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INTERLEUKIN 1 INDUCES FERRITIN HEAVY CHAIN IN HUMAN MUSCLE CELLS

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Interleukin 1 alpha (IL-1) and tumor necrosis factor alpha (TNF) are two monokines which play a prominent role in the response to inflammation and injury. We recently observed that TNF leads to an increase in the synthesis of the heavy chain of ferritin, suggesting that TNF may be involved in iron homeostasis (Torti *et al.* (1988) J. Biol. Chem. 263, 12638-12644). The experiments reported here demonstrate that in cultured human muscle cells, IL-1 induces ferritin H mRNA and protein as effectively as TNF. TNF and IL-1 were additive in their effects on ferritin H expression, and IL-1 induction of ferritin H was not blocked by anti-TNF antibodies. Ferritin H induction was a specific response not observed with beta or gamma interferon, nor with transforming growth factor beta. Both differentiated myotubes as well as myoblasts responded to IL-1 with the induction of ferritin H. These results suggest that monokine-mediated alterations in the subunit composition of the ferritin molecule may be of biological relevance in the response to inflammation and injury.

Tumor necrosis factor alpha (TNF/cachectin) is a protein produced by activated macrophages which has been implicated in the syndrome of cachexia as well as in septic shock (1,2). We recently observed that TNF induces ferritin heavy chain in human muscle (3). This observation suggests that ferritin, the major intracellular iron binding protein, comes under the regulatory influence not only of iron, but of a cytokine implicated in the systemic response to infection and injury. Since alterations in iron metabolism are common accompaniments to systemic inflammation (4), it is perhaps not surprising that cytokines involved in the host response to injury and inflammation should exert an effect on a key protein in iron homeostasis. However, to assess the biological significance of the increase in ferritin H it is important to determine whether it is a specific response to TNF, or a more general response that can be provoked by other inflammatory mediators. In this report we explore the relationship between ferritin and interleukin 1 alpha (IL-1), a monokine produced after infection, injury or antigenic challenge and implicated as a mediator of the acute phase response (5). We demonstrate that interleukin 1, like TNF, selectively induces the heavy chain of ferritin in both myoblasts and fully differentiated myotubes.

Abbreviations. TNF -- tumor necrosis factor alpha; IL-1 -- interleukin 1 alpha; ferritin H -- heavy, heart, acidic ferritin; TGF-β -- transforming growth factor beta; FeNTA -- ferric nitrilotriacetate; Ara-c -- cytosine arabinoside.

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MATERIALS AND METHODS

Human muscle cultures and treatment. Human myoblasts were a gift of H. Blau, Stanford University. Their isolation, growth, and differentiation has been described in detail (6). Briefly, myoblasts were grown in growth medium (F-10 supplemented with 15% FBS and 0.5% chick embryo extract). To induce muscle fusion and differentiation, cultures were incubated in starvation medium (DME with 1 μM insulin and 2.5 μM dexamethasone) for approximately 18 hours. Differentiated cultures were maintained in fusion medium (starvation medium supplemented with 2% horse serum) which was replaced daily. The day at which differentiation is initiated is referred to as day 0. Muscle cells were fully differentiated by day 6, as judged morphologically and by immunofluoresence of myosin heavy chain (6). To examine the effect of IL-1, TNF or TGF-β on muscle differentiation, treatment of myoblasts was initiated 3 days prior to the shift to differentiation conditions. In some experiments (see text) cytosine arabinoside (Ara-c, 10^{-5}M) was added on day 3 followed by the addition of TNF, IL-1 or both on day 7. In all cases, fresh medium containing the monokines was added daily.

Chemicals and reagents. Recombinant human TNF alpha (TNF, cachectin) was a gift from Cetus Corp., Emeryville, CA. Recombinant human IL-1 alpha was a gift from Peter Lomedico at Hoffman-La Roche Inc., Nutley, New Jersey. Rabbit anti-TNF antibody was obtained from Cetus Corp. and used at a 1:250 dilution (neutralizes 1600 U/ml). Transforming growth factor beta (unfractionated) was obtained from R & D Systems, Inc., Minneapolis, MN. Human recombinant gamma interferon was a gift from AMGen, Thousand Oaks, CA. Human recombinant beta interferon was a gift from Triton Biosciences Inc., Alameda, CA.

