ANNALS OF SURGERY
Vol. 219, No. 4, 389–399

Autoregulation by Eicosanoids of Human Kupffer Cell Secretory Products

A Study of Interleukin-1, Interleukin-6, Tumor Necrosis Factor-α, Transforming Growth Factor-β, and Nitric Oxide

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Objective
Methods employed previously to analyze the secretory behavior of rodent Kupffer cells (KC) were used to examine the human KC's secretory response to lipopolysaccharide (LPS).

Summary Background Data
As the resident hepatic macrophage, the KC resides at the interface between the portal and systemic circulations. Consequently, this cell may play an integral role in the immune response to antigens and bacteria in the sinusoid. Study of cytokine production by the KC has relied predominantly on the rat as the source of these cells. Whether human KCs respond similarly to rat KCs after LPS stimulation has been a matter of speculation.

Methods
Kupffer cells obtained from seven human livers were tested under conditions identical to those used to study rat KCs. Kupffer cells rested for 12 hours after isolation were stimulated with LPS (2.5 μg/mL). Arginine concentration in the culture medium varied from 0.01 to 1.2 mM. To examine the role of eicosanoids, parallel culture wells received indomethacin (10 μM). Culture supernatants were assayed for interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), prostaglandin E_2 (PGE_2), and nitric oxide.

Results
Similar to the rat KC, LPS-stimulated human KCs released IL-1, IL-6, TNF-α, TGF-β, and PGE_2. However, unlike rat KCs, nitric oxide could not be detected, regardless of whether the human KCs were exposed to LPS, interferon-γ (INF-γ), or LPS + IFN-γ. Similar to rat KCs, indomethacin prevented PGE_2 release while significantly upregulating TNF-α, IL-1, and IL-6, but not TGF-β, consistent with an autoregulatory control of eicosanoids over proinflammatory cytokines. As has been shown in the rat, physiologic levels of L-arginine (0.01 mM) significantly enhanced LPS-induced PGE_2 secretion relative to the response in medium containing standard L-arginine concentration (1.2 mM); however, unlike the rat KC, the human's cytokine response to LPS was not downregulated by this enhanced PGE_2 release.

Conclusions

Although many functional features are shared by rat and human KCs, significant differences do exist. Such discrepancies reinforce the need to proceed with caution when generalizing from the results obtained in other species to human physiology.

The resident macrophages of the liver, known as Kupffer cells (KCs), project into the vascular space of the hepatic sinusoids and, as a result, are exposed to all substances passing from the splanchnic bed into the portal venous circulation. The anatomic location of KCs at the interface of the portal and systemic circulations is consistent with their function as the predominant phagocytic component of the reticuloendothelial system (RES).[1] The gastrointestinal tract is a reservoir of viable bacterial organisms and biologically active microbiologic products, such as endotoxin or its purified form, lipopolysaccharide (LPS). Endotoxin, a component of the cell wall of gram-negative bacteria and a physiologic constituent of portal blood in healthy humans,[2,3] is a major mediator of endotoxic shock. In addition, LPS has been detected in the portal circulation of patients with gastrointestinal diseases,[4] alcohol ingestion,[4] and mesenteric ischemia.[5] Kupffer cells bear the major responsibility for the clearance of circulating LPS and appear to be uniquely adapted to this role by internalizing LPS through absorptive pinocytosis, which does not exhibit saturation kinetics.[2] Absorption of LPS by KCs has been proposed as a mechanism for preventing the development of LPS-mediated systemic injury — e.g., adult respiratory distress syndrome.[6] However, during pathologic states producing a high burden of endotoxin, LPS stimulation of KCs is believed to have the potential for causing local and distant damage because of the release of potent KC inflammatory products.[7,8] Studies of isolated and *in situ* animal KCs have defined the large secretory repertoire of this macrophage, which includes IL-1-α, IL-1-β, IL-6, TNF-α, TGF-β, eicosanoids, nitric oxide, platelet activating factor, collagenase, leukotrienes, lysosomal enzymes, free oxygen radicals, and fibronectin, many released in response to LPS stimulation.[8]

Although certain similarities have been demonstrated between human and animal KCs,[9–16] such as the internalization of LPS,[11] viral particles,[12] or latex beads,[12] and the release of IL-1-β,[13] TNF-α,[13,14] IL-6,[15] and prostaglandin E_2 (PGE_2),[16] much remains undefined about the human KC, especially when compared with the more extensively studied rodent KC.[2,6,8,17–20] For example, a well-defined mechanism of autoregulatory control of rat KC cytokine production is through the coincident release of PGE_2 by the KC.[8,17,20] Cytokines inhibited in this fashion include TNF-α,[8,18,20] IL-1,[19] and IL-6.[17] In addition, it has been demonstrated by our laboratory that the release of PGE_2 by the rat KC is sensitive to ambient arginine concentrations to the extent that a reduction in arginine availability can augment PGE_2 production to downregulate the LPS-stimulated release of TNF-α.[20] Such a regulatory function of PGE_2 could have important consequences during portal endotoxemia to limit the pro-inflammatory response of KCs to endotoxin. The modulation of PGE_2 by ambient arginine may be relevant particularly in the liver, the primary organ of the urea cycle, because arginine levels are lowered within the liver by high hepatic arginase activity,[21] and by inducible nitric oxide synthase (iNOS).[22] Whether similar regulatory mechanisms are present in human KCs has not been addressed previously. Thus, these studies examined the effect of arginine availability and cyclooxygenase blockade on the *in vitro* release of IL-1-β, TNF-α, IL-6, PGE_2, TGF-β, and nitric oxide by cultured adult human KCs stimulated with LPS. As in the rodent model, LPS-mediated release of IL-1-β, TNF-α, and IL-6 by human KCs was sensitive to inhibition by eicosanoid. In contrast, nitric oxide release could not be stimulated, even under *in vitro* conditions known to maximally stimulate iNOS induction in rodent cells.[22]

