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Interferon-γ induces dipeptidylpeptidase IV expression in human glomerular epithelial cells

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SUMMARY

Because dipeptidylpeptidase IV (DPP IV) is present *in vivo* on glomerular visceral epithelial cells and possesses immunogenic properties, as shown by the capability of anti-DPP IV antibody to induce the Heymann model of glomerulonephritis, we studied the expression and regulation of DPP IV in cultured human glomerular visceral epithelial cells. DPP IV is an ectoenzyme, as indicated by the rapid detection of the product of the reaction in the incubation medium of intact cells and the staining of paraformaldehyde-fixed cells in the presence of a specific anti-DPP IV antibody. DPP IV activity was inhibited by diisopropylfluorophosphate and phenylmethyl sulphonylfluoride. Its optimum pH was alkaline (7·7–8) and it exhibited a K_m value of 0·94 mM. DPP IV expression was induced in cells treated by interferon-γ (IFN-γ). The effect was significant after a 3-day treatment with 100 U/ml. It increased with time, reaching a plateau after 11 days, and was dose-dependent with a maximum at a concentration of 1000 U/ml. Staining of the cells with anti-DPP IV antibody was also increased after a 6-day treatment with 100 U/ml IFN-γ. It was shown by Northern analysis that, after 24 hr of exposure to 500 U/ml of IFN-γ, DPP IV mRNA transcript was stimulated. Transcriptional activation by IFN-γ did not require new protein synthesis. Interleukin-1 (IL-1) and cyclic AMP had a small stimulatory effect, whereas dexamethasone and phorbol esters were inefficient. These results suggest that DPP IV of glomerular epithelial cells may be up-regulated by IFN-γ from activated T lymphocytes in glomerular diseases and during lymphocyte-mediated graft rejection.

INTRODUCTION

Dipeptidylpeptidase IV (DPP IV; EC 3.4.14.5) is a serine exopeptidase that preferentially cleaves peptides containing Gly-Pro or Ala-Pro sequences at their N-terminal part. This enzyme has recently been cloned and its primary structure deduced from its cDNA both in rat[1] and in the human species.[2] DPP IV is an intrinsic membrane glycoprotein with most of its protein mass, including the catalytic moiety, protruding on the extracellular side. A number of cell-surface antigens, such as gp90,[3] gp108,[4] Tp103[5] and CD26,[6] initially recognized by specific monoclonal antibodies, have been identified eventually as DPP IV. Extensive evidence has been provided recently that DPP IV plays a role in the immune system. An anti-DPP IV monoclonal antibody was found to have T-cell activating property.[4] Proliferation of peripheral blood lymphocytes induced by mitogenic lectins and production of immunoglobulins by pokeweed mitogen-stimulated lymphocytes were inhibited by DPP IV inhibitors or anti-DPP IV antibody.[7] Production of interleukin-2 (IL-2) and interferon-γ (IFN-γ) by mitogen plus phorbol ester-stimulated human mononuclear cells was reduced by these same agents.[8] DPP IV also participated in tumour necrosis factor-α (TNF-α) degradation in the human monocytic lineage cell line U937, as well as in peripheral monocytes and macrophages.[9] In the rat kidney, DPP IV, reported as a 90,000 MW antigen by Ronco *et al.*[3] or a 108,000 MW antigen by Natori *et al.*,[4] is a major immunogen of the renal tubular membrane fraction of the rat. Injection of anti-gp90 or anti-gp108 into rats induced proteinuria and granular deposits along the glomerular capillary walls.[3,10] DPP IV has been localized in the rat kidney on glomerular visceral epithelial cells, endothelial cells, and the proximal tubule brush border by Chatelet *et al.*[11] using the anti-gp90 antibody.

The purpose of the present study was: (1) to verify that cultured human glomerular visceral epithelial cells express DPP IV; (2) to characterize the enzyme; and (3) to examine the regulation of its expression. Evidence is provided that intact subcultured glomerular epithelial cells exhibit DPP IV activity *in vitro* and that this enzyme is up-regulated by IFN-γ. The basis for this increased activity has been attributed to an increase of DPP IV mRNA transcript levels.

