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Production of angiogenic activity by human monocytes requires an L-arginine/nitric oxide-synthase-dependent effector mechanism

(macrophage/stimulation/endothelium/cytokines/chemotaxis)

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ABSTRACT **Human monocytes (Mϕ) require stimulation with substances such as bacterial endotoxin [LPS (lipopolysaccharide)] to produce angiogenic activity. In this study, we report that stimulation of Mϕ with LPS (5 μg/ml) in the absence of L-arginine greatly reduced their production of angiogenic activity, as assessed *in vivo* in rat corneas and *in vitro* by chemotaxis of human umbilical vein endothelial cells (HUVECs). D-Arginine did not substitute for L-arginine in the production of angiogenic activity. The nitric oxide synthase (NO synthase, EC 1.14.13.39) inhibitors N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine methyl ester (L-NAME) both inhibited the production of angiogenic activity by LPS-stimulated Mϕ in the presence of L-arginine, suggesting the involvement of this enzyme in the pathway that generates angiogenic activity. Neither of these substances directly inhibited the Mϕ-derived angiogenic activity. LPS-induced production of the cytokines tumor necrosis factor α (TNF-α) and interleukin 8 (IL-8) was not significantly reduced when Mϕ were incubated in the absence of L-arginine. Similarly, L-NMMA and L-NAME did not significantly reduce the LPS-induced production of these cytokines by Mϕ in the presence of L-arginine. These results suggest that the LPS-stimulation-dependent generation of angiogenic activity by Mϕ requires an L-arginine-dependent NO-synthase effector mechanism that may be independent of the generation of TNF-α and IL-8.**

Monocytes (Mϕ) and macrophages play a key role in fibroproliferative responses such as wound repair, inflammation, and solid tumor development by producing angiogenic factors, which induce the growth of new microvascular blood vessels (1–9). While unstimulated Mϕ do not express the angiogenic phenotype, microenvironmental influences such as endotoxin [LPS (lipopolysaccharide)], low oxygen tension, and select cytokines stimulate Mϕ and macrophages and induce expression of the angiogenic phenotype (4–9). The process of Mϕ stimulation is complex and involves several interacting sequential stages (10, 11). Early, rapidly induced events result in the production of oxygen free radicals via the NADP-dependent hexose monophosphate shunt (12). Early changes in gene transcription of several protooncogenes and cytokines result in the later increased translation and production of cytokines such as interleukin 1, tumor necrosis factor α (TNF-α), and interleukin 8 (IL-8) (13–15). These substances play important roles in modulating the inflammatory response and wound repair. In addition, activation of macrophages induces the production of nitric oxide (NO) (16, 17). NO is produced from the amino acid L-arginine by an inducible enzyme, NO synthase (EC 1.14.13.39), using NADPH and molecular oxygen as cofactors (18, 19). NO has been shown to be an important mediator of blood vessel relaxation, platelet function, inflammation, neurotransmission, and lymphocyte proliferation, as well as contributing to the cytotoxic action of macrophages against microbes and tumor cells (20–25).

We have shown previously that production of angiogenic activity by human Mϕ is inhibited by oxygen free radical scavengers, thiol-containing compounds, and compounds that disrupt Mϕ cellular redox balance (26–28). In the present study, we examined the role of an L-arginine-dependent inducible NO-synthase pathway of Mϕ in the generation of angiogenic activity. These studies confirm that this pathway exists in human Mϕ, and they suggest an important role for this pathway in the production of Mϕ-dependent angiogenic activity (MDAA).

MATERIALS AND METHODS

Preparation of Human Mϕ. Human Mϕ were isolated from buffy coats of normal volunteers by centrifugation on Lymphoprep (Nycomed, Oslo) followed by Sepracell-MN (Sepratech, Oklahoma City), as described previously (27, 28). Mϕ were purified further by overnight adherence of 1×10^6 cells per ml to 100-mm tissue culture dishes at 37°C in RPMI-1640 medium containing 10% fetal calf serum (FCS) and gentamycin (50 μg/ml). The adherent cells were washed with phosphate-buffered saline. Mϕ obtained in this manner were >95% pure, as estimated morphologically and by esterase staining (Sigma). Relevant test media, described below, were then added to the Mϕ.