METHODS

Human Liver Tissue

Human liver tissue samples were obtained from six patients undergoing liver resection for neoplastic disease (Table 1). At the time of surgery, 100 to 200 g of grossly uninvolved peripheral liver tissue were immediately removed from the resected specimen and chilled to 4 C for transport to the laboratory in Hanks' balanced salt solution (HBSS). An additional specimen (patient 1) came from a donor cadaveric liver stored at 4 C in University of Wisconsin preservative solution for 6 hours before KC isolation. None of the patients had been exposed to drugs, such as cyclooxygenase inhibitors, which can alter arachidonic acid metabolism.

Supported by grant RO1 AI28480, the American Liver Foundation, and the Association for Academic Surgery.

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Accepted for publication October 11, 1993.

Kupffer Cell Isolation

The method for isolating human KCs was based on the protocol used in our laboratory to obtain rodent KCs.[17] In brief, small pieces of liver tissue were forced through a 250 μm sieve (U.S. Standard Sieve, #60) using a glass stopper. The resulting tissue homogenate was washed with HBSS supplemented with penicillin (10^5 U/L), streptomycin (10^5 μg/L), and HEPES buffer (10 mM). The pellet obtained after a 10-minute, 500 × g spin, was suspended again in HBSS containing 0.1% collagenase (CLS-1, Worthington, Freehold, NJ), 0.01% $CaCl_2$, and 0.1% DNAse (Type I, Sigma, St. Louis, MO). After a 45-minute incubation in a 37 C rocking water bath, the digested connective tissue was separated from cells by centrifugation at 500 × g for 10 minutes. Erythrocytes were lysed by a 3-minute incubation of the cells in Tris-NH_4Cl. Cells adhered to 100-mm tissue culture grade petri dishes (#3100, Costar, Cambridge, MA) containing 5 mL of RPMI-1640 supplemented with 5% fetal calf serum (FCS) (low endotoxin, Hyclone Labs, Inc., Logan, UT), penicillin (10^5 U/L), streptomycin (10^5 μg/L), HEPES buffer (10 mM), and L-glutamine (2 mM) over 3 hours in an incubator at 37 C with 5% CO_2. Nonadherent cells were removed with three consecutive washes with warm HBSS. Adherent cells were dislodged after a 30-minute period at 4 C by gentle scraping with a rubber policeman. Phagocytosis of latex beads was consistently 90% to 95%, indicating the purity of this macrophage preparation. More than 90% of cells isolated in this manner were viable, as determined by trypan blue exclusion.

Culture Protocol

After isolation, human KCs were suspended in RPMI-1640-based complete culture medium (GIBCO Laboratories, Grand Island, NY) containing HEPES (10 mM), nonessential amino acids (1%), L-glutamine (2 mM), penicillin (10^5 U/L), streptomycin (10^5 μg/L), and 10% FCS. Arginine concentration in the culture medium was either 1.2 mM, as in standard RPMI-1640-supplemented with 10% FCS, or 0.01 mM, which is thought to reproduce the low arginine intrahepatic environment.[20] The latter concentration was attained by supplementing arginine-free RPMI-1640 (Tissue Culture Center, Washington University School of Medicine, St. Louis, MO) with 10% FCS. Kupffer cells were seeded in 24-well plates (#3424, Costar, Cambridge, MA) at a density of 5 × 10^5/mL/well. After overnight culture, the wells were replenished with fresh medium, and LPS (*E. coli* type 0111:B4, Difco Laboratories, Detroit, MI) was added for a final concentration of 2.5 μg/mL. Some wells also received recombinant human interferon-γ (rhIFN-γ; Genzyme, Cambridge, MA) at 100 U/mL with or without additional LPS (2.5 μg/mL). Cyclooxygenase or iNOS was inhibited in selected wells with indomethacin (10 μM) or N^G-methyl-L-arginine (NMMA) (0.5 mM) (Sigma, St. Louis, MO), respectively. Supernatants were collected at timed intervals, filtered (0.2 μm Acrodisc, Gelman Sciences, Ann Arbor, MI), and stored at −80 C until analysis.

Table 1. PATIENT CHARACTERISTICS

Patient No.	Age	Sex	Diagnosis
1	22	M	Liver donor (death by asphyxiation)
2	48	F	Metastatic ovarian adenocarcinoma
3	53	F	Metastatic colon adenocarcinoma
4	70	M	Hepatocellular carcinoma
5	65	M	Metastatic colon adenocarcinoma
6	50	F	Metastatic colon adenocarcinoma
7	82	M	Metastatic colon adenocarcinoma

Prostaglandin E_2 Radioimmunoassay

Supernatants were combined with ^{3}H-labeled PGE_2 (New England Nuclear, Boston, MA) and a specific rabbit antiserum to PGE_2 (provided by Dr. Aubrey Morrison, Department of Pharmacology, Washington University). After a 12-hour incubation at 4 C, the excess ^{3}H-PGE_2 was removed with a charcoal-dextran mixture and the bound portion quantitated by liquid scintillation spectroscopy. To determine the concentration of PGE_2 (ng/mL), triplicate values were averaged and compared with a standard curve using PGE_2 (Advanced Magnetics, Cambridge, MA) performed with each assay.

