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Interferon-gamma, Tumor Necrosis Factor-alpha, and Lipopolysaccharide Promote Chitotriosidase Gene Expression in Human Macrophages

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Human chitotriosidase (Chit), a chitinolytic enzyme, is a member of the chitinase family. In human plasma, Chit activity has been proposed as a biochemical marker of macrophage activation in several lysosomal diseases. Recently we found that Chit activity is higher in patients affected by *Plasmodium falciparum* malaria infection, suggesting that Chit may reflect induction of an immunological response. To assess this hypothesis, we evaluated the CHIT1 mRNA levels in human monocytes/macrophages (HMMs) following treatment with interferon-gamma (IFNγ), tumor necrosis factor-alpha (TNFα), and lipopolysaccharide (LPS). Stimulation of macrophages with INF-γ, TNF-α, and LPS resulted in an increase in Chit activity as well as the levels of CHIT1 mRNA as measured by quantitative real-time PCR. The data presented in this article show that Chit plays a role in the response to the activation of INF-γ-, TNF-α-, and LPS-driven macrophages, suggesting that the production of Chit by macrophages could have biological relevance in the immune-response. J. Clin. Lab. Anal. 19:128–132, 2005. © 2005 Wiley-Liss, Inc.

Key words: human monocyte/macrophages; chitotriosidase; cytokines; immune response

INTRODUCTION

Human chitotriosidase (Chit) is a member of the chitinase family, a group of enzyme 18 of glycosyl hydrolase with the capability to hydrolyze chitin. Chitin is a glycopolymer that is present as a structural component in the coating of many living species, such as the cell wall of fungi (1), the sheath of parasitic nematodes (2), and in the lining of gut of many insects (3). Human Chit exhibits a remarkable sequence homology among other chitinases from plants, bacteria, fungi, nematodes, and insects (4,5).

The CHIT1 gene is localized in the chromosome 1q31–q32 (6); the gene consists of 12 exons and spans about 20 kilobases of genomic DNA (4). Chit is mainly secreted as a 50-kDa active enzyme containing a C-terminal chitin binding domain (7). In macrophages, this protein is proteolytically processed to a C-terminally truncated 39-kDa isoform, characterized by hydrolase activity and accumulated in the lysosomes (7). In addition, the 50-kDa Chit form is synthesized by neutrophilic granulocytes progenitors and stored in their granules (4,8). A recessive inherited deficiency in Chit activity is frequently encountered in different populations. A 24-basepair duplication in exon 10 results in the activation of a cryptic 3′ splice site, generating an abnormally-spliced mRNA with an in-frame deletion of 87 nucleotides (9). The spliced mRNA encodes an enzymatically inactive protein that lacks an internal stretch of 29 amino acids (9). This CHIT1 mutant allele has been found in 33 and 35% of Ashkenazi Jewish and Dutch individuals, respectively, whereas both populations were about 6% homozygous for this allele (9). Additional studies performed in different populations confirmed the presence of the

Abbreviations: Chit, chitotriosidase; HMMs, human monocytes/macrophages; IFN-γ, interferon-gamma; IL-12, interleukin-12; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-alpha.

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24-basepair duplication in individuals completely deficient in enzymatically-active Chit (9).

The Chit enzyme is of interest for clinical reasons, since it has been proposed as a biochemical marker of macrophage activation in several lysosomal diseases (6), particularly as a valuable diagnostic tool to monitor the efficacy of therapy in Gaucher diseases or beta-glucocerebrosidase deficiency (10), a disorder characterized by the presence of large amounts of activated, lipid-laden macrophages in spleen, liver, and other tissues (10). More modest elevations are found in plasma of patients with sarcoidosis (10). Recombinant CHIT1 has been found to inhibit hyphal growth of chitin-containing fungi (1), suggesting a physiological role in defense against chitin-containing pathogens (11). Moreover, Chit activity is increased in patients affected by malaria infection and in other hematological disorders, in which activated macrophages are involved (12–13).

On the basis of these findings, it can be hypothesized that Chit activity increases in response to various immunological stimuli. It is noteworthy that macrophages play an important role in defending the body against bacterial and fungal infections and malignancy. In response to various stimuli such as interferon-gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and bacterial lipopolysaccharide (LPS), they produce reactive oxygen metabolites used for killing of microorganisms and tumor cells (14,15). However, nothing is known about the immunological stimuli that can induce Chit synthesis by activated macrophages.

In order to further approach this issue, we analyzed Chit activity and CHIT1 mRNA levels in macrophages following treatment with IFN-γ, TNF-α, and LPS.

Our results show that an elevation in the levels of Chit activity and CHIT1 mRNA accumulation is induced by IFN-γ, TNF-α, and LPS, suggesting that Chit is not only a biochemical marker of macrophage activation in several lysosomal and hematological diseases, but that it can also be regarded as a component of the immunological response.

