Immunology 1997 **91** 354–360

Inhibitors of dipeptidyl peptidase IV induce secretion of transforming growth factor-β_1 in PWM-stimulated PBMC and T cells

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SUMMARY

Various studies have shown that the membrane ectoenzyme dipeptidyl peptidase IV (DP IV; CD26), expressed on T, natural killer (NK) and B cells in the immune system, is involved in the regulation of DNA synthesis and cytokine production. We show that the specific DP IV inhibitors Lys[Z(NO_2)]-thiazolidide, Lys[Z(NO_2)]-piperidide, and Lys[Z(NO_2)]-pyrrolidide inhibit DNA synthesis as well as production of interleukin-2 (IL-2), IL-10, IL-12, and interferon-γ (IFN-γ) of pokeweed mitogen (PWM)-stimulated purified T cells. Most importantly, these inhibitors induce a three- to fourfold increased secretion of latent transforming growth factor-β_1 (TGF-β_1) by PWM-stimulated peripheral blood mononuclear cells (PBMC) and T cells, as measured with a specific TGF-β_1 enzyme-linked immunosorbent assay and in the Mv1Lu bioassay. As we could demonstrate previously, TGF-β_1 exhibits the same inhibitory effects as DP IV inhibitors on DNA synthesis and cytokine production (*Cytokine* 1994, **6**, 382–8; *J Interferon Cytokine Res* 1995, **15**, 685–90). A neutralizing chicken anti-TGF-β_1 antibody was capable of abolishing the DP IV inhibitor-induced suppression of DNA synthesis of PWM-stimulated PBMC and T cells. These data suggest that TGF-β_1 might have key functions in the molecular action of DP IV/CD26 in regulation of DNA synthesis and cytokine production.

INTRODUCTION

The dipeptidyl peptidase IV (DP IV, EC.3.4.14.3) is a transmembrane type II glycoprotein that is present on most mammalian cells.[1] DP IV is a serine peptidase that catalyses the release of N-terminal dipeptides from peptides and proteins, preferentially proline, hydroxyproline and alanine at the penultimate position.[2] In the plasma membrane, DP IV occurs as a dimer with a total molecular mass of 220 000–240 000. The 4th Workshop on Leucocytes' Differentiation Antigens subsumed a number of monoclonal antibodies recognizing DP IV under the term CD26.[3]

DP IV has been shown to be an activation marker for various types of immune cells. Surface expression of CD26 is up-regulated after mitogenic, anti-CD3 or interleukin-2 (IL-2) stimulation of T cells, *Staphylococcus aureus* protein stimulation of B cells and IL-2 stimulation of natural killer (NK) cells.[4–7] Data from several groups have provided evidence that DP IV plays an intergral role in the regulation of differentiation and growth of lymphocytes.[1,8,9] DP IV seems to be involved in CD3/T-cell receptor (TCR)-mediated signal transduction.[10,11]

Using specific inhibitors of DP IV, it has been demonstrated that DP IV is involved not only in the regulation of DNA synthesis, but also in production of various cytokines.[12–15]

Flad *et al.*[16] reported that IL-2 production by T cells is associated with the expression of DP IV. Schön *et al.*[12] demonstrated that the production of interleukin-2 (IL-2) and interferon-γ (IFN-γ) by phytohaemagglutinin/phorbol myristate acetate (PHA/PMA)-stimulated peripheral blood mononuclear cells (PBMC) diminishes in the presence of DP IV inhibitors. Using a CD26-transfected Jurkat T-cell line which lacked DP IV enzyme activity but still reacted with CD26 antibodies, Tanaka *et al.*[17] showed that DP IV enzyme activity enhances IL-2 production induced via CD26-related and unrelated pathways. They found that transfectants expressing DP IV activity on their surface produced substantially more IL-2 than did transfectants with proteolytically inactive DP IV after triggering by a combination of anti-CD26 and anti-CD3 or anti-CD3 antibodies plus PMA. These results are disputed by other authors.[1]

Recently, we could show that synthetic DP IV inhibitors suppress DNA synthesis as well as IL-2 and IL-6 production in pokeweed mitogen (PWM)-stimulated PBMC. The release

Received 25 November 1996; revised 9 February 1997; accepted 26 March 1997.

Abbreviations: DP IV, dipeptidyl peptidase IV; TGF-β, transforming growth factor-β.