Interleukin-6 Bioassay

Interleukin-6 activity in the supernatants was measured by proliferation of the IL-6-responsive B9.9 cell line (originating from Dr. Lucian Aarden, the Netherlands, and provided by Dr. Richard Nordan, National Institutes of Health, MD). Serially diluted supernatant samples were incubated in triplicate at 37 C in 5% CO_2 for 44 hours with 2 × 10^3 B9.9 cells. ^{3}H-thymidine (2 μCi/well; ICN, Irvine, CA) was added for the final 12 hours of culture. Incorporated ^{3}H-thymidine was harvested at 56 hours with an automatic cell harvester (Brandel, Gaithersburg, MD) onto glass fiber filters. Radioactivity was determined by liquid scintillation spectrometry. Unknown supernatant IL-6 (pg/mL) levels were determined by comparison to a standard curve of

B9.9 proliferation to known quantities of recombinant human IL-6 (Genzyme, Boston, MA).

Interleukin-1 Bioassay

The murine D10.G4.1 cell line, which recognizes conalbumin A (Sigma) in context of I-A^k (provided by Dr. Thomas Ferguson, Department of Ophthalmology, Washington University), was used to measure IL-1 in the supernatants from human KCs. Serially diluted supernatant samples in triplicate were incubated for 48 hours at 37 C in 5% CO_2 with 2×10^4 D10.G4.1 cells/well and 2.5 μg/mL conalbumin A (Sigma). ^{3}H-thymidine (0.4 μCi/well) was added for the final 12 hours of the culture. DNA was harvested at 72 hours after culture initiation on glass fiber filters, and ^{3}H-thymidine incorporation was determined by liquid scintillation spectrometry. Unknown supernatant IL-1 levels (pg/mL) were determined by comparison with a standard curve of D10.G4.1 proliferation induced by known quantities of recombinant murine IL-1 (supplied by Dr. David Chaplin, Department of Medicine, Washington University).

Transforming Growth Factor-β Bioassay

TGF-β activity was measured by inhibition of proliferation of the TGF-β-responsive MV-1-Lu mink lung cell line (American Type Culture Collection, Rockville, MD). Serially diluted supernatant samples in triplicate were incubated at 37 C in 5% CO_2 for 24 hours with 1×10^5 mink lung cells in 96-well plates. ^{3}H-thymidine (2 μCi/well) was added for the final 4 hours of culture. Incorporated ^{3}H-thymidine was harvested on glass fiber filters and quantitated by liquid scintillation spectrometry. Unknown TGF-β levels (pg/mL) were determined by comparison to a standard curve of inhibition of MV-1-Lu proliferation with known quantities of recombinant murine TGF-β (Genzyme, Boston, MA).

Assay of Nitric Oxide

To assess the production of nitric oxide by human KCs, concentrations of nitrite (NO_2), a rapidly formed and stable metabolite of nitric oxide,[23] were measured by the Griess reaction. Fifty μL of supernatant were incubated at room temperature with 100 μL of a 1:1 mixture of 1% sulfanilamide in 30% acetic acid with 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride in 60% acetic acid. After 20 minutes, absorbance at 540 nm was measured using a Dynatech MR700 plate reader (Dynatech Laboratories, Inc., Alexandria, VA). Nitrite present in each well was quantified by comparison to a standard curve of sodium nitrite ($NaNO_2$).

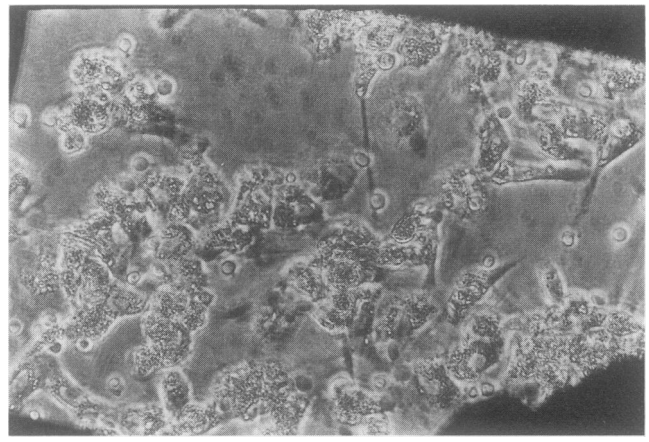

Figure 1. Appearance of human KCs after 24-hour culture in standard complete medium (magnification × 10). Morphologically distinct features include granularity, heterogeneic cell size, and long processes.

Tumor Necrosis Factor-α Bioassay

Tumor necrosis factor-α levels were determined by adding serially diluted culture supernatant in duplicate to 7.5×10^5 TNF-α-sensitive L929 fibroblasts (American Type Culture Collection) that had been treated for 2 hours with 2 μg/mL Actinomycin D (Sigma). After 18 hours at 37 C in 5% CO_2, cell viability was determined by staining the cells with crystal violet stain and reading the absorbance at 540 nm using a Dynatech MR700 plate reader. Units of TNF-α per mL were calculated by comparing the unknowns against the response of the L929 cells to a murine TNF-α standard (provided by Dr. Robert Schreiber, Department of Pathology, Washington University).