MATERIALS AND METHODS

Materials

Materials were obtained from the following suppliers: RPMI-1640 medium, HEPES and 0·05% trypsin-0·02% EDTA from

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Flow Laboratories (Irvine, U.K.); fetal calf serum, penicillin G and streptomycin sulphate from Gibco (Grand Island, NY); plastic plates and dishes from Nunc (Roskilde, Denmark) or Costar (Cambridge, MA); collagenase, gly-pro p-nitroanilide, 8-bromo cyclic AMP, 8-bromo cyclic GMP, *Escherichia coli* lipopolysaccharide, A23187 Ca^{2+} ionophore, prostaglandin E_2 (PGE_2), dexamethasone, forskolin, phorbol 12 myristate 13 acetate (PMA), diisopropylfluorophosphate (DFP), bestatin, phenylmethyl sulphonylfluoride (PMSF), antipain and leupeptin from Sigma (St Louis, MO); recombinant interferon-γ (rIFN-γ) and recombinant human interleukin-1β (rIL-1β) from Genzyme (Boston, MA). All other chemicals were of reagent grade and were used without further purification. The cDNA probe specific for human DPP IV was a gift from Dr Trugnan (Paris, France) and has been described previously.[12]

Epithelial cell culture

Isolation and characterization of human glomerular epithelial cells were performed as described previously.[10] Glomeruli were obtained by differential sieving and centrifugation from human cadaver kidneys judged to be unsuitable for transplantation. A homogenous population of epithelial cells was obtained by collagenase digestion of isolated glomeruli. These cells were cultured at 37° in RPMI-1640 medium buffered with 20 mM HEPES to pH 7·4 and supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine under an atmosphere of 5% CO_2/95% air. Human glomerular epithelial cells reached confluence after 10–15 days. They were subcultured and studied after one to three passages. Cells exhibited typical morphological and biochemical features of epithelial cells as described previously.[13]

Enzyme assays

Surface DPP IV activity was determined on confluent cells in 24-well plates. Cells were rinsed three times and incubated at 37° in 50 mM Tris-HCl buffer, pH 8·0, containing 130 mM NaCl and 1 mM $MgCl_2$. The enzymatic reaction was started by addition of 1·5 mM gly-pro p-nitroanilide. Incubation was carried out for 5–15 min with gentle agitation under zero-order kinetic conditions. The amount of p-nitroanilide formed was measured in the supernatant by reading at an OD of 405 nm. Cell-free and substrate-free blanks were run in parallel. Total enzyme activity was measured in cells which were sonicated using a Branson sonifier (model W 185 D; Branson Sonic Power Co., Danbury, CT) at position 5. The tubes containing the cells were immersed in an ice-water bath and the sample was sonicated for 60 seconds. Enzyme activity was expressed as nmol p-nitroanilide formed per min and per mg of cell protein.

Surface DPP IV of human glomerular epithelial cells was characterized by its susceptibility to various inhibitors: DFP, a serine protease inhibitor, PMSF, a protease inhibitor, antipain, and leupeptin, two serine protease inhibitors of microbial origin, and bestatin, an aminopeptidase-specific inhibitor. Cells were preincubated with inhibitor at the concentration stated for 10 min at 37° before substrate was added. Incubation was then carried out for 15 min. The optimum pH for glomerular epithelial cell DPP IV was determined over the range 6·2–8·5 using 50 mM Tris-HCl buffer containing 130 mM NaCl and 1 mM $MgCl_2$, or phosphate-buffered saline supplemented with 1 mM $MgCl_2$. The apparent K_m value was calculated by measuring the initial reaction velocities (V) over a substrate concentration (S) range of 0·25–5 mM. The data obtained were plotted according to Hofstee (V versus V/S).

After appropriate digestion with 1 M NaOH, cell protein was determined according to Lowry *et al.*[14] using bovine serum albumin as standard.

