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Granulocyte-colony stimulating factor inhibits inducible nitric oxide synthase gene expression in pulmonary epithelial cells in vitro

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Abstract

Excessive release of nitric oxide (NO) is most likely a crucial pathophysiological pathway leading to pulmonary dysfunction. Thus, repression of supranormal NO production might be one beneficial mechanism of granulocyte-colony stimulating factor (G-CSF) in inflammatory processes. The aim of this study was to investigate the influence of G-CSF on inducible nitric oxide synthase gene expression in the alveolar epithelial cell line L2. We show that G-CSF suppresses interferon-γ/tumor necrosis factor-α (TNF-α) induced inducible nitric oxide synthase gene expression detected as inducible nitric oxide synthase cDNA (cDNA concentration was 633 ± 38 amol/μg total RNA following 24 h incubation with 100 U/ml interferon-γ/500 U/ml TNF-α, and 440 ± 14 amol/μg total RNA following 24 h incubation with 250 U/ml G-CSF + 100 U/ml interferon-γ/500 U/ml TNF-α, respectively). In addition, application of G-CSF resulted in a decreased synthesis of inducible nitric oxide synthase protein and diminished NO release mediated by the cytokines. The suppression of inducible nitric oxide synthase gene expression in L2 cells by G-CSF may represent a beneficial counterregulatory effect on excessive NO synthesis induced by proinflammatory cytokines in the lung.

Keywords: Endotoxin shock; Nitric oxide (NO); Inflammatory mediator; Cytokine

1. Introduction

Sepsis is a frequently fatal inflammatory response to infection that is believed to be induced mainly by the release of endotoxins, exotoxins, and/or other bacterial products. The following cascade of events culminate in severe changes in hemodynamic and respiratory functions. Thus, sepsis is known to be a major risk factor for the development of the acute respiratory distress syndrome in the adult (Fein et al., 1983). A number of studies indicate that the production of nitric oxide (NO) is involved in these cytotoxic and tissue damaging events (Wright et al., 1992). NO is synthesized by several cell types including inflammatory and epithelial cells. It is generated by the conversion of L-arginine to L-citrulline, a reaction which is catalyzed by the enzyme nitric oxide synthase (NOS) (Palmer et al., 1988). Currently three isoforms of nitric oxide synthase have been cloned and characterized mainly classified to be either constitutive or inducible. Although it is conceivable that NO derived from each nitric oxide synthase isoform could be involved in pathophysiological processes, it has been suggested that only the large amounts of inducible nitric oxide synthase-generated NO mediate the vascular changes and tissue injuries that occur in septic shock (Evans and Cohen, 1996). In the lung, the source of NO in pathological processes is assumed to be of macrophage as well as pulmonary endothelial and epithelial origin (Gustafson et al., 1991; Cremona et al., 1995). Recent studies indicate that type II alveolar epithelial cells (ATII cells) are a primary generator of NO. Inducible nitric oxide synthase was detected in freshly isolated rat fetal as well as adult ATII cells (Punjabi et al., 1994; Gutierrez et al., 1995), in the murine lung adenoma cell line LA-4 (Robbins et al., 1994), and in the human pulmonary carcinoma cell line A549 (Adcock et al., 1994). Additionally, we found that L2 pneumocytes, a non-tumor derived alveolar epithelial cell line expresses the inducible nitric oxide synthase gene upon costimulation with inter-

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feron-γ and tumor necrosis factor-α (TNF-α) (Hoffmann et al., 1995). Since bacterial infections are associated with the production and secretion of interferon-γ from CD^{4+}-lymphocytes and TNF-α from monocytes/macrophages, the resulting activation of inducible nitric oxide synthase gene expression and NO-production in ATII cells may be involved in pulmonary dysfunction.