Statistics

Parametric data were analyzed by Student's t test for single comparisons. The level of significance was determined at $p < 0.05$. Data are reported as mean ± standard deviation (SD).

RESULTS

Cell Morphology

Between 2×10^6 and 6×10^6 human KCs were obtained with each liver isolation. These KCs appeared larger and more granular by light microscopy (Fig. 1) than our preparations of either murine or rat KCs. Similar to rat and mouse KCs, human KCs change their shape from spherical to stellate or spindle-shaped after being in culture for 24 hours. These observations are consistent with those of Professor Kupffer from the last

Table 2. PEAK CONCENTRATIONS IN RESPONSE TO LPS BY HUMAN KUPFFER CELLS

	Culture Duration	LPS 2.5 μg/mL	Patient No. 1	2	3	4
IL-1 (pg/mL)	72 hrs	−	2.0 ± 0.4	0.8 ± 0.1	1.4 ± 0.3	1.5 ± 0.4
		+	5.1 ± 0.8	1.6 ± 0.3	2.7 ± 0.4	2.5 ± 0.3
			(0.0039)*	(0.0119)	(0.0108)	(0.0257)
IL-6 (pg/mL)	72 hrs	−	245 ± 25	95 ± 10	147 ± 12	195 ± 20
		+	510 ± 57	200 ± 17	456 ± 45	388 ± 42
			(0.0018)	(0.0008)	(0.0003)	(0.002)
PGE_2 (ng/mL)	48 hrs	−	3 ± 0.6	0.7 ± 0.1	2.1 ± 0.1	2.5 ± 0.3
		+	15.4 ± 1.8	7.1 ± 1.1	12.2 ± 2.0	13.0 ± 1.4
			(0.0003)	(0.0006)	(0.0009)	(0.0002)
TNF-α (U/mL)	12–24 hrs	−	0.4 ± 0	0.12 ± 0	<0.1	0.3 ± 0.09
		+	21.2 ± 4.0	0.39 ± 0.19	9.47 ± 3.0	1.5 ± 0.25
			(0.0008)	(0.0696)	(0.0054)	(0.0014)
TGF-β (pg/mL)	72 hrs	−	0.8 ± 0.1	0.2 ± 0.005	0.55 ± 0.4	0.6 ± 0.2
		+	3.7 ± 0.7	1.6 ± 0.7	2.1 ± 0.6	2.0 ± 0.5
			(0.0021)	(0.0257)	(0.0204)	(0.0108)
NO_2 (μM)	72 hrs	−	ND	ND	ND	ND
		+	ND	ND	ND	ND

Mean ± standard deviation.
* Statistics by two-tailed unpaired Student's t test.
ND = baseline level at 48 hrs culture.
Responses were obtained from 5×10^5 KC cultured in complete medium containing 1.2 mM arginine.

century[9] and more recent electron microscopy studies comparing animal and human KCs.[10,11]

Kinetics of Cytokine Release

The addition of LPS (2.5 μg/mL) to cultured human KCs (patients 1 through 4) resulted in a significant increase of mediators measured in the culture supernatant when compared against equal numbers of time-matched, unstimulated KCs (Table 2). The only exception to this was nitric oxide, which was not synthesized by LPS-stimulated human KCs as determined by nitrite level measurements. Although the patterns of response to LPS were consistent among the individual KC preparations, concentrations of measured KC products ranged widely. The earliest cytokine response was that of TNF-α (Fig. 2A), which peaked between 12 and 24 hours after LPS; maximal PGE_2 levels were detected 48 hours after LPS stimulation (Fig. 2B). Interleukin-1 (Fig. 2C), IL-6 (Fig. 2D), and TGF-β (Fig. 2E) peaked later at 72 hours after LPS. Although the response kinetics generally were similar to those reported in the rat,[17,20,23] the rat and human KCs appear to differ because maximal levels of human KC TNF-α were achieved approximately 12 hours later than with rat KCs.[20]

Eicosanoid Inhibition of Inflammatory Cytokines

Prostaglandin E_2 is a well-characterized immunosuppressive product of both rodent[6,17,18,20] and human[16] KCs. Lipopolysaccharide (2.5 μg/mL) induced a significantly higher release of PGE_2 by human KCs in standard medium at all time points after LPS when compared against time-matched, nonstimulated KCs (Fig. 2B). Because PGE_2 release by rodent KCs is regulated directly by ambient arginine levels,[20] we also measured the effects of arginine availability on the human KCs' response to LPS. Similar to the behavior by cultured rat KCs,[17,20] a reduction of arginine levels in the medium to 0.01 mM was associated with significantly enhanced PGE_2 release at all time points relative to the response of cells cultured in high arginine (1.2 mM) medium (Fig. 3A). Maximal PGE_2 concentration was attained by 24 hours after LPS addition with both arginine concentrations.

The augmentation of PGE_2 by limiting arginine availability is sufficient to downregulate rat KC release of IL-1, IL-6, and TNF-α in an autocoid fashion.[17,20] However, human KCs cultured in low (0.01 mM) or standard (1.2 mM) arginine media and stimulated with LPS did not respond in a similar fashion, i.e., a reduction in arginine concentration was unexpectedly associated with an in-

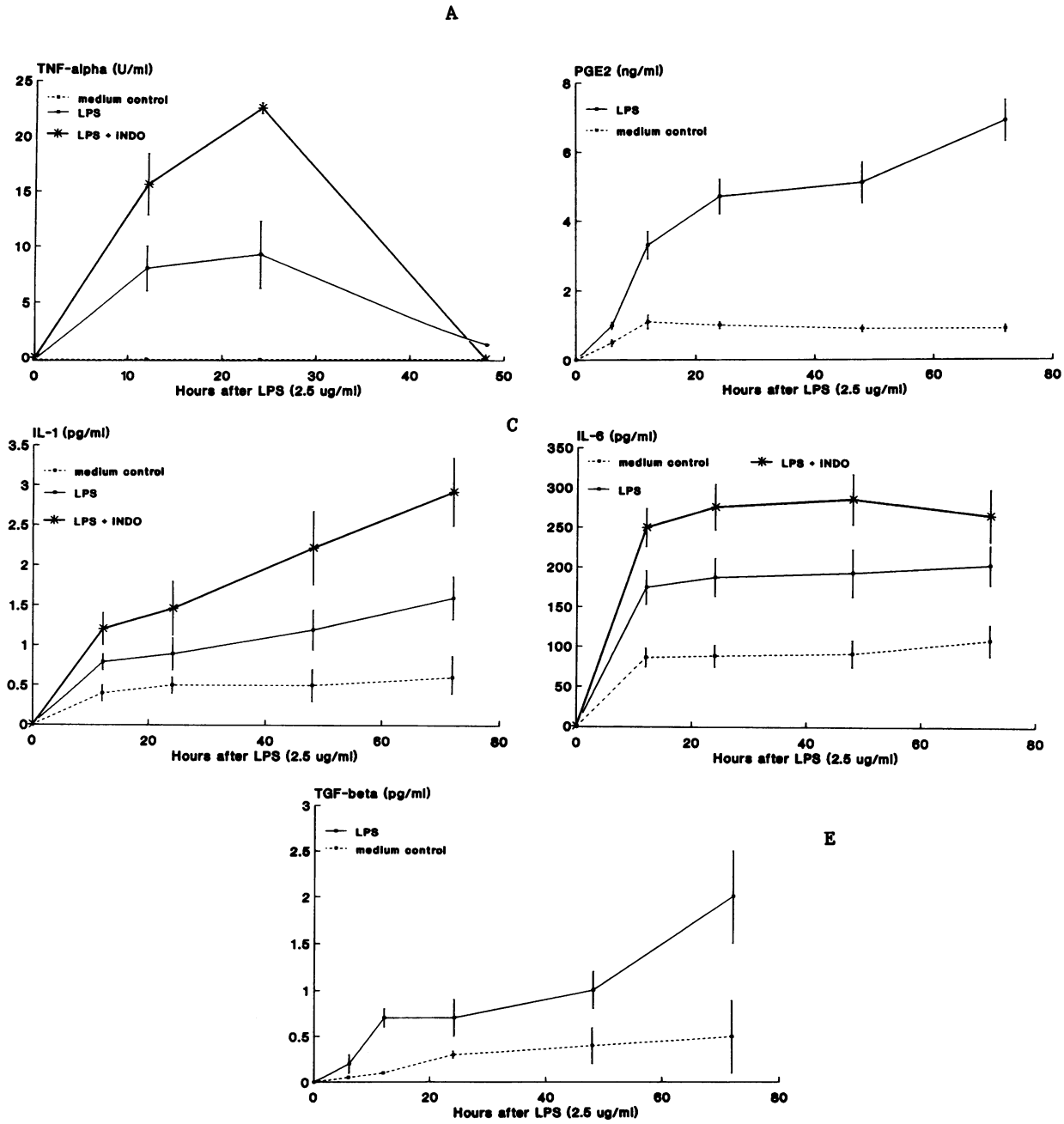

Figure 2. The kinetics of the cytokine response of 5×10^5 human KCs to LPS (2.5 μg/mL) in the absence or presence of indomethacin (10 μM). Kupffer cells were cultured in standard medium containing 1.2 mM L-arginine. The data shown are representative for the responses of patients 1 through 4. (A) TNF-α release is downregulated significantly by indomethacin ($p < 0.02$). (B) Prostaglandin E_2 release is increased significantly above baseline as early as 12 hours after LPS ($p < 0.001$). No PGE_2 was detectable by radioimmunoassay when indomethacin was added at the time of LPS (not shown). (C) Interleukin-1 levels were significantly above baseline as early as 12 hours after LPS ($p < 0.01$). Indomethacin added at the time of LPS stimulation resulted in a significant increase ($p < 0.03$) in IL-1 activity in supernatants at 48 and 72 hours. (D) Interleukin-6 concentrations were significantly above background as early as 12 hours after LPS ($p < 0.01$). Indomethacin was associated with a significant increase ($p < 0.02$) in IL-6 activity in supernatants collected at 12, 24, and 48 hours. (E) Transforming growth factor-β supernatant concentrations beyond 6 hours after culture initiation were significantly enhanced ($p < 0.03$) by the addition of LPS. Indomethacin was not found to affect the release of LPS-stimulated TGF-β (not shown).

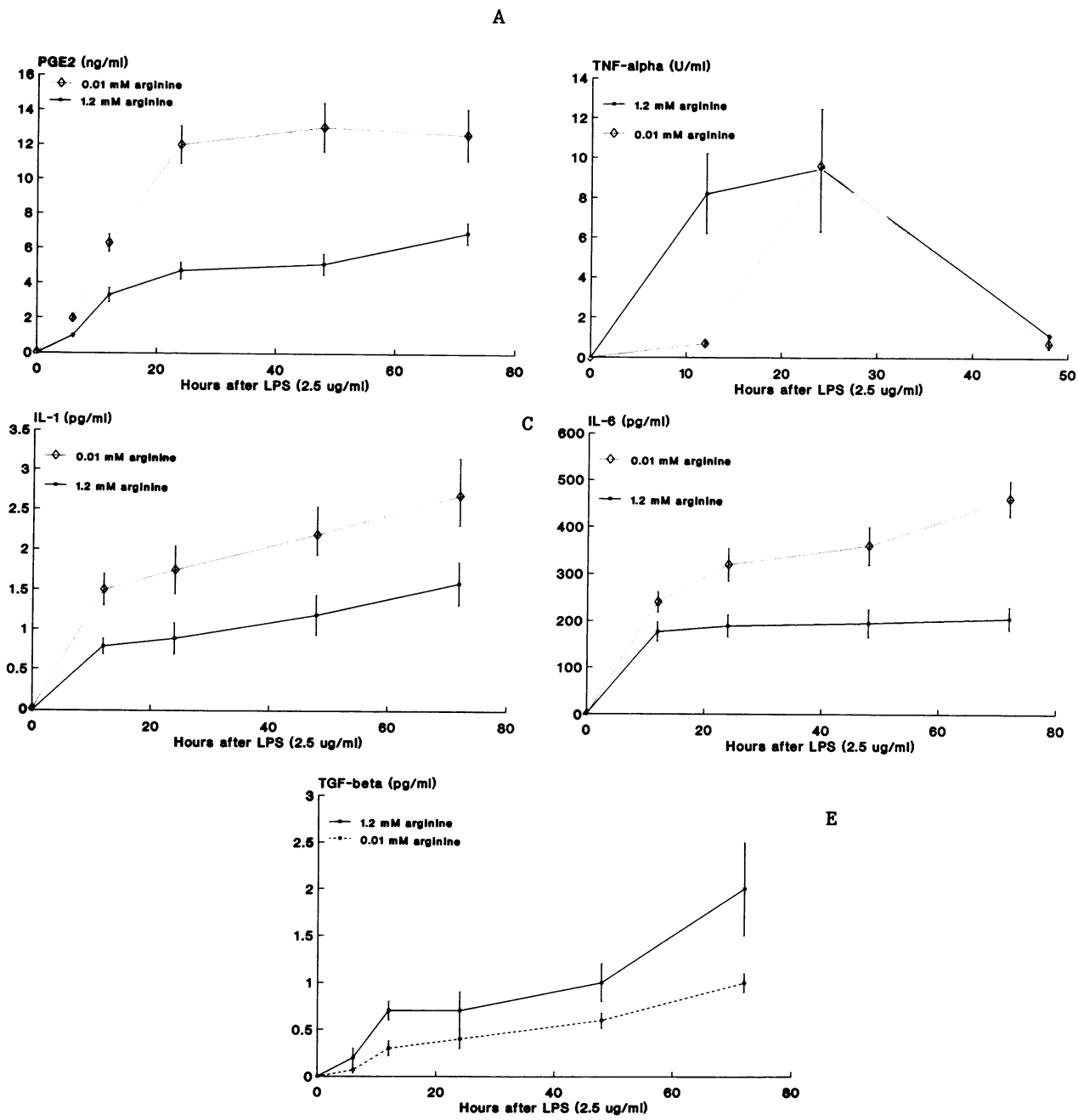

Figure 3. Effect of reducing L-arginine concentration in the culture medium from 1.2 mM to 0.01 mM on the PGE_2 and cytokine responses to LPS by KCs from patients 1 through 4. (A) The reduction in L-arginine concentration significantly enhanced ($p < 0.02$) the LPS-mediated release of PGE_2. Both LPS-induced IL-1 (C) and IL-6 (D) activities in supernatants were significantly enhanced ($p < 0.02$) when KCs were cultured in low arginine medium. A consistent effect of L-arginine restriction on the TNF-α response to LPS (B) was not observed. (E) Transforming growth factor-β levels were decreased slightly, but significantly ($p < 0.03$), as a function of L-arginine restriction.

crease in IL-1 (Fig. 3B) and IL-6 (Fig. 3C). The effect of limiting L-arginine was variable on the TNF-α response (Fig. 3D); TGF-β release was decreased slightly, although significantly, by limiting L-arginine availability (Fig. 3E).

Indomethacin was added to parallel wells at a dose of 10 μM as another approach to define the possible role of eicosanoids in the autoregulation of human KC products. Cyclooxygenase in LPS-activated human KC was

blocked completely because PGE_2 levels in these indomethacin-supplemented wells were consistently below the detection limits of our assay (data not shown). That eicosanoids autoregulate the secretory output of human KCs is suggested by the significantly increased release of IL-1 (Fig. 2C), IL-6 (Fig. 2D), and TNF-α (Fig. 2A) by LPS-stimulated human KCs cultured in standard medium (1.2 mM L-arginine), to which indomethacin was added. TGF-β was not affected by inhibition of cyclooxygenase (data not shown).

