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Dynamic production of tumour necrosis factor-α (TNF-α) messenger RNA, intracellular and extracellular TNF-α by murine macrophages and possible association with protein tyrosine phosphorylation of STAT1α and ERK2 as an early signal

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SUMMARY

Tumour necrosis factor-α (TNF-α), an important mediator in both immune and inflammation responses, is one of the major cytokines released by activated macrophages. The present study shows that, during macrophage activation, protein tyrosine phosphorylation of STAT1α and ERK2 occurred as an immediate early signal, whereas maximum TNF-α mRNA transcription appeared at 3 hr, precursor TNF-α formation at 3 to 4 hr, and TNF-α release at 5 to 6 hr after stimulation of an RPMI-1640-based induction medium containing lipopolysaccharide (100 ng/ml), interferon-γ (100 U/ml), and 0·5% bovine serum albumin. Herbimycin A, a tyrosine kinase inhibitor, suppresses protein tyrosine phosphorylation of STAT1α and ERK2 and also blocks TNF-α production by resident peritoneal macrophages from BALB/c mice, suggesting a possible association between protein tyrosine phosphorylation of STAT1α and ERK2 and macrophage activation resulting in TNF-α production.

INTRODUCTION

Tumour necrosis factor-α (TNF-α) was discovered initially as a result of the ability of an endotoxin-induced serum factor or supernatant fluid from macrophage cultures to destroy malignant tumours *in vivo* and *in vitro*.[1] Recent studies have shown that TNF-α is one of the primary mediators involved in the pathogenesis of inflammation, cachexia, septic shock and tissue injury. TNF-α production is most commonly induced by stimulation of macrophages with bacterial lipopolysaccharide (LPS) although several other TNF-α inducers have been reported, including phorbol myristate acetate (PMA),[2] phytohaemagglutinin (PHA),[3] taxol,[4,5] Sendai virus,[6] herpes simplex virus-1,[7] and *Candida albicans* in yeast and hyphal forms.[8]

Induction of TNF-α mRNA is rapid, but transient in LPS-activated macrophages. The maximal production of TNF-α mRNA occurs between 1 and 6 hr post-stimulation.[9,10] Others have reported that maximum TNF-α bioactivity is detectable only at 6–8 hr after LPS stimulation of macrophages.[11,12] Since the time correlation of TNF-α mRNA transcription and TNF-α protein maturation and release by LPS-stimulated macrophages has not been systematically described, many reports on TNF-α studies use samples collected from macrophages at a very late activation period and thus provide misleading results.[13–16]

The present study provides evidence showing the kinetics of TNF-α mRNA transcription, TNF-α protein maturation and release by murine peritoneal resident macrophages. We have shown that protein tyrosine phosphorylation of STAT1α (signal transducer and activator of transcription 1α, or so called previously interferon-stimulated gene factor 3 p91 [ISGF-3 p91])[17] and ERK2 (extracellular signal-regulated kinase 2)[18] is required as an immediate early event for macrophage activation and TNF-α production.

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MATERIALS AND METHODS

Macrophage culture and TNF induction

BALB/c mouse (8–12-week-old) resident peritoneal macrophages were obtained by washing the peritoneal cavity with 5 ml Hanks' balanced salt solution (Gibco, Grand Island, NY) buffered with 50 mM HEPES (pH 7·4). The cells were washed once by centrifugation (200 *g* for 10 min) and resuspended in RPMI-1640 medium (Gibco). Macrophages were then counted, adjusted to 2×10^6 cells/ml, and plated in six-well plates at 3 ml/well or in 24-well plates at 0·5 ml/well (Costar, Cambridge, MA). After incubation for 2 hr at 37° in an atmosphere of 5% CO_2, 95% air, non-adherent cells were removed by changing medium with RPMI-1640 medium. The adherent cells were examined under a light microscope and the remaining adherent cells in consistence in each well were then treated with induction