Granulocyte-colony stimulating factor (G-CSF), an acidic glycoprotein, plays a crucial role in maintaining the blood polymorphonuclear count. Additionally, it regulates the response of neutrophils in inflammatory and infectious diseases (Dale et al., 1995). In lipopopolysaccharide-challenged rodents, recombinant G-CSF prevented the expected increase in TNF-α serum concentrations and suppressed the lethal effects of lipopolysaccharide (Görgen et al., 1992). In other studies, administration of recombinant G-CSF reduced the mortality in neonatal rats inoculated with group B streptococci (Cairo et al., 1990a,b). Recently, application of recombinant G-CSF proved to be beneficial as adjunctive therapy in the treatment of patients with pneumonia (Nelson et al., 1996a,b). These findings suggest that G-CSF may prevent or attenuate the potentially lethal effects of inflammatory mediators during sepsis. However, the precise mechanism(s) of these ameliorating effects are at present unclear. Assuming that excessive NO-release is a major pathophysiological pathway leading to pulmonary dysfunction, inhibition of NO-production might be one beneficial mechanism of G-CSF in severe inflammatory processes. Therefore, the aim of the present study was to investigate the influence of G-CSF on cytokine-induced inducible nitric oxide synthase gene expression and NO-production in L2 cells.

2. Experimental procedures

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM) with phenol red was purchased from ccPro, Karlsruhe, Germany. Recombinant rat interferon-γ and TNF-α were from IC Chemicals, Ismaning, Germany. Recombinant murine G-CSF was from R&D Systems, Wiesbaden, Germany. DMEM without phenol red, L-arginine, L-glutamine, *N*-(1-naphthyl-ethylene)-diamine, β-NADPH, phenol–chloroform–isoamylalcohol mixture, and actinomycin D were from Sigma, Deisenhofen, Germany. Fetal calf serum, penicillin–streptomycin, phosphate buffered saline, trypsin–EDTA, and M-MLRV superscript reverse transcriptase were purchased from Gibco Life Technologies, Eggenstein, Germany. Sulfanilamide was from Serva, Heidelberg, Germany. Oligo-$(dT)_{15}$ and dNTP-mix were from Amersham Buchler, Braunschweig, Germany. Taq polymerase and primer sets were obtained from Biometra, Göttingen, Germany. Guanidine isothiocyanate and rotiphorese gel 40 were from Roth, Karlsruhe, Germany; and nitrate reductase was from Boehringer Mannheim, Germany. Inducible nitric oxide synthase antibody (rabbit polyclonal immunoglobulin G) and inducible nitric oxide synthase control peptide were purchased from Santa Cruz Biotechnology, Santa Cruz, CA; goat anti-rabbit antibody was obtained from BioRad, München, Germany.

2.2. Characteristics and culture of L2 cells

The lung epithelial cell line, L2 (adult female Lewis strain rat), was purchased from American Type Culture Collection (Rockville, MD, USA). This cell line was described by Douglas and Farrell (1976) and characterized to be of type II pneumocyte origin because of the observed osmiophilic lamellar inclusions in early passages. Culture of L2 cells was performed as described recently (Hoffmann et al., 1995). In brief, L2 cells were grown in 25 cm^2 culture flasks in DMEM containing fetal calf serum (10%), L-glutamine (2 mM), and penicillin–streptomycin (100 U/ml–100 μg/ml). Semi-confluent L2 cells were washed with phosphate buffered saline prior to incubation. Passages 45 to 55 were used for the present studies.

2.3. Nitrite and nitrate assay

Synthesis of the stable NO-metabolites nitrite and nitrate was determined in the cell-free culture supernatants incubated for 24 h in L-arginine enriched medium (final concentration: 2 mM) without phenol red. Nitrate was reduced to nitrite by nitrate reductase (0.4 U/ml), in the presence of 10 mM β-NADPH. Total nitrite accumulation was assayed by the Griess reaction (Green et al., 1982).

2.4. RNA isolation and polymerase chain reaction

At the end of the 24-h experiments, cells were washed with sterile phosphate buffered saline and lysed with 4 M guanidine isothiocyanate containing 0.1 M 2-mercaptoethanol. Total RNA was isolated by acid phenol–chloroform extraction according to the method of Chomczynski and Sacchi (1987) redissolved in water and the concentration determined photometrically at a wavelength of 260 nm. One μg total RNA was reverse-transcribed into first strand cDNA using oligo $(dT)_{15}$ as primer for reverse transcriptase (RT). RT-generated cDNA encoding for rat inducible nitric oxide synthase was amplified using polymerase chain reaction (PCR) as described recently (Schobersberger et al., 1995). Expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as control.

