Decreased Concentrations of Tumor Necrosis Factor-α in Supernatants of Monocytes From Homozygotes for Hereditary Hemochromatosis

By Victor R. Gordeuk, Stanley Ballou, Gerard Lozanski, and Gary M. Brittenham

To determine whether release of tumor necrosis factor-α (TNF-α), a cytokine that affects iron homeostasis, may be selectively altered in hereditary hemochromatosis, we measured concentrations of TNF-α and interleukin-1β (IL-1β) in supernatants of cultured peripheral blood monocytes from 11 homozygotes for hereditary hemochromatosis, 11 healthy individuals, and five patients with iron-loading anemia. The gene for hereditary hemochromatosis is tightly linked to the HLA locus on chromosome 6, but its exact site and product are not known. The gene for TNF-α also is located within the HLA region. Monocytes were incubated from 4 to 36 hours in medium alone or with added lipopolysaccharide. Mean concentrations of immunoreactive TNF-α in supernatants were significantly lower for subjects with hereditary hemochromatosis as compared to healthy controls (P < .037) and patients with iron-loading anemia (P < .006); differences between homozygotes for hemochromatosis and healthy controls were up to 4.5-fold at 4 hours (P = .008), 1.9-fold at 12 hours (P = .036), and 7.0-fold at 36 hours (P = .001). Importantly, concentrations of IL-1β in supernatants were not significantly different among the three groups. We conclude that release of TNF-α by monocytes may be selectively impaired in hereditary hemochromatosis. Deficient activity of TNF-α may contribute to the disordered iron metabolism of this disease.

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MATERIALS AND METHODS

The study was approved by the Committee on Investigation in Humans of MetroHealth Medical Center, Case Western Reserve University, and informed consent was obtained from all participants.

Study participants. Eleven homozygotes for hereditary hemochromatosis, 11 healthy control volunteers, and five patients with iron-loading anemias were studied. The diagnosis of hereditary hemochromatosis was established by standard criteria.29 Nine of the patients with hereditary hemochromatosis initially presented because of symptoms related to the disease or abnormal laboratory tests, and two were ascertained because they were first-degree relatives of probands with the disease.

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relatives of an individual known to be affected. Most had substantial iron burdens at the time of diagnosis as documented by therapeutic phlebotomy (range of 5 to 30 g). Ten of the subjects were enrolled in the study after removal of excess body iron and one volunteer was studied at the beginning of his phlebotomy program. The patients with iron-loading anemia included two with refractory sideroblastic anemia, two with myelodysplasia, and one with congenital dyserythropoietic anemia. All of the healthy controls and patients with iron-loading anemia were unrelated. Nine of the patients with hereditary hemochromatosis were unrelated.

Indirect measures of iron status. Serum iron and total iron binding capacity (TIBC) were determined by methods recommended by the International Committee for Standardization in Haematology with modifications for small quantities of serum. Serum ferritin was measured by an enzyme immunoassay (Ramco Laboratories, Inc, Houston, TX).