Immunofluorescence on confluent cultures

Expression of DPP IV at the cell surface was detected using a specific monoclonal antibody raised in the laboratory.[15,16] MOPC 21, a monoclonal antibody devoid of reactivity with renal tissue, was used as control. After three washings in phosphate-buffered saline (PBS), cells were fixed in 4% paraformaldehyde for 15 min at room temperature. They were then rinsed in 0·1 M glycine and incubated for 30 min with 100 μg/ml anti-DPP IV antibody. After three washings in PBS, cells were incubated with fluorescent sheep anti-mouse IgG for another 30-min period and again washed in PBS. Cells were observed with a Leitz microscope equipped with epifluorescence optics.

Northern blot analysis of DPP IV mRNA

Cells were incubated in medium with 10% fetal calf serum in the presence of 500 U/ml of IFN-γ for various periods of time. When necessary, 10 μg/ml of cycloheximide were added 20 min before adjunction of IFN-γ. Total RNA was extracted from human glomerular epithelial cells by the phenol/chloroform method and precipitated by LiCl as described by Genton *et al.*[17] Twenty micrograms of RNA was separated on a 0·9% agarose gel containing 20% formaldehyde and then transferred onto a nylon membrane (Gene Screen Plus, NEN, Boston, MA). Filters were prehybridized for 6 hr at 42° in a buffer containing 50% formamide, 600 mM NaCl, 0·5% sodium dodecylsulphate (SDS), 50 mM Tris-HCl (pH 7·4), 5 mM EDTA, 1 × Denhardt's (100 mg Ficoll, 50 mg polyvinylpyrrolidone and 50 mg bovine serum albumin in 10 ml H_2O) and then hybridized for 20 hr with a ^{32}P-labelled cDNA probe specific for human DPP IV under the same conditions. Filters were washed three times in 2 × SSC (1 × SSC is 0·15 M NaCl and 0·015 M sodium citrate, pH 7·0) and 0·1% SDS for 20 min at 42°. Equal loading of gels was assessed by methylene blue staining of the 28 S and 18 S RNA. Autoradiography was performed using a Fuji film and bands were quantified using a densitometric scanner (Appligene, Paris, France). Results are expressed as the ratio of the values found under experimental and control conditions.

Statistical analysis

Results are expressed as mean ± SE. Comparisons between groups were made using Student's *t*-test or variance analysis. Correlations between two parameters were estimated by regression analysis.

RESULTS

Characterization of DPP IV

When human glomerular epithelial cells were incubated for 5–15 min with gly-pro p-nitroanilide, a linear accumulation of p-nitroanilide in the medium was recorded. No measurable product was formed when gly-pro p-nitroanilide was incubated without cells. Immunohistochemistry on cells fixed with paraformaldehyde using a specific monoclonal antibody to DPP IV confirmed that DPP IV was present at the cell surface (Fig. 1).

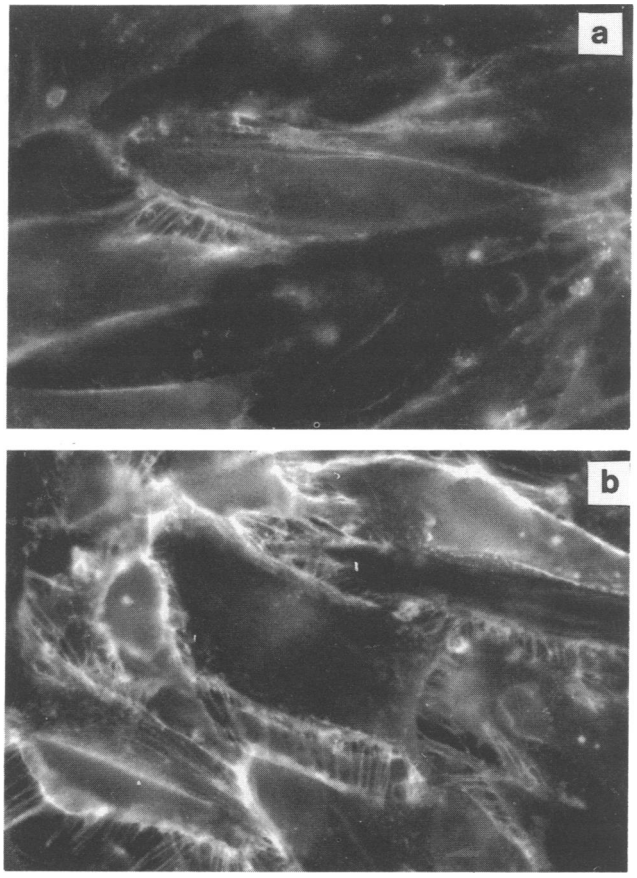

Figure 1. Expression of DPP IV in human glomerular epithelial cells. The enzyme was detected by immunofluorescence using a specific monoclonal antibody. Cells were fixed with 4% paraformaldehyde before incubation with the antibody under control conditions (a) or after exposure to 100 U/ml of IFN-γ for 6 days (b). Films for immunofluorescence pictures (Ilford HP5 plus, 400 ASA) were exposed for 18 seconds to the illuminated microscope field and the resulting negatives were printed under the same conditions. Note increased intensity of fluorescence in IFN-γ-treated cells (b).

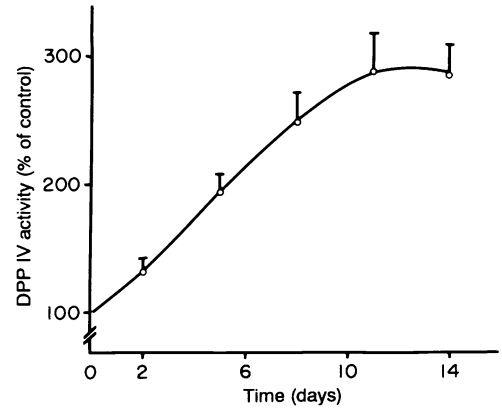

Figure 2. Time-course of DPP IV activity of human glomerular epithelial cells cultured with 100 U/ml of IFN-γ for 2–14 days. Means ± SE of four determinations are shown. Regression analysis showed a significant correlation ($P < 0.01$) between DPP IV and time.

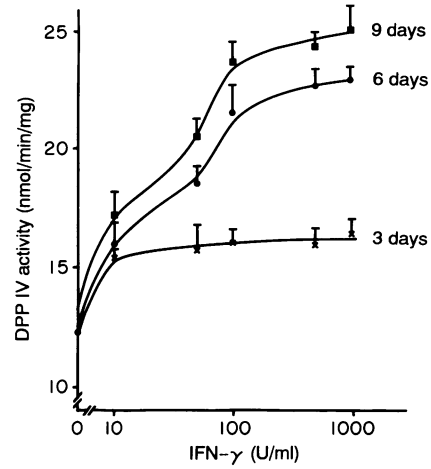

Figure 3. DPP IV activity of human glomerular epithelial cells as a function of increasing concentrations of IFN-γ. Cells were exposed to IFN-γ for 3 (crosses), 6 (closed circles) or 9 (closed squares) days. Means ± SE of four determinations are given. Two-way analysis of variance showed that DPP IV activity varied significantly ($P < 0.001$) with time and IFN-γ concentration.

Table 1. Effect of protease inhibitors on DPP IV activity of human glomerular epithelial cells

	DPP IV activity	
Inhibitor	nmol/min/mg	% of control
None	9·3 ± 0·4	100
Bestatin (100 μM)	9·6 ± 0·7	102·9
Antipain (1 mM)	9·8 ± 0·2	105·1
Leupeptin (1 μM)	9·0 ± 0·2	96·5
PMSF (1 mM)	6·6 ± 0·2**	70·8
DFP (1 mM)	1·02 ± 0·03***	10·9

Values are mean ± SE of four determinations. Control DPP IV activity is taken as 100%. The effects of inhibitors were compared with control using Student's *t*-test.

**$P < 0.01$; ***$P < 0.001$.

Activity of the ectoenzyme represented a substantial fraction of the total enzyme activity (8·9 ± 0·4 versus 23·3 ± 1·4 nmol/mg/min).

