Enhanced Endogenous Leukotriene Biosynthesis in Patients Treated With Granulocyte-Macrophage Colony-Stimulating Factor

By C. Denzlinger, A. Kapp, M. Grimbarg, H.H. Gerhartz, and W. Wilmanns

The hematopoietic cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) is being used in clinical trials for its potential in the treatment of hematopoietic insufficiency due to various causes. Involvement of leukotrienes in the effects of GM-CSF is suggested by analytical and pharmacologic evidence obtained in vitro. However, until now no data in support of a role of leukotrienes in GM-CSF action in vivo have been presented. In the present investigation this question was approached by measurement of endogenous cysteinyl leukotriene formation in patients treated with the cytokine for cytopenia induced by cytostatic drugs or for refractory anemia with excess of blasts (RAEB). Endogenous cysteinyl leukotriene formation was assessed by determination of urinary leukotriene metabolites using combined high-performance liquid chromatography and radiimunoassay analysis. After GM-CSF administration a distinct increase in urinary cysteinyl leukotrienes was found in the cytopenic and the RAEB patients that ranged from 2.3- to 57-fold and 2.4- to 333-fold, respectively. In the cytopenic patients the increase in leukotriene production was correlated to an expansion of peripheral blood leukocytes; RAEB patients responded to GM-CSF with enhanced leukotriene biosynthesis even if the peripheral leukocytes decreased, possibly due to an abnormal number and/or irritability of leukotriene-producing cells. The increase in endogenous leukotriene production during therapy with GM-CSF may indicate that leukotrienes play a role in GM-CSF action in vivo.

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PATIENTS AND METHODS

Patients and controls. Urinary cysteinyl leukotrienes were determined in patients treated with GM-CSF for cytopenia induced by cytostatic drugs or for RAEB. GM-CSF was given subcutaneously (SC). Nine healthy individuals (five male, four female; age range 20 to 39 years) served as controls. In the group of cytopenic patients 12 cases were included (9 high-grade lymphomas, 2 osteosarcomas, 1 colon carcinoma; 5 male, 7 female; age range 16 to 63 years). Eight of these patients were treated in the context of a dose-finding study, four on the basis of compassionate need use. Cytopenia was caused by standard chemotherapy protocols. Before GM-CSF therapy was started, leukocyte counts were 200 to 1,500/µL. The cytokine was given to these patients at a dose of 2 to 16 µg/kg (mean 6 µg/kg) for 5 subsequent days except for two patients who were treated for 8 and 9 days, respectively. RAEB patients (three male, three female; age range 52 to 76 years) were treated according to an EORTC protocol. Four of these patients were treated with 6 µg/kg of GM-CSF for 7 subsequent days. One patient received 3 µg/kg of GM-CSF for 14 subsequent days; in one patient treatment was discontinued after 2 days for local side effects (edema and erythema). In addition to GM-CSF, RAEB patients were given LD-Ara-C (20 mg/m²/d SC) for 14 days either before (four cases) or simultaneously with (two cases) the cytokine according to the study protocol.

Renal and liver functions were tested in all patients receiving GM-CSF and were found to be within the normal range with few exceptions. One patient in the cytopenia group suffered from...
decompensated renal insufficiency necessitating hemodialysis. One RAEB patient developed compensated renal failure during treatment with GM-CSF with an increase in serum creatinine up to 140 μmol/L. Two patients in the cytopenia group had cholestatic liver disease due to their malignant disorder as indicated by the elevated serum concentrations of bilirubin (27 and 61 μmol/L), of alkaline phosphatase (1,047 and 605 U/L) and of γ-glutamyl transferase (442 and 214 U/L, respectively). Patients with uncontrolled infections were not included in the present investigation.