Measurement of cytokine levels in supernatants of peripheral blood monocytes. Venous blood was drawn into heparin (30 U/mL) and centrifuged at 300g for 10 minutes to separate platelet-rich plasma. The cells were diluted with 1 vol of Hanks' Balanced Salt Solution (HBSS) and layered over Ficoll-Paque (Pharmacia, Piscataway, NJ). After centrifugation at 1,000g for 30 minutes the mononuclear cell layer was carefully aspirated, washed three times in HBSS, resuspended in RPMI 1640 with 20% fetal calf serum (FCS), and incubated in culture dishes at 4°C. The monocytes were washed three times in HBSS, resuspended at 1 x 10^6 cells/mL in RPMI 1640 supplemented with 2% FCS, streptomycin, and penicillin, and cultured in 16-mm wells (0.5 mL/well) at 37°C, 5% CO₂, and 95% humidity. The nonadherent cells were aspirated and the adherent cells ("monocytes") were released by vigorous pipetting after a 20-minute incubation in calcium- and magnesium-free HBSS at 4°C. The monocytes were washed three times in HBSS, resuspended in RPMI 1640 supplemented with 2% FCS, streptomycin, and penicillin, and cultured in 16-mm wells (0.5 mL/well) at 37°C, 5% CO₂, in the presence of medium alone or medium plus 0.1, 1.0, or 10.0 μg/mL bacterial lipopolysaccharide (LPS) (Escherichia coli 026:B6; Sigma, St Louis, MO) for 4 to 36 hours. After culture, supernatants were carefully aspirated, centrifuged at 10,000g for 5 minutes, and stored at -70°C for subsequent assays. The viability of the cultured monocytes, as assessed by Trypan blue exclusion, was greater than 95% both at the outset and the completion of the culture periods. Concentrations of TNF-α and interleukin-1β (IL-1β) were measured in monocyte supernatants using specific enzyme-linked immunoassays according to instructions supplied by the manufacturers (TNF-α: T-Cell Sciences, Cambridge, MA; IL-1β: Cistron Biotechnology, Pine Brook, NJ). The detection limit for the TNF-α assay was 10 pg/mL and for the IL-1β assay was 20 pg/mL. All assays were performed in duplicate. We used high concentrations of LPS in the culture system because in preliminary studies we observed differences in concentrations of TNF-α in supernatants of monocytes between homozygotes for hereditary hemochromatosis and controls both when LPS was not present and when it was added at a concentration of 0.1 μg/mL; in the present study we wished to determine if large amounts of LPS could overcome any apparent decrease in production or release of the cytokine.

Statistical analysis. Cytokine concentrations in monocyte supernatants were found to have a log normal distribution and were transformed logarithmically for further analysis. To determine whether the increasing concentrations of LPS used (0.1, 1.0, and 10.0 μg/mL) resulted in greater levels of cytokines, concentrations of cytokines measured in supernatants of monocytes cultured at these three levels of stimulation were compared using repeated measures analysis of variance. At each time point, mean cytokine concentrations measured at four levels of stimulation (no added LPS as well as concentrations of 0.1, 1.0, and 10.0 μg/mL) were compared among patients with hereditary hemochromatosis, healthy controls, and subjects with iron-loading anemia using repeated measures analysis of variance (Fig 1).³³

RESULTS

The clinical features of the study participants are summarized in Table 1. The mean serum ferritin was in the normal range for all but one of the homozygotes for hereditary hemochromatosis, suggesting excess storage iron had been

![Fig 1. Concentrations of cytokines in supernatants of peripheral blood monocytes cultured over 4 to 36 hours at four levels of stimulation with LPS. Means and standard errors are depicted. Statistical comparisons for TNF-α-hemochromatosis versus healthy controls: P < .01 at 4, 16, 24, and 36 hours; P = .014 at 8 hours; P = .036 at 12 hours; hereditary hemochromatosis versus iron-loading anemia: P > .005 at each time point. Statistical comparisons for IL-1β-hemochromatosis versus healthy controls: P > .8 for each time point; hereditary hemochromatosis versus iron-loading anemia: P > .25 for each comparison.](image-url)
DECREASED CONCENTRATIONS OF TNF-α

Table 1. Clinical Characteristics of Participants in the Study

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Sex (M:F)</th>
<th>Age (yr)</th>
<th>Serum Iron (μg/dL)</th>
<th>TIBC (μg/dL)</th>
<th>Transferrin Saturation (%)</th>
<th>Serum Ferritin* (μg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hereditary hemochromatosis</td>
<td>11</td>
<td>5:6</td>
<td>45 ± 16</td>
<td>174 ± 53</td>
<td>235 ± 42</td>
<td>77 ± 26</td>
<td>63 (12-321)</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>11</td>
<td>7:4</td>
<td>41 ± 15</td>
<td>103 ± 31</td>
<td>307 ± 61</td>
<td>35 ± 14</td>
<td>34 (29-40)</td>
</tr>
<tr>
<td>Iron-loading anemia</td>
<td>5</td>
<td>3:2</td>
<td>60 ± 24</td>
<td>217 ± 85</td>
<td>236 ± 85</td>
<td>92 ± 13</td>
<td>1,222 (698-2,138)</td>
</tr>
</tbody>
</table>

(normal range)

Results are mean ± SD.