MATERIALS AND METHODS

Cells

Human monocyte-macrophages (HMMs) were isolated from fresh buffy coat of healthy volunteers, in which DNA analysis to select the subjects homozygous for the wild-type CHIT allele was preliminarily performed. The buffy coat was diluted with phosphate-buffered saline (PBS) supplemented with 2.5 mM ethylenediaminetetraacetic (EDTA) and layered onto Ficoll-Hypaque gradients (Invitrogen, Milan, Italy). After 30 min of centrifugation at 400g at room temperature, the mononuclear cells were collected, washed twice with PBS, and placed in plastic Petri dishes at a concentration of $1–2 \times 10^6$ cells/cm surface areas in Iscove's medium supplemented with 2 mM glutamine, and 50 mg/mL of penicillin/streptomycin.

The cells were further purified using CD14 magnetic beads (Miltenyi Biotec, Bologna, Italy) according to the manufacturer's instructions. The HMMs were cultured in Iscove's medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, and 1% of penicillin/streptomycin (Invitrogen, Milan, Italy).

Cell Treatment

Cells were divided in several groups; for each experimental group we used 100-mm Petri dishes. The cells were cultured for 7 days; thereafter, cell groups were treated for 2, 4, 8, and 24 hr, respectively, with 100 U/mL of human recombinant IFN-γ (Sigma), with 100 U/mL TNF-α (Sigma, Milan, Italy), and with 50 ng/mL LPS (Sigma). Untreated cells were used as negative control.

Quantitative Real-Time PCR

Real-time fluorescence PCR, based on SYBR Green, was carried out in a 30 μL final volume containing 1 × SYBR Green PCR Master Mix (Applied Biosystems, Monza, Italy), 200 nM forward and 200 nM reverse primers (Table 1) and 20 ng of cDNA. Thermal cycling was performed for each gene (CHIT1) in triplicate on cDNA samples in MicroAmp Optical 96-well reaction plate (Applied Biosystems) with MicroAmp optical caps (Applied Biosystems) using the ABI PRISM 7700 sequence detection system (Applied Biosystems). Amplification was carried out with the following conditions: 50°C for 2 min, 95°C for 10 min, and 40 cycles each of 95°C for 15 sec and 60°C for 1 min. The levels of CHIT1 mRNA were normalized to levels of glyceraldeyde-3-phosphate dehydrogenase (GAPDH). All data were captured using Sequence Detector Software (Applied Biosystems).

Chit Activity Determination

For Chit assay, cells were harvested using cell lysis buffer (10 mM HEPES pH 7.9, 1 mM EDTA pH 8, 60 mM KCl, 1 mM PMSF, 0.5% NP40). The lysates were

TABLE 1. Synthetic primers for RT-PCR for gene verifications

Gene	Primer	Sequence 5′–3′	Fragment length
Target gene CHIT1	Forward	CTGCATCAT GGTGCGGTC	210
	Reverse	CAACCACCAGCTGAGCAC	
GAPDH	Forward	ACTCCCATTCCTCCACCTTT	144
	Reverse	TTACTCCTTGGAGGCCATGT	

used for determination by a fluorimetric method (16). Chit activity was measured by incubating 5 μL of undiluted plasma with 100 μL of a solution containing 22 μmol/L of the artificial substrate 4-methylumbelliferyl-β-d-N,N′,N″triacetylchitotriose (Sigma) in 0.5 M citrate-phosphate buffer pH 5.2, for 15 min at 37°C. The reaction was stopped by using 2 mL of 0.5 mol/L Na_2CO_3-$NaHCO_3$ buffer, pH 10.7. The fluorescence was read by a Perkin Elmer fluorimeter (Perkin Elmer, Norwalk, CT), at 365 nm excitation and 450 nm emissions. Chit activity was measured as nanomoles of substrate hydrolyzed per milliliter per hour (nmol/mL/hr). Samples with a Chit levels >110 nmol/mL/hr were measured again after a dilution of 10-fold or 50-fold with distilled water.

Statistical Analysis

Data are expressed as mean ± standard error (SE). Significance was assessed by one-way analysis of variance (ANOVA) and Student's *t*-test. $P<0.05$ was considered to be statistically significant.