DPP IV activity was almost completely inhibited by pre-treatment of the cells with 1 mM DFP. Another serine-protease inhibitor, PMSF, also inhibited surface DPP IV activity but to a lesser extent (30%). In contrast, antipain and leupeptin, two microbial serine protease inhibitors, as well as bestatin, an aminopeptidase inhibitor, were ineffective (Table 1). Optimum pH was 7·7 in phosphate-buffered saline and 8·0 in Tris-HCl buffer. The apparent K_m value calculated from the Hofstee's transformation of the velocity versus substrate concentration curve was 0·94 mM.

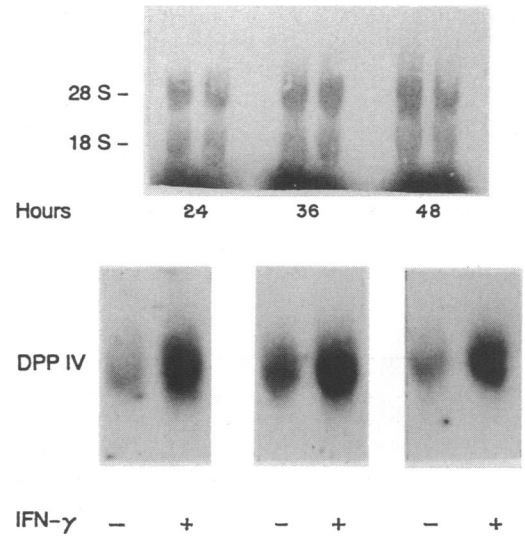

Figure 4. Effect of 500 U/ml of IFN-γ on DPP IV mRNA level in human glomerular epithelial cells after increasing times of incubation (24–48 hr). Methylene blue staining of the 28 S and 18 S RNA is shown in the upper part of the figure.

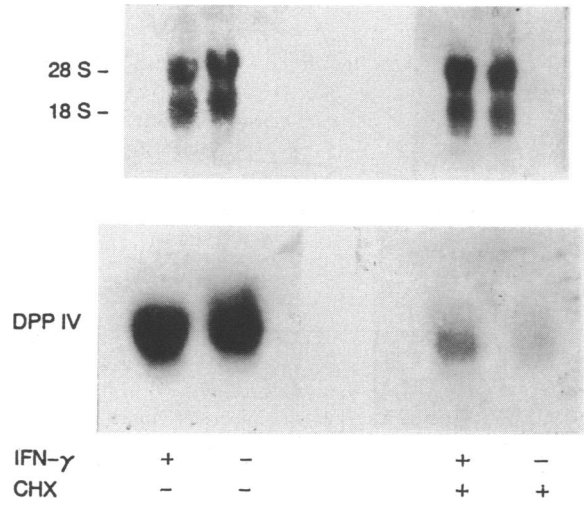

Figure 5. Effect of cycloheximide (CHX) on DPP IV mRNA level. Human glomerular epithelial cells were exposed to 10 μg/ml of cycloheximide for 36 hr in the presence or absence of IFN-γ (500 U/ml). Methylene blue staining of the 28 S and 18 S RNA is shown in the upper part of the figure.