Preparation of Mϕ-Conditioned Media. To test the requirement for L-arginine on the production of MDAA, Mϕ were cultured in: (*i*) minimal essential Eagle's medium (MEM) prepared without L-arginine, containing 0.5% FCS; (*ii*) MEM/0.5% FCS containing L-arginine at 105 μg/ml (0.6 mM); or (*iii*) MEM/0.5% FCS with D-arginine (105 μg/ml) in place of L-arginine. Incubations were continued for 48 hr at 37°C in a humidified incubator gassed with 95% air/5% CO_2. Since production of MDAA requires stimulation, Mϕ were incubated with bacterial LPS (phenol-extracted preparation of *Escherichia coli* O55:B5 lipopolysaccharide, Sigma), added at 5 μg/ml 30 min after the addition of the specific test

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Abbreviations: Mϕ, monocytes; LPS, endotoxin (lipopolysaccharide); TNF-α, tumor necrosis factor α; IL-8, interleukin 8; MDAA, Mϕ-dependent angiogenic activity; FCS, fetal calf serum; LDH, lactate dehydrogenase; L-NAME, N^G-nitro-L-arginine methyl ester; L-NMMA, N^G-monomethyl-L-arginine; HUVEC, human umbilical vein endothelial cell.

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medium to the Mϕ cultures. LPS was then present throughout the incubations. The conditioned media were then harvested. Since the determinations of nitrite, nitrate, and lactate dehydrogenase (LDH) concentrations in conditioned media described below all involved colorimetric assays, media without phenol red were used throughout these experiments. For the assay of angiogenic activity, media were concentrated (20-fold) by centrifugation through Centricon 3 filters (3000-Da cutoff; Amicon).

To determine the requirement for NO synthase in the production of MDAA, Mϕ were cultured as described above in MEM containing L-arginine, together with the specific inhibitors of NO synthase N^G-nitro-L-arginine methyl ester (L-NAME) or N^G-monomethyl-L-arginine (L-NMMA) at 0.6 mM (19–23). This concentration of inhibitors is equivalent to a 1:1 molar ratio of inhibitor to L-arginine, and it was selected in preliminary experiments as the concentration giving maximal inhibition of NO_2^- and NO_3^- production, while not affecting Mϕ viability.

To determine the direct effects of L-NMMA and L-NAME on MDAA itself, these compounds were added directly to conditioned media from LPS-stimulated Mϕ prepared in MEM with L-arginine. This medium was demonstrated to be angiogenically active in the corneal bioassay and the human umbilical vein endothelial cell (HUVEC) chemotaxis assay (see below).

Determination of Nitrite and Nitrate Concentration in Conditioned Media. The oxidation of L-arginine by NO synthase results in the production of citrulline and the highly reactive, transient, unstable molecule NO. NO is rapidly converted to nitrite (NO_2^-) and nitrate (NO_3^-). To determine whether the reaction conditions used in our experiments modified NO production by human Mϕ in the predicted manner, NO_2^- and NO_3^- concentrations in the Mϕ conditioned media were determined.

NO_2^- concentrations in conditioned media were determined by using a colorimetric assay based on the method of Griess (29, 30). Briefly, samples (0.25 ml of unconcentrated medium) were added on ice to an equal volume of 350 mM NH_4Cl buffer, pH 9.6. Griess reagent [1 mM sulfanilic acid/1 mM *N*-1-(1-naphthyl)ethylenediamine in acetic acid] (0.5 ml) was added, the samples were mixed and incubated at room temperature for 10 min, and the color developed was read at 555 nm. NO_2^- concentrations were calculated from a standard curve, using $NaNO_2$ as a standard.

NO_3^- concentrations were determined by using the *Aspergillus* nitrate reductase method (47), involving the reduction of NO_3^- to NO_2^- by the enzyme nitrate reductase. The reaction results in the oxidation of NADPH to $NADP^+$ and measures the change in absorbance at 340 nm. Briefly, the following reagent mixture was prepared: 4 ml of 0.14 M potassium phosphate buffer at pH 7.5, 3 ml of water, 0.25 ml of 0.1 M FAD (Sigma), and 1 mg of NADPH (Sigma). Next, 0.25-ml samples or a standard was added to 0.733 ml of reagent mix, and absorbance at 340 nm was measured for 2 min, to ensure stability. Then 16.7 μl of *Aspergillus* nitrate reductase (3.5 units/ml in 0.042 M potassium phosphate buffer, pH 7.5) (Sigma) was added. Absorbance at 340 nm was read for 10 min. NO_3^- concentrations were determined from a standard curve, prepared with $NaNO_3$.

