Streptokinase Promotes Development of Dipeptidyl Peptidase IV (CD26) Autoantibodies after Fibrinolytic Therapy in Myocardial Infarction Patients

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Dipeptidyl peptidase IV (DPP IV) (CD26) plays a critical role in the modulation and expression of autoimmune and inflammatory diseases. We recently reported that sera from patients with rheumatoid arthritis and systemic lupus erythematosus contained low levels of DPP IV and high titers of anti-DPP IV autoantibodies of the immunoglobulin A (IgA) and IgG classes and found a correlation between the low circulating levels of DPP IV and the high titers of anti-DPP IV autoantibodies of the IgA class. Since streptokinase (SK) is a potent immunogen and binds to DPP IV, we speculated that patients with autoimmune diseases showed higher DPP IV autoantibody levels than healthy controls as a consequence of an abnormal immune stimulation triggered by SK released during streptococcal infections. We assessed this hypothesis in a group of patients suffering from acute myocardial infarction, without a chronic autoimmune disease, who received SK as part of therapeutic thrombolysis. Concomitant with the appearance of anti-SK antibodies, these patients developed anti-DPP IV autoantibodies. These autoantibodies bind to DPP IV in the region which is also recognized by SK, suggesting that an SK-induced immune response is responsible for the appearance of DPP IV autoantibodies. Furthermore, we determined a correlation between high titers of DPP IV autoantibodies and an augmented clearance of the enzyme from the circulation. Serum levels of the inflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) increased significantly after 30 days of SK administration, while the levels of soluble IL-2 receptor remained unchanged during the same period, suggesting a correlation between the lower levels of circulating DPP IV and higher levels of TNF-α and IL-6 in serum in these patients.

Dipeptidyl peptidase IV (DPP IV) (CD26) is a widely distributed, multifunctional, highly glycosylated membrane-bound ectoenzyme (10) that cleaves X-Pro dipeptides from the NH₂ terminus of a number of proteins (31). Expression of DPP IV is highly associated with cell differentiation and activation, and it is involved in T-lymphocyte activation and migration across the extracellular matrix (1, 28). DPP IV is also found in human plasma (24), where its enzymatic activity is correlated with the activity of the enzyme in normal T lymphocytes (37). Not only are plasma DPP IV isoforms analogous to isoforms found in T lymphocytes (23), but they bind adenosine deaminase with similar specificity and affinity (21), suggesting that the plasma enzyme originates from T lymphocytes (22, 38).

Due to the key role that the membrane-bound DPP IV plays in T-cell-mediated immune responses and lymphokine synthesis (8), the enzyme has been studied in several autoimmune disorders such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). In both RA and SLE there is a reduction in serum DPP IV activity (11, 29, 30, 41). In a recent report (6), we demonstrated that reduction of serum DPP IV activity in RA patients was due to hypersialylation of the enzyme, whereas in SLE patients a similar reduction in activity was possibly the result of increased clearance of DPP IV from circulation due to the high titers of circulating anti-DPP IV autoantibodies of the immunoglobulin A (IgA) class (6).

In previous studies we found that streptokinase (SK), a protein secreted by streptococci which facilitates the development of focal infection in association with plasminogen (Pg) of the host, can induce anti-Pg autoantibodies (16). We also demonstrated that SK binds to DPP IV expressed by rheumatoid synovial fibroblasts (17). Given these observations and since SK is a very potent immunogenic protein, we hypothesized that high titers of DPP IV autoantibodies in plasma in patients with autoimmune diseases could possibly result from immune stimulation by bacterial proteins such as SK. We assessed this hypothesis in a group of patients without chronic autoimmune disease who had suffered from acute myocardial infarction and had received SK as part of therapeutic thrombolysis.

We analyzed the expression and titers of anti-DPP IV antibodies in serum for 90 days after administration of SK and found that these autoantibodies bind preferentially to an epitope in DPP IV which is also recognized by SK. We also analyzed serum levels of the inflammatory cytokines tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), and soluble interleukin 2 receptor (IL-2 sRα), which are sensitive to DPP IV levels in the circulation (21). Since superantigens of bacterial origin have been postulated as participants in the pathogenesis of autoimmune disease in humans (32), our data suggest a potential role for SK in these processes.
MATERIALS AND METHODS