Nitric Oxide and the Human Kupffer Cell

Although human monocytes[24] and hepatocytes[25] are known to release nitric oxide, the iNOS activity of the human KC has not been determined. Consequently, we tested human KCs (patients 1 through 4) under conditions that reliably stimulate nitric oxide production in rat KCs. An increase in supernatant nitrite levels over 72 hours (data not shown) could be attributed only to spontaneous formation of nitric oxide in the medium. There was no inhibition of enzymatic generation of nitric oxide with NMMA, and nitrite concentrations were identical whether wells contained LPS-exposed KCs, quiescent KCs, or medium only. Recognizing that maximal iNOS induction in various animal[22] and human[24,25] models requires more than one signal, human KCs (from patients 5 through 7) were exposed to LPS (2.5 μg/mL), rhIFN-γ (100 U/mL), or LPS + rhIFN-γ. None of these culture conditions were associated with an increase in nitrite above baseline at 72 hours, when nitric oxide induction reaches a plateau (Fig. 4). Eicosanoids were not responsible for this lack of response because the addition of indomethacin also was not associated with LPS-induced nitric oxide generation (data not shown).

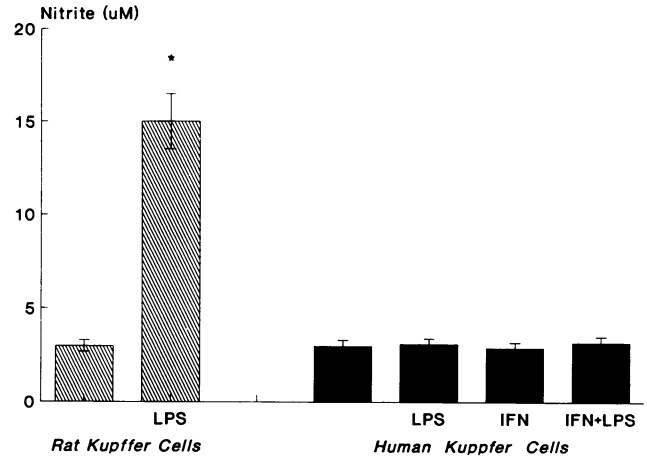

Figure 4. Nitric oxide, as determined by nitrite concentration, in 72-hour culture supernatant. Medium used for these experiments contained 1.2 mM L-arginine. Human KCs (patients 5 through 7; solid bars) were exposed to LPS (2.5 μg/mL), rhIFN-γ (100 U/mL), or rhIFN-γ plus LPS. No increase above baseline was observed. For comparison, a typical rat KC response (hatched bars; $p < 0.001$) to LPS is shown.

DISCUSSION

The earliest published report[9] on the *Sternzellen* (star cells) of the liver by Carl Kupffer (in 1876) included a description of the morphology and microanatomic location of these cells in "a healthy liver from a patient who had been killed violently a few hours [before the liver] became available." However, modern study of the human KCs did not begin until Wisse and Knook[11,16] and others[10] were able to isolate KCs from human livers, confirming Kupffer's original assertion that the morphology of rat and human KCs is similar. Analysis of eicosanoid release by human KCs demonstrated that LPS induces a dose-dependent release of multiple eicosanoids, including PGE_2.[16] In addition, it has been shown that human fetal KCs respond to LPS by producing IL-6,[26] IL-1,[13] and TNF-α.[13] Recently, our laboratory extended the observation of LPS-induced IL-6 production, and Roh et al. of TNF-α production,[14] to the adult human KC.[15] The LPS-mediated release of TGF-β by human KCs has not been reported previously.

Although we detected significant increases in all of these substances after LPS stimulation, the range of maximal levels varied. The physical properties of the liver samples, e.g., degree of fibrosis, which varied among the samples studied, could have influenced the functional subclass of KC isolated and mediators released.[27,28] Nonetheless, marked variability of *in vitro* PGE_2 responses to LPS has been observed by other investigators.[16] In addition, normal human subjects administered a fixed dose of endotoxin will release widely disparate levels of TNF-α into the plasma.[29] Similarly, *in situ* Northern blot analysis of human liver IL-1, IL-6, and TNF messages also has revealed tenfold differences in absolute amounts of mRNA between samples.[30] The range of responses observed among our KC preparations may reflect either the disease state of the liver or normal biologic variability among individuals, a problem not encountered when studying the KC of inbred rodent strains, because the genes encoding TNF-α and the major histocompatibility complex are linked closely.[31] The variable cytokine responses to LPS by the KCs from various individuals may reflect their genetic polymorphism.

Either LPS or TNF-α can stimulate the release of PGE_2, one of several eicosanoids synthesized by prostaglandin H synthase of KCs.[8] From animal studies, it appears that the primary biologic importance of PGE_2 in the liver is to counter the excessive production of poten-

tially harmful KC products. Specifically, in an autocoid fashion, PGE_2 downregulates TNF-α,[8,18,20] IL-1,[17,19] and IL-6[17] release by rodent KCs stimulated with LPS *in vitro*. Before similar regulation can be attributed to human cells, identical culture conditions to those employed in rat experiments must be used for human cells.[17,20] The present data indicate that a negative feedback mechanism similar to that observed in the rat may also occur in the human KC because inhibition of cyclooxygenase significantly upregulated the LPS-stimulated release by human KCs of TNF-α, IL-1, and IL-6, known mediators in the physiologic derangements of septic shock.[29,32-36] Because indomethacin prevents the synthesis of prostaglandins of the D, E, and F series, it is not possible to ascertain from our data which eicosanoid was specifically responsible for the autocoid control of cytokines. This model of autoregulated cytokine production could explain why portal endotoxemia usually does not result in either liver damage or a septic response.[6,20]

A previous report from this laboratory demonstrated a marked augmentation of PGE_2 production by rat KCs when cultured in low arginine RPMI-1640-based medium.[20] The mechanism by which reduction of arginine to 0.01 mM enhances PGE_2 release by cultured rat KCs has not been ascertained, but this concentration of arginine more accurately reflects arginine availability in the microenvironment of the liver than does the supraphysiologic level of 1.2 mM. (unpublished data)[21] The augmentation of KC PGE_2 release by low arginine conditions is sufficient to significantly suppress TNF-α[20] and IL-6[17] secreted by LPS-stimulated rat KCs. In our study, we demonstrated that human KCs respond identically to rat KCs by significantly increasing LPS-mediated PGE_2 release when cultured in low arginine medium. However, unlike the rat experiments, this enhanced PGE_2 response to LPS was not sufficient to inhibit IL-1, IL-6, or TNF-α in an autocoid fashion. These results suggest that other, as yet unidentified, signals are required *in vivo* for human KC regulation of these mediators.

The importance of arginine in the immune functions of the liver is indicated by its influence over PGE_2 release by cultured KCs and by its role as the substrate of iNOS.[22] Inducible nitric oxide synthase (iNOS), whose synthesis is initiated by LPS, converts arginine to L-citrulline and nitric oxide, the latter being a labile compound converted rapidly to nitrite (NO_2) and nitrate (NO_3) in fixed proportions.[22] Nitric oxide has been implicated as a cytotoxic effector produced by activated macrophages[37] and as a KC-derived inhibitor of hepatocyte protein synthesis.[38] Its correlation with the human sepsis syndrome is strong, given its elevation in the circulation of patients with septic shock.[39] In addition, *in vitro* stimulation of nitric oxide production by human monocytes[24] and hepatocytes[25] raises the possibility that the source of nitric oxide during human sepsis could be from both of these cell populations. Because of the ability of these human cells[24,25] and rat KCs[22] to produce nitric oxide, it was surprising that, when using culture conditions which consistently result in fourfold increases in nitrite release from LPS-stimulation rat KCs, no elevation in nitrite levels above baseline could be measured as late as 72 hours after LPS stimulation of human KCs. The considerable contamination of human liver samples with erythrocytes, a technical problem noted by other investigators,[16] may have released sufficient hemoglobin into the culture supernatant of some preparations to bind free nitric oxide.[39] Nevertheless, even those KC preparations devoid of significant erythrocyte contamination (Fig. 1) failed to produce measurable nitrite levels. In addition, although rat KC iNOS is induced readily with LPS alone, human hepatocytes require a mixture of LPS, TNF-α, and IFN-τ for optimum iNOS induction.[25] Consequently, we exposed human KCs to LPS and IFN-τ. (Because KCs produce their own TNF-α and IL-1 in response to LPS,[8,17,20] these cytokines were not added.) Despite these optimal conditions, no nitrite levels were detectable. These results serve as an example of how generalizations between species must be made with caution. Although an additional autocrine signal necessary for iNOS induction in the human KC may have been missing in the culture wells, the results imply that the human KC is not the source of elevated circulating nitric oxide in the human sepsis syndrome.[40]

Transforming growth factor-β is a recently described family of peptide growth factors synthesized by essentially all cells and for which most cells have receptors.[41] The source of this peptide within the human liver has been localized to nonparenchymal sinusoidal cells; however, definitive proof that human KCs produce TGF-β has been lacking.[42,43] The kinetics of the human TGF-β response to LPS are similar to those shown in rat KCs[23] with significant release of this factor within 12 hours. In addition to inhibiting liver regeneration[42] and promoting fibrogenesis,[43] it is possible that KC TGF-β acts in an autocrine loop to enhance PGE_2 production. This has been shown to be the case with monocytes of patients manifesting post-trauma immunosuppression.[44] In addition, the known TGF-β functions of upregulating cytokine production, e.g., IL-6, IL-1, and TNF,[45,46] may have bearing on the functional significance of TGF-β released by human KC. Finally, because TGF-β is not considered one of the classical proinflammatory macrophage mediators, it is not surprising that TGF-β was not found to be under the autocoid control of eicosanoids

We have found that, in addition to TNF-α, IL-1, IL-6, and PGE_2, it is possible to add TGF-β to the growing

list of mediators released by the human KC. However, unlike rat KCs, human KCs are resistant to the induction of iNOS *in vitro*. These data support a model, originally suggested by the secretory behavior of rodent KCs, that eicosanoids, e.g., PGE_2, may play a role in downregulating proinflammatory KC cytokines in an autocoid manner. Because the KC is positioned strategically to be the primary fixed-tissue macrophage encountering gut-derived endotoxin, such a regulatory mechanism appears to have evolved to protect the organism from the deleterious effects of LPS during portal bacteremia. In the absence of PGE_2-mediated counterregulation of the LPS-stimulated KC, normally benign portal endotoxemia may result in excessive proinflammatory cytokine release causing local damage, as well as in spillage to extrahepatic tissues, such as to the pulmonary bed.

Acknowledgments

The authors thank Michael Murphy for performing the PGE_2 radioimmunoassay and Ms. Theresa Belgeri for assisting with manuscript preparation. They also thank Dr. Todd Howard for providing hepatic tissue from patient 7.

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