RNA analysis and cDNA probes. RNA was isolated by guanidium thiocyanate lysis and centrifugation through CsCl as previously described (6). 10 μg total RNA from each sample was applied to formaldehyde gels and analyzed by northern blotting. Blots were washed free of residual radioactivity using two successive 15 minute washes in 0.1X SSC (1X SSC is 0.15 M sodium chloride, 0.015 M sodium citrate) plus 50% formamide at 70°C. cDNA for ferritin H chain has previously been described (3). β-actin cDNA (7) was obtained from L. Kedes, and used to determine whether equal amounts of RNA were loaded and transferred to nitrocellulose filters. cDNA probes were ^{32}P-labeled by random priming (8).

Ferritin protein synthesis. Ferritin protein was analyzed by immunoprecipitation following metabolic labeling of control or treated cells with [^{35}S]-methionine as previously described (3). Following lysis of cells, total incorporation was measured by trichloroacetic acid precipitation, and equivalent amounts of lysate (5 X 10^6 trichloroacetic acid precipitable counts per minute), were immunoprecipitated by anti-ferritin antibody (rabbit anti-human ferritin, Dako Corp., Santa Barbara, CA.) in each case. The immunoprecipitates were collected following incubation with protein A agarose and analyzed by SDS PAGE.

RESULTS

IL-1 induces ferritin H mRNA and protein in cultured muscle cells

Ferritin is composed of two types of subunits, termed H (heavy or heart) and L (light or liver). Twenty-four of these associate in a ratio determined by tissue type and physiological state to form the apoferritin protein (see [9] for review). We had previously observed that TNF induces ferritin heavy chain in adipocytes and muscle cells (3). In order to test whether this is a unique response to TNF, proliferating myoblasts were treated for 24 hours with increasing doses of IL-1 alpha. Duplicate cultures were treated with TNF alpha at 10 ng/ml (a concentration of TNF previously shown to maximally induce ferritin H in myoblasts [6]). RNA was isolated and levels of ferritin H mRNA assessed by Northern blot analysis. As a control, expression of β-actin was also measured. The resulting autoradiograms were quantitated by scanning densitometry to determine the magnitude of the effect of IL-1 or TNF on ferritin H expression. Human myoblasts responded to IL-1 treatment in a dose dependent manner with an increase in the level of ferritin H mRNA (figure 1). At maximal doses of either TNF or IL-1, ferritin H expression was stimulated approximately 3-fold.