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of IL-1 and tumour necrosis factor-α (TNF-α), however, was not influenced under these conditions.[14]

The molecular mechanisms underlying the regulatory effects of DP IV/CD26 on DNA synthesis and cytokine production are not yet clear. In the present study, we demonstrate that the synthetic competitive DP IV inhibitors Lys[Z(NO_2)] (Lys[Z(NO_2)])-thiazolidide, Lys[Z(NO_2)]-piperidide, and Lys[Z(NO_2)]-pyrrolidide significantly suppress production of IL-2, IL-10, IL-12, and IFN-γ in PWM-stimulated T cells. Under the same conditions, the inhibitors are capable of inducing a three- to fourfold increase in secretion of the latent form of the 'immunoinhibitory' cytokine transforming growth factor-β_1 (TGF-β_1). A neutralizing anti-TGF-β_1 antibody abolished the DP IV inhibitor-induced suppression of DNA synthesis by PWM-stimulated PBMC and T cells. These data suggest that the immunosuppressive effects of DP IV inhibitors are at least partially mediated by TGF-β_1 and that this cytokine is directly involved in the DP IV-dependent regulation of cell growth.

MATERIALS AND METHODS

Cells

PBMC were isolated from the heparinized venous blood of healthy donors by density gradient centrifugation over Ficoll–Paque gradients (Pharmacia LKB, Uppsala, Sweden), as described by Bøyum.[18] Monocytes and B cells were depleted by passage through nylon wool columns (Polysciences, Warrington, PA). PBMC or T lymphocytes (90–95% T cells) were suspended in serum-free CG medium (Vitromex, Vilshofen, Germany).

Proliferation assay

PBMC and purified T lymphocytes (10^5 cells/100 μl) were stimulated in serum-free CG medium with PWM (2 μg/ml; Serva, Heidelberg, Germany) in the presence of different concentrations of the synthetic reversible inhibitors of DP IV, Lys[Z(NO_2)]-thiazolidide, Lys[Z(NO_2)]-piperidide, and Lys[Z(NO_2)]-pyrrolidide.[14,19] In defined experiments, cells were incubated with PWM and TGF-β_1 (R&D systems, Minneapolis, MN) or with PWM and DP IV inhibitors (10^{-5} M) in combination with a neutralizing chicken anti-TGF-β_1 antibody (R&D systems). After 90 hr the cultures were pulsed for an additional 6 hr with [^{3}H]methyl-thymidine ([^{3}H]TdR, 0·2 μCi per well; Amersham, Braunschweig, Germany). Cells were harvested onto glass fibre filters, and the incorporated radioactivity was measured by scintillation counting.[14]

MTT reaction

Cell viability was measured using a modified MTT assay.[14] The tetrazolium salt MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, Serva) was dissolved in CG medium at 5 mg/ml. Twenty-five microlitres of this stock solution was added to 100 μl of cell culture, and the plates were incubated at 37° for 4 hr. Following centrifugation of the plates at 600 *g* for 10 min, the medium was removed by aspiration. To each well 100 μl acidified isopropyl alcohol/dimethylformamide solution (0·04 m HCl in isopropanol/dimethylformamide 1:1) was added. The formazan concentrations were measured with an enzyme-linked immunosorbent assay (ELISA) reader (570 nm wavelength filter).

Cytokine induction

PBMC and T cells were induced to produce cytokines (IL-2, IL-10, IL-12, IFN-γ, and TGF-β_1) by stimulation with PWM as described previously.[20] Briefly, 10^6 cells/ml were incubated in serum-free CG medium with 2 μg/ml PWM and different concentrations of the DP IV inhibitors Lys[Z(NO_2)]-thiazolidide, Lys[Z(NO_2)]-piperidide, and Lys[Z(NO_2)]-pyrrolidide. After 4, 24, 48 and 72 hr, the cell culture supernatants were harvested for cytokine determination and stored at − 70°.

ELISA for IL-2, IL-10, IL-12, IFN-γ and TGF-β_1

The IL-2, IL-10, IL-12 and IFN-γ concentrations of cell culture supernatants were determined with commercially available 'sandwich' ELISA (Biosource, Camarillo, CA and R & D systems).

