV substrate (Fig. 3A). As expected the inhibitor suppressed the enzymatic DP IV activity of these cells (Fig. 3B).

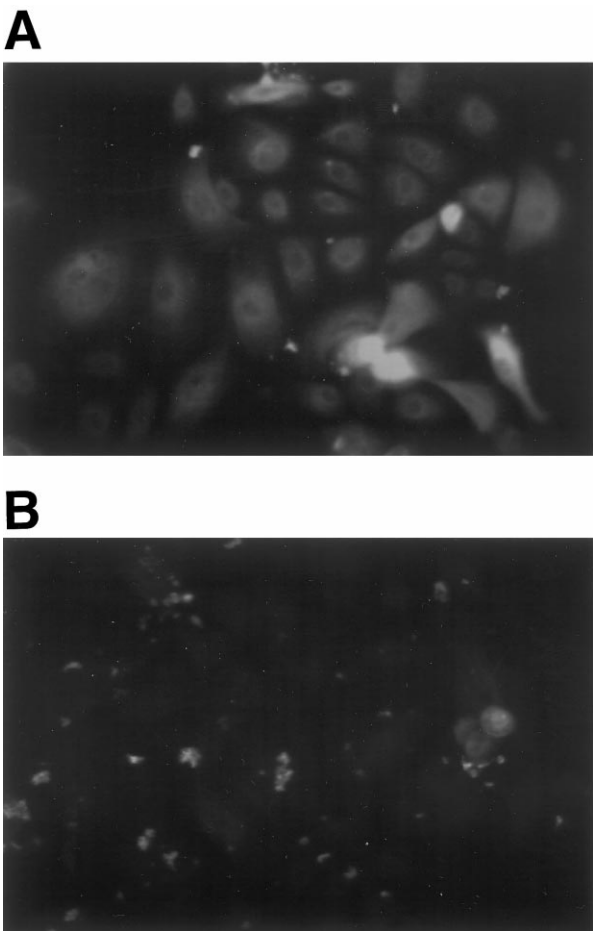

Fig. 3. Enzymatic staining of human keratinocytes using the DP IV substrate (Gly-Pro)$_2$-Rhodamine 110. NHEK Ad keratinocytes were cultured for 72 h on chamber slides at 37°C, 5% CO_2 in serum-free KGM-2 medium. For enzymatic staining, 50–70% confluent cells were washed, incubated for 30 min at 37°C with the DP IV substrate (Gly-Pro)$_2$-Rhodamine 110 (0.02 mM) in absence (A) and presence (B) of the DP IV inhibitor Lys[Z(NO$_2$)]-thiazolidide (10^{-5} M).

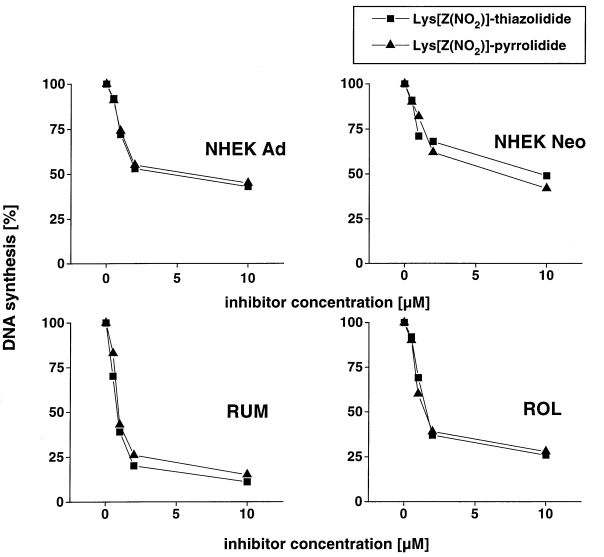

Fig. 4. Influence of DP IV inhibitors on DNA synthesis of human keratinocytes. Human keratinocytes (10^4 cells/100 μl) were incubated with different concentrations of the synthetic DP IV inhibitors Lys[Z(NO$_2$)]-thiazolidide, and Lys[Z(NO$_2$)]-pyrrolidide. After 56 h, the cultures were pulsed with ^{3}H-methyl-thymidine for a further 16 h. [^{3}H]dThd incorporation is indicated as mean from three different experiments. The values are expressed as % [^{3}H]dThd incorporated in relation to control cultures without inhibitor ([^{3}H]dThd incorporation in control cultures: NHEK Ad = 6550 ± 710 cpm; NHEK Neo = 4520 ± 420 cpm; RUM = 5610 ± 650 cpm; ROL = 4400 ± 380 cpm). The standard deviation from three experiments was less than 18%.