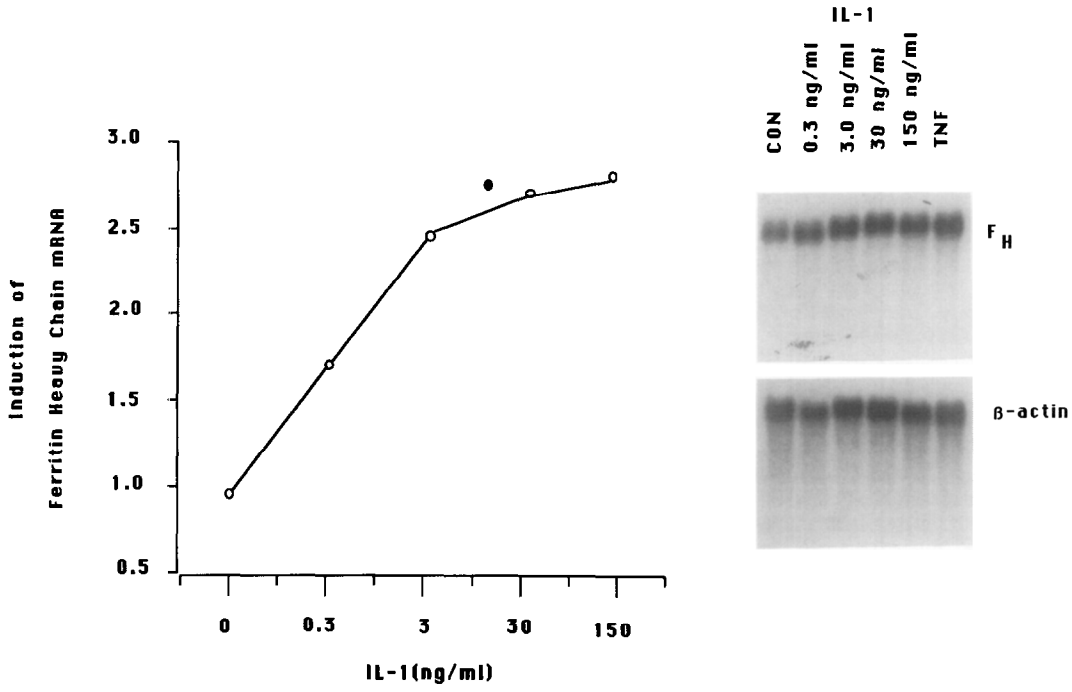

Figure 1. IL-1 induces ferritin heavy chain expression in a dose-dependent manner. Proliferating myoblasts were incubated with IL-1 at the indicated concentrations or with 10 ng/ml TNF for 24 h. RNA was isolated and examined by Northern blot analysis. The filter was hybridized first with ferritin H cDNA and then washed and hybridized with β-actin. Autoradiograms were scanned by densitometry and the results were normalized to the ferritin H/β-actin hybridization seen in control (untreated) cultures. Fold induction in IL-1 treated cultures (0) was compared to cultures treated with TNF (●) at 10 ng/ml, a concentration which maximally induces ferritin H (F_H) transcripts. The northern blot is shown at right; results of scanning this blot are depicted graphically at left. This experiment was repeated twice with virtually identical results.

To examine the effect of TNF and IL-1 on ferritin protein synthesis, myoblasts were pulse-labeled with [^{35}S]-methionine following treatment with either TNF or IL-1. Treatment with iron (ferric nitrilotriacetate, FeNTA), a known inducer of ferritin synthesis (10), was used as a positive control. Cell lysates were prepared and analyzed by immunoprecipitation and electrophoresis. IL-1 (like TNF) selectively induced the synthesis of the ferritin H subunit (figure 2). In contrast, iron stimulated the synthesis of both the H and L subunits of ferritin.

When added to cultures of proliferating myoblasts, both TNF (6) and IL-1 (Miller and Torti, unpublished observations) inhibit differentiation into myotubes. In order to test whether all agents which inhibit muscle differentiation induce ferritin H, muscle cells were treated with TGF-β at concentrations sufficient to inhibit differentiation (11,12). Figure 3 compares effects of an 8 hour treatment of TGF-β, IL-1 and TNF on ferritin H mRNA levels. In contrast to the induction of ferritin H observed with IL-1 and TNF, TGF-β did not affect ferritin H expression. Nor did prolonged TGF-β treatment cause delayed appearance of augmented ferritin H transcripts; extended (96 hr) treatment with TGF-β did not induce ferritin H, although muscle differentiation was completely blocked (data not shown). In separate experiments, we also tested recombinant gamma (100 ng/ml) and beta (4.5×10^3 units/ml) interferons and found no effect of these proteins on ferritin H expression (data not shown).

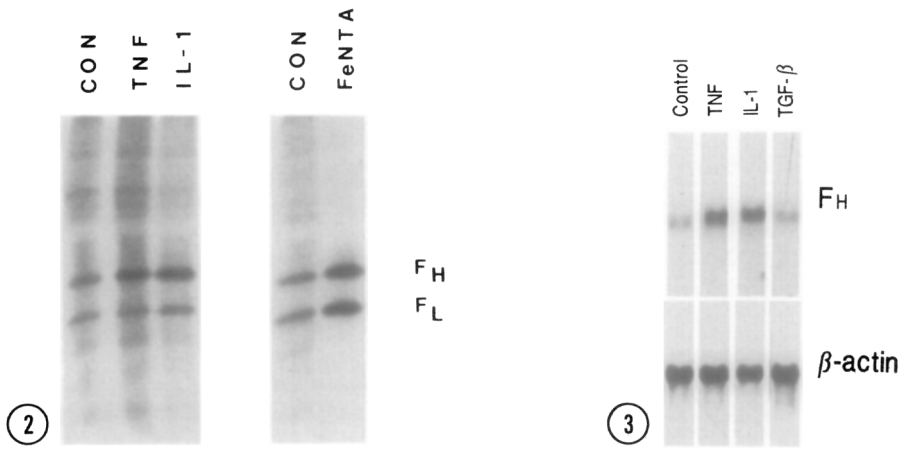

Figure 2. IL-1 and TNF selectively increase the synthesis of the heavy chain of ferritin. Human myoblast cultures were incubated with medium alone (con), or medium containing TNF (10 ng/ml) or IL-1 (30 ng/ml). In a separate experiment, cultures were treated with 200 μM ferric nitrilotriacetate (FeNTA). Cultures were incubated for 5 h and proteins were labeled during a subsequent 2 h incubation with 50 μCi/ml [^{35}S]-methionine in methionine-free medium containing TNF, IL-1 or FeNTA as appropriate. Samples were lysed and equivalent amounts of total protein immunoprecipitated with an anti human ferritin antibody. The heavy (F_H) and light (F_L) subunits of human ferritin are indicated.