Active TGF-β_1 was measured with an ELISA as described by Danielpour using a mouse monoclonal anti-TGF-β_1, β_2, β_3 antibody (Genzyme, Cambridge, MA) and a chicken anti-TGF-β_1 antibody (R & D Systems).[21] This assay is sensitive to 100 pg of TGF-β_1 activity per ml. Samples were tested before and after transient acidification (reduction of the pH to 1·5 by addition of 5 M HCl for 30 min at 37° and neutralization with 1·4 M NaOH in 0·7 M HEPES).[22]

TGF-β bioassay

Mv1Lu mink lung epithelial cells (CCL64, American Type Culture Collection, Bethesda, MD) were used as target cells for the detection of TGF-β bioactivity in supernatants from PBMC and T cells as described by Massague.[23] Briefly, 10^4 Mv1Lu cells/well were seeded on flat-bottom 96-well plates in Dulbecco's medium (IMDM, GibcoBRL, Eggenstein, Germany) containing 10% fetal calf serum (GibcoBRL). After 20 hr of culture, supernatants were added to the cells in twofold serial dilutions. After 72 hr the cultures were pulsed for an additional 6 hr with [^{3}H]TdR (0·2 μCi per well; Amersham). Cells were harvested onto glass fibre filters, and the incorporated radioactivity was measured by scintillation counting.[20] Samples were tested before and after transient acidification. TGF-β activity was calculated using probit analysis to compare a standard of natural human TGF-β_1 with the sample dilutions producing a 50% proliferation.

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°). 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° until use.

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 dithiothreitol (DTT), 50 mmol/l random hexanucleotides

(Boehringer Mannheim) and 50 units of placenta RNase inhibitor (Ambion, Austin, TX) during a 1 hr incubation at 37°. The enzyme was inactivated by a 10 min incubation at 65° and the reaction mixture was kept frozen at −70° until enzymatic amplification.

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 (Eurogentec, 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°. Each cycle consisted of annealing for 0·7 min at 60°, elongation for 72° for 1·0 min, and denaturing at 96° for 0·3 min. The final elongation step was extended to 3·0 min. Ten microlitres 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.

RESULTS

DP IV inhibitors suppressed DNA synthesis, as well as IL-2, IL-10, IL-12 and IFN-γ production in PWM-stimulated purified T cells

We have demonstrated elsewhere that the DNA synthesis of PWM-stimulated PBMC was strongly inhibited by the synthetic competitive DP IV inhibitors Lys[Z(NO_2)]-thiazolidide and Lys[Z(NO_2)]-piperidide.[14] A similar suppressive effect on proliferation of PWM-stimulated purified T cells was found after incubation with these two DP IV inhibitors as well as with Lys[Z(NO_2)]-pyrrolidide (Fig. 1a). Neither thiazolidine, piperidine, nor pyrrolidine had an influence on DNA synthesis in these cell systems (data not shown). Moreover, to exlude possible cytotoxic effects of the DP IV inhibitors, we measured the viability of cell cultures by tetrazolium salt MTT reaction. Previously, we had shown that the MTT reaction is highly sensitive to toxic agents, and the data obtained with this method correlated well with those obtained in other viability tests. In no case was the MTT reaction impaired by the DP IV inhibitors (data not shown).

To address the question whether the suppressive effect of DP IV inhibitors on DNA synthesis of PBMC and T lymphocytes correlates with a decrease in production and secretion of different cytokines, we measured the concentrations of the cytokines IL-2, IL-10, IL-12 and IFN-γ in supernatants of PWM-stimulated T cells in the presence or absence of DP IV inhibitors. T lymphocytes were incubated with 2 μg/ml PWM together with LYys[Z(NO_2)]-thiazolidide, Lys[Z(NO_2)]-piperidide, and Lys[Z(NO_2)]-pyrrolidide. The concentrations of cytokines released after 24, 48 and 72 hr were measured by enzyme immunoassays. As shown in Fig. 1(b), the production of all four cytokines was significantly inhibited by DP IV inhibitors at concentrations of 10^{-5} M.