Chemotaxis of HUVECs. The chemotactic activity of Mϕ-conditioned media for HUVECs was determined by measuring the number of cells that migrated across gelatin-coated Nuclepore polycarbonate membranes (8-μm pore size) in a 48-well microwell chemotaxis chamber (Neuroprobe, Cabin John, MD). Briefly, early-passage subconfluent cultures of HUVECs were trypsinized, washed, and resuspended at 1×10^6 cells per 1.5 ml in medium containing 0.1% FCS. The cell suspensions to be tested (25 μl) were plated into each well of the chemotaxis chamber and the gelatin-coated filter was positioned above the wells. The top half of the chamber was reattached, and the chamber was incubated inverted at 37°C in 95% air/5% CO_2 for 2 hr to allow even attachment of the HUVECs to the filter. The test or control media (50 μl) were then added to the top section wells, and the chambers were wrapped in Parafilm and incubated for an additional 2 hr at 37°C. The filters were removed from the chamber, fixed, stained and mounted on glass slides. Cells that had migrated through the filter pores were counted under a 40× microscope objective.

Corneal Bioassay of Angiogenesis. Twentyfold-concentrated Mϕ-conditioned medium (5 μl) was incorporated into equal volumes of slow-release Hydron (12% wt/vol in 95% ethanol) (Interferon Sciences) and allowed to dry. Hydron pellets were implanted aseptically into pockets within the rat corneal stroma, 2 mm from the limbal vasculature, as described previously (1, 3, 4, 31). Corneas were examined daily for 7 days with a stereomicroscope and were perfused with colloidal carbon at the end of the observation period to provide a permanent record of the angiogenic responses. Corneas were examined histologically for any evidence of nonspecific inflammation.

Determination of TNF-α and IL-8 Concentrations in Conditioned Media. TNF-α levels in conditioned media were determined by using a sandwich enzyme-linked immunosorbent assay (ELISA) technique with a commercially available kit (BioSource International, Camarillo, CA), following the instructions of the manufacturer. Each sample was assayed in duplicate. Concentrations of TNF-α in the test samples were determined from a standard curve established with recombinant human TNF-α. This assay is specific for the measurement of biologically active TNF-α and has a minimum sensitivity of 4 pg/ml. When necessary, appropriate dilutions of conditioned media were made to ensure that TNF-α levels were in the linear range of sensitivity of the assay.

IL-8 levels were determined by using a commercially available sandwich ELISA kit (R & D Systems), as described by the manufacturer. Each sample was assayed in duplicate, and concentrations in the test samples were determined from a standard curve. When necessary, appropriate dilutions of the conditioned media were made to bring the IL-8 concentrations into the linear range of sensitivity (4–2000 pg/ml) of the assay kit.

Determination of Mϕ Viability in Culture. Viability of Mϕ under the various conditions of culture was assessed by determining (*i*) the percentage of cells that excluded trypan blue and (*ii*) LDH release, using a commercially available kit (Sigma). This procedure uses a colorimetric method, involving the measurement of the LDH-catalyzed reduction of pyruvate to lactate. Positive controls for LDH release were Mϕ frozen and thawed five times to induce total cell lysis.

RESULTS

Angiogenic Activity of Mϕ-Conditioned Media. The angiogenic responses induced in rat corneas by the conditioned media prepared from human Mϕ under the various conditions of culture are shown in Table 1.

Medium from nonstimulated Mϕ cultured in MEM in the presence of L-arginine was not angiogenic, while medium from LPS-stimulated Mϕ under identical conditions was potently angiogenic. In contrast, medium from LPS-stimulated Mϕ cultured in MEM lacking L-arginine showed a greatly reduced capacity to induce an angiogenic response. Medium prepared with D-arginine in place of L-arginine also showed a greatly reduced angiogenic response, indicating the stereospecific requirement for L-arginine in this process. These results suggested that production of angiogenic activity by Mϕ was dependent on the presence of L-arginine in the

Table 1. Effects of arginine and NO synthase inhibitors on angiogenic activity induced in rat corneas by Mϕ conditioned media

Group*	Angiogenic response,† no. of corneas assayed		
	−	±	+
Conditioned media from Mϕ incubated in MEM			
A. − LPS, + L-Arg	8	0	0
B. + LPS, − L-Arg	5	3	0
C. + LPS, + L-Arg	0	0	8
D. + LPS, + D-Arg	4	1	0
E. + LPS, + L-Arg, + L-NAME	6	3	0
F. + LPS, + L-Arg, + L-NMMA	5	3	0
G. + LPS, + L-Arg, + L-NAME, + L-NMMA	7	2	0
Medium from group C; test compounds added to conditioned medium			
H. + L-NAME	0	0	7
I. + L-NMMA	0	0	6
J. + L-NAME, + L-NMMA	0	0	7