For quantitation of inducible nitric oxide synthase cDNA, a competitive PCR was performed using a DNA

fragment derived from the viral oncogene V-erb B to which the inducible nitric oxide synthase primer template sequences have been added (Competitive DNA MIMIC, Clontech, Heidelberg, Germany), as described by Siebert and Larrick (1992). Densitometric analysis of the scan photographs was done by using the Imaging densitometer GS 670 (BioRad) with regard to the different amount of ethidium-bromide intercalation according to the length of the fragments (length of inducible nitric oxide synthase cDNA is 498 bp, length of competitor fragment is 400 bp, respectively). Results were calculated by detecting the point of equivalence between competitor and inducible nitric oxide synthase cDNA according to their respective band intensities. Results are expressed as amol inducible nitric oxide synthase-cDNA/μg total RNA taking into consideration that 1 μl of the 25 μl RT reaction yield was used per quantitative PCR. Oligonucleotide primers for inducible nitric oxide synthase and internal standard were: 5′-CCCTTCCGAAGTTTCTGGCAGCAGG-3′ (upstream), and 5′-GGCTGTCAGAGCCTTGTGCCTTTGG-3′ (downstream) corresponding to the murine macrophage inducible nitric oxide synthase (Lyons et al., 1992). Oligonucleotide primers for GAPDH were: 5′-GCAGGGGGAGCCAAAAGGG-3′ (upstream) and 5′-TGCCAGCCCCAGCGTCAAAG-3′ (downstream) corresponding to the human GAPDH gene (Platzer et al., 1994).

2.5. Preparation of cytosolic extracts

Cytosolic extracts were prepared according to a method described by Hoppe-Seyler et al. (1991). Following a 24-h and 48-h incubation, cells were rinsed with cold phosphate buffered saline solution and lysed directly on the culture dishes in 1 ml cold lysis buffer (0.6% Nonidet P-40, 150 mM NaCl, 10 mM Tris pH 7.9, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride). Lysed cells were transferred into a 2-ml Eppendorf tube and incubated for 5 min on ice. The nuclei were pelleted (1250 g, 4°C, 5 min) and the supernatant was collected for determination of protein content.

2.6. Western blot analysis

Inducible nitric oxide synthase from cytosolic extracts was detected by immunoblotting. Sample protein content was determined using bovine serum albumin as a standard according to the method of Bradford (1976). Equal protein amounts from each sample were separated by sodium dodecyl-sulfate-polyacrylamide gel electrophoresis using an 8% acrylamide gel as described by Laemmli (1970). Separated proteins were transferred on a nitrocellulose membrane for 1 h at 250 mA by using a BioRad Transblot