Induction of DPP IV by rIFN-γ and other agents

DPP IV activity of human glomerular epithelial cells treated by 100 U/ml of rIFN-γ over a period of 14 days increased progressively with time. A significant effect was obtained from the second day of treatment but the maximum effect was reached only after 11 days. There was no further increase over the three following days (Fig. 2). DPP IV activity also increased as a function of the concentration of rIFN-γ after various periods of treatment. A plateau at 34% above basal value was rapidly reached after a 3-day treatment for concentrations between 10 and 1000 U/ml. More marked effects occurred after 6- or 9-day treatments. The highest DPP IV activities observed under these conditions with 1000 U/ml of rIFN-γ were 1·9 and 2 times basal value, respectively (Fig. 3). IFN-γ stimulated both surface and total enzyme activities. Total basal DPP IV activity (23·3 $\pm$ 1·4 nmol/mg/min) increased up to 31·1 $\pm$ 1·7 and 33·6 $\pm$ 1·9 nmol/mg/min after a 6-day treatment by 100 and 500 U/ml rIFN-γ, respectively. The stimulatory effect of IFN-γ on DPP IV expression at the surface of glomerular epithelial cells was confirmed by immunofluorescence. Staining with an anti-DPP IV monoclonal antibody showed an increase of DPP IV expression at the cell surface after a 6-day treatment with 100 U/ml of rIFN-γ (Fig. 1). We next performed Northern analysis to test the possibility that the primary mechanism of IFN-γ up-regulation of DPP IV activity occurred at the transcriptional level. Control cells constitutively exhibited DPP IV mRNA. Transcript was detected as a single band of approximately 4 kilobases. IFN-γ markedly stimulated the DPP IV transcript after 24 hr exposure to 500 U/ml (2·4 times basal value). The effect was still apparent after incubations of 36 and 48 hr (1·4 and 1·9 times basal value, respectively) (Fig. 4). After hybridization, the blots were stained by methylene blue. Results shown in Fig. 4 (upper part) indicate that the same amount of mRNA was loaded on the gel. To determine whether induction of DPP IV mRNA was a primary effect or required intermediate protein synthesis, we examined the effect of treating the cells with 10 μg/ml of cycloheximide for 36 hr (Fig. 5). Cycloheximide alone decreased DPP IV mRNA by 50%. When cells were treated with both IFN-γ and cycloheximide, stimulation by IFN-γ still persisted (1·5 times the value obtained with cycloheximide alone).

The effect of other agents on DPP IV activity was also studied. No stimulation was observed with PMA after a 3-day treatment over a concentration range of 1–25 ng/ml or with dexamethasone after a 1–6-day treatment over a concentration range of 0·1–10 μM (data not presented). Similarly, PGE_2, *E. coli* lipopolysaccharide, A23187 calcium ionophore and 8-bromo-cyclic GMP had no effect (Table 2). Exposure of the cells to 500 μM 8-bromo-cyclic AMP for 3 days produced a slight but significant effect (30·3% above basal value; $P < 0{\cdot}01$). A similar effect was obtained with 1 U/ml rIL-1β (27·5% above basal

Table 2. Effect of various agents on DPP IV expression at the surface of human glomerular epithelial cells

Agent	DPP IV activity	
	nmol/min/mg	% of control
None	8·5 $\pm$ 0·3	100
PGE_2 (10 μM)	8·8 $\pm$ 0·2	103·3
E. coli lipopolysaccharide (10 μg/ml)	8·9 $\pm$ 0·2	103·9
A23187 calcium ionophore (1 μM)	8·6 $\pm$ 0·2	100·5
8-bromo-cyclic AMP (500 μM)	11·1 $\pm$ 0·2**	130·3
8-bromo-cyclic GMP (500 μM)	9·1 $\pm$ 0·2	107·3
IL-1β (1 U/ml)	10·8 $\pm$ 0·4**	127·5

Values are means $\pm$ SE of four determinations. Control DPP IV activity is taken as 100%. Cells were exposed to the agents indicated for 3 days. The effects of treatments were compared with control using Student's *t*-test. **$P < 0{\cdot}01$.

value; $P<0{\cdot}01$). For these latter two agents, there was no increase in their stimulatory effect when the cells were treated for up to 6 days. Activation of DPP IV after a 3-day treatment was dose-dependent for 8-bromo-cyclic AMP. In contrast, there was a plateau with rIL-1β between 1 and 5 U/ml.

DISCUSSION

This study indicates that intact human glomerular epithelial cells in culture exhibit DPP IV activity, are positive for the DPP IV antigen when exposed to a DPP IV monoclonal antibody (anti-gp90) and express constitutively DPP IV mRNA. The detection of the reaction product, p-nitroanilide, in the extracellular medium without any delayed period and the staining of paraformaldehyde-fixed cells which are not permeable to antibodies confirm the presence of the enzyme at the cell surface. Therefore this characteristic of podocytes, already demonstrated *in vivo*,[11] is maintained *in vitro* and can be used to identify this cell type. Moreover, we have recently shown that the DPP IV antigen was also present at the surface of a simian virus 40-transformed human podocyte cell line presenting all the characteristics of the non-transformed parental cells.[18] The properties of the enzyme that we have described in human podocytes are similar to those already reported in other preparations. The K_m value of 0·94 mM is almost identical to that found in murine thymocytes[19] and close to that of the purified enzyme excreted in normal human urine,[20] estimated as 0·95 and 0·13 mM, respectively. Inhibition of glomerular epithelial cell DPP IV activity by PMSF is in agreement with the results of Bauvois[19] in murine thymocytes.