*Geometric mean and standard deviation range.

removed by phlebotomy in all but the single patient recently begun on phlebotomy. The mean serum iron and transferrin saturation were elevated, as is characteristic for hereditary hemochromatosis. Both serum ferritin and transferrin saturation were markedly elevated in the patients with iron-loading anemia, indicating the presence of substantial iron overload.

TNF-α and IL-1β were detected in some of the supernatants of monocytes cultured without added LPS, probably reflecting trace amounts of LPS in the culture medium or FCS. With increasing amounts of LPS, 0.1 μg/mL, 1.0 μg/mL, and 10.0 μg/mL, added to the monocyte cultures there were significant increments in the levels of cytokines in the supernatants (P < .001 for both TNF-α and IL-1β). The concentrations of TNF-α and IL-1β in supernatants of monocytes cultured for periods of 4 to 36 hours are shown in Fig 1. At each timepoint, concentrations of TNF-α were significantly lower in monocyte supernatants from homozygotes for hereditary hemochromatosis as compared to healthy controls (P < .037) and subjects with iron-loading anemia (P < .005). The differences in mean TNF-α concentrations between homozygotes for hereditary hemochromatosis and healthy controls were up to 4.5-fold at 4 hours (P = .008), 1.9-fold at 12 hours (P = .036), and 7.0-fold at 36 hours (P = .001). In contrast, concentrations of IL-1β were not significantly different between homozygotes for hereditary hemochromatosis and healthy controls (P > .8) or patients with iron-loading anemia (P > .25).

DISCUSSION

In these experiments, mean concentrations of TNF-α in supernatants of cultured monocytes from patients with hemochromatosis were substantially lower than levels in monocyte supernatants from healthy controls and patients with iron-loading anemia at each time point measured over 4 to 36 hours and at each degree of stimulation with LPS. In contrast, concentrations of IL-1β, another cytokine that helps to modulate inflammatory changes in iron metabolism, were not different from controls (Fig 1). In addition to accumulation of newly released TNF-α, the available data indicate that monocyte supernatant concentrations of the cytokine reflect the following processes: binding and dissociation of the cytokine to TNF-α receptors on the surface of cultured monocytes, internalization and degradation of the receptor-ligand complexes, and binding and dissociation of TNF-α by soluble receptors that have been shed from the cell surface. Also, levels of cytokines in monocyte supernatants might be affected by stability of the molecules and the presence of proteolytic enzymes.

Following or during stimulation of monocytes and HL-60 cells by LPS, there is an increase in TNF-α mRNA and cell-associated protein that is maximal within 2 to 3 hours. Thus, the TNF-α concentrations in monocyte supernatants measured at 4 hours in our experiment, in which levels were lower in homoyzogotes with hereditary hemochromatosis to a highly significant degree, represent the findings in our study most temporally related to mRNA transcription and translation. At the 4-hour time point, it seems most likely that the reduced concentrations of TNF-α in monocyte supernatants represent decreased release of the cytokine.

Recognizing that our measurements of cytokine concentrations are influenced by complex processes over the 36-hour experimental period, we believe that our observations suggest that the release of TNF-α by monocytes from homozygotes for hereditary hemochromatosis may be selectively impaired.