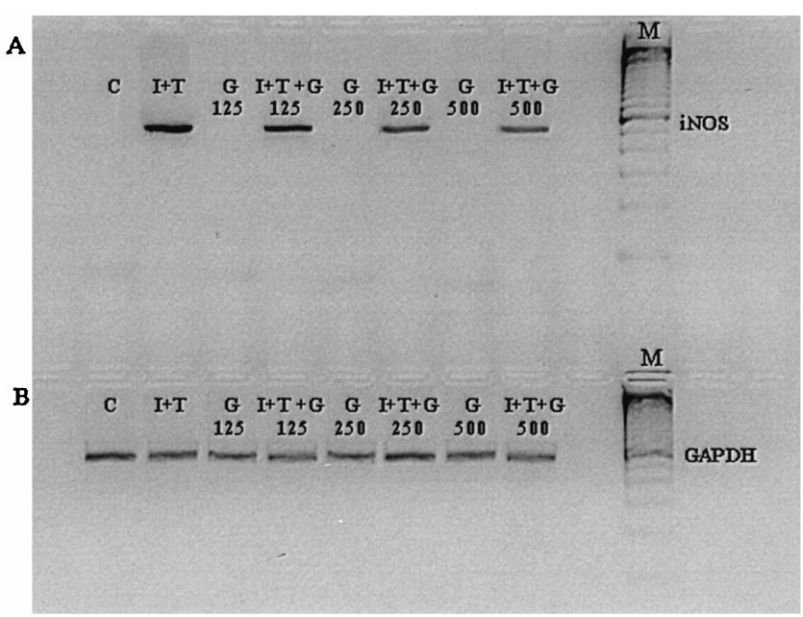

Fig. 1. Panel A of the figure shows the results of the qualitative analysis of inducible nitric oxide synthase mRNA expression detected as inducible nitric oxide synthase cDNA (fragment size: 498 base pairs) following 24-h incubations of L2 cells with: 100 U/ml interferon-γ/500 U/ml tumor necrosis factor-α (I + T), 125 U/ml granulocyte-colony stimulating factor (G 125), 100 U/ml interferon-γ/500 U/ml tumor necrosis factor-α + 125 U/ml granulocyte-colony stimulating factor (I + T + G 125), 250 U/ml granulocyte-colony stimulating factor (G 250), 100 U/ml interferon-γ/500 U/ml tumor necrosis factor-α + 250 U/ml granulocyte-colony stimulating factor (I + T + G 250), 500 U/ml granulocyte-colony stimulating factor (G 500), and 100 U/ml interferon-γ/500 U/ml tumor necrosis factor-α + 500 U/ml granulocyte-colony stimulating factor (I + T + G 500), respectively. Lane C shows the result of a control experiment in unstimulated cells, lane M indicates a size standard (100 base pair ladder). Panel B shows the corresponding GAPDH bands (fragment size: 567 bp). Results are representative of three different experiments.

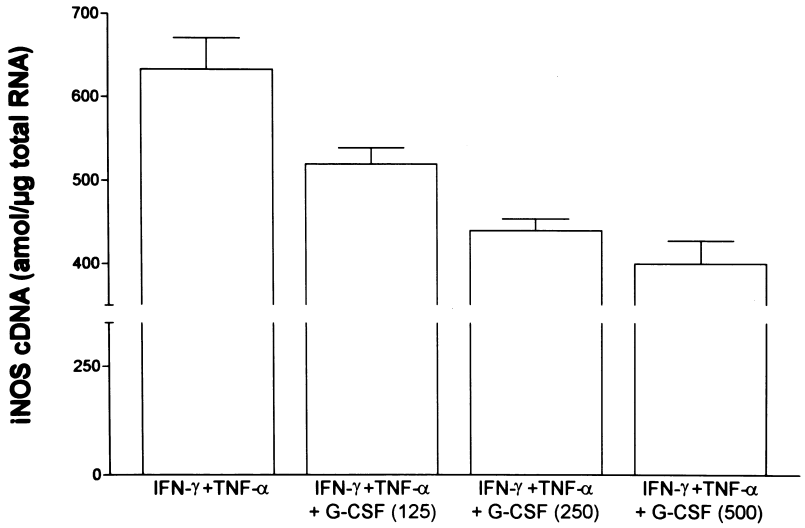

Fig. 2. Quantitative analyses of inducible nitric oxide synthase mRNA expression detected as inducible nitric oxide synthase cDNA following 24-h incubations of L2 cells with: 100 U/ml interferon-γ/500 U/ml tumor necrosis factor-α (IFN-γ/TNF-α), 100 U/ml interferon-γ/500 U/ml tumor necrosis factor-α + 125 U/ml granulocyte-colony stimulating factor (IFN-γ/TNF-α + G-CSF (125)), 100 U/ml interferon-γ/500 U/ml tumor necrosis factor-α + 250 U/ml granulocyte-colony stimulating factor (IFN-γ/TNF-α + G-CSF (250)), and 100 U/ml interferon-γ/500 U/ml tumor necrosis factor-α + 500 U/ml granulocyte-colony stimulating factor (IFN-γ/TNF-α + G-CSF (500)), respectively. Results are representative of three different experiments.

apparatus. Washed filters were incubated for 12 h with a polyclonal rabbit anti-rat inducible nitric oxide synthase antibody (mouse and rat reactive) corresponding to amino acids 1126–1144 mapping at the carboxy terminus of the enzyme. An anti-rabbit secondary antibody was used to visualize the inducible nitric oxide synthase antibody complex by an alkaline phosphatase reaction catalyzing 5-bromo-4-chloro-3-indolyl-phosphate as a substrate.