DP IV inhibitors stimulated secretion of latent TGF-β_1 in PWM-stimulated PBMC and purified T cells

To determine whether the 'immunoinhibitory cytokine' TGF-β_1 secreted by T cells is involved in the DP IV inhibitor-induced suppression of DNA synthesis and cytokine production, the concentrations of this cytokine in supernatants of PWM-stimulated PBMC and T cells were assayed in the presence or absence of DP IV inhibitors. PBMC and T lymphocytes were incubated with 2 μg/ml PWM together with Lys[Z(NO_2)]-thiazolidide, Lys[Z(NO_2)]-piperidide, and Lys[Z(NO_2)]-pyrrolidide (10^{-5} M) and the cell culture supernatants were harvested after 4 hr. TGF-β_1 concentrations were determined using an enzyme immunoassay. Samples were tested before (active TGF-β_1) and after transient acidification (latent TGF-β_1) of the supernatants. In acid-treated cell culture supernatants of PWM-stimulated PBMC and T cells obtained after 4 hr of incubation, 1·2 ± 0·3 ng/ml (PBMC) and 0·6 ± 0·2 ng/ml (T cells) were found (Fig. 2). Measurement of untreated culture supernatants revealed that only a small amount (10–20%) of the TGF-β_1 was present in the active 25000 MW form (data not shown). Interestingly, the DP IV inhibitors were capable of inducing a three- to fourfold increase in secretion of latent TGF-β_1 on both PWM-stimulated PBMC and purified T cells after a period of 4 hr (Fig. 2). These findings were confimed in a TGF-β-specific bioassay using the Mv1Lu cell line (data not shown). Control experiments showed that thiazolidine, piperidine and pyrrolidine as well as the aminopeptidase inhibitors amastatin, actinonin and probestin had no effect on TGF-β_1 secretion by PWM-stimulated PBMC and T lymphocytes (data not shown). As expected, TGF-β_2 which is not produced by human lymphocytes was not found in supernatants of the investigated cell cultures.

To clarify whether the DP IV inhibitor-induced secretion of latent TGF-β_1 was the result of increased TGF-β_1 mRNA levels, enzymatic amplification was performed on cDNA derived from unstimulated PBMC or T cells and from PBMC and T cells stimulated with PWM in the presence or absence of 10^{-5} M Lys[Z(NO_2)]-thiazolidide (Fig. 3). Total RNA was isolated after 3 hr from stimulated cells, reverse transcribed, and polymerase chain reaction (PCR) amplified using primers specific for TGF-β_1. In the presence of the DP IV inhibitor Lys[Z(NO_2)]-thiazolidide, levels of TGF-β_1 mRNA were found to be significantly increased in both PWM-stimulated PBMC and T cells (Fig. 3).

An anti-TGF-β_1 antibody neutralized the inhibitory effect of DP IV inhibitors on DNA synthesis of PWM-stimulated PBMC and purified T cells

In further experiments we investigated whether the amounts of TGF-β_1 measured in cell culture supernatants are capable of modulating DNA synthesis by PWM-stimulated PBMC and T cells. As shown in Fig. 4, active TGF-β_1 did suppress DNA synthesis significantly (more than 50% inhibition) at concentrations between 1 and 5 ng/ml when added 24 hr after PWM to PBMC or T cells.

Additional evidence on the crucial role of TGF-β_1 was provided by a specific TGF-β_1 antibody which is capable of neutralizing the biological activities of TGF-β_1. As shown in Fig. 5, this polyclonal chicken anti-TGF-$\beta_1$antibody abolished the DP IV inhibitor-induced suppression of DNA synthesis

