AUGMENTED EXPRESSION OF A MYELOID-SPECIFIC PROTEIN TYROSINE KINASE GENE (*hck*) AFTER MACROPHAGE ACTIVATION

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Mononuclear phagocytes directly mediate the destruction of pathogens through ingestion, and can also be stimulated to produce potent immunoregulatory molecules, including IL-1α, IL-1β, and TNF. These diverse functions of phagocytic cells are modulated by a large set of cell surface receptors that probe the extracellular milieu. Priming of tissue macrophages by T cell–derived IFN-γ results in increased hydrogen peroxide production, increased killing of intracellular pathogens, and increased expression of class II cell surface glycoproteins (1–4). A second activating stimulus, for example that provided by bacterial LPS, augments the tumoricidal activity of primed macrophages and provokes the expression of TNF and IL-1 (5–8). Although LPS by itself can trigger release of these cytokines, LPS and IFN-γ administered together provide a synergistic stimulus that is believed to mimic physiologic activation (2, 9, 10).

To better understand the regulation of macrophage activity, considerable effort has been directed towards characterizing changes in gene expression subsequent to LPS or IFN-γ treatment of phagocytic cells. LPS stimulation of macrophages results in rapid but transient increases in the levels of several mRNAs including those encoded by the KE, JC, *myc*, and *fos* genes (11, 12), transcripts that characteristically accumulate in a variety of cell types after mitogen stimulation. In contrast, IFN-γ treatment of macrophages does not induce expression of these "competence" genes. IFN-γ does provoke a slow increase in intracellular Ca^{2+} concentration in macrophages, and also stimulates increases in protein kinase C activity. These observations have provided a biochemical basis for distinguishing between different macrophage activation protocols. Nevertheless, the mechanisms whereby signals are transmitted from a large array of cell surface receptors to the macrophage interior are completely obscure.

Among the possible signal transduction elements implicated in the regulation of myeloid cell behavior are protein tyrosine kinases. For example, the colony-stimulating factor (CSF-1) receptor, which promotes differentiation in monocytes, is a transmembrane protein tyrosine kinase that resembles other growth factor receptors in structure and molecular physiology (13). Another set of membrane-associated protein

This work was supported in part by grants CA-45682 (R. M. Perlmutter) and HD-18184 and AI-16760 (C. B. Wilson) from the National Institutes of Health. Address correspondence to Roger M. Perlmutter, Howard Hughes Medical Institute, University of Washington, SL-15, Seattle, WA 98195.

tyrosine kinases, also implicated in the control of cell differentiation, is encoded by members of the *src* family of proto-oncogenes. Physiologic regulators of *src*-like kinase activity in any cell type are unknown. Nevertheless, they are considered attractive candidates for components of a signal transduction cascade since it is clear that mutated versions of these molecules with increased kinase activity can stimulate neoplastic growth in many cell types (14–17). The *hck* gene encodes an *src* family protein tyrosine kinase that is expressed primarily in myeloid cells (18, 19). The human *hck* gene, for example, is expressed at highest levels in granulocytes (18). Since these cells are terminally differentiated phagocytes, it is likely that the *hck* product participates in the regulation of some behavior other than replication. Recent studies of a related element, the lymphocyte-specific protein tyrosine kinase gene *lck*, indicate that stimuli which induce lymphokine secretion also modulate *lck* gene expression (20). These observations together suggest that the *hck* gene product, when expressed in macrophages, may assist in mediating macrophage activation.

To better assess the role of *src* family protein tyrosine kinases in the regulation of myeloid cell behavior, we have developed antisera to the product of the *hck* gene. We have shown that this product ($p59^{hck}$) and the transcripts that encode it are upregulated in LPS-stimulated human macrophages. Our results indicate that the *hck* gene product is required late in the macrophage activation pathway. In contrast, we find that the *fgr* gene, which is structurally related to the *hck* gene and is also expressed primarily in myeloid cells (21), is downregulated during macrophage activation. It thus appears that these two closely related gene products participate in functionally distinct signaling pathways.

Materials and Methods

Blood-derived Cells. Human peripheral blood monocyte-derived macrophages were isolated as previously described (22, 23). Briefly, monocytes were purified from human peripheral blood mononuclear cells by attachment to plastic dishes. Cells were cultured in RPMI 1640 containing 2 mM L-glutamine, 22 mM Hepes, 50 μg/ml penicillin G, 50 μg/ml streptomycin, and 10% human AB serum at 37°C. Cells were cultured in vitro for 6 d before stimulation.

Cell Stimulations. Cells were stimulated with LPS (Sigma Chemical Co.), at either 10 μg/ml or 10 ng/ml, for varying time intervals. IFN-γ (a gift of Genentech, Inc., San Francisco, CA) was added at a final concentration of 300 U/ml.

RNA Analysis. RNA was isolated from cultured human macrophages by guanidinum isothiocyanate/cesium chloride centrifugation (24). 10 μg of each RNA sample was electrophoresed through agarose gels containing 2.2 M formaldehyde (25) and transferred to Nytran membranes (Schleicher & Schuell, Keene, NH) before hybridization, washing, and autoradiography as previously described (18). The 2.0-kb *hck* transcript was identified using a 3′ untranslated region probe as previously described (18). A human c-*fgr* probe was provided by Dr. Cheryl Willman, University of New Mexico, Albuquerque, NM (21). RNA probes for human TNF (an 800-bp Eco RI fragment of the cDNA; reference 8) and IL-1β (a 570-bp Sst I/Pvu II fragment from the cDNA; reference 8) were labeled with ^{32}P by random oligonucleotide priming as described (8). Relative levels of these transcripts were quantitated by laser densitometric analysis of the autoradiograms.

Expression of the Human hck Gene in Murine Fibroblasts. To construct *hck* expression plasmids, a previously described human *hck* cDNA clone was engineered, using oligonucleotide reconstruction, to contain a 5′ Eco RI site and a satisfactory initiation codon containing the sequence AGG*ATG*GGG. This reconstructed cDNA was inserted into the pNUT vector (26) in both sense and antisense orientations to permit expression in eukaryotic cells under control of the murine metallothionein I promoter. NIH 3T3 cells were cotransfected with the

pNUT-*hck* constructs and with the plasmid pSV-Hygro (a kind gift of Paul Berg, Stanford University, Stanford, CA), which confers resistance to the drug hygromycin. Transfected cells were selected with hygromycin (400 μg/ml; CalBiochem-Behring Corp., La Jolla, CA) and cloned lines were isolated using cloning cylinders (17).

Generation of Anti-hck Antiserum and Immunoblotting. A portion of the *hck* cDNA clone (18) encoding residues 34–198 was inserted into the pATH10 vector which was used to direct the synthesis of a *trp*E/*hck* fusion protein in *Escherichia coli* (27). Insoluble *trp*E/*hck* protein was used to immunize rabbits by subcutaneous injection of 1-mg aliquots emulsified in CFA (first immunization), then in IFA (second immunization), and thereafter in saline at 1-wk intervals. For immunoblotting, total cellular membranes from transfected cell lines and human macrophages were isolated by hypotonic swelling followed by dounce homogenization and centrifugation (20). Membrane preparations containing 20–30 μg of total protein, as shown by Bradford assay (28), were solubilized in 1% Triton X-100 before separation by SDS-PAGE. The separated proteins were transferred to nitrocellulose (20) and reacted with the rabbit anti-*trp*E/*hck* serum at a dilution of 1:1,500. Antibody-reactive proteins were visualized using an alkaline phosphatase–conjugated secondary antibody followed by reaction with the chromogenic substrates NBT and BCIP (Bio-Rad Laboratories, Richmond, CA). As shown in Fig. 1, immune serum from rabbit 6168 specifically detected a species of 59 kD in the NIH 3T3 cells expressing the pNUT-*hck* construct in the sense orientation, thus defining the product of the *hck* gene (hereafter referred to as $p59^{hck}$). This antiserum failed to detect this polypeptide in cells expressing the pNUT-*hck* construct in the antisense orientation (Fig. 1), nor did it detect $p56^{lck}$ or $p60^{src}$ (data not shown).

Results

Augmented hck Expression in Stimulated Human Macrophages. To better assess the importance of the *hck* gene in the regulation of myeloid cell behavior, we first examined *hck* gene expression in functionally stimulated human peripheral blood monocyte–derived macrophages using RNA blotting techniques. Monocytes were purified from human peripheral blood and cultured in vitro for 6 d (see Materials and Methods). Under these conditions the monocytes differentiate into cells that closely resemble resting tissue macrophages (23, 29, 30). Macrophages were exposed to 10 μg/ml LPS, an optimal dose to induce cytokine production, for varying time intervals. RNA was isolated from the cells and analyzed using probes for TNF (as a measure of cell activation) and *hck*. As shown in Fig. 2, TNF mRNA was rapidly induced after this treatment, reaching maximal levels in 1–3 h and declining thereafter. A similar pattern was observed using a probe for human IL-1β, although the IL-1β mRNA remained at a high level for at least 24 h (data not shown). These results are consistent with previously published studies of cytokine expression in stimulated human macrophages (6, 8, 31). Intriguingly, steady-state levels of *hck* mRNA were also in-

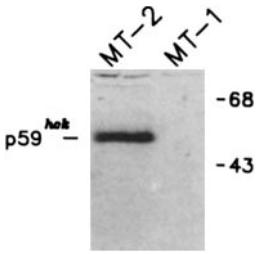

FIGURE 1. Immunoblot of NIH 3T3 cells expressing pNUT *hck* constructs. Membrane proteins (30 μg) from the cell lines MT-1 (*hck*-anti sense orientation) and MT-2 (*hck*-sense orientation) were separated on SDS-polyacrylamide gels and analyzed by immunoblotting as described in Materials and Methods. The positions of OVA (43 kD) and BSA (67 kD) on the immunoblot are marked.

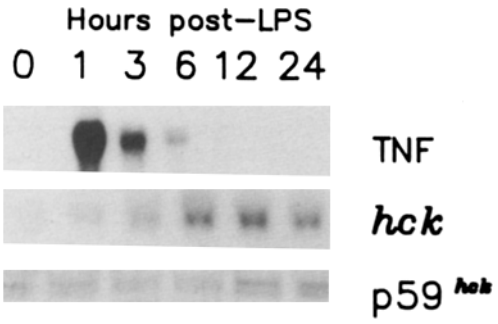

FIGURE 2. Expression of *hck* and TNF in LPS-stimulated human macrophages. Human monocyte-derived macrophages were treated with LPS (10 μg/μl) for the indicated times and RNA and membranes were isolated. For RNA analysis, 10 μg of total RNA was electrophoresed through agarose gels containing 6% formaldehyde, transferred to nytran filters, and hybridized with either a TNF probe or an *hck* probe. For protein analysis, membrane proteins were extracted with 1% Triton X-100 and 30 μg were separated and treated as described in Materials and Methods. Densitometric analysis revealed that levels of *hck* mRNA increased 8.7-fold at 24 h, while $p59^{hck}$ levels increased 4.8-fold.

creased more than eightfold after LPS treatment, but with a much slower time course beginning at ~6 h (Fig. 2). Thus, changes in *hck* gene expression are a relatively late event in macrophage activation.

Using the *trp*E-*hck* antiserum, it was possible to analyze the effect of LPS treatment on levels of $p59^{hck}$ in cultured macrophages. Fig. 2 shows that some $p59^{hck}$ is present in resting cells, and that LPS activation is associated with a fivefold increase in the level of $p59^{hck}$ protein that parallels the accumulation of *hck* mRNA, but as expected, it occurs somewhat later. Interestingly, although $p59^{hck}$ is monomorphic when expressed in transfected fibroblasts (Fig. 1), our *hck*-specific antisera detect a closely spaced doublet in immunoblot analyses of membrane proteins from mouse and human myeloid cell lines. The nature of the presumed posttranslational modification responsible for this effect is unknown.

Priming of Macrophages Does Not Alter hck Expression. Stimulation of macrophages is a multistep process, involving both priming and activating signals (1, 2, 9, 10). We therefore analyzed cells either treated with a simple priming stimulus (IFN-γ)

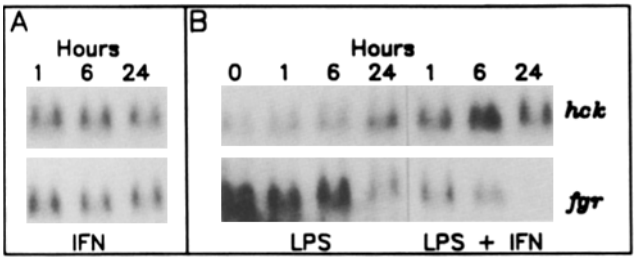

FIGURE 3. RNA analysis of primed and activated human macrophages. Human macrophages were treated with (*A*) IFN-γ (300 U/ml), or (*B*) LPS (10 ng/ml), or both IFN-γ and LPS (300 U/ml and 10 ng/ml, respectively) for the indicated times, after which RNA was extracted and analyzed by blot hybridization using either *hck* or *fgr* probes. Densitometric analysis showed that *hck* mRNA increased 3.5-fold with low-dose LPS alone and 7.0-fold with LPS plus IFN-γ. Levels of *fgr* mRNA decrease 7.5-fold with LPS and 10-fold with LPS plus IFN-γ. IFN-γ did not appreciably alter the levels of either transcript.

or an activating stimulus (LPS, at an optimal dose), or the synergistic combination of INF-γ with LPS (provided at a suboptimal dose), hoping in this way to be able to associate changes in *hck* transcript abundance with cell behavior. Fig. 3 demonstrates that *hck* mRNA levels are augmented by macrophage activation and not by macrophage priming. Levels of *hck* mRNA increased only minimally in macrophages analyzed 1, 6, or 24 h after treatment with IFN-γ (Fig. 3 *A*) or a suboptimal dose of LPS (less than twofold and approximately threefold, respectively). However, the synergistic combination of IFN-γ and LPS resulted in a maximal increase in *hck* mRNA levels of more than sevenfold (Fig. 3). Thus, increased expression of *hck* mRNA is a characteristic of fully activated but not primed macrophages.

Differential Regulation of hck and fgr Expression in Stimulated Macrophages. The *hck* and *fgr* genes encode structurally related protein tyrosine kinases and are both preferentially expressed in myeloid cells (18, 19, 21). To test whether increased expression of *src* family kinases is a characteristic feature of macrophage activation, we compared *hck* and *fgr* gene expression in samples from IFN-γ and/or LPS-stimulated cells. Fig. 3 *A* shows that resting macrophages have easily detectable levels of *fgr* transcripts, and that IFN-γ has little effect on *fgr* expression. Surprisingly, levels of *fgr* transcripts are reduced by about eightfold in macrophages treated with an optimal stimulating regimen of IFN-γ and low-dose LPS (Fig. 3 *B*). Thus levels of *fgr* transcripts decline as levels of *hck* transcripts are increasing.

The reciprocal relationship between *hck* and *fgr* expression patterns during macrophage activation is further illustrated in Fig. 4. In this experiment, resting macrophages were again stimulated with LPS, IFN-γ, or with both together. As before, LPS alone augments *hck* expression, with maximal mRNA accumulation at about 24 h. At the same time, the level of *fgr* transcripts declines dramatically. While stimulation of macrophages with IFN-γ by itself results in little modulation of *hck* levels and provokes only a small decline in *fgr* expression even after 48 h, synergistic stimulation with IFN-γ and LPS boosts the level of *hck* and reduces the level of *fgr* transcripts. We conclude that *hck* and *fgr* are differentially regulated during macrophage activation.

Discussion

The *src* gene family includes seven well-characterized elements: *fgr, fyn, hck, lck, lyn, src,* and *yes* (32). All encode membrane-associated protein tyrosine kinases of

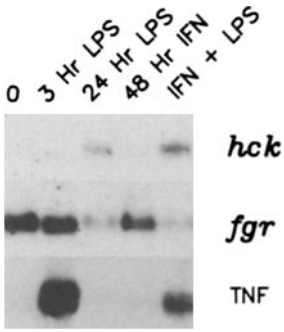

FIGURE 4. Expression of *hck* and *fgr* in activated human macrophages. Human macrophages were treated with LPS (10 μg/ml) or IFN-γ (300 U/ml) for the indicated times, and IFN-γ (300 U/ml) for 48 h and LPS (10 ng/ml) for 24 h, and RNA was analyzed as in Figs. 2 and 3. Levels of *hck* mRNA increased 7.7-fold after 24 h with 10 μg/ml LPS and 8.3-fold with LPS plus IFN-γ. Levels of *fgr* mRNA decreased 4.9-fold with 10 μg/ml LPS and 3.5-fold with LPS plus IFN-γ.

between 505 and 543 residues composed of conserved COOH-terminal tyrosine kinase and phospholipase C-like domains (33, 34) joined to an NH_2-terminal domain that differs completely from one kinase to the next (32). These protein tyrosine kinases are implicated in the transduction of proliferative signals by virtue of their transforming activity when inappropriately expressed (35) or when expressed in mutated form (14–17, 36). Thus it is provocative that many of the cell types that express high levels of *src* family kinases have little replicative potential. For example, $p60^{src}$ is found in neurons and even in platelets (37, 38). In many cases, the *src* family kinases are expressed in a lineage-restricted manner. This is best illustrated in the case of *lck* transcripts that accumulate exclusively in lymphoid cells in mouse and man (39, 40). Alterations in *lck* gene expression can be provoked by combinations of stimuli that induce lymphokine expression (20), which suggests that this gene may assist in regulating the process of lymphocyte activation. These observations prompted us to examine the pattern of expression of a new member of the *src* gene family, *hck*. Transcripts from the *hck* gene accumulate primarily in cells of myeloid origin, including granulocytes. Since granulocytes are post-mitotic, terminally differentiated cells, the *hck* gene product almost certainly regulates features of myeloid cell behavior unrelated to cell division. We therefore asked whether *hck* mRNA and/or protein levels were affected by macrophage activation protocols.

To investigate the potential role of the *hck* gene in regulating myeloid cell activity we studied cultured human peripheral blood monocytes that closely resemble resting tissue macrophages (23, 29, 30). In these cells, and in fresh monocytes as well (data not shown), increased accumulation of *hck* mRNA is a characteristic feature of LPS-induced activation, and is accompanied by a parallel increase in $p59^{hck}$, defined here for the first time using heterologous antisera. Changes in *hck* transcript abundance are not provoked by IFN-γ treatment, indicating that activation, and not priming, of macrophages is responsible for the increase in *hck* expression. At the same time, increased levels of $p59^{hck}$ are not absolutely required for cytokine expression since TNF and IL-1β mRNA levels (Fig. 1), and levels of secreted cytokines (8), peak before *hck* transcript abundance is substantially augmented. In addition, PMA treatment, which results in the production of significant amounts of TNF and IL-1β, actually decreases the level of *hck* mRNA (data not shown). Thus the requirement for increased *hck* expression in LPS-stimulated macrophages is imposed by some later feature of the activation pathway. These observations further support the recent suggestion that the LPS- and PMA-mediated stimulation pathways in myeloid cells are biochemically distinct (41).

Interestingly, the *hck* and *fgr* genes are differentially regulated. Myelomonocytic cells express several protein tyrosine kinases, including at least four members of the *src* gene family, *fgr, hck, lyn,* and *src* (Fig. 3, and Cooke, M., C. Willman, S. Ziegler, and R. Perlmutter, unpublished observations). There is clearly no simple relationship among these genetic elements. LPS treatment of macrophages induces IL-1, TNF, and *hck* expression, reduces *fgr* expression, and leaves *lyn* expression unchanged (Figs. 1 and 3, and data not shown). Therefore, despite the close structural relationships among the *src* family kinases, they probably subserve very different functions.

It is worth emphasizing that these changes in kinase gene expression occur in populations of nondividing cells. Thus, if these alterations in transcript levels are biologically meaningful, they again imply that the *src* family kinases act at least in part

as components of metabolic pathways that can be modulated in terminally differentiated cells. Willman et al. (21) have previously reported that *fgr* transcripts accumulate within 8 h after CSF-1 treatment of cultured mouse monocytes, and that the c-*src* and c-*yes* genes were not induced by this treatment (21). Since CSF-1 promotes differentiation within the myeloid lineage (42), but does not activate macrophages to become microbicidal (43), it is possible that subpopulations of myeloid cells with different functional properties will vary significantly with respect to levels of *src* family kinases. Indeed, we have observed characteristic variations in the ratio of *hck* to *fgr* expression in human leukemic cells distinguished on the basis of differentiative phenotype (defined using cell surface markers), and in similarly fractionated normal human peripheral blood cells (Willman, C., S. Ziegler, C. Stewart, and R. Perlmutter, manuscript in preparation).

Exposure of macrophages to activating stimuli dramatically alters the metabolism and behavior of these cells. Many of these changes might be regulated by modulation of the activity of a *src*-family protein tyrosine kinase. The *src* family kinases have been implicated in the processes of granule fusion and exocytosis (44), and in the modification of cell morphology generally (33), key components of the activated macrophage phenotype. In addition, $p60^{src}$ expression has been reported to regulate small molecule diffusion through membrane channels in communicating cells (45). We have shown that the activation of macrophages profoundly alters the relative abundance of transcripts encoding two myeloid-specific protein tyrosine kinases, *fgr* and *hck*. Artificial manipulation of the *fgr/hck* ratio, through the introduction of appropriate expression constructs, may provide an additional approach to the biochemical dissection of macrophage activation pathways.

Summary

Protein tyrosine kinases are thought to participate in signal transduction pathways in a variety of cell types. Recent studies have identified a new *src* family protein tyrosine kinase (*hck*) that is preferentially expressed in myeloid cells. To examine the hypothesis that this kinase may regulate myeloid cell activity, antisera were generated that define the 59-kD product of the *hck* gene. Functional activation of human cultured macrophages with LPS augmented the expression of *hck* transcripts and of $p59^{hck}$, but decreased the level of transcripts encoded by the closely related c-*fgr* protooncogene. Thus these two structurally similar *src* family kinases almost certainly subserve distinct functions. Reasoning from the known properties of the *src* family protein tyrosine kinases, it is likely that the products of these two protooncogenes assist in regulating the behavior of activated phagocytes.

We thank Yim-Foon Lee for oligonucleotide synthesis, Barry Davison for the pNUT vector, Mike Weaver for technical assistance and our colleagues for helpful discussions.

Received for publication 27 May 1988.

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