Materials. Human recombinant GM-CSF, fully glycosylated as derived from a Chinese hamster ovary carcinoma cell line was provided by Sandoz Ltd. (Basel, Switzerland) and Schering-Plough (Kenilworth, NJ). Unlabeled leukotrienes C₄, D₄, and E₄ were purchased from Paeisel, Frankfurt, FRG; the respective 14,15-[³H] labeled leukotrienes (0.74 to 2.22 PBq/mmol) were obtained from Amersham, Buckinghamshire, England ([³H]LT₄C, and [³H]LT₄D,) or duPont, Boston, MA ([³H]LT₄E, N-acetyl LT₄E, and N-acetyl [³H]LT₄E, were synthesized from LT₄E, and [³H]LT₄ as described.<ref>14</ref>

HPLC was used to control the purity of leukotriene standards and to restore them if they were found to be less than 97% pure. The concentration of unlabelled leukotrienes was determined by absorbance measurements at 280 nm. 4-Hydroxy-2,2,6,6,-tetramethylpiperidine-1-oxyl (HTMP), dextran (approximately 70 Kd), and activated, neutralized charcoal were bought from Sigma, St Louis, MO. The rabbit cysteiny1 leukotriene antiserum was kindly donated by Professor B.A. Peskar, Ruhr-Universität, Bochum, FRG.

Urine collection and deproteinization. Urine was obtained from spontaneous micturition or via urinary catheter. The day before GM-CSF treatment and on the last treatment day urine was collected for 12 hours (8 PM to 8 AM). In some patients additional samples were obtained several times before, during, and after GM-CSF treatment. Urine was stored at −4°C until assay. Each urine sample was screened (Combur'Test, Boehringer, Mannheim, FRG) for pathologic constituents (leukocytes, erythrocytes, protein, etc). Creatinine concentrations in urine were determined by the Jaffé reaction. For deproteinization, a 5-mL aliquot of each urine sample was added to 40 mL of 90% aqueous methanol containing 0.5 mmol/L EDTA and 1 mmol/L HTMP, pH 7.4. The suspension was stored at −40°C for at least 12 hours and then centrifuged at 2,500g for 10 minutes at −10°C. The supernatant was added to charcoal (0.5% charcoal, 0.5% dextran in phosphate buffer, pH 7.4). Leukotriene standards were dissolved in 30% aqueous methanol containing 1 mmol/L EDTA, and phosphatase buffer, pH 7.4. The flow rate was 1 mL/min. HPLC fractions were collected in 0.5-minute intervals. An aliquot of these fractions was counted for tritium radioactivity to determine retention times of [³H]leukotriene standards. Another aliquot was stored at −20°C under argon for further analysis by RIA.

RIA. A 200-μL aliquot of the HPLC fractions was evaporated to dryness and reconstituted in 200 μL of assay buffer (0.9% NaCl, 0.1% gelatin, 10 mmol/L EDTA, 0.1% sodium azide in 10 mmol/L phosphate buffer, pH 7.4). Leukotriene standards were dissolved in 200 μL of assay buffer together with the residue of 200 μL of evaporated leukotriene-free HPLC buffer. Antiserum (100 μL) diluted 1:5,700 in assay buffer was added to the samples and standards. After mixing, the tubes were preincubated for 30 minutes at room temperature. Then [³H]LT₄C (130 Bq) was added in 100 μL of assay buffer, mixed, and tubes incubated at 4°C for 16 to 20 hours. Unbound [³H]LT₄C was precipitated by addition of 500 μL of charcoal suspension (0.5% charcoal, 0.5% dextran in 10 mmol/L phosphate buffer pH 7.4) and subsequent centrifugation at 1,400g for 15 minutes at 4°C. The supernatant was added to 10 mL of scintillation fluid. The lower detection limit of the assay system was below 10 fmol for LT₄C, LT₄D, LT₄E, and LT₄NAC; the relative cross-reactivities on a molar basis at 50% binding were 100%, 68%, 53%, and 82%, respectively. LT₄B does not cross-react in this system at amounts of up to 1 μmol.

Recovery of leukotrienes from urine and reproductibility. Recovery of leukotrienes from urine was tested by adding defined amounts of standard LT₄E, to urines followed by processing and analysis by HPLC and RIA as described above. The amount of endogenously produced LT₄E, was determined in parallel and subtracted from the value obtained for the urine containing standard LT₄E. The difference indicated recoveries greater than 85% (87% to 109%, n = 5). Replicate determinations of LT₄E, and LT₄E, NAc in urine samples differed by less than 15% (2% to 14%, n = 4) when separate deproteinizations and HPLC fractionations with subsequent RIA were compared.

Statistics. The Wilcoxon test for paired observations was used to analyze significance between urinary leukotriene concentrations before and after GM-CSF treatment. Correlations were estimated by calculating product-moment correlation coefficients and rank correlation coefficients (Spearman, Kendall).

Ethical approval. Ethical approval for the GM-CSF treatment studies was obtained from the ethics committee at the Klinikum Grosshadern, Ludwig-Maximilians University, Munich.

RESULTS

Measurements of urinary cysteiny1 leukotrienes. Determination of urinary cysteiny1 leukotrienes by combined use of HPLC and RIA showed LT₄E, as the major and LT₄E, NAc as the minor detectable metabolite (Fig 1). The identity of the endogenous leukotriene metabolites was confirmed by comigration with authentic [³H]-labeled leukotriene standards in HPLC systems with mobile phases varying in methanol content and pH (not shown). Concentrations of LT₄C, and LT₄D in urine were below the detection limit, apart from patients with urinary tract infections not included in the present study. LT₄E plus LT₄E, NAc, corrected for the urinary creatinine concentration may, therefore, be considered as an appropriate measure for urinary cysteiny1 leukotrienes in this investigation.

Healthy volunteers secreted small amounts of cysteiny1 leukotrienes into urine (2.8 to 8, median 3.8 nmol LT₄E, plus LT₄E, NAc/mol creatinine) (Fig 2). No significant effects of sex, age, or diurnal sampling period of urine were observed in this population.

In cytopenic patients (leukocyte counts 200 to 1,500/μL) urinary cysteiny1 leukotriene concentrations were within the range of controls except for patients with liver or renal disease (Fig 2). The patient with terminal renal insufficiency had lower urinary cysteiny1 leukotriene concentrations (0.7 nmol LT₄E, plus LT₄E, NAc/mol creatinine). In the two patients with cholestatic liver disease higher values were determined (70 and 141 nmol LT₄E, plus LT₄E, NAc/mol creatinine, respectively).
LEUKOTRIENE PRODUCTION IN GM-CSF TREATMENT

was collected the day before GM-CSF treatment and on the last times of tritium leukotriene standards added to the deproteinized with GM-CSF. GM-CSF was administered subcutaneously (SC) for combined use of HPLC and RIA before (B) and after (A) treatment. Determination of cysteinyl leukotriene was performed by application of radioimmunologic analysis to the HPLC fractions as described in detail in the methods section. Arrows indicate retention times of urinary leukotriene standards added to the deproteinized urines before HPLC.

In the RAEB patients, before GM-CSF therapy urinary cysteinyl leukotrienes were within the range of healthy controls except for a patient who suffered concomitantly from asthma bronchiale necessitating bronchodilatator therapy (Fig 2). Four of the six RAEB cases had been treated for 7 days with LD-Ara-C before GM-CSF was administered. This pretreatment was without significant effect on urinary cysteinyl leukotrienes as assessed by determinations of leukotrienes before and during treatment with LD-Ara-C in three patients.

In both the cytopenic and RAEB patients urinary cysteinyl leukotrienes were strongly enhanced after GM-CSF treatment (Figs 1 and 2; significance in the Wilcoxon test for paired observations, P < .001). In the group of cytopenic patients the increase was 2.3- to 57-fold, resulting in concentrations of up to 350 nmol LTE₄ plus LTE₄NAc/mol creatinine, corresponding to 2 nmol LTE₄ plus LTE₄NAc eliminated with urine during the 12-hour period. In the patient with terminal renal insufficiency the increase in urinary cysteinyl leukotrienes was minimal (1.1-fold). In the two patients with cholestatic liver disease the increase in urinary cysteinyl leukotrienes was also low (2.1- and 5.5-fold, respectively), but originating from elevated pretreatment values. An osteosarcoma patient who was operated on during GM-CSF treatment exhibited an increase in urinary cysteinyl leukotrienes within the range of the other cytopenic patients. In the group of RAEB patients urinary cysteinyl leukotrienes increased 2.9- to 333-fold, resulting in concentrations of up to 2,320 nmol LTE₄ plus LTE₄NAc/mol creatinine, corresponding to 18 nmol LTE₄ plus LTE₄NAc eliminated with urine during 12 hours. One patient in this group developed compensated renal insufficiency during the course of GM-CSF therapy. He had lower urinary cysteinyl leukotrienes after GM-CSF treatment compared with pretreatment values. In both the cytopenic and RAEB patients elevation of urinary cysteinyl leukotrienes was observed as early as 24 hours after start of GM-CSF treatment and persevered for at least 48 hours after discontinuation of the therapy.

Correlation of urinary cysteinyl leukotriene with increase in leucocyte counts during GM-CSF treatment. Changes in urinary cysteinyl leukotrienes were tested for correlation with GM-CSF dose, incidence of side effects, total leucocyte counts, changes in granulocyte, monocyte, and eosinophil counts, and relative increase in leucocyte counts. A statistically significant correlation was found only with the latter parameter and only in the group of cytopenic patients, provided the patients with renal or liver disease and

![Fig 1. Determination of urinary cysteinyl leukotrienes by combined use of HPLC and RIA before (B) and after (A) treatment with GM-CSF. GM-CSF was administered subcutaneously (SC) for 6 days at a dose of 12 μg/kg to a patient who was cytopenic after standard treatment with cytostatic drugs for osteosarcoma. Urine was collected the day before GM-CSF treatment and on the last treatment day. Normal renal and hepatic functions were ascertained and urogenital infections excluded by laboratory tests. Determination of cysteinyl leukotrienes was performed by application of radioimmunologic analysis to the HPLC fractions as described in detail in the methods section. Arrows indicate retention times of tritium leukotriene standards added to the deproteinized urines before HPLC.](fig1)

![Fig 2. Urinary cysteinyl leukotrienes in healthy individuals and in cytopenic and RAEB patients before and after GM-CSF treatment. Urinary concentrations of LTE₄ plus LTE₄NAc corrected for urinary creatinine are given on a logarithmic scale. The cytopenic and RAEB group values before GM-CSF treatment are indicated on the left and values after GM-CSF treatment on the right side. Analyses of cysteinyl leukotrienes were performed as depicted in Fig 1. Closed circles (●) represent data from patients with normal renal and liver functions and without surgical intervention during the time of observation. Horizontal bars represent median values from these data. Open triangles in the cytopenic group represent data from patients with cholestatic liver disease (△), alkaline phosphatase 606 U/L, bilirubin 61 μmol/L; ▼, alkaline phosphatase 1,047 U/L, bilirubin 27 μmol/L). Open squares in the cytopenic group and open circles in the RAEB group represent data from patients with renal insufficiency ([□], creatinine in serum > 500 μmol/L, urea > 30 mmol/L; hemodialysis; [◇], creatinine in serum 140 μmol/L). Closed triangles (▲) in the cytopenia group represent data from a patient undergoing amputation of one arm during GM-CSF treatment.](fig2)
were omitted (Fig 3). The product-moment correlation coefficient in the group of cytopenic patients was .88 in the Spearman rank test and statistically significant according to Kendall.

**DISCUSSION**

Studies in vitro have shown that GM-CSF stimulates leukotriene biosynthesis in eosinophils and neutrophils. CSF-induced in vitro clonal growth of granulocyte–macrophage progenitor cells is dependent on leukotriene biosynthesis as demonstrated by suppression of colony growth in the presence of lipoxigenase inhibitors. Unlike the in vitro situation, GM-CSF acts in vivo as one component of a closely regulated network of cytokines. To assess the relevance of leukotrienes in vivo with regard to the action of pharmacological doses of GM-CSF, determination of endogenous leukotriene biosynthesis provides important information.

Determination of endogenous leukotriene biosynthesis was achieved in the present study in patients treated with the cytokine for cytopenia induced by intensive chemotherapy or for RAEB, a subtype of the myelodysplastic syndromes. Determination of urinary cysteinyl leukotrienes served as a measure for endogenous leukotriene biosynthesis, an approach recently approved in studies with patients suffering from asthma, hepato renal syndrome, and coronary heart disease.

In agreement with these studies, urinary cysteinyl leukotrienes were found to be low in healthy controls (Fig 2) and LTE4 was the major metabolite detected (Fig 1). In cytopenic patients, before GM-CSF treatment urinary cysteinyl leukotriene concentrations were within the range of controls. This group of patients includes different stages of myelosuppression, as reflected by different peripheral leukocyte counts. In two cytopenic patients with cholestatic liver disease pretreatment values of urinary LTE4 plus LTE4NAc were elevated above the level of healthy controls. This can probably be explained by deviation of hepatic to urinary leukotriene secretion, because these two routes are alternative pathways in primates for leukotriene elimination from circulating blood. Elimination of leukotrienes via urine is clearly dependent on renal function as demonstrated by a cytopenic patient with chronic decompensated renal failure who had subnormal urinary LTE4 concentrations (Fig 2). These observations underline the importance of close controls of renal and liver functions if urinary cysteinyl leukotrienes are used as a measure of endogenous leukotriene production.