*When present, LPS was 5 μg/ml; L- and D-arginine (L-Arg and D-Arg) were 0.6 mM, and L-NAME and L-NMMA were 0.6 mM.
†Angiogenic responses were graded as follows: +, strong growth of a dense network of capillary buds and sprouts from the limbal vasculature toward the Hydron implant. By day 7 after implantation, the new vessels had reached and invaded the implant: ±, occasional sprouts from limbal vasculature observed by day 3, usually not progressing further by day 7; and −, no capillary sprouting from limbal vasculature.

culture medium. To determine if this requirement for L-arginine was dependent on the activity of the enzyme NO synthase, experiments incorporating specific inhibitors of NO synthase, L-NMMA and L-NAME, were carried out. At a concentration of 0.6 mM (1:1 molar ratio of inhibitors to L-arginine), these inhibitors, either alone or in combination, strongly inhibited the production of angiogenic activity by the LPS-stimulated Mϕ. Preliminary experiments indicated that the inhibition was dose dependent, with lower concentrations being less effective. No decrease in Mϕ viability, assessed by determining trypan blue exclusion and the release of LDH, was observed under the various test conditions (data not shown).

To determine whether L-NMMA and L-NAME had a direct effect on MDAA, the inhibitors were added directly to angiogenically active conditioned media prepared from LPS-stimulated Mϕ prepared in MEM containing L-arginine. These media were then tested in the corneal bioassay. No inhibition of the angiogenic responses was observed, indicating that their effect was to inhibit the generation of angiogenic activity by Mϕ rather than to inhibit the Mϕ-derived angiogenic factor(s) directly.

Chemotaxis of HUVECs by Mϕ-Conditioned Media. The ability of the various Mϕ-conditioned media to induce migration of HUVECs across 8-μm pore size gelatin-coated polycarbonate membranes was tested, and the results are presented in Fig. 1.

As was observed in the *in vivo* corneal angiogenesis assay, chemotaxis of HUVECs was also dependent on the presence of L-arginine in the Mϕ culture medium. Media from Mϕ cultured in the absence of L-arginine or in the presence of D- rather than L-arginine did not induce HUVEC migration. Both L-NMMA and L-NAME inhibited production of chemotactic activity by LPS-stimulated Mϕ but did not directly inhibit the Mϕ-derived chemotactic activity.

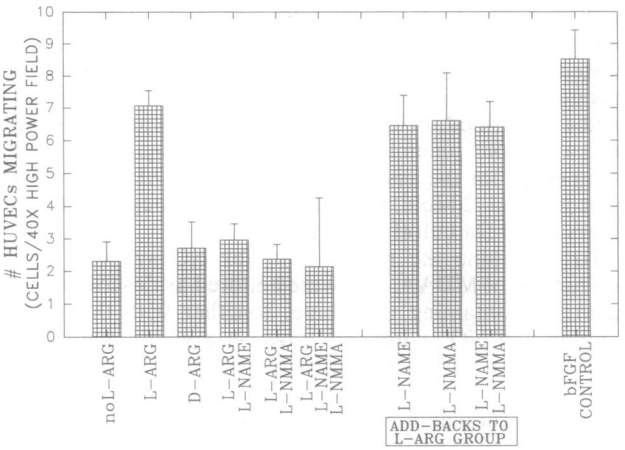

FIG. 1. Chemotaxis of HUVECs in response to human Mϕ-conditioned media. Migration of HUVECs across gelatin-coated 8-μm polycarbonate filters in a microwell chemotaxis chamber in response to conditioned media from Mϕ stimulated with LPS (5 μg/ml) was determined as described in the text. Filters were examined under a 40× objective in the light microscope. The number of cells that had migrated through the pores across the membrane was counted. Each test sample was assayed in quadruplicate, and at least three high-power fields per well were counted. Results shown are the mean ± SEM of three experiments. Basic fibroblast growth factor (bFGF) (60 nM) (R & D Systems) was used as a positive control. L-NAME and L-NMMA were used at 0.6 mM.

Nitrite and Nitrate Concentrations of Mϕ-Conditioned Media. L-Arginine is a substrate for the production of NO by NO synthase. NO is then converted nonenzymatically to NO_2^- and NO_3^- (16, 20). To determine whether the NO-synthase pathway was indeed functional in human Mϕ under the conditions used in our experiments, we measured the production of these ions under the various conditions of Mϕ culture. The results for the levels of NO_2^- and NO_3^- in the Mϕ-conditioned media are shown in Fig. 2.