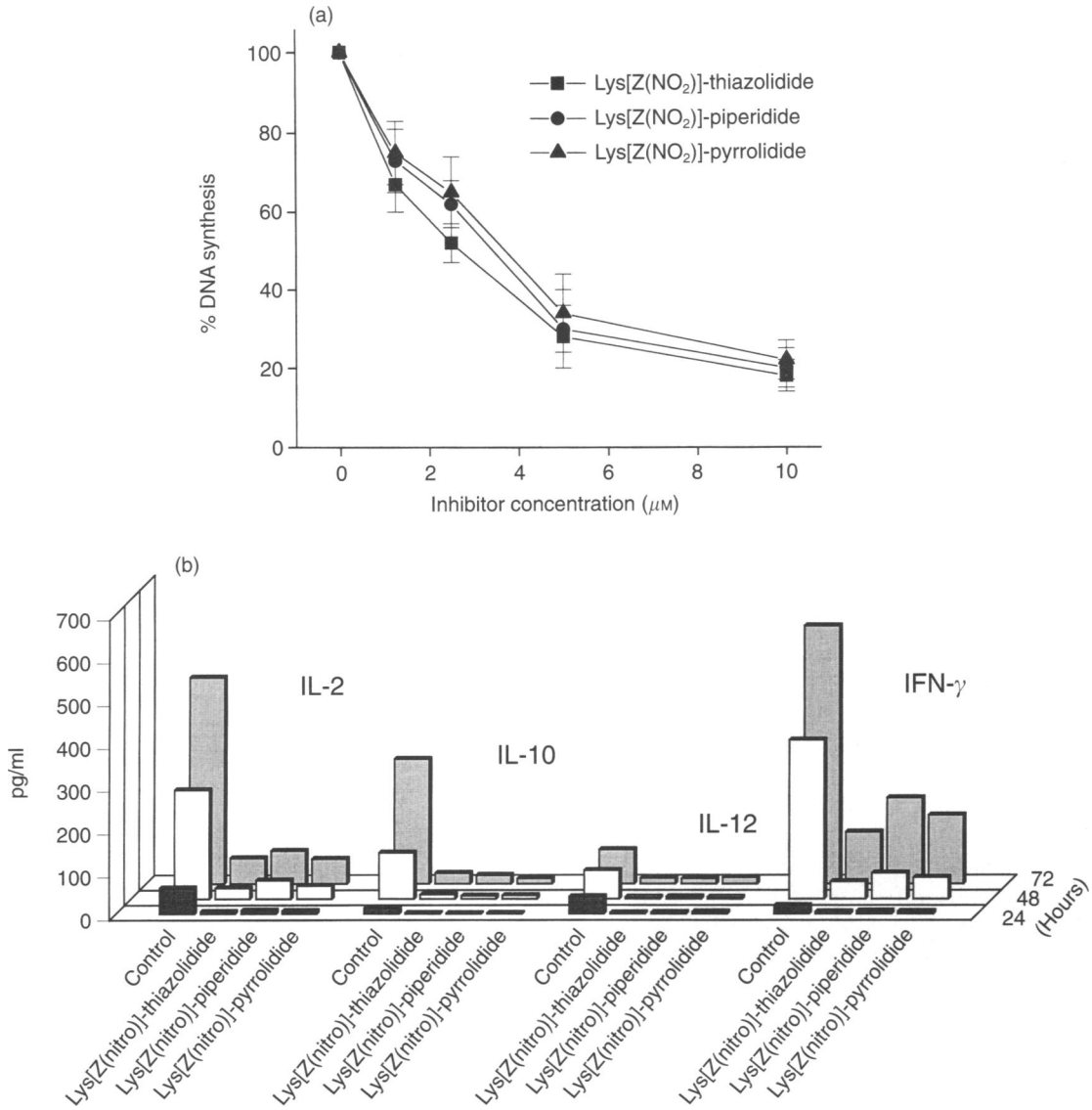

Figure 1. Influence of DP IV inhibitors on DNA synthesis and IL-2, IL-10, IL-12 and IFN-γ production in PWM-stimulated purified T cells. (a) T cells (10^5 cells/100 μl) were incubated with PWM (2 μg/ml) and different concentrations of the synthetic competitive DP IV inhibitors Lys[Z(NO_2)]-thiazolidide, Lys[Z(NO_2)]-piperidide and Lys[Z(NO_2)]-pyrrolidide. After 90 hr, the cultures were pulsed with[^{3}H]TdR for a further 6 hr. [^{3}H]TdR incorporation in c.p.m. is indicated as mean $\pm$ SD from four independent experiments. The values are expressed as percentage [^{3}H]TdR incorporated in relation to control cultures without inhibitor [^{3}H]TdR incorporation in control cultures 32 700 $\pm$ 4200. (b) The same cells (10^6 cells/ml) were incubated with PWM (2 μg/ml) and Lys[Z(NO_2)]-thiazolidide, Lys[Z(NO_2)]-piperidide and Lys[Z(NO_2)]-pyrrolidide (10^{-5} M). After 24, 48 and 72 hr, the supernatants were harvested, stored at $-70°$, and the cytokine levels were analysed in enzyme immunoassays. Results are indicated as the mean of three different experiments. The SD was less than 15%.

by PWM-stimulated PBMC and purified T cells almost completely.