In the RAEB patients, before GM-CSF therapy urinary cysteinyl leukotriene concentrations were within the control range irrespective of pretreatment with LD-Ara-C. An elevated concentration probably not related to the hematologic disease was observed only in one RAEB patient with asthma (Fig 2). Elevated concentrations of urinary LTE4 have recently been shown to occur in asthma patients.

During and after GM-CSF treatment a dramatic increase in urinary cysteinyl leukotrienes was observed (Fig 2). This applies to the cytopenic and to the RAEB patients with the exception of an RAEB patient who suffered from decreasing renal function during therapy with the cytokine.

Hematologic responses to GM-CSF in the cytopenic patients were compatible with results obtained in previous studies. Data on hematologic effects of GM-CSF plus LD-Ara-C in myelodysplastic patients are just being collected in the context of a phase II study. In agreement with earlier investigations, side effects of GM-CSF were infrequent and usually mild in the dose range applied.

Leukotriene production in vivo may be expected to depend on the number of leukotriene-producing cells and their functional activity. It has further been shown that the capacity for leukotriene production is related to differentiation of hematopoietic cells. GM-CSF is known to influence all three of these parameters; it induces proliferation, differentiation, and enhanced functional activity of cell lines, namely leukocytic, with the potential to produce leukotrienes. Moreover, GM-CSF can induce production of other cytokines such as tumor necrosis factor (TNF). This might also contribute to leukotriene production during GM-CSF therapy in vivo because TNF has been shown to enhance leukotriene biosynthesis.

From our data it is not possible to evaluate the exact impact of these single parameters on endogenous leukotriene production. In the patients treated with GM-CSF for cytopenia induced by cytostatic drugs, expansion of the peripheral leukocyte pool seems to represent the predominant factor.
leading to the increase in endogenous cysteinyl leukotrienes. A linear correlation was observed if urinary cysteinyl leukotrienes were compared with the relative increase of leukocyte counts in blood, provided that the patients with pathologic renal or liver functions and the patient undergoing surgery during GM-CSF treatment were omitted (Fig 3). Omission of the latter patient seems justified in this respect, because an operative trauma represents a stimulus for endogenous leukotriene biosynthesis by itself23 and may be expected, on the other hand, to cause wasting of leukocytes.

No correlation between urinary cysteinyl leukotrienes and leukocytes in blood was found in the RAEB patients. One patient responded to GM-CSF with an increase in leukotrienes although the peripheral leukocytes decreased in number (Fig 3). In the RAEB patients the dramatic increase in endogenous leukotriene production may be due to the presence of an abnormal number and/or irritability of leukotriene-producing cells, such as monocytes and eosinophils. Alternatively, GM-CSF–induced differentiation and concomitant acquisition of the capacity of immature cells to produce leukotrienes could be a decisive factor. Combination of GM-CSF with LD-Ara-C might have influenced the effect of the cytokine on endogenous leukotriene production as well, although the drug administered alone had no effect on urinary cysteinyl leukotrienes.

The observed increase in urinary cysteinyl leukotrienes during treatment with GM-CSF appears to reflect an effect of pharmacologic doses of the cytokine. Endogenous production of hematopoietic factors has been documented to occur in both cytopenic1 and myelodysplastic26 patients. However, GM-CSF generated in vivo is undetectable with current assays, and in the patients investigated in the present study there was no indication for remarkable changes in urinary cysteinyl leukotrienes during myelosuppression, spontaneous regeneration from cytopenia, or in the natural course of RAEB. This does not exclude local biosynthesis of small amounts of leukotrienes stimulated by endogenous hematopoietins that may escape detection in urine by local oxidative inactivation.27 The pharmacologic doses of GM-CSF applied in the present study (2 to 16 μg/kg), in contrast, stimulate endogenous leukotriene biosynthesis sufficient to be demonstrable by the determination of urinary leukotriene metabolites.

Our finding that GM-CSF treatment enhances endogenous cysteinyl leukotriene generation in patients provides evidence for a role of leukotrienes in GM-CSF action in the therapeutic situation in vivo.

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