Figure 3. TNF and IL-1, but not TGF-β, induce ferritin heavy chain in myoblasts. RNA was isolated from myoblast cultures which were treated for 8 h with TNF (10 ng/ml); IL-1 (30 ng/ml); or TGF-β (7 ng/ml). These concentrations completely inhibited differentiation of these primary human myoblasts (data not shown). 10 μg of RNA from control and treated samples were used to prepare a northern blot. The blot was hybridized sequentially to ferritin H and β-actin cDNA.

Anti-TNF antibody inhibits TNF, but not IL-1 effect on ferritin H expression

TNF synthesis is not limited to macrophages: for example, both TNF transcripts and protein have been demonstrated in human embryonic lung fibroblasts (13). Further, cytotoxic effects of activated macrophages induced by IL-1 are actually mediated by TNF (14). We therefore considered the possibility that the induction of ferritin H by IL-1 might be a consequence of its induction of TNF. To test this possibility, anti-TNF antibody was added directly to fresh medium prior to supplementation with TNF or IL-1. The TNF effect on ferritin H expression was completely abolished in cultures which received the anti-TNF antibody (Table I). In contrast, IL-1 mediated induction of ferritin H was unaffected by incubation with the anti-TNF antibody. These results suggest that the IL-1 effect on ferritin H is not mediated by the secretion of TNF in response to IL-1.

TNF and IL-1 are additive in their effect on ferritin H

Figure 4 shows the results obtained from cultures treated for 21 hours with TNF (10 ng/ml), IL-1 (30 ng/ml), or both cytokines at doses sufficient to cause maximal induction (see figure 3) of ferritin H. Quantitation of these results by scanning densitometry demonstrates that the induction of ferritin H mRNA is additive when cultures were treated with the combination of TNF and IL-1 (Ferritin H expression after normalization to β-actin, expressed in arbitrary units, following treatment with TNF = 2.53; IL-1 = 2.57; and IL-1 plus TNF = 4.59).

TABLE I. Anti-TNF antibody does not inhibit the IL-1 effect on Ferritin H expression

SAMPLE	TNF Ab	FERRITIN H
CON	-	2.26
	+	2.24
TNF	-	5.35
	+	2.66
IL-1	-	5.40
	+	5.38

Myoblast cultures were incubated in medium containing TNF (10 ng/ml) or IL-1 (30 ng/ml) with or without anti-TNF antibody (1:250 dilution; neutralizes 1600 U/ml of TNF). Cultures were incubated for 8 hours and RNA was isolated and examined by Northern analysis. Autoradiograms were scanned by densitometry and the results were normalized to β-actin.

Time course and magnitude effect of IL-1 and TNF on ferritin H mRNA

Myoblast cultures were treated for 4, 8, 24, 48 hours with either TNF (10 ng/ml) or IL-1 (30 ng/ml). RNA was isolated and analyzed for presence of ferritin H transcripts (figure 5). After 4 hours of treatment both monokines stimulated the expression of ferritin H equally. The level of ferritin H mRNA increased with time of treatment and reached a plateau which stayed relatively constant after 8 hours. (In separate experiments, we have shown persistent induction of ferritin H transcripts by IL-1 and TNF for up to 96 hours [not shown]).

Differentiated myotubes augment ferritin H synthesis in response to IL-1

Myoblasts with proliferative capacity represent a relatively small fraction of skeletal muscle in the intact animal; *in vivo*, muscle mass is largely comprised of terminally differentiated myotubes. In order to model the *in vivo* situation more accurately, we next examined whether myotubes as well as myoblasts responded to IL-1 with elevated synthesis of ferritin H. For these experiments human muscle cultures were differentiated into multinucleated myotubes

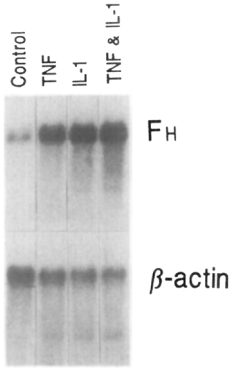

Figure 4. Myoblasts treated with both TNF and IL-1 express ferritin H in an additive fashion. Proliferating myoblast cultures were treated for 21 h with TNF (10 ng/ml), IL-1 (30 ng/ml), or both monokines. RNA was isolated, a Northern blot prepared, and the blot hybridized sequentially with cDNA to ferritin H and β-actin. mRNA levels were quantitated by scanning densitometry.