DISCUSSION

Interest in the investigation of DP IV/CD26 has increased in recent years due to their presence on the surface of T cells, B cells and NK cells.[1,6,7] Evidence has also accumulated that this peptidase plays an important role in growth regulation and other functions of immune cells. Intriguingly, DP IV is an 'activation marker' of T cells suggesting that it may be involved in the processes of T-cell activation and in the regulation of the immune response.[1,2] The precise role(s) played by this enzyme in regulating these processes is unclear; however, the fact that specific synthetic inhibitors of DP IV can also inhibit T-cell growth suggests it may interact with, or influence, components of the immune cell cytokine network.[12,14,15,17] One of the main questions in defining the modes of action of DP IV is whether its enzyme activity is in fact necessary for these processes.

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 histiocytic

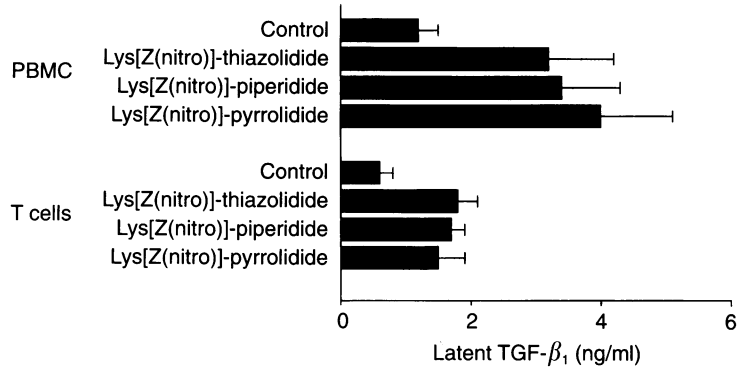

Figure 2. Influence of DP IV inhibitors on production of latent TGF-β_1 in PWM-stimulated PBMC and T cells. PBMC and T cells (10^6 cells/ml) were incubated with PWM (2 μg/ml) and the synthetic DP IV inhibitors Lys[Z(NO_2)]-thiazolidide, Lys[Z(NO_2)]-piperidide and Lys[Z(NO_2)]-pyrrolidide (10^{-5} M). After 4 hr, supernatants were harvested, stored at $-70°$, and the TGF-β_1 concentrations were measured with an enzyme immunoassay. Samples were tested after transient acidification (latent TGF-β_1). Results are expressed as mean $\pm$ SD of three independent experiments.

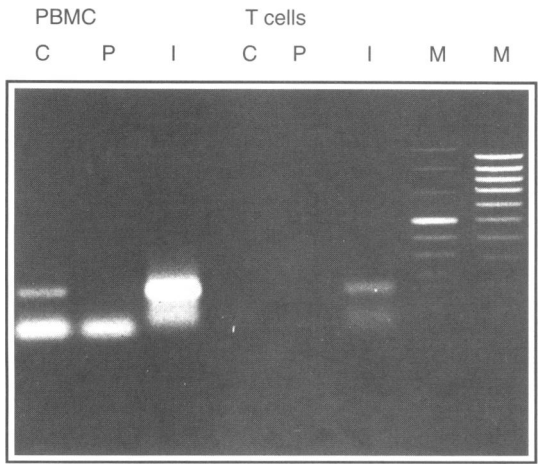

Figure 3. Influence of DP IV inhibitors on TGF-β_1 mRNA expression in PWM-stimulated PBMC and T cells. Enzymatic amplification of TGF-β_1 cDNA derived from untreated PBMC or T cells (C), cells stimulated with PWM (P), and cells stimulated with PWM in the presence of Lys[Z(NO_2)]-thiazolidide (10^{-5} M, I). Stimulation was allowed to proceed for 3 hr.

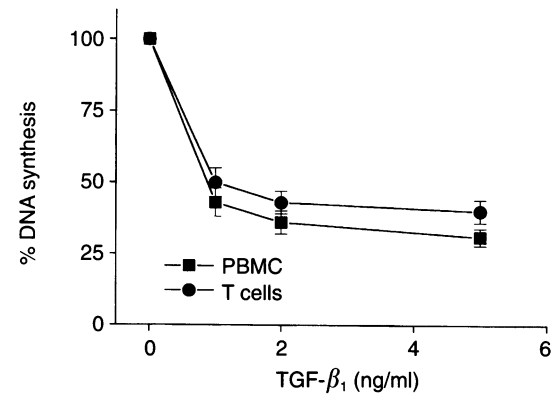

Figure 4. Influence of TGF-β_1 on DNA synthesis of PWM-stimulated PBMC and purified T cells. PBMC and purified T cells (10^6 cells/ml) were incubated with PWM (2 μg/ml). After 24 hr various concentrations of human natural TGF-β_1 were added. [^{3}H]TdR incorporations in c.p.m. are indicated as mean $\pm$ SD from three independent experiments. The values are expressed as percentage of [^{3}H]TdR incorporated in relation to control cultures without TGF-β_1 ([^{3}H]TdR incorporation in control cultures: 54 700 $\pm$ 4800 c.p.m. on PBMC; 28 400 $\pm$ 3100 c.p.m. on purified T cells).