2.7. Statistical analysis

Results are expressed as mean values ± standard error of the mean (S.E.M.). To test for significance of differ-

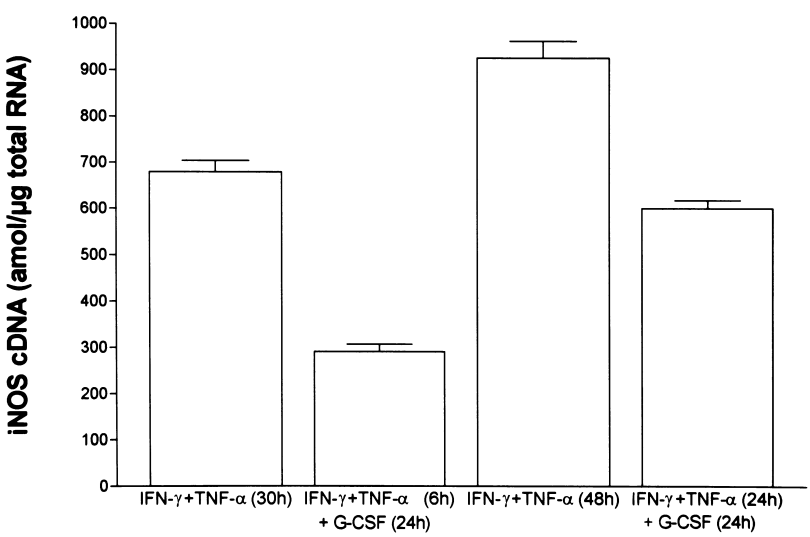

Fig. 3. Quantitative analyses of inducible nitric oxide synthase mRNA expression detected as inducible nitric oxide synthase cDNA following the L2 preincubation experiments: 100 U/ml interferon-γ/500 U/ml tumor necrosis factor-α for 30 h (IFN-γ/TNF-α (30 h)), 100 U/ml interferon-γ/500 U/ml tumor necrosis factor-α for 6 h and subsequent addition of 250 U/ml granulocyte-colony stimulating factor for 24 h (IFN-γ/TNF-α (6 h) + G-CSF (24 h)), 100 U/ml interferon-γ/500 U/ml tumor necrosis factor-α for 48 h (IFN-γ/TNF-α (48 h)), and 100 U/ml interferon-γ/500 U/ml tumor necrosis factor-α for 24 h and subsequent addition of 250 U/ml granulocyte-colony stimulating factor for 24 h (IFN-γ/TNF-α (24 h) + G-CSF (24 h)), respectively. Results are representative of three different experiments.

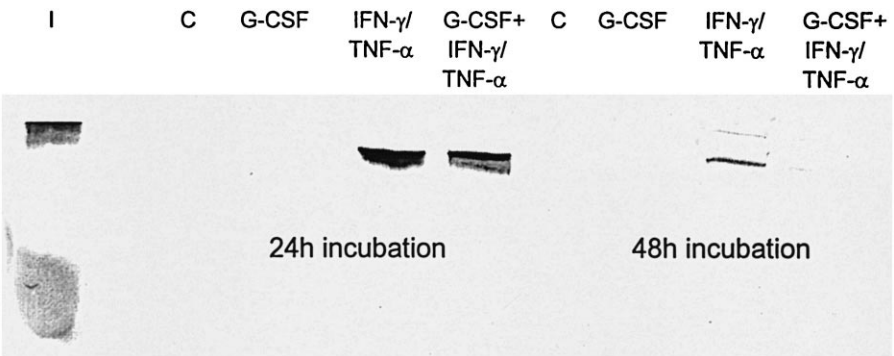

Fig. 4. Western Blot analysis of inducible nitric oxide synthase protein in cytosolic extracts of L2 cells following 24 h as well as 48 h incubations with: 250 U/ml granulocyte-colony stimulating factor (G-CSF), 100 U/ml interferon-γ/500 U/ml tumor necrosis factor-α (IFN-γ/TNF-α), and 250 U/ml granulocyte-colony stimulating factor + 100 U/ml interferon-γ/500 U/ml tumor necrosis factor-α (G-CSF + IFN-γ/TNF-α), respectively. Lane C shows the results of the control experiments in unstimulated cells. Lane I indicates inducible nitric oxide synthase control peptide that served as a molecular mass marker (size: 117 kDa). Results are representative of three different experiments.

ences between the mean value of a control vs. the mean value of treated cells, the Student's *t*-test was used. *P*-values of < 0.05 were considered to be significant.