We also demonstrate that exposure to rIFN-γ induces expression of DPP IV in human glomerular epithelial cells. This effect is time- and concentration-dependent. It is noteworthy that the maximum effect required a long period of treatment. Northern blot analysis also showed an effect of IFN-γ on DPP IV mRNA after 24 hr incubation which was still present at 48 hr. This is in accordance with the variability in the speed and the persistence of the response to IFN-γ as reported in the literature for the expression of various genes. For example, increased mRNA transcripts of xanthine dehydrogenase-xanthine oxidase in cultured rat pulmonary endothelial cells were observed after exposure to IFN-γ between 4 and 48 hr, a maximum being reached by 48 hr.[21] Induction of DPP IV mRNA was inhibited by cycloheximide, suggesting that transcription of the gene or stability of the mRNA depends on synthesis of an intermediate stimulatory protein. In contrast, cycloheximide did not block IFN-γ dependent stimulation of DPP IV mRNA expression, indicating a direct effect of IFN-γ on the transcriptional rate or mRNA half-life. Since IFN-γ markedly increased DPP IV mRNA and since IFN-γ induced both total and surface DPP IV activity, it is likely that this cytokine did not modify the intracellular distribution of the enzyme, but increased its synthesis. IFN-γ, a cytokine produced by activated T lymphocytes, modulates several aspects of the immune response.[22] It induces expression of a variety of membrane proteins and, particularly, class I and class II antigens of the major histocompatibility complex.[23,24] This effect has been described in epithelial cells of renal allografts. IFN-γ also augments the expression of intercellular adhesion molecule-1 (ICAM-1) in renal epithelial cells.[25] Such cells thus acquire immunogenic properties and may play a direct role in the lymphocyte-mediated graft rejection.[26] Recently, Mendrick *et al.*[27] demonstrated that glomerular visceral epithelial cells, after having been cultured for 4–5 days in the presence of 100 U/ml IFN-γ, were capable of processing and presenting complex antigens to T-cell hybridomas. Only cells exposed to the cytokine could act as antigen-presenting cells. Over-expression of DPP IV may represent a supplementary mechanism by which IFN-γ contributes to the differentiation of epithelial cells in renal allografts or glomerular diseases. In accordance with this hypothesis, DPP IV was found to be induced in podocytes in various renal diseases,[28] and urinary excretion of DPP IV was increased in both glomerular and tubulointerstitial diseases.[29]

In contrast with IFN-γ, we did not find any effect of dexamethasone on DPP IV activity. This result is in agreement with the findings of Ronco *et al.*,[30] who found that total DPP IV activity of cultured renal proximal tubular cells was unchanged when a serum-enriched incubation medium was replaced by a serum-free defined medium containing dexamethasone. In contrast, dexamethasone enhanced the expression of DPP IV in transfected chinese hamster ovary cells,[31] indicating a different control of the enzyme expression in various tissues.

Comparison of the results of the present study with those obtained in human glomerular cells for other peptidases shows that these enzymes are under different regulations. Aminopeptidase A expression depends on cyclic AMP and dexamethasone,[32] whereas aminopeptidase N is induced by IFN-γ and mitogens such as phorbol esters.[33] In contrast with the latter enzyme, DPP IV activity is not stimulated by PMA but appears to be essentially dependent on IFN-γ. The knowledge of the mechanisms by which the synthesis of these enzymes is regulated is of particular interest because it has been demonstrated that ectopeptidases play a major role in cell communication by processing peptide hormones and local mediators, and in cell activation by as yet unknown mechanisms.

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