lymphoma cell line U937, one expressing high levels (U937-H), the other low levels (U937-L) of CD26.[15] 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.[15] Moreover, we showed previously that Lys[Z(NO_2)]-thiazolidide and Lys[Z(NO_2)]-piperidide did not influence the IL-1 and TNF-α production of PWM-stimulated PBMC.[14] These results also convincingly demonstrated that these inhibitors do not impair the cellular function via non-specific cytotoxic actions.

Because of the specificity of these DP IV inhibitors, their inhibitory activity on lymphocyte proliferation and release of cytokines should be considered to be the consequence of a specific interaction with the enzyme on the surface of T lymphocytes.

The twofold effects of DP IV inhibitors on DNA synthesis on the one hand and the release of various 'immunostimulatory' and 'immunoinhibitory' cytokines on the other have not previously been studied in parallel in the same T-cell system. In the present report, we demonstrate that the inhibitors of DP IV Lys[Z(NO_2)]-thiazolidide, Lys[Z(NO_2)]-piperidide, and Lys[Z(NO_2)]-pyrrolidide inhibit the DNA synthesis of purified T lymphocytes in a dose-dependent manner. These findings confirm our former observations that DP IV inhibition results in impaired DNA synthesis and IL-2 and IL-6 production by PWM-stimulated PBMC.[14] In the present study, moreover, we could show for the first time that these DP IV inhibitors suppress the production of IL-2, IL-10, IL-12 and IFN-γ in PWM-stimulated purified T lymphocytes.

Additional experiments were carried out to determine whether the DP IV inhibitor-induced suppression of DNA synthesis and cytokine production may be due to the effect of another 'immunoinhibitory' cytokine, such as TGF-β_1 secreted

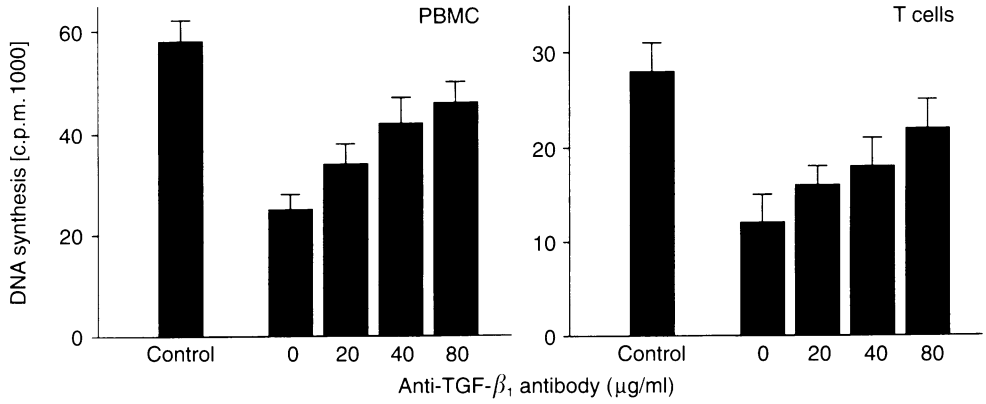

Figure 5. The effect of DP IV inhibitors on DNA synthesis of PWM-stimulated PBMC and T cells is partly neutralized by an anti-TGF-β_1 antibody. PBMC and T cells (10^5 cells 100 μl) were incubated with PWM (2 μg ml) in absence (control) and presence of the synthetic DP IV inhibitor Lys[Z(NO_2)]-thiazolidide (10^{-5} M) and different concentrations of a neutralizing chicken anti-TGF-β_1 antibody (R & D systems, BDA 19). After 90 hr, the cultures were pulsed with [^{3}H]TdR for a further 6 hr. [^{3}H]TdR incorporation in c.p.m. is indicated as mean ± SEM from three independent experiments.

by T cells after inhibition of DP IV activity. TGF-β_1 is known to have similar effects on the proliferation and cytokine production of PWM-stimulated PBMC and T cells, as has been described for the DP IV inhibitors we applied.[20,24]

Interestingly, the DP IV inhibitors were capable of inducing a three- to fourfold increase in the secretion of latent TGF-β_1 by both PWM-stimulated PBMC and purified T cells within a period of 4 hr. Furthermore, the TGF-β_1 mRNA levels in PWM-stimulated PBMC and T cells were strongly increased in the presence of the DP IV inhibitor Lys[Z(NO_2)]-thiazolidide after a period of 3 hr.