Patients. The protocol was approved by the Ethics Committees of both participating centers. After informed consent was obtained, a group of 10 patients who were treated with SK due to myocardial infarction during 1994 and 1995, at the University of Chile Clinical Hospital, were studied to assess the induction of anti-SK and anti-DPP IV autoantibodies. All of them were men, with a mean age of 47 years (age range, 39 to 72 years). Seven patients were chronic cigarette smokers. Three of the patients had arterial hypertension; two of them were being treated with atenolol and hydrochlorothiazide, and the third one was receiving enalapril. One of the patients had diabetes mellitus and was being treated with tolbutamide. None of them had a history of streptococcal infection during the 6 months previous to the acute myocardial infarction. They received thrombolytic therapy as a single intravenous dose of 1.5 × 10^9 UF of SK. All patients received aspirin, 250 mg/day, after administration of SK. Analyses of the levels of anti-DPP IV IgA, IgG, or IgM were performed with serum samples collected immediately before administration of SK. Additional serum samples were collected at 10, 30, and 90 days after administration of the thrombolytic agent. None of the patients had a prior history of connective tissue disease or suffered from preexisting lymphoproliferative diseases. No one developed arthritis or any signs of connective tissue disease during the 2 years of follow-up.

Peptides. The octapeptides LITSRAPGH and its randomized sequence APHLSTGR were purchased from Multiple Peptide Systems, San Diego, Calif.

Proteins. DPP IV from a pool of human plasma (2 liters) was isolated by sequential purification using DEAE-Sepharose ion-exchange chromatography, Gly-Leu affinity chromatography, and gel filtration over Sephacryl S-200 as described by De Meester et al. (38). The polyclonal anti-DPP IV IgG antibodies were prepared in rabbits according to standard protocols (19). The polyclonal anti-DPP IV IgG, IgA, and IgM were purified from patient sera by chromatography on maltose binding protein-Sepharose (35), followed by immunoadsorption of DPP IV coupled to Sepharose 4B. The monoclonal anti-DPP IV IgG, IgA, and IgM were purified by affinity chromatography on protein A-Sepharose (13) followed by immunoabsorption to DPP IV coupled to Sepharose 4B. The monoclonal anti-DPP IV IgG, IOA26-clone BAS, was purchased from AMAC, Inc. (Westbrook, Maine). Human IgG Fc fragment and goat affinity-purified F(ab')2 fragments against human IgA, IgG, and IgM were purchased from ICN Pharmaceuticals, Inc. (Aurora, Ohio). Secondary antibodies to human IgA, IgG, and IgM were purchased from Sigma Chemical Co. Human anti-DPP IV IgA was purified from patient sera by chromatography on Jacalin-Sepharose (33) followed by immunoadsorption to DPP IV coupled to Sepharose 4B. Human anti-DPP IV IgG was purified from patient sera by chromatography on protein A-Sepharose (13) followed by immunoadsorption to DPP IV coupled to Sepharose 4B. Human anti-DPP IV IgM was purified from patient sera by ion-exchange chromatography on maltose binding protein-Sepharose (35), followed by immunoabsorption to DPP IV coupled to Sepharose 4B.