3. Results

3.1. Effects of G-CSF on inducible nitric oxide synthase gene expression

Panel A of Fig. 1 summarizes the results of the qualitative analysis of inducible nitric oxide synthase mRNA expression measured as inducible nitric oxide synthase cDNA following 24 h incubations of L2 cells. Interferon-γ (100 U/ml)/TNF-α (500 U/ml) and the combination of interferon-γ (100 U/ml)/TNF-α (500 U/ml) and different concentrations of G-CSF (125 U/ml, 250 U/ml, and 500 U/ml, respectively) increased inducible nitric oxide synthase mRNA expression while no inducible nitric oxide synthase cDNA was found in control incubations (C) and in cells treated with G-CSF alone. Panel B of Fig. 1 shows the corresponding GAPDH bands. RT-PCR for GAPDH indicated no significant change in GAPDH cDNA levels under each of the experimental conditions. The results of the quantitative analyses of iNOS cDNA from cells incubated with interferon-γ/TNF-α and interferon-γ/TNF-α + increasing amounts of G-CSF are presented in Fig. 2. Incubation of L2 with interferon-γ/TNF-α + G-CSF led to significantly lower amounts of iNOS cDNA as compared to the interferon-γ/TNF-α treatments. Application of interferon-γ/TNF-α resulted in 633 ± 38 amol iNOS cDNA/μg total RNA ($n = 3$) following the 24-h incubation whereas coincubations with interferon-γ/TNF-α + G-CSF reduced iNOS cDNA values in a dose-dependent manner to: 520 ± 19 amol/μg total RNA (125 U/ml G-CSF, n = 3), 440 ± 14 amol/μg total RNA (250 U/ml G-CSF, $n = 3$), and 400 ± 28 amol/μg total RNA (500 U/ml G-CSF, $n = 3$). Thus, the percentual reduction of iNOS cDNA due to coincubation with G-GSF was −18% (125 U/ml G-CSF), −31% (250 U/ml G-CSF), and −37% (500 U/ml G-CSF), respectively. To investigate whether there is a time-dependent effect of G-CSF on iNOS gene expression, a potent G-CSF concentration (250 U/ml) was used in a set of preincubation experiments.

Table 1
Nitrite/nitrate levels measured as accumulated nitrite (nmol/10^6 cells) in cell-free culture supernatants following 24 h incubations

Unstimulated controls	4.8 ± 0.3
Tumor necrosis factor-α (500 U/ml) + interferon-γ (100 U/ml)	106.1 ± 2.5*
Granulocyte-colony stimulating factor (125 U/ml)	5.1 ± 0.6
Tumor necrosis factor-α (500 U/ml) + interferon-γ (100 U/ml) + granulocyte-colony stimulating factor (125 U/ml)	81.7 ± 1.5*,#
Granulocyte-colony stimulating factor (250 U/ml)	3.6 ± 0.3
Tumor necrosis factor-α (500 U/ml) + interferon-γ (100 U/ml) + granulocyte-colony stimulating factor (250 U/ml)	66.9 ± 1.6*,#
Granulocyte-colony stimulating factor (500 U/ml)	4.7 ± 0.9
Tumor necrosis factor-α (500 U/ml) + interferon-γ (100 U/ml) + granulocyte-colony stimulating factor (500 U/ml)	72.1 ± 1.9*,#

Data are expressed as means ± S.E.M., $n = 8$.
* $P < 0.05$ as compared to unstimulated controls.
# $P < 0.05$ as compared to the respective incubations without granulocyte-colony stimulating factor.

The results of the quantitative analyses of iNOS cDNA are shown in Fig. 3. L2 cells pretreated with interferon-γ/TNF-α for 6 h with a subsequent addition of G-CSF expressed markedly lower concentrations of iNOS cDNA (291 ± 16 amol/μg total RNA, $n = 3$) as compared to cells treated with interferon-γ/TNF-α for the complete incubation time of 30 h (680 ± 24 amol/μg total RNA, $n = 3$). Preincubation of cells with interferon-γ/TNF-α for 24 h led to comparable results (iNOS cDNA concentration following 24 h preincubation with interferon-γ/TNF-α and subsequent addition of G-CSF for another 24 h was 601 ± 18 amol/μg total RNA; iNOS cDNA concentration following the complete 48 h incubation with interferon-γ/TNF-α was 928 ± 36 amol/μg total RNA, $n = 3$, respectively).

3.2. Modulation of inducible nitric oxide synthase protein synthesis by G-CSF

Fig. 4 shows inducible nitric oxide synthase protein in cytosolic extracts of L2 cells detected by Western Blot analysis. The 24 h stimulation of cells with interferon-γ/TNF-α and interferon-γ/TNF-α + G-CSF (250 U/ml) resulted in enhanced cytosolic inducible nitric oxide synthase protein contents. With regard to band intensities, inducible nitric oxide synthase protein levels seem to be higher in those cells incubated with interferon-γ/TNF-α as compared to L2 cells coincubated with interferon-γ/TNF-α + G-CSF (250 U/ml). Following 48 h treatments, no inducible nitric oxide synthase protein could be labelled with its specific antibody in cells treated with interferon-γ/TNF-α + G-CSF (250 U/ml), whereas the enzyme was still detectable in those cells exposed to interferon-γ/TNF-α alone.

3.3. Influence of G-CSF on nitric oxide release

Nitrite/nitrate determinations in cell-free culture supernatants following 24 h incubations revealed higher concentrations of NO metabolites in the environment of cells treated with interferon-γ/TNF-α as compared to the coincubations with interferon-γ/TNF-α + increasing concentrations of G-CSF (Table 1). This indicates that L2 cells were stimulated to produce and to release NO by interferon-γ/TNF-α, but NO-production was remarkably lower in the presence of G-CSF. Similar to the effects of G-CSF on iNOS cDNA expression, the inhibition of NO release is more pronounced when L2 cells were pretreated with interferon-γ/TNF-α prior to the addition of G-CSF. Nitrite/nitrate concentrations after 6 h preincubation of L2 cells with interferon-γ/TNF-α followed by an additional incubation with G-CSF for 24 h was 47.8 ± 1.7 nmol/10^6 cells. This was significantly lower as compared to the values obtained by incubation of cells with interferon-γ/TNF-α for the complete incubation time of 30 h (119.7 ± 3.8 nmol/10^6 cells, $P < 0.05$; $n = 8$). Nitrite/nitrate concentrations following 24 h preincubation with interferon-γ/TNF-α and subsequent addition of G-CSF for another 24 h averaged 103.6 ± 2.2 nmol/10^6 cells whereas treatment of cells with interferon-γ/TNF-α for 48 h resulted in 158.4 ± 5.4 nmol/10^6 cells ($P < 0.05$; $n = 8$).

4. Discussion

The main finding of the present study is that different amounts of G-CSF suppress the effect of interferon-γ/TNF-α on inducible nitric oxide synthase gene expression in alveolar epithelial cells in vitro. Since additional studies with actinomycin D revealed no differences in mRNA half time between cytokine- and cytokine + G-CSF-treated L2 cells, G-CSF exhibits its inhibitory effects most likely at the transcriptional level. Moreover, application of G-CSF resulted in a decreased interferon-γ/TNF-α mediated synthesis of inducible nitric oxide synthase protein as well as NO production and release.