TGF-β_1 is a multipotent peptide that appears to be a potent immunosuppressive agent. Its capability to inhibit lymphocyte proliferation at femtomolar concentrations proves it to be significantly more potent than T-cell-specific immunosuppressants, such as cyclosporin A.[25] Various reports have suggested that TGF-β_1 is involved in the regulation of the cytokine network and the cytokine-mediated communication between immune cells.[20,24–27] In the present article we demonstrated that human TGF-β_1 inhibits DNA synthesis by both PWM-stimulated PBMC and purified T cells in a dose-dependent manner. We had previously found that TGF-β_1 significantly suppresses the release of IL-2, IL-6 and IL-10 by PWM-stimulated PBMC and T cells.[20,24]

We could also show here that a chicken anti-TGF-β_1 antibody neutralizes the DP IV inhibitor-induced suppression of DNA synthesis on PWM-stimulated PBMC and purified T cells, demonstrating the crucial role of TGF-β_1 in these processes.

We conclude that at least some of the immunosuppressive effects of DP IV inhibitors are mediated by TGF-β_1, which is secreted by T cells shortly after inhibition of the enzymatic activity.

As already mentioned, the particular role of DP IV/CD26 in the molecular mechanisms of immune response is not understood as yet. It is conceivable but not proven in detail that DP IV may play a crucial role in the cytokine cascade regulating the immune response. Interestingly, most of the immunostimulating cytokines, secreted in the early phase of immune activation, such as IL-1β, IL-2, IL-6, TNF-β, granulocyte and granulocyte–macrophage colony-stimulating factors, IL-11 and IL-13, are characterized by the particular N-terminal structure with proline in the second position which is susceptible for DP IV.[28,29] This suggests direct interactions of such bioactive peptides and cytokines with DP IV on the surface of activated immune cells. Thus, regulatory effects of DP IV might be associated with the modification of the biological activities of cytokines by limited proteolysis. Previously reported experimental approches revealed that DP IV/CD26 is capable of removing N-terminal X-Pro-peptides from synthetic oligopeptides with sequences analogous to the N-terminal part of natural and recombinant cytokines *in vitro*.[29]

The competetive inhibitors Lys[Z(NO_2)]-thiazolidide and Lys[Z(NO_2)]-piperidide used in the present investigations might function in the same manner as potential substrates of the DP IV. They bind to the catalytically active site of the enzyme and may be displaced by substrates with a higher affinity to the enzyme. In the present paper we could show that the incubation of DP IV-expressing cells with the competitive inhibitors results in the release of the immunosuppressive cytokine TGF-β_1. Assuming that immunostimulating cytokines or their fragments in fact are substrates of DP IV it seems to be possible that DP IV is involved in the induction of immunosuppression in order to limit excessive immune activation.

Zhou *et al.* could show that high concentrations of IL-6 (a potential substrate of the DP IV) induce the release of TGF-β_1.[30] This observation was confirmed by our own investigations in patients with limited inflammatory processes: excessively high concentrations of IL-6 were found to be associated with high levels of TGF-β_1 *in vivo* (unpublished preliminary results).

However, the proteolytic enzyme DP IV might be one of the regulatory elements between immunostimulating and immunosuppressing cytokines – controlling the balance of cellular immune response.

In summary, our findings show that the suppressive effect of DP IV inhibitors on DNA synthesis and cytokine production (IL-2, IL-10, IL-12 and IFN-γ) by PWM-stimulated PBMC and T cells may result from the induction of the inhibitory cytokine TGF-β_1. It remains for additional experi-

ments to determine whether this is the only mechanism responsible for these DP IV inhibitor-induced effects.

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft, SFB 387, grants A1, A4, A5 and by the Ministerium für Wissenschaft und Forschung des Landes Sachsen-Anhalt, grant 823 A 10313. The authors thank Ms Silke Möhring and Mrs Karin Frank for their skilful technical assistance.

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