medium, an RPMI-1640 medium containing *Salmonella enteritidis* LPS (100 ng/ml) (Sigma, St Louis, MO), mouse interferon-γ (IFN-γ) (100 U/ml) (Genzyme, Cambridge, MA), and 0·5% bovine serum albumin (BSA, Sigma).[19] Supernatant fluids from macrophage cultures were collected at the desired times and stored at −20° for TNF-α bioactivity assay. The remaining cells were directly lysed into sodium dodecylsulphate (SDS)-sample buffer for Western blot assay or into TRI REAGENT™ (a guanidinium–phenol–containing extraction buffer, Molecular Research Center [MRC], Inc., Cincinnati, OH) for Northern blot analysis.

Titration of TNF-α by bioassay

Lysis of a TNF-sensitive murine cell line, WEHI-164, using a ^{51}Cr-release cytotoxicity assay[20,21] was used to measure TNF-α activity.[22]

TNF-α immunocytochemical staining

Immunocytochemical staining for TNF-α was modified from publications[23,24] and performed as follows. Macrophage monolayers on chamber slides were fixed for 5 min in 4% paraformaldehyde in phosphate-buffered saline (PBS) and/or methanol and rinsed twice with PBS. The fixed cells were treated for 5 min with 3% hydrogen peroxide in Tris–HCl (50 mM, pH 7·3) at room temperature to inactivate any remaining peroxidase activity and incubated with normal, non-immune rabbit serum (1 : 50 dilution) for 10 min at 37° to block non-specific binding of secondary antibodies and then with monoclonal hamster anti-murine TNF-α (Genzyme, 7 μg/ml in PBS) for 10 min at 37°. The slides were rinsed three times with PBS and overlaid with rabbit anti-hamster IgG-horseradish peroxidase (Accurate Chemical and Scientific Corp., Westbury, NY) (1 : 500 in PBS) for 10 min at 37°. After rinsing three times with PBS, the slides were stained for 5 min at room temperature with 3,3′-diaminobenzidine tetrahydrochloride (DAB) solution (0·06% DAB, 50 mM Tris–HCl [pH 7·3], 0·03% hydrogen peroxide, 0·03% nickel chloride), then counterstained for 30 seconds with 0·5% Giemsa working solution, dehydrated in 70%, 95%, 100% ethanol, respectively, and finally examined by light microscopy using an high dry objective.

TNF-α immunoblotting assay

Macrophages (6×10^6/well) were directly lysed in SDS sample buffer. The soluble cell extracts were boiled for 5 min and then resolved by 12% SDS–polyacrylamide chinos. The gel was equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8·3) for 30 min. Then, electrophoretic transfer at 20 V onto nitrocellulose membranes (Bio-Rad, Richmond, CA) was conducted overnight in a Bio-Rad transblotting cell. The blot membranes were briefly washed in water, blocked in Tris-buffered saline (TBS) (10 mM Tris–HCl, 154 mM NaCl, pH 7·4) containing 3% BSA (Promega, Madison, WI) and 1% ovalbumin (ICN Biochemicals, Inc., Costa Mesa, CA) for 2–4 hr at room temperature, and incubated with a 1 : 250 dilution of rabbit anti-mouse TNF-α antibodies (Genzyme) for 1 hr. After washing three times in TBS/0·1% Tween 20 (TTBS), membranes were incubated with goat anti-rabbit IgG-horseradish peroxidase (1 : 3000) (Bio-Rad) for 1 hr, washed three times again in TTBS, and then detected by incubation with fresh DAB (ICN) solution (50 mM Tris–HCl [pH 7·3], 0·03% nickel chloride, 0·005% DAB, 0·03% hydrogen peroxide) at room temperature until coloured bands developed and a background began to be detectable. After removing the membranes from the container and washing them in another reservoir containing deionized water to halt colour development, the blot membranes were air-dried and stored in a plastic bag in the dark for further analysis.