ELISA. Enzyme-linked immunosorbent assays (ELISA) were performed in 96-well culture plates. Quantification of anti-SK antibodies was performed in 96-well culture plates coated with SK as previously described (15). For quantification of DPP IV, plates were coated first with 200 μl of a solution containing 5 μg of an anti-DPP IV monoclonal antibody (MAb) (IOA26-clone BAS) per ml in 0.1 M Na2CO3 and incubated overnight at 4°C. The MAb anti-DPP IV clone BAS is well characterized and has been used for immunoprecipitations and Western blot analyses of full-length DPP IV (20). After coating, plates were rinsed with 200 μl of 10 mM sodium phosphate–0.1 M NaCl (pH 7.4) containing 0.05 Tween 80 (PBS-Tween) to remove unbound protein. Nonspecific sites were blocked by incubating with PBS-Tween containing 2% bovine serum albumin (PBS-Tween–2% BSA) at room temperature for 1 h. Plates were washed again with PBS-Tween, air dried, and stored at 4°C. For assays, increasing concentrations of sera were added in triplicate in a 200-μl final volume of PBS-Tween–2% BSA and incubated at 37°C for 2 h, followed by rinsing with PBS-Tween and incubation with an affinity-purified polyclonal anti-DPP IV rabbit IgG (100 ng/ml) at 37°C for 1 h. Plates were then washed with PBS-Tween containing 200 μl of alkaline phosphatase substrate (1 mg/ml p-nitrophenylphosphate) in 0.1 M glycine–1 mM MgCl2–1 mM ZnCl2 (pH 10.4), was added to the plate, and absorbance was monitored at a wavelength of 405 nm using an Anthos Labtec Kinetic Plate reader. Bound DPP IV was expressed as ΔA405/min. Concentrations of DPP IV were calculated from a calibration curve constructed with affinity-purified DPP IV. The concentrations of anti-DPP IV IgA, IgG, and IgM in sera of patients were determined by ELISA using 96-well culture plates coated with DPP IV. Provisions were made to avoid reactivity of specific DPP IV autoantibodies with rheumatoid factors. Plates were incubated with serum samples (1:200 dilution) in the presence of IgG Fc fragments (1 μg/ml) at 37°C for 2 h, rinsed extensively with PBS-Tween, and finally incubated with alkaline phosphatase-conjugated anti-human IgA, IgG, and IgM F(ab')2 fragments at 37°C for 1 h, and the concentrations of bound immunoglobulins were calculated from calibration curves expressing the rate of hydrolysis of the alkaline phosphatase substrate p-nitrophenylphosphate (ΔA405/min) versus the concentration of pure immunoglobulins of the three classes. The total concentration of these immunoglobulins in patient sera was determined by ELISA in 96-well culture plates coated with F(ab')2 specific anti-human IgA, IgG, and IgM. After incubation of sera in the presence of Fc fragments (1 μg/ml) to avoid cross-reactivity of immunoglobulins, the plates were rinsed with PBS-Tween and incubated with Fc-specific alkaline phosphatase-conjugated anti-human IgA, IgG, and IgM. Final concentrations were calculated from calibration curves constructed with pure human IgA, IgG, and IgM. For peptide competition experiments, plates coated with DPP IV were first incubated with increasing concentrations of the octapeptides LITSRAPGH or its randomized sequence APHLSTGR in PBS- Tween–2% BSA and incubated for 1 h at 37°C before addition of a single concentration (100 ng/ml) of anti-DPP IV autoantibodies. Concentrations of bound antibodies were determined as described above.

Serum cytokine assays. Concentrations of TNF-α, IL-6, and IL-2 sR in serum were determined by quantitative sandwich ELISA (R&D Systems, Minneapolis, Minn.) according to manufacturer’s instructions. Assays of sample reproducibility showed intra-assay coefficients of variation for TNF-α, IL-6, and IL-2 sR equal to or less than 4.2, 2.0, and 4.6%, respectively, while interassay coefficients of variation were equal to or less than 3.8, 2.6, and 1.8%, respectively.

RESULTS

Anti-SK antibody levels in patients who received SK therapeutically. Analysis of anti-SK IgA, IgG, and IgM levels (Fig. 1) shows that IgA and IgG titers peaked 30 days after administration of SK, followed by a slow increase up to 90 days after the infusion of the thrombolytic agent. There were statistically significant differences between the IgA baseline levels and those at 10, 30, and 90 days (medians, 34.99, 72.02, 74.41, and 60.98 μg of anti-SK IgA/mg of IgA, respectively; P < 0.0003 overall and P < 0.005 for each comparison). The differences between the baseline IgG anti-SK antibodies and those at 10, 30, and 90 days were also statistically significant (medians, 20.86, 64.83, 78.04, and 74.38 μg of anti-SK IgG/mg of IgG, respectively; P < 0.0002 overall, and P < 0.008 for each comparison). No statistically significant differences between baseline and 10, 30, and 90 days were observed for IgM anti-SK autoantibodies (medians, 28.88, 29.37, 26.99, and 27.32 μg of IgM/mg, respectively).

Anti-DPP IV autoantibodies levels in patients who received SK therapeutically. Concomitant with the increase of anti-SK IgA and IgG in serum, we observed a statistically significant increase of anti-DPP IV IgA from the baseline to 10, 30, and 90 days after SK administration (median concentrations in serum, 14.76, 24.34, 27.97, and 27.13 μg of anti-DPPV IV
IgA/mg of IgA respectively; \( P < 0.0002 \) overall and \( P < 0.0015 \) for all comparisons) (Fig. 2A). Anti-DPP IV autoantibodies of the IgG class were also increased 10 days after SK administration, reaching statistically significant levels at 30 and 90 days after SK administration (median concentrations in serum, 0.72, 0.78, 0.90, and 0.93 \( \mu \)g of anti-DPP IV IgG/mg of IgG, respectively; \( P < 0.004 \) overall; \( P < 0.057 \) at 10 days; \( P < 0.012 \) at 30 and 90 days) (Fig. 2B). No statistically significant increase in DPP IV autoantibodies of the IgM class was found over time (median concentration in serum, 28.87, 25.45, 24.03, and 25.96 \( \mu \)g of anti-DPP IV IgM/mg of IgM, respectively) (Fig. 2C).