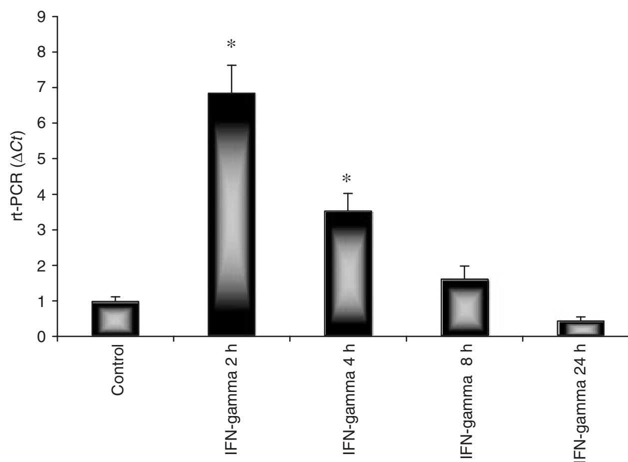

Fig. 1. Detection of CHIT1 expression by quantitative real-time PCR of RNA obtained from HMMs untreated and treated with IFN-γ (100 U/mL). All rt-PCR values are given as ΔC_t values, which correspond to the difference of the maximum number of rt-PCR cycles (40) and the value obtained for each individual sample. Statistical analysis was performed by Student's *t*-test; $^*P<0.05$, of treated cells vs. control. Data are representative of three independent experiments.

RESULTS

CHIT Expression in Macrophages Following IFN-γ, TNF-α, and LPS Exposure

In the first experiments, we evaluated the effect of IFN-γ, TNF-α, and LPS on CHIT1 gene expression by measuring CHIT mRNA levels in human monocytes of peripheral blood cultured for 7 days in order to mature into macrophages. The total mRNA of macrophages treated at different times (2, 4, 8, and 24 hr) was subjected to quantitative real-time PCR. Figure 1 shows that CHIT mRNA levels were significantly induced at 2 hr (5.8-fold increase compared to untreated control cells) after stimulation with 100 U/mL of IFN-γ and decreased gradually thereafter. We also observed a similar pattern of CHIT mRNA accumulation (4.9-fold increase compared to untreated control) after the treatment with 100 U/mL TNF-α for 2 hr (Fig. 2). Whereas in macrophages, following LPS (50 ng/mL) treatment CHIT mRNA levels reached the higher expression (6.1-fold increase compared to untreated control) at 4 hr after stimulation (Fig. 3). After 24 hr of IFN-γ, TNF-α, and LPS exposure, expression was remarkably lower compared to untreated controls.

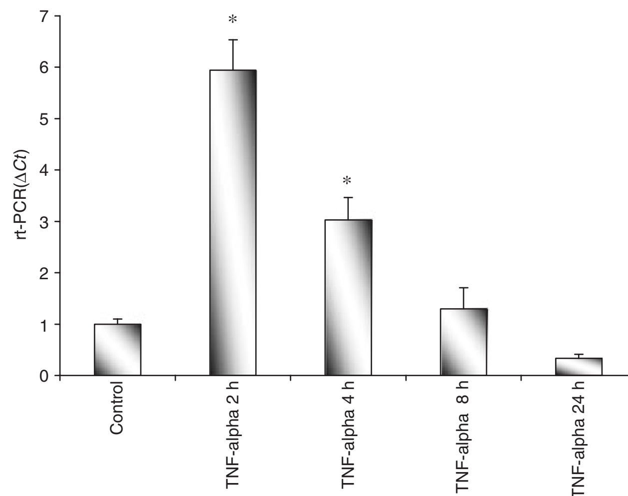

Fig. 2. Detection of CHIT1 expression by quantitative real-time PCR of RNA obtained from HMMs untreated and treated with TNF-α (100 U/mL). All rt-PCR values are given as ΔC_t values, which correspond to the difference of the maximum number of rt-PCR cycles (40) and the value obtained for each individual sample. Statistical analysis was performed by Student's *t*-test; $^*P<0.05$, of treated cells vs. control. Data are representative of three independent experiments.

Chitotriosidase Activity Determination in Macrophages Following IFN-γ, TNF-α, and LPS Exposure

We next examined the Chit activity in lysates of human macrophages cultured for 7 days and thereafter treated at different times of culture (2, 4, 8, and 24 hr) and in the absence or presence of IFN-γ, TNF-α, and LPS. As seen in Fig. 4, total Chit activity in macrophages was increased by 224-fold after 2 hr of stimulation with IFN-γ, as compared with control cells. This activity showed a marked decreased (151-fold) after

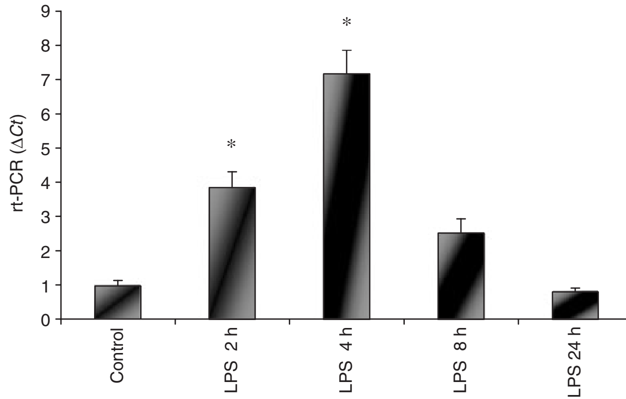

Fig. 3. Detection of CHIT1 expression by quantitative real-time PCR of RNA obtained from HMMs untreated and treated with LPS (50 ng/mL). All rt-PCR values are given as ΔC_t values, which correspond to the difference of the maximum number of rt-PCR cycles (40) and the value obtained for each individual sample. Statistical analysis was performed by Student's *t*-test; $^*P<0.05$, of treated cells vs. control. Data are representative of three independent experiments.