RNA extraction and Northern blot analysis

Isolation of total cellular RNA was performed by a single-step method.[25] Macrophages (6×10^6/well) were directly lysed in 1 ml of TRI REAGENT™ (MRC). The homogenized samples were stored for 5 min at room temperature and mixed with 0·2 ml of chloroform for phase separation. RNA in the aqueous phase was precipitated by mixing with 0·5 ml of isopropanol for 5–10 min at room temperature and then centrifuged at 12 000 r.p.m. for 10 min at 4°. After washing the RNA pellet once with 1 ml of cold 75% ethanol, the RNA pellet was briefly air-dried, solubilized in 50 μl of FORMAzol™ (MRC), and incubated at 55° for 10 min. The RNA extracts were then checked for quantity and purity by a Shimadzu Model UV160 U spectrophotometer and stored at −70°.

Northern blot analysis was performed as described previously using ^{32}P-labelled murine TNF-α oligonucleotide probe cocktail (R & D Systems, Inc., Minneapolis, MN).[19]

Anti-phosphotyrosine, anti-STAT1α and anti-ERK2 immunoblotting assay

Macrophages at 6×10^6 cells/well in a six-well plate were cultured for 3 days in standard RPMI-1640 medium (no serum) and then treated with RPMI-1640 medium containing IFN-γ (100 U/ml) for STAT1α tyrosine phosphorylation or LPS (1 μg/ml) for ERK2 tyrosine phosphorylation. A higher concentration of LPS (1 μg/ml) and an extended time stimulation of macrophages were used in order to obtain a strong ERK2 phosphorylation. Macrophage sample preparation and anti-phosphotyrosine immunoblotting assay were performed as described.[26] These procedures were also used for anti-ERK2 and anti-STAT1α immunoblot assay. After blocking and washing as described,[26] the membrane were probed for 1 hr with monoclonal murine anti-ERK2 or anti-STAT1α (1 μg/ml in TTBS, Transduction Laboratories, Lexington, KY), incubated with goat anti-mouse IgG-horseradish peroxidase (1 : 3000 in TTBS), and visualized using the ECL Western blotting detection system (Amersham). If the membrane was first probed with monoclonal PY20 (anti-phosphotyrosin) antibody for ECL Western blotting assay, the same membrane was then stripped for 10 min at room temperature with stripping solution (7 M guanidine hydrochloride, 50 mM glycine [pH 8·4], 0·05 mM EDTA, 0·1 M KCl, 20 mM mercaptoethanol),[27] blocked with TTBS containing 3% BSA and 1% ovalbumin for 2 hr, and reprobed using another antibody for the second immunoblot assay.

RESULTS

Protein tyrosine phosphorylation of STAT1α and ERK2 in LPS/IFN-γ-stimulated macrophages

It was noted in our previous study[26] that protein tyrosine phosphorylation appeared as an early (within minutes)