3.2. DP IV inhibitors suppressed DNA synthesis

We have demonstrated recently that the DNA synthesis of human PWM-stimulated PBMC and purified T cells was strongly inhibited by the synthetic competitive DP IV inhibitors Lys[Z(NO$_2$)]-thiazolidide and Lys[Z(NO$_2$)]-pyrrolidide [30].

The effect of DP IV inhibitors on DNA synthesis of human keratinocytes was determined by incubation of viable cells for 72 h in the presence and absence of various concentrations of Lys[Z(NO$_2$)]-thiazolidide and Lys[Z(NO$_2$)]-pyrrolidide. As shown in Fig. 4, the DNA synthesis of all four cell systems was strongly suppressed.

To exclude possible cytotoxic effects of the DP IV inhibitors, we measured the viability of the cell culture using the EZ4U assay. In no case the EZ4U assay was impaired by the DP IV inhibitors (data not shown).

4. Discussion

DP IV/CD26 has been shown to be an activation marker for various types of immune cells. Surface expression of CD26 is upregulated after mitogenic, anti-CD3 or IL-2 stimulation of T cells, St. aureus protein stimulation of B cells and IL-2 stimulation of NK cells [1,3,4,27]. Data from several groups have provided evidence that DP IV plays an integral role in the regulation of differentiation and growth of lymphocytes [1,30–32]. Studying the influence of specific synthetic inhibitors of DP IV, we and other laboratories have demonstrated previously that DP IV is involved in the regulation of DNA synthesis and production of various cytokines in immune cells [22,30,31,33,34].

Using RT-PCR, flow cytometry, enzymatic staining and an enzymatic DP IV assay, here we could show for the first time that this enzyme is highly expressed also on human keratinocytes. Moreover, the specific DP IV inhibitors Lys[Z(NO_2)]-thiazolidide, Lys[Z(NO_2)]-pyrrolidide were found to inhibit the enzymatic activity of these cells in a dose-dependent manner.

Most importantly, we found that these DP IV inhibitors do suppress also the DNA synthesis of human keratinocytes dose dependently.

To determine the specific action of synthetic DP IV inhibitors and to exclude cytotoxic effects, we recently studied the influence of Lys[Z(NO_2)]-thiazolidide and Lys[Z(NO_2)]-piperidide on two different clones of the human histocytic lymphoma cell line U937, one expressing high levels (U937-H), the other low levels (U937-L) of CD26 [23]. Using these cell models, we could show that Lys[Z(NO_2)]-thiazolidide and Lys[Z(NO_2)]-piperidide diminished DNA synthesis and production of IL-1β, but increased the secretion of the IL-1 receptor antagonist (IL-1RA) and of TNF-α in U937-H cells which strongly express CD26. The inhibitors did not influence the cytokine production in U937-L cells with weak CD26 expression [23]. Thus, because of the specificity of these DP IV inhibitors, their inhibitory activity on cell proliferation must be considered to be the consequence of a specific interaction with the enzyme on the cell surface.

Keratinocytes actively participate in the immune response and interact with infiltrating mononuclear cells, either by releasing pro-inflammatory cytokines or via intercellular adhesion reactions.

Novelli and colleagues [35] investigated CD26 expression on keratinocytes, in both normal and pathological skin, using immunohistochemical techniques. They found only a sporadic focal CD26 positivity in normal skin. Interestingly, increased CD26 expression was shown in cutaneous T-cell lymphomas and in inflammatory skin diseases, e.g. psoriasis, lichen planus and spongiotic dermatitis [35]. In this study, keratinocyte expression of CD26 was not restricted to a specific skin disease. The authors proposed that CD26 expression is associated with the presence of a T lymphocyte intraepidermal infiltrate and that the CD26 molecule may function as a keratinocyte activation antigen.

In our experiments considerable expression of CD26 and functional activity could be detected on human keratinocytes in absence of potential sources of cytokines like T lymphocytes. As cultured human keratinocytes are in a highly proliferative activated state, partially due to special media ingredients, i.e. low Ca^{2+} concentration (0.15 mM), our observations correspond well to Novelli's.

Taken together, our results suggest that DP IV plays a crucial role in the regulation of DNA synthesis and cellular proliferation in cultured human keratinocytes of different origin. Further investigation is needed not only to determine the function of CD26 expression in keratinocytes but to illuminate CD26 activity by keratinocytes in different environments and i.e. inflammation or wound repair in skin related processes.

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