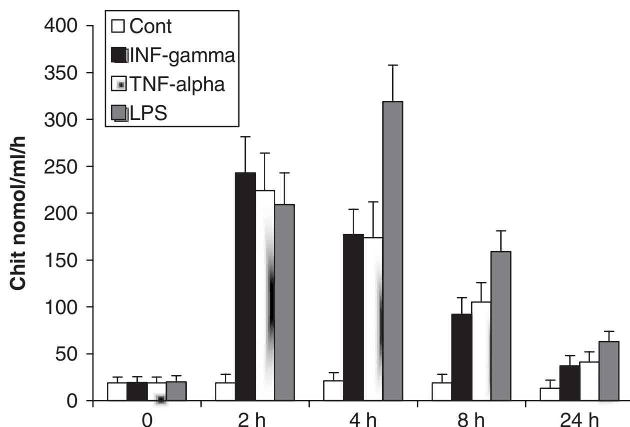

Fig. 4. Time-course of Chit activity in HMMs following treatment with IFN-γ (100 U/mL), TNF-α (100 U/mL), and LPS (50 ng/mL). Data are representative of three independent experiments.

8 hr of IFN-γ treatment. TNF-α led to about a 205-fold increase in Chit activity after 2 hr; this effect decreased to about 119-fold after 8 hr. In contrast, in cells treated with LPS, Chit augmented about 300-fold after 4 hr compared to untreated cells.

These results confirm the quantitative real-time PCR analysis.

DISCUSSION

In the present study, we described, for the first time, that Chit is involved in cellular response elicited by regulatory cytokines and by bacterial lipopolysaccharide, as evidenced by the levels of CHIT1 gene expression following IFN-γ, TNF-α, and LPS treatment (Figs. 1–3). These results were also confirmed by Chit activity.

Chit is not a housekeeping enzyme (4); the macrophages are able to produce large amounts of this enzyme under specific circumstances. One such condition is the prolonged culture of macrophages derived from peripheral blood monocytes (4). In fact, Boot et al. (4) assumed that CHIT1 mRNA comprise approximately 0.1% of the total mRNA in macrophages cultured for 3 weeks. High levels of CHIT1 mRNA are found in Gaucher disease, in which an inherited deficiency in glucocerebrosidase activity causes lysosomal storage of glucosylceramide in macrophages. This is accompanied by greatly enhanced levels of Chit in plasma (9). Macrophages are generally considered to be important elements in natural resistance in most, if not all, organs and are strategically placed to protect the microenvironment in which they are situated. In this contest, great attention must be given to the fact that this enzyme is produced by the macrophages themselves. Nevertheless, the physiological role of human chitinase still has to be established.

Our study provides direct evidence that IFN-γ, TNF-α, and LPS upregulate CHIT1 gene expression. Since it is known that macrophages treated with IFN-γ and TNF-α develop an increased cytocidal activity against intracellular microorganisms and tumor cells, we hypothesize that Chit could be involved in a cellular response elicited by regulatory cytokine.

In addition, this high expression is consistent with the activity of this enzyme in children with acute *Plasmodium falciparum* malaria, reported in a previous study in which we suggested that Chit could have a role in the immune response and in the outcome of malaria infection (12). Further, in chimpanzee, it has been demonstrated that IL-12 injection is associated with an enhanced Chit activity (17). This finding is important since a recent study has shown that protective immunity in malaria is mediated by a cascade of events involving IL-12 (18,19) which is a potent immunomodulatory cytokine effective in conferring protection against viral, bacterial, and intracellular parasitic infections (20). This cytokine not only enhances cell-mediated immune response, but also affects humoral immunity by inducing isotype switching through both IFN-γ dependent and independent mechanisms (21,22). Thus, we cannot rule out the possibility that the elevated levels of IL-12 modulate the macrophage activity stimulating their microbicidal function through a pathway also involving Chit activity, as a direct consequence of IFN-γ induction.

In conclusion, macrophages are an important source of Chit under physiological or pathological conditions. We suggest that this neglected production of Chit by macrophages may have biological relevance for the host immune response in the early phases of infections.

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