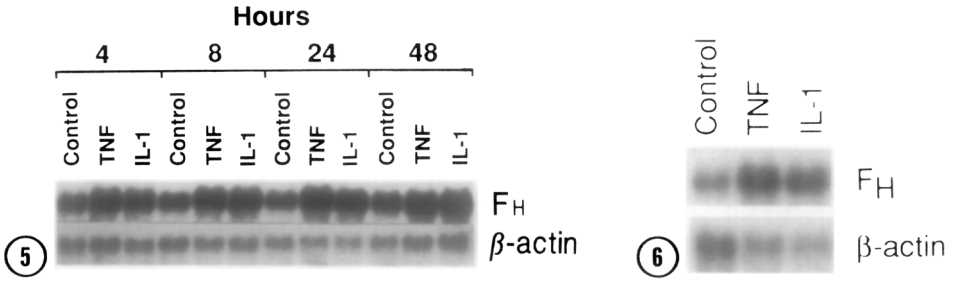

Figure 5. Time-course of IL-1 versus TNF effect on ferritin H expression. Myoblast cultures were incubated with 10 ng/ml TNF or 30 ng/ml of IL-1 and RNA was isolated at the indicated times. The results from Northern analysis are shown.

Figure 6. IL-1 and TNF induce ferritin H mRNA in differentiated human myotubes. Myoblast cultures were differentiated (see Methods) and Ara-c (10^{-5} M) was added on day 3 to remove residual dividing cells. TNF (10 ng/ml) or IL-1 (30 ng/ml) were added on day 7. Fresh medium containing Ara-c, TNF or IL-1 was replaced daily. Cultures were harvested for RNA analysis after 48 h treatment on day 9 and examined for ferritin H and β-actin mRNA.

(see Methods). Cytosine arabinoside (Ara-c) was then used to selectively eliminate the population of dividing cells. This treatment has previously been shown to yield a virtually pure population of myotubes (6). As shown in fig. 6, human myotubes respond to both TNF and IL-1 with the induction of ferritin H.

DISCUSSION

The experiments reported here demonstrate that treatment of human muscle cells with interleukin 1 results in increased levels of mRNA for ferritin heavy chain and increased synthesis of the ferritin heavy chain protein. In this regard, IL-1 behaves similarly to TNF. Although the precise nature of the interaction between TNF, IL-1 and muscle is still under investigation, these results are in concert with other reports which suggest that both *in vivo* (15,16) and *in vitro* (6,17), muscle interacts with the cytokine network.

Observations in the literature that in some cells IL-1 induces TNF (14) prompted us to first test whether the effect of IL-1 on ferritin H might be secondary to its ability to induce TNF. However, we found that the induction of ferritin H by IL-1 is not mediated through secreted TNF, but is an independent response to IL-1 not blocked by anti-TNF antibodies (Table I). The ability of TNF and IL-1 to evoke distinct responses not dependent on their ability to induce each other is further supported by experiments testing the effects of a combination of TNF and IL-1: these treatments led to additive effects on ferritin H at maximal concentrations of each cytokine.

Although TNF and IL-1 induce ferritin H and prevent muscle differentiation, the induction of ferritin is not an obligatory correlate of inhibition of muscle differentiation *per se*. For example, TGF-β, which completely inhibits the differentiation of muscle cell lines (11,12) and human myoblasts (Miller and Torti, unpublished observations) has no effect on ferritin H (Fig. 3). We therefore consider elevated synthesis of ferritin H to be a specific response to cytokine treatment rather than a non-specific response to a block in differentiation.

It is intriguing that both TNF and IL-1 appear to exert a selective effect on the H subunit of ferritin. Although the consequences of this selective increase on iron metabolism in cytokine-treated cells has not yet been assessed, the subunit composition of ferritin is known to influence its function. L-rich ferritins predominate in organs specialized for long term iron storage, such as liver and spleen (18,19); biochemical properties of L-rich ferritins are also consistent with a role in long term iron storage (18,20). In contrast, H-rich ferritins occur in a broader variety of tissues (e.g. heart [18,20], pancreas, kidney [19], myelopoetic cells [21]) and confer a greater capacity for rapid iron uptake (22,23). IL-1 and TNF action could therefore favor rapid sequestration of iron in H-rich ferritin rather than long-term iron storage as appears mediated by ferritin L. Although the degree to which paracrine and endocrine factors modulate ferritin composition *in vivo* remains to be documented, *in vivo* consequences of increased sequestration of iron to ferritin might be expected to include local reductions in ferrous iron concentrations. This might in turn lead to reduction of hydroxyl radical formation (24) and attendant tissue damage. An increase in ferritin H might thus exemplify a mechanism employed by the host to protect itself from its own defenses. Although further experiments will be required to clarify the role played by ferritin H at both a cellular and organismal level, the observation that an increase in ferritin H is provoked independently by two inflammatory mediators suggests that cytokine-mediated alterations in the subunit composition of the ferritin molecule may be of biological relevance, perhaps in the response to inflammation and injury.

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