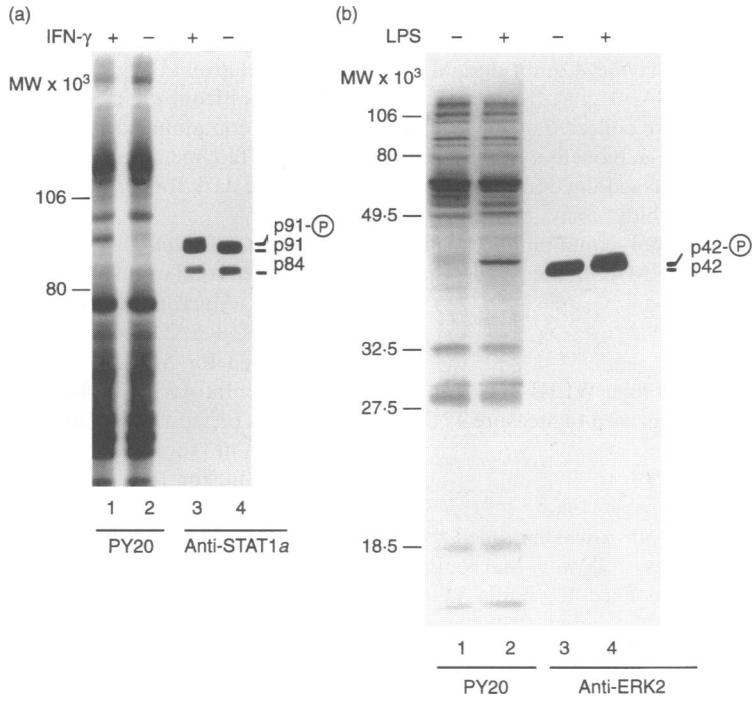

Figure 1. Protein tyrosine phosphorylation (arrow) of STAT1α and ERK2 during macrophage activation. Macrophages at 6×10^6 cells/well in a six-well plate were cultured for 3 days in standard RPMI-1640 medium (no serum) and then treated with RPMI-1640 medium containing IFN-γ (100 U/ml) (a) for 15 min or LPS (1 μg/ml) (b) for 30 min as indicated in the Figure. The macrophages then were washed and lysed into the lysis buffer as described.[26] Cell lysates (30 μg/lane) were run on 7·5% (a) or 12·5% (b) SDS–PAGE, transferred onto nitrocellulose membranes, and immunoblotted with monoclonal anti-phosphotyrosine antibody PY20 (ICN) and then stripped and reprobed with monoclonal antibody against STAT1α (p91) or ERK2 (p42).

cascaded signal of macrophage activation during LPS/IFN-γ stimulation, in that some of the tyrosine-phosphorylated proteins were observed earlier than others. The present study demonstrated that LPS and IFN-γ, when used alone for induction of protein tyrosine phosphorylation in peritoneal macrophages, yielded different results. Tyrosine phosphorylation of STAT1α (p91) was induced by IFN-γ treatment of macrophages and was easily detectable within 30 min after the initiation of IFN-γ stimulation. This protein was further identified as tyrosine-phosphorylated STAT1α by Western blot analysis (Fig. 1a). However, the tyrosine-phosphorylated p42 was usually induced by LPS, not by IFN-γ. This protein was identified as an ERK2 protein which appeared later (detectable between 30 and 60 min) during macrophage activation (Fig. 1b).

Kinetics of TNF-α mRNA transcription by LPS-stimulated macrophages

Total RNA extracted from LPS/IFN-γ activated macrophages was used for this study. There was no detectable TNF-α mRNA in non-stimulated resident peritoneal macrophages (Fig. 2). However, TNF-α mRNA was detectable at 1 hr and reached a maximum level at 3 hr post LPS/IFN-γ stimulation. Thereafter, TNF-α mRNA declined to a very low level at 6 hr and completely disappeared by 9 hr after LPS/IFN-γ stimulation in these studies.