The production of NO_2^- and NO_3^- by Mϕ was dependent on stimulation by LPS (5 μg/ml) and on the presence of L-arginine in the culture medium. D-Arginine did not substitute for L-arginine as a substrate for the production of these ions, as indicated by the low level of production of NO_2^- and NO_3^- in this group. Both L-NMMA and L-NAME, either alone or in combination, inhibited production of NO_2^- and NO_3^- by

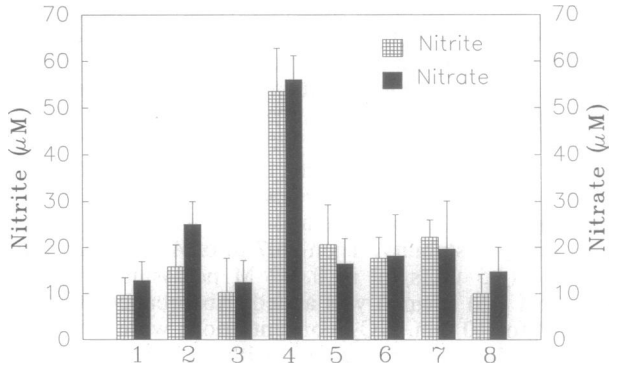

FIG. 2. Production of NO_2^- and NO_3^- by human Mϕ. Purified adherent Mϕ were incubated for 48 hr in the media indicated below. LPS (5 μg/ml) and L-NAME and L-NMMA (0.6 mM) were added to the incubation media as indicated. Media were harvested and assayed for nitrite and nitrate as described in the text. 1, No LPS, no L-arginine; 2, LPS, no L-arginine; 3, no LPS, L-arginine; 4, LPS, L-arginine; 5, LPS, L-arginine, L-NAME; 6, LPS, L-arginine, L-NMMA; 7, LPS, L-arginine, L-NAME, L-NMMA; 8, LPS, D-arginine. Each sample was assayed in triplicate. Results are the mean ± SEM of three separate experiments.

LPS-stimulated Mϕ cultured in the presence of L-arginine (0.6 mM). This inhibition was dose dependent, with lower concentrations of L-NMMA or L-NAME exhibiting less inhibition of NO_2^- and NO_3^- production.

TNF-α and IL-8 Concentrations of Mϕ-Conditioned Media. Since both TNF-α and IL-8 have been implicated as important angiogenic mediators produced by stimulated Mϕ (32–34), we assayed the production of these cytokines under the various culture conditions, to determine if cytokine production was dependent upon L-arginine or modified by the inhibitors of NO synthase. The concentrations of TNF-α and IL-8 in the various Mϕ-conditioned media are shown in Fig. 3. Results shown are the mean ± SEM for three separate experiments.

Production of both TNF-α and IL-8 was dependent upon stimulation of Mϕ with LPS. Conditioned media from nonstimulated Mϕ did not contain demonstrable levels of these cytokines, while conditioned medium from LPS-stimulated Mϕ prepared in MEM plus L-arginine contained about 800 pg of TNF-α per ml and 400 ng of IL-8 per ml. In the absence of L-arginine, however, TNF-α or IL-8 production was not reduced significantly. Also, the NO-synthase inhibitors L-NMMA and L-NAME did not significantly decrease the levels of these cytokines in Mϕ-conditioned media in the presence of L-arginine.

DISCUSSION

In this study we show that the production of angiogenic activity by human Mϕ is dependent on the presence of L-arginine in the culture medium. Also, production of angiogenic activity requires the activity of the enzyme NO synthase. This was indicated by the specific inhibition of production of both angiogenic activity and NO_3^- and NO_2^- by the NO-synthase inhibitors L-NMMA and L-NAME. We have assayed angiogenesis *in vivo* in this study by using the corneal implant technique (1, 3–5, 31) and *in vitro* by the chemotaxis of endothelial cells across gelatin-coated 8-μm pore size polycarbonate membranes (7, 32, 34, 35). In both these assays, a clear dependence of Mϕ on the presence of L-arginine and the action of NO synthase for the production of activity was demonstrated (Table 1 and Fig. 1).

As we and others have reported previously, production of angiogenic and chemotactic activity is dependent on stimulation of Mϕ. In the experiments reported here, we stimulated Mϕ with LPS at 5 μg/ml, which consistently induced Mϕ expression of angiogenic activity. Omitting L-arginine from the culture medium during the incubation of Mϕ with LPS markedly reduced the production of angiogenic and chemotactic activity. D-Arginine was not able to substitute for L-arginine in this system, indicating the stereospecificity of the reaction. In the presence of L-arginