A granulocyte inhibitory protein overexpressed in chronic renal disease regulates expression of interleukin 6 and interleukin 8

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Communicated by Tadeus Reichstein, October 4, 1993 (received for review August 29, 1993)

ABSTRACT Growing evidence suggests that cytokine expression is influenced by locally produced mediators, thus modifying the pluripotential effects of cytokines toward a tissue-specific inflammatory reaction. The granulocyte inhibitory protein (GIP), a 23-kDa protein found to be significantly overexpressed in patients with chronic renal failure, increases autocrine transcription and expression of interleukin (IL) 6 and IL-8 in human mesangial cells. Moreover, GIP alone induced the transcription of c-jun mRNA; however, in combination with IL-6, it stimulated de novo synthesis of DNA and the transcription of both c-jun and c-fos genes. The data suggest that the overall effect of GIP results in the modulation of the glomerular response to injury and contributes to the progression of glomerulosclerosis.

Increased proliferation of mesangial cells (MCs) and expansion of the extracellular mesangial matrix are striking features of acute and progressive glomerular disease in humans regardless of the primary stimulus (1). MCs play an important role in the modulation of glomerular injury since they produce and react to numerous inflammatory mediators, including platelet-derived growth factor (2, 3), interleukin (IL) 6 (4–6), and IL-8 (7). Overexpression of IL-6, IL-8, and extracellular matrix proteases are characteristic features associated with glomerular inflammation in humans (8–10). While extracellular matrix proteins have an impact on cell functions such as adherence, growth, and secretion or deposition of cellular products, IL-6 serves to regulate the immune response, acute phase reactions, and hematopoiesis (11, 12). IL-8 is capable of activating and eliciting selective diapedesis of polymorphonuclear leukocytes (PMNs), which participate in the development of glomerulonephritis (13).

We have recently characterized a granulocyte inhibitory protein (GIP) from the ultrafiltrate of patients with renal failure that blocked chemotaxis, labeled hexose uptake, and intracellular killing of bacteria in PMNs (14). GIP is efficacious at nanomolar concentrations, which is in the range of other cytokines. The existence of GIP in patients with chronic renal failure could well account for their increased susceptibility to infectious diseases. Sequence analysis of the amino terminus of the peptide did not show homology to known growth hormones and/or cytokines. The observation that GIP affected cell-mediated immune functions (14) suggested to us a potential role of the substance in the regulation of chronic renal inflammation. To characterize the potential role of GIP in the pathogenesis of chronic progressive glomerular disease, we studied its effect on the expression of genes that have been found to be important in glomerular inflammation.

We report here that GIP (i) increases autocrine expression of IL-6 and IL-8 in human MCs and (ii) induces the transcription of c-jun mRNA. (iii) However, GIP, in combination with exogenous IL-6, stimulates de novo synthesis of DNA and transcription of both c-fos and c-jun.

METHODS

Human MCs. Primary human MCs were isolated from human kidney specimens after nephrectomy as described (15). The cells were cultivated in RPMI 1640 plus 0.5% fetal calf serum (FCS), 8 μg/ml, and insulin-transferrin-selenite additive (10 μl/mliter). Two days prior to experiments, 1 × 10^6 MCs (passage 4–6) were seeded into a 750-ml culture flask (Falcon); the culture medium was exchanged later with RPMI 1640 supplemented with 0.1% FCS (low serum condition) to bring the cells to a quiescent state.

Assay for Mitogenicity. The mitogenic effect was measured by the amount of [3H]thymidine incorporated into DNA of human MCs by the procedure of Chesterman et al. (16). In brief, cells were stimulated for 20 hr with either GIP (5 or 10 μg/ml) and/or IL-6 (1 nM) followed by a 4-hr addition of [3H]thymidine (1 μCi/ml; 1 Ci = 37 GBq). The cells were washed in phosphate-buffered saline; proteins were precipitated by addition of 10% (wt/vol) trichloroacetic acid, washed in ethanol/ether (2:1, vol/vol), and dissolved in 0.5 M NaOH. The experiments were performed in triplicate for each condition.

Extraction of Total RNA and Northern Blot Analysis. Total RNA was extracted by a modified protocol of Chomczynski and Sacchi (17), as described (18). The RNA was quantitated by spectrophotometry (A260-A280). The transfer of RNA to nylon membranes was accomplished by capillary blotting overnight, and then the RNA was fixed to the membranes by UV irradiation. The hybridization of the radioactive probes was performed at the appropriate temperature (45°C) overnight or for at least 16 hr (19). To determine the binding of the radiolabeled probes to the corresponding mRNA band, the membranes were washed under stringent conditions [for IL-6 and IL-8 oligonucleotides, two 15-min washes in 2.5× saline citrate (SSC) at 55°C and 5× SSC at 45°C; or for the c-fos oligonucleotide, two 15-min washes in 2.5× SSC at 75°C; and for 28S oligonucleotide, two 20-min washes in 5× SSC and 0.5× SSC at 40°C]. The oligonucleotide probes for IL-6 and IL-8 were from British Biotechnology (Oxford, U.K.), the 28S probe was from Amgen Biologicals. The membranes were then exposed to x-ray film (X-Omat AR; Kodak) for 1–7 days at −70°C. The intensity of the mRNA bands was analyzed by a computerized screen-scanning system (Apple, Macintosh).

RNAse Protection Assay. The template for the c-jun RNA probe was obtained by Pvu II digestion of a mouse c-jun

Abbreviations: MC, mesangial cell; IL, interleukin; GIP, granulocyte inhibitory protein; FCS, fetal calf serum; PMN, polymorphonuclear leukocyte.

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cDNA (American Type Culture Collection) in pGEM2 containing 452 nt of the original sequence including the T7 RNA polymerase promoter. In vitro transcription and labeling was performed with Escherichia coli DNA-dependent T7 RNA polymerase (Boehringer Mannheim) in a 10-μl reaction at 37°C for 60 min followed by gel purification of the RNA probe in a 6% urea/SDS/acylamide gel. The labeled probe was eluted overnight at 37°C in a solution containing 2 M NH₄CH₂-COOH, 1% SDS, and 0.1% EDTA (pH 7.5) plus 20 μg of tRNA, precipitated with 100% ethanol (30 min, −70°C), and redissolved in 80% (vol/vol) formamide hybridization buffer. For each test sample, 10 μg of total MC RNA or 10 μg of RNase-free yeast tRNA as specificity control was precipitated, redissolved in 30 μl of hybridization buffer containing 90,000 cpm/μl, and incubated for 12 hr at 50°C. Nonhybridized RNA was then digested with RNase T₁ (10 μg/ml) (Boehringer Mannheim) for 1 hr at 30°C. The reaction was stopped by addition of 5 M guanidium isothiocyanate/1 M sodium citrate/20% sarcosyl; the RNA was then again phenol extracted and finally precipitated with 2 M sodium acetate at pH 4.5, and tRNA (25 μg final) was stored in 100% isopropl alcohol at −20°C. After washing in 70% ethanol and a 3-hr separation in a 6% urea/1% SDS/acylamide gel (300 V), the hybridized RNA was visualized by exposure of the dried gel to x-ray film (X-Omat AR; Kodak) for 2 days at −70°C.

Protein Expression of IL-6 and IL-8. Human MCs were grown to subconfluence, synchronized as described above, and challenged with IL-6 (1 nM) with and without GIP (10 μg/ml) for 1 hr, followed by exchange of medium to replace the stimulating agents, and an additional incubation for 6, 12, and 24 hr. Protein expression of IL-6 and IL-8 in the supernatant was measured using the IL-6 and IL-8 enzyme immunoassay from Advanced Magnetics (Cambridge, MA) according to the manufacturer’s instructions. Briefly, 100 μl of supernatant or control standard was added to the precoated wells, mixed and incubated for 1 hr at 37°C, washed three times in phosphate-buffered saline, followed by addition of the antibodies and incubation for 30 min. After washing, conjugate and substrate were added sequentially and incubated for 30 min. Results were read on a Bio-Rad automated ELISA reader. The values are corrected for the amount of total protein. The experiments were performed in triplicate for each condition.

RESULTS

GIP Augments IL-6-Induced DNA Synthesis de Novo. To mimic pathophysiological conditions associated with glomerular inflammation, MCs were grown on collagen I and then exposed to GIP. While the addition of GIP alone to the cells did not change [3H]thymidine incorporation, GIP caused a dose-dependent augmentation of IL-6-induced DNA synthesis (Fig. 1). This effect could be reversed upon washing and restoration of MCs in culture. Moreover, the viability of the cells was not affected when GIP was tested at a concentration 100 times the ED₅₀ using trypan blue exclusion (data not shown).

GIP Stimulates Transcription and Expression of IL-6 and IL-8 mRNAs. The biosynthesis of IL-6 is regulated by both autocrine and paracrine mechanisms (5, 20). To examine the mechanism by which GIP augments the expression of IL-6, we tested the effect of the peptide on autocrine secretion of IL-6 in human MCs. In the absence of IL-6, GIP induced the transcription of IL-6 mRNA by a factor of 2 above basal level (Fig. 2). The transcription started to increase at 3 hr, peaked at 6 hr, and declined thereafter to basal level. This effect was similar to that induced by the addition of exogenous IL-6. However, the combination of both agents resulted in markedly increased expression of IL-6 mRNA that was about 5-fold greater than that achieved by either substance.

It has been demonstrated that IL-6 not only stimulates autocrine secretion of IL-6 but also induces transcription and translation of IL-8. Taking into account that the induction of
IL-6 mRNA is amplified by GIP, we hypothesized that the peptide should also influence the transcription of IL-8 mRNA. Therefore, we next examined the ability of GIP to induce IL-8 mRNA. As depicted in Fig. 3, GIP induced the transcription of the IL-8 gene. Furthermore, analogous to its effect on the gene coding for IL-6, GIP clearly amplified the transcription of IL-8 that was induced by exogenous IL-6. The effects of GIP at the level of gene transcription were parallel to those obtained for the expression of the proteins using specific monoclonal antibodies. It should be noted, however, that the expression of IL-8 was much more pronounced than that of IL-6 (Fig. 4).

When testing the effects as a function of increasing dosage of GIP (2.5–10 μg/ml) in the presence of fixed concentrations of IL-6 (1 nM), there was a dose-dependent increase in gene transcription (data not shown). The effects of all substances on the IL-6 and IL-8 genes were abolished by pretreatment of MCs with actinomycin D (5 μg/ml, 30 min), an inhibitor of RNA synthesis, which suggests that the changes occurred at the mRNA level.

**Effect of GIP on Transcription of c-jun and c-fos mRNAs.** The activation of the gene encoding IL-6 involves, at least in part, the formation of the AP-1 or c-fos (serum responsive element) transcription factor (21). Since GIP modulated the transcription and expression of IL-6, we studied its effect on transcription of c-jun and c-fos mRNAs, both of which constitute the AP-1 transcription factor. Fig. 5A illustrates the effect of IL-6 and GIP on the transcription of c-jun; in contrast to IL-6, GIP alone induced an increase in c-jun mRNA whereas c-fos was only marginally transcribed (Fig. 5B). However, the simultaneous presence of the peptide and exogenous IL-6 markedly amplified the transcription of both early response genes.

**DISCUSSION**

Nanomolar concentrations of GIP induced the transcription and expression of IL-6 and IL-8, both of which are involved in the formation of synchiae or crescents and in tubulointerstitial and glomerular injury. Studies on the mechanism of gene transcription indicate a specific ability of GIP to modulate the transcription of c-jun and c-fos.

We have previously shown that GIP, at concentrations that can be obtained under clinical circumstances, is capable of inhibiting effector cell functions of cell-mediated immunity, which could, at least in part, explain the increased susceptibility to infections frequently found in patients with chronic renal failure (14). The consequences of our present finding, however, are more far reaching. It demonstrates that GIP is a pluripotential modulator of inflammatory responses that specifically promotes and/or amplifies the transcription of genes such as IL-6 and IL-8.

Several studies have clarified the role of IL-6 and its relation to inflammation and disease. Thus, deregulated IL-6...
gene expression has been implicated in the pathogenesis of autoimmune diseases and plasma cell neoplasias (22–24). In fact, IL-6-transgenic C57BL/6 mice showed a massive polyclonal plasmacytosis with production of autoantibodies and mesangial proliferative glomerulonephritis (25). Our observation that GIP, a peptide that is overexpressed in chronic renal disease, amplifies the autocrine effect of IL-6 could delineate a mechanism by which glomerular damage may persist after an initial injury and result in progressive glomerular sclerosis.

IL-8 is thought to act as the major stimulus for glomerular recruitment of inflammatory cells such as PMNs. In addition to IL-6, IL-1α, tumor necrosis factor, or lipopolysaccharide stimulated the transcription and expression of IL-8 in MCs. Infusion of the latter cytokines increased the severity of glomerular injury in a PMN-dependent model of glomerulonephritis (7). The fact that GIP, either alone or in combination with IL-6, augments the transcription and expression of IL-8 gene suggests a potential paracrine function of the peptide to initiate and sustain inflammatory lesions associated with glomerular injury.

What could be the mechanism of action by which GIP stimulates expression of IL-6 and IL-8? (i) IL-6 acts by binding to its receptor, which consists of two molecules: a ligand-binding 80-kDa or a non-ligand-binding transducer, gp130, both of which belong to the cytokine family (26, 27). Thus, GIP could facilitate the binding of IL-6 to either receptor site (by heterologous regulation), leading to intensification of transcription of IL-6 mRNA. (ii) Our observation that GIP induced c-jun, and also c-fos in combination with exogenous IL-6, agrees with the notion that activation of transcription of IL-6 gene involves Fos and Jun (28). Analogously, prolonged transcription of c-jun and c-fos has been found in the phase of cell proliferation and differentiation after tissue injury (29). (iii) A nuclear factor responsible for IL-6 expression, NF-IL-6, was shown to bind to the regulatory regions of various acute-phase proteins and cytokines such as IL-8 (30). Consequently, a GIP-dependent increase of transcription of IL-6 would result in an increased transcription of IL-8 mRNA. The presence of GIP could, therefore, exaggerate MC responses to inflammatory stimuli by activating autocrine secretion of IL-6 and IL-6-dependent induction of IL-8.

Beyond the scope of these investigations, an exact characterization of the role of GIP for the pathogenesis of inflammatory mechanisms of the kidney requires the cloning of its complete gene.

This work was supported by a grant from the Alfred Krupp Foundation.