Jacob et al reported variability in concentrations of TNF-α in supernatants of cultured peripheral blood mononuclear cells according to sex and HLA class II antigen, and it is important to consider whether the differences in the levels of TNF-α that we observed could be related to these factors. The variability according to sex was found only in premenopausal females with TNF-α factor levels fluctuating when the test was repeated on different days, but mean values did not differ significantly from males. In our study, three of the homozygotes for hemochromatosis were premenopausal females as were three of the healthy controls and one of the patients with iron-loading anemia; it is unlikely that variability in TNF-α release in the premenopausal females could account for the decreased TNF-α concentrations in subjects with hereditary hemochromatosis in our study. In the investigation of Jacob et al, TNF-α concentrations in monocyte supernatants tended to be lower among individuals with HLA-DR2 or HLA-DQw1 as compared with other class II antigens. In our study, 25 of the 27 participants were unrelated and the magnitude of the difference in TNF-α concentrations in monocyte supernatants between subjects with hereditary hemochromatosis.

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and controls appears larger than the difference between individuals with DR2 and DQw1 antigens and the general study population of Jacob et al.48 Although HLA typing was not a part of our study, it is unlikely that class II-related variability could account for the magnitude of the difference in TNF-α levels that we observed. The report of decreased TNF-α concentrations in association with HLA-DR2 is interesting because HLA-DR2 is linked to the hereditary hemochromatosis locus in certain pedigrees.49,50

Our data are limited because they were derived from studies on peripheral blood monocytes in vitro. Furthermore, the decreased TNF-α concentrations observed might only represent an epiphenomenon of hereditary hemochromatosis. Nevertheless, the apparent defect in TNF-α release by peripheral blood monocytes from homozygotes for hereditary hemochromatosis could be present in other cells of the mononuclear-phagocyte system such as Kupffer cells, bone marrow reticulum cells, and tissue macrophages. Given this possibility, decreased TNF-α release by cells of the mononuclear-phagocyte system might be involved in the pathogenesis of the disorder. Reduced TNF-α activity could then be responsible for the relatively decreased ferritin content and reduced amounts of mononuclear-phagocyte iron that have been described in hereditary hemochromatosis. Furthermore, a deficiency of TNF-α leading to inadequate ferritin synthesis might be related to the increased release of mononuclear-phagocyte iron found in hereditary hemochromatosis as well as to the increased serum iron and hepatocyte iron-loading that are characteristic of the condition.

The decreased concentrations of TNF-α we observed in monocyte supernatants from homozygotes for hereditary hemochromatosis might reflect decreased transcription of mRNA or reduced translational activity. The gene for TNF-α is located approximately 200 kb centromeric to the HLA-B gene on chromosome 6.53 The gene for hereditary hemochromatosis is also linked to the HLA locus on chromosome 6. An allele of the TNF-α gene with lowered responsiveness to inflammatory stimuli might have segregated along with the hemochromatosis allele on chromosome 6 but have no relationship to the pathogenesis of the condition. On the other hand, given the profound effects of TNF-α on certain aspects of iron metabolism, the TNF-α gene, or an associated regulatory locus, might be affected by the gene responsible for hereditary hemochromatosis or be the gene itself. Simon et al proposed that the gene for hereditary hemochromatosis is located more closely to the HLA-A locus than the HLA-B locus because of findings in the French population that HLA-A3 is an independent marker for hereditary hemochromatosis whereas HLA-B7 and B14 are not. On the basis of pedigrees with hemochromatosis in which recombination events are thought to have occurred, investigators have assigned a position for the hereditary hemochromatosis gene centromeric to the HLA-A locus but telomeric to the HLA-B locus,54-57 or alternatively telomeric to the HLA-A locus.58 The known location of the gene for TNF-α does not coincide with these proposed sites for the gene for hereditary hemochromatosis. However, the precise location of the hemochromatosis gene in relation to the HLA-A and B loci remains to be determined and it would be of interest to examine the TNF-α locus and associated regulatory loci in pedigrees with hereditary hemochromatosis.

In conclusion, we found evidence that the release of TNF-α, but not IL-1β, may be impaired in patients with hemochromatosis. This alteration in cytokine release may provide a clue to the pathogenesis of hereditary hemochromatosis.

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DECREASED CONCENTRATIONS OF TNF-α

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Decreased concentrations of tumor necrosis factor-alpha in supernatants of monocytes from homozygotes for hereditary hemochromatosis

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