**FIG. 1.** Levels of anti-SK antibodies in serum from patients \( (n = 10) \) who received SK therapeutically. Serum samples (1:500 dilution) in 0.2 ml of PBS-Tween were added separately to 96-well microtiter plates coated with SK and analyzed as described in Materials and Methods. (A) Anti-SK IgA titers; (B) anti-SK IgG titers; (C) anti-SK IgM titers. n.s., not significant.

**DPP IV levels and activity over time in sera of patients who received SK therapeutically.** The median serum DPP IV levels from a group of patients \( (n = 10) \) who received SK therapeutically (Fig. 3) show a continuous decrease in the enzyme concentration between 0 and 90 days after SK administration (1.9, 1.12, 1.12, and 0.80 \( \mu \)g/ml at 0, 10, 30, and 90 days, respectively). The difference between 0 and 90 days is statistically significant \( (P < 0.0042) \). However, measurements of DPP IV enzymatic activity during the same period (data not shown) show no statistically significant changes.

**Binding specificity of anti-DPP IV autoantibodies.** SK binds to DPP IV via the LTSRPA amino acid sequence (17). Therefore, if interactions with SK are involved in the mechanisms responsible for the production of anti-DPP IV autoantibodies, either SK or the LTSRPA peptide should be able to interfere with autoantibody binding to DPP IV. A series of ELISA experiments was carried out to establish the binding specificity of the anti-DPP IV autoantibodies. Plates coated with DPP IV were incubated with a single aliquot (5 \( \mu \)l), in triplicate, from each SK-treated patient in the presence of increasing concentrations of the octapeptide LTSRPAHG or its randomized sequence APHLSTGR. The concentrations of anti-DPP IV autoantibodies of the IgA and IgG classes bound under these conditions were determined as described in Materials and Methods. Since the inhibition curves were similar for all the sera tested (data not shown), we purified anti-DPP IV IgA and IgG from one serum with the highest titers. Both purified anti-DPP IV antibodies react with the immobilized antigen in a dose-dependent manner (Fig. 4). Incubation of DPP IV plates with a single concentration of either IgA (Fig. 5A) or IgG (Fig. 5B) anti-DPP IV autoantibody (500 ng/ml) in the presence of increasing concentrations of the peptide LTSRPAHG, inhibited binding of both classes of autoantibodies, whereas its randomized sequence APHLSTGR produced no effect.

**Serum cytokine levels.** Analyses of levels of TNF-\( \alpha \), IL-6, and IL-2 sR in serum after SK administration (Fig. 6A) show that TNF-\( \alpha \) increased from a median of 1.55 pg/ml at baseline to 1.86 pg/ml at 10 days and 7.04 pg/ml at 30 days, returning to near baseline (1.50 pg/ml) at 90 days after SK administration. With regard to serum IL-6 levels (Fig. 6B), their medians also increased from 36.91 pg/ml at baseline to 93.57 and 117.80 pg/ml at 10 and 30 days after SK administration, respectively. The median serum IL-6 levels returned close to baseline (38.86 pg/ml) at 90 days. For both these cytokines, the differences between their levels at baseline and 10 and 30 days post-SK administration were statistically significant, with a \( P \) of \( <0.02 \) and \( <0.006 \) for TNF-\( \alpha \) at 10 and 30 days, respectively, and a \( P \) of \( <0.006 \) and \( <0.006 \) for TNF-\( \alpha \) at10 and 30 days, respectively. The median IL-2 sR levels in serum changed from 610.68 pg/ml at baseline to 694.84, 676.41, and 655.03 pg/ml at 10, 30, and 90 days after SK administration, respectively (Fig. 6C). None of these differences were statistically significant.
In addition to its multiple roles in the expression or modulation of autoimmune and inflammatory responses (29), DPP IV functions as a receptor for both Pg (14) and SK (17). In a previous study (16), we found evidence suggesting that high titers of anti-Pg autoantibodies in sera from patients with RA and SLE may be the result of associations between SK (liberated during streptococcal infections) and Pg of the host (16). Recently, we reported the presence of anti-DPP IV autoantibodies in sera of patients with several autoimmune disorders.

**DISCUSSION**

In addition to its multiple roles in the expression or modulation of autoimmune and inflammatory responses (29), DPP IV functions as a receptor for both Pg (14) and SK (17). In a previous study (16), we found evidence suggesting that high titers of anti-Pg autoantibodies in sera from patients with RA and SLE may be the result of associations between SK (liberated during streptococcal infections) and Pg of the host (16). Recently, we reported the presence of anti-DPP IV autoantibodies in sera of patients with several autoimmune disorders.
The inflammatory cytokines TNF-α and IL-6 in serum were significantly elevated after 30 days of SK administration, while the levels of IL-2 sRα remained unchanged during the same period, suggesting a correlation between the lower levels of circulating DPP IV and high levels of TNF-α and IL-6 in these patients.

Newly synthesized DPP IV is cleared from circulation by the liver basolateral endosomes in association with polymeric IgA receptors, responsible for the transportation of IgA to bile, via a joint transcytotic process (2). This process has been also suggested as a major mechanism to clear harmful antigens from the circulation in the form of IgA-antigen complexes via bile secretion (4) and clears both the polymeric IgA receptors and the DPP IV together with the same kinetics (2). In this context, we should mention that SK is also rapidly removed from the circulation via secretion into the bile (5).

Our study also demonstrates that the IgA class antibody response against SK and DPP IV persists for at least 90 days after thrombolytic therapy with SK. Taking into consideration the short half-life of IgA in circulation, these findings suggest that SK persists for long periods in the host tissues, although it is not clear whether it remains intact or degraded. It has been postulated that a significant elevation of IgA antibody titers may result from enterobacterial antigenic stimulation via the gastrointestinal tract (39). In our study, intravenous administration of SK, without any evidence of mucosal participation, produced a predominant IgA anti-DPP IV response, thereby suggesting that high IgA circulating levels do not necessarily involve the gut as the site of infection. In this regard, it may be noteworthy that a large proportion of autoantibodies developed against plasma proteins after SK administration appear not to be of IgA class. Proteins like Pg (16) and lactate dehydrogenase M (33) have in common their reactivity with SK. Most streptococcal infections of humans occur at skin or mucosal surfaces without causing bacteremia. Therefore, if the original immunogenic stimulus by streptococci is at a mucosal surface, the humoral immune response is primarily IgA.

The role of DPP IV in immune function is very complex. In some experimental models, such as adjuvant-induced arthritis (41) specific inhibition of DPP IV activity suppresses disease expression, pointing to a role for DPP IV activity in the pathogenesis of experimentally induced arthritis. However, in rheumatoid synovial fibroblasts (17), SK binds to DPP IV and induces an increase in intracellular calcium independently of DPP IV enzymatic activity, thereby suggesting that its role in the pathogenesis of arthritis is not confined to only its enzymatic activity. DPP IV also has a key regulatory role in the metabolism of peptide hormones involved in psychoneuroendocrine, nutrition, and immune functions (21). Therefore, an increase in the clearance rate of circulating DPP IV may have either negative or positive pathogenic implications. For example, since DPP IV inactivates TNF-α (3), lower levels of DPP IV may increase bioactive levels of this cytokine in the circulation, thereby promoting TNF-α-induced tissue damage (21). Conversely, in patients with inflammatory bowel disease, reduced levels of circulating DPP IV lead to increased levels of bioactive glucagon-like peptide-2, facilitating enhanced repair of the intestinal mucosal epithelium in vivo (46).

Although SK administration induced anti-DPP IV autoantibodies and higher circulating levels of TNF-α and IL-6, none...
of these patients developed an autoimmune disease at the beginning or during 2 years after the thrombolytic therapy with SK. Work by other investigators (40) shows that myocardial infarction patients have high titers of anti-SK antibody up to 90 months after SK administration, but their immune functions were similar to those of the general population. Based on the experimental and clinical data available, the apparently contradictory effects on the immune system may be explained by a bimodal action of DPP IV in immune function; immune reactions that have been initiated by other mechanisms are supported by DPP IV enzymatic activity, while other immune reactions are rather reduced, thus focusing the immunosurveillance on processes that are already under way (21).

A direct participation of bacterial components in the onset of arthritis has been reported, but the mechanisms involved are unclear (18, 25, 26, 36, 43, 45). It appears that exposure to a single isolated bacterial peptide is insufficient to trigger an autoimmune disease. Other factors such as up-regulation of

FIG. 6. Analyses of levels of TNF-α, IL-6, and IL-2 sRα in serum. The concentrations of the three cytokines in serum samples (n = 10) from patients who received SK therapeutically were determined by an ELISA method as described in Materials and Methods. (A) TNF-α titers; (B) IL-6 titers; (C) IL-2 sRα titers. n.s., not significant.
the immunostimulatory cytokines TNF-α, IL-6, or IL-1 by microbial entry into phagocytic cells (44); a distinct genetic profile between streptococcal strains (12); or autoimmune genetic susceptibility of the host (25) may also be necessary for this process.

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REFERENCES