G-CSF is an important hemopoietic factor known to be involved in neutrophil precursor cell proliferation as well as differentiation. Moreover, the actions of G-CSF enhance specific neutrophil functions, e.g., chemotaxis, superoxide generation and phagocytotic activity (Dale et al., 1995). A significant increase in circulating G-CSF is a well known host response in severe inflammatory diseases, e.g., sepsis, in order to improve distinct immune functions. In this context, excessive release of G-CSF may act as a double edged sword: On the one hand, it induces neutrophilia, enhances the adhesion of neutrophils to endothelial cell surfaces and stimulates the release of superoxide anions, thus improving local host defense mechanisms against invading pathogens. On the other hand, the same effects could lead to inadvertent tissue damage and subsequent organ dysfunction, promoting multiple organ failure. These potential disadvantages of G-CSF represent a critical factor in the clinical use of this cytokine. Several investigations of the effects of prior treatment with recombinant G-CSF on responses to endotoxins have been published. In rodents, injection of recombinant G-CSF prevented the lethality induced by lipopolysaccharide which was accompanied by a significant reduction of TNF-α synthesis (Görgen et al., 1992). Kanazawa et al. (1992) reported that pretreatment of guinea pigs with recombinant G-CSF attenuated neutrophil-dependent acute lung injury to endotoxin. In addition, Fink et al. (1993) found no recombinant G-CSF-induced adverse effects on pulmonary and hemodynamic responses of pigs to intravenous endotoxin. Recently performed clinical studies confirmed the mainly positive influences of recombinant G-CSF in sepsis and inflammatory pulmonary diseases. Infusion of recombinant G-CSF in postoperative/posttraumatic patients at risk of and with sepsis counterregulated hyperactivation of proinflammatory processes (Weiss et al., 1996). In patients with

mutilobar pneumonia recombinant G-CSF hastened radiographic and clinical resolution of pneumonia and reduced acute respiratory distress syndrome and sepsis-induced complications (Nelson et al., 1996a,b).

The possible mechanism(s) of the beneficial effects of G-CSF are at present unclear. There remains some controversy about the presence and the action of G-CSF receptors on non-hematopoietic cells. Although G-CSF receptors could be detected in various non-hematopoietic cell lines including epithelial cells (Bussolino et al., 1989; Uzumaki et al., 1989; Avalos et al., 1990), the number of G-CSF receptor copies seems to be rather low (<500 receptors/cell). Since we were able to detect G-CSF receptor gene expression in L2 cells by RT-PCR (data not shown), L2 cells seem to be susceptible to G-CSF. The signal transduction mechanisms activated by the G-CSF receptor are poorly understood. It has been demonstrated that receptor and non-receptor protein tyrosine kinases are involved in G-CSF receptor signaling (Tweardy et al., 1993). The anti-inflammatory effects of G-CSF receptor activation might be mediated by the downregulation of pro-inflammatory cytokines (TNF-α, IL-1, interferon-γ) and/or the upregulation of their antagonists (soluble TNF-receptor I and II, IL-1 receptor antagonists). As our data suggest, another property of G-CSF might be the inhibition of interferon-γ/TNF-α-induced inducible nitric oxide synthase gene expression and NO production in L2 cells. The suppressive effect of G-CSF was more pronounced when L2 cells were pretreated with the proinflammatory cytokines prior to the addition of G-CSF. Thus, one may speculate that G-CSF interacts with a distal oriented part of the inducible nitric oxide synthase/NO inducing pathway of interferon-γ/TNF-α gaining a higher impact on NO synthesis when added subsequent to the stimuli. Excessive NO generation in alveolar epithelial cells following bacterial infection is involved in the dysregulation of several pulmonary functions. Concurrent synthesis of NO and NO-derived reactive oxygen species like peroxynitrite lead to impaired production of surfactant proteins and phospholipids (Haddad et al., 1994, 1996; Miles et al., 1996), thus exacerbating the risk of acute respiratory distress syndrome. In addition, peroxynitrite generated by NO inhibits sodium transport by injuring apically located amiloride-sensitive Na^+ channels in ATII cells (Hu et al., 1994). In a recent study, we could show that NO-donors inhibit the activity of L-type Ca^{2+} channels in L2 cells which might affect various Ca^{2+}-dependent intracellular processes (Schobersberger et al., 1997). Therefore, the inhibition of inducible nitric oxide synthase gene expression in L2 cells by G-CSF may represent a beneficial counterregulatory effect on excessive NO-generation by proinflammatory cytokines in the lung. If this effect of G-CSF is not specific for alveolar type II cells, it is conceivable that cytokine-induced NO release can be suppressed by G-CSF in other organ systems, too. This is in accordance with previous observations of a reduced NO production in vascular smooth muscle cells stimulated with interferon-γ/TNF-α (Hoffmann et al., unpublished data).

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