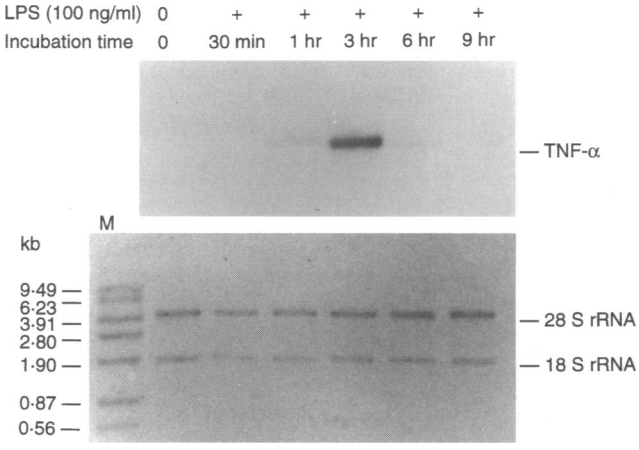

Figure 2. Time–course of TNF-α mRNA transcription in LPS-stimulated macrophages. Macrophages at 6×10^6/well in six-well plates were cultured for 2 days in standard RPMI-1640 medium and then activated by incubation in RPMI-1640 medium containing LPS (100 ng/ml), IFN-γ (100 U/ml), and 0·5% BSA. After incubation for the indicated time, as shown in the Fig. at 37° in 5% CO_2, the macrophage cultures were washed and then lysed in 1 ml TRI REAGENT™ for total cellular RNA extraction. RNA extracts (5 μg/lane) were denatured in sample buffer containing formaldehyde, run on 1·2% agarose/formaldehyde gel, transferred to a Zeta-probe GT membrane, and hybridized to ^{32}P-labelled murine TNF-α probe cocktail. M, RNA marker.

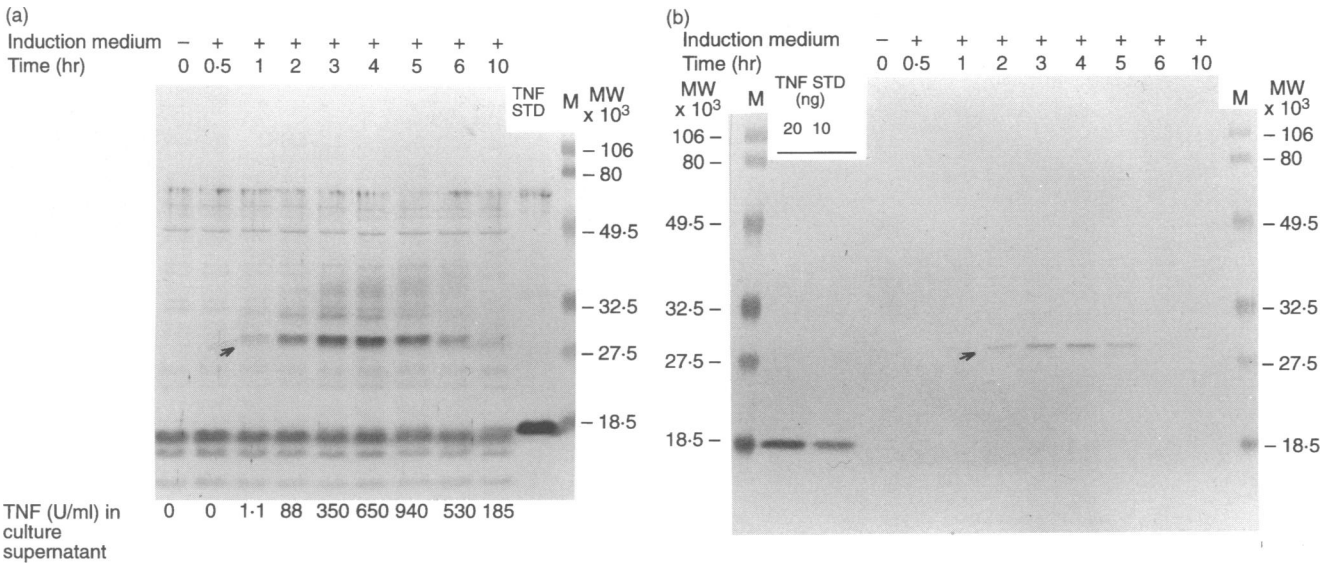

Figure 3. Time–course of intracellular TNF-α precursor (arrow) synthesis and its release by LPS-stimulated macrophages. Macrophages at 6×10^6/well in six-well plates were cultured for 2 days in standard RPMI-1640 medium and then activated as shown in Fig. 2. After incubation for the indicated time-point at 37° in 5% CO_2, the macrophage culture supernatants were collected for TNF-α bioactivity assay. The cell lysates (30 μg/lane) were separated using 12% SDS–PAGE, transferred to nitrocellulose membranes, probed with rabbit anti-TNF-α polyclonal antibody (a) or monoclonal hamster anti-TNF-α antibody (b), and detected with goat anti-rabbit (a) or rabbit anti-hamster (b) IgG–horseradish peroxidase.

Kinetics of intracellular and extracellular TNF-α production

The pattern of intracellular TNF-α precursor production as shown in Fig. 3 is very similar to that of TNF-α mRNA as shown in Fig. 2. The precursor protein, with a molecular weight of 29 000, was detectable at 1 hr, reached a maximum level at 3 to 4 hr, started to decline at 5 to 6 hr, and was almost completely gone by 10 hr (the latest time-point of the study) after LPS stimulation in the presence of 0·5% BSA. Moreover, the production pattern of intracellular TNF-α precursor was compared with the extracellular TNF-α profile by analysis of TNF-α bioactivity in macrophage culture supernatant fluids. The results revealed a very closely related profile between the precursor synthesis and mature TNF-α release by macrophages, with approximately a one hour lag for release (Fig. 3). In addition, TNF-α release by LPS-stimulated macrophages was observed under these induction conditions by using an immunocytochemical technique (Fig. 4). It was noted at 4 hr after LPS stimulation in the presence of 0·5% BSA that LPS-activated macrophages lead to a 'firework-like' TNF-α release phenomenon. During TNF-α release, it was common that one activated macrophage could trigger its nearby cells, resulting in a group of the cells releasing TNF-α to form a firework-like plaque. Thus, a group of cells releasing TNF-α together was frequently observed as visualized using a light microscope.

Requirement of protein tyrosine phosphorylation in TNF-α production by macrophages

Herbimycin A (HA), a specific protein tyrosine kinase inhibitor, was found not only to inhibit LPS/IFN-γ-induced ERK2 and STAT1α protein tyrosine phosphorylation, but also to suppress TNF-α production by macrophages (Fig. 5). Used at concentration which were non-cytotoxic for macrophages, HA at 5 μg/ml showed a blockade of the protein tyrosine phosphorylation of both STAT1α (Fig. 5a) and ERK2 proteins (Fig. 5b). HA at 0·5 μg/ml also revealed a significant inhibitory effect on TNF-α production and at 5 μg/ml completely suppressed TNF-α secretion by macrophages. In addition, a stronger effect on TNF-α production by macrophages was observed when 0·1 μg HA/ml was included in the culture medium for 3 hr before LPS-IFN-γ activation of macrophages and then simultaneously added to the induction medium again during 6 hr macrophage stimulation (Fig. 5). TNF-α production was completely blocked when 0·5 μg HA/ml was used.

DISCUSSION

The present study shows that protein tyrosine phosphorylation of STAT1α and ERK2 is an immediate early event during macrophage activation by LPS/IFN-γ. Subsequently, the maximum production of TNF-α mRNA, intracellular 29 000 molecular weight TNF-α precursor, and extracellular TNF-α was followed, respectively, at 3 hr, 3 hr to 4 hr, and 5 hr to 6 hr post-LPS/IFN-γ stimulation. The results suggest that STAT1α and ERK2 tyrosine phosphorylation, TNF-α mRNA, TNF-α precursor and its maturation and release are a closely related sequence during macrophage activation. Such extensive systemic study on signal initiation, TNF-α transcription and maturation and release has not been reported in literature even though in a few reports either TNF-α mRNA[9,10] or TNF-α bioactivity in culture supernatant fluids[11,12] have been documented. In addition, the observation also provides a useful guide for sampling TNF in future studies.

A firework-like TNF-α release from a single or a group of activated macrophages was directly visualized using a light microscope and an immunocytochemical staining method,

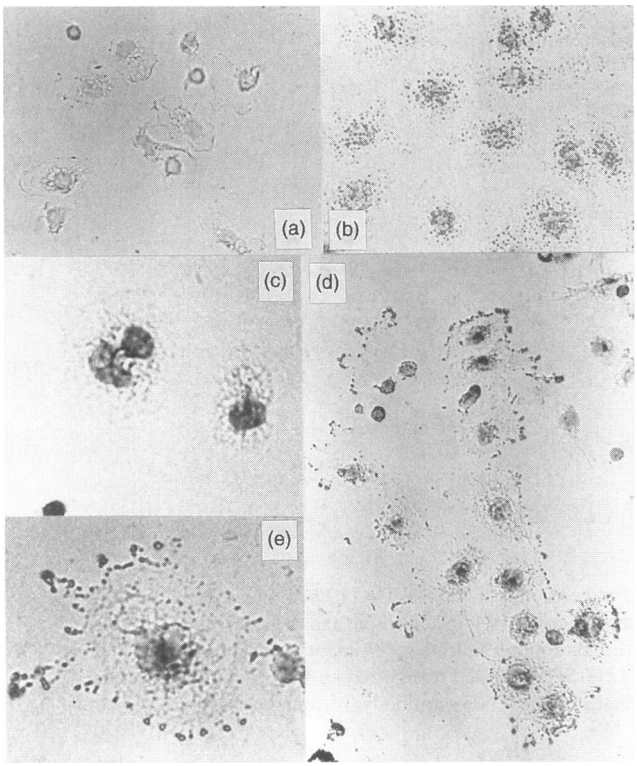

Figure 4. Immunocytochemical staining of membrane-bound TNF-α release by LPS-stimulated macrophages. All cells in panels b, c, d, e were stimulated by induction medium as described in Fig. 2. The cells in panels a, c, d, and e were fixed using 2% paraformaldehyde. (a) Unstimulated macrophages (15×); (b) cytoplasmic TNF-α in stimulated cells fixed by methanol (75×); (c) a stimulated macrophage with no TNF-α (300×); (d) firework-like plaque (75×); (e) a stimulated macrophage with TNF-α releasing in a firework-like plaque (300×).

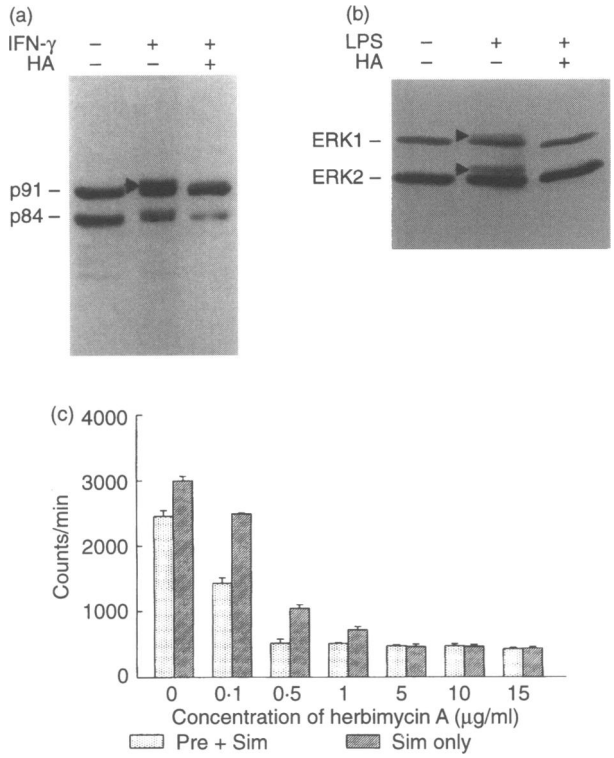

Figure 5. Effect of herbimycin A (HA) on STAT1α and ERK2 protein tyrosine phosphorylation and TNF-α production by macrophages. Macrophages at 6×10^6 cells/well in a six-well plate were cultured for 3 days in standard RPMI-1640 medium (no serum) and then treated with the medium containing IFN-γ (100 U/ml) for 15 min (a) or LPS (1 μg/ml) for 1 hr (b), with or without HA (5 μg/ml) as indicated in the Figure. The macrophages then were washed and lysed into the lysis buffer as described.[26] The cell lysates (30 μg/lane) were run on 7·5% (a) or 12·5% (b) SDS–PAGE, transferred onto nitrocellulose membranes, and immunoblotted with monoclonal antibody against STAT1α (a) or ERK2 (b). Arrows indicate the tyrosine-phosphorylated proteins. Cross-reaction to STAT1β (p84) or ERK1 (p44) of anti-STAT1α or anti-ERK2 monoclonal antibody was also noted as indicated by arrows. For the analysis of the effect of HA on TNF-α production, macrophages at 1×10^6 cells/well in 24-well plates were subjected either to a 3 hr HA treatment before TNF-α induction and/or directly to a 4 hr simultaneous treatment with TNF-α induction medium (Fig. 2) plus HA as indicated in the Fig. (c). Each culture used for HA pretreatment received the same concentration of HA in the simultaneous HA treatment. The culture supernatants were collected for TNF-α bioassay and three individual cultures were used for these experiments.

suggesting that TNF-α release by one macrophage may have an influence on the behaviour of neighbouring cells. Thus, although all cells have been exposed to the same stimulation medium, some cells appear more sensitive than others to LPS triggering. So far, there has been no such report describing this phenomenon during macrophage activation except the localization of TNF-α in macrophages using similar methods.[23,24]

Since IFN-γ is necessary for priming macrophages for LPS triggering,[28,29] it also was included in the induction medium to enhance TNF-α production by activated macrophages. Although the present study has demonstrated that the macrophages primed by IFN-γ could induce tyrosine phosphorylation of STAT1α protein as an immediate early process, IFN-γ itself, as reported elsewhere,[28,30,31] could not induce macrophages to produce TNF-α mRNA or secrete TNF-α (data not shown). Therefore, enhancement by IFN-γ of TNF-α production by macrophage must be though an indirect pathway during LPS stimulation. Since ERK2 tyrosine phosphorylation induced by LPS stimulation is somewhat later than STAT1α tyrosine phosphorylation induced by IFN-γ in the present study, the tyrosine-phosphorylated STAT1α might be one of the important priming signals induced by IFN-γ for LPS triggering.

STAT1α is an important regulatory protein.[32] After activation by tyrosine phosphorylation induced by IFN-γ[33] in the cytoplasm, STAT1α could be then translocated to the nucleus to take part in transcriptional regulation. ERK proteins also are important regulatory intermediates in signal transduction pathways[18] and their active forms with tyrosine phosphorylation are initiated by many types of cell surface receptors including the LPS receptor.[34–36] These phosphorylated ERK proteins have been shown to phosphorylate and thereby activate many well studied regulatory proteins located in diverse cellular compartments, including nuclear transcription factors.[37] The present study has confirmed the previous observations[33–36] and has compared the time required for tyrosine phosphorylation of ERK proteins induced by LPS to that of protein tyrosine phosphorylation of STAT1α induced by IFN-γ. The results indicate that tyrosine phosphorylation of

STAT1α precedes that of ERK proteins. It remains unclear how the tyrosine-phosphorylated STAT1α correlates to the function of EKR proteins to enhance TNF-α production during macrophage activation. Nevertheless, finding that protein tyrosine phosphorylation of STAT1α and ERK2 was induced by LPS/IFN-γ as early events during TNF-α induction suggests that protein tyrosine phosphorylation is required for TNF-α production by macrophages. The demonstration that HA, a broad tyrosine kinase inhibitor,[38] also inhibited TNF-α production provides further evidence in support of such an association. However, more evidence is needed to establish such relationship between the tyrosine phosphorylation of STAT1α/ERK2 and TNF-α production since HA used in the present study lacks specificity as a tyrosine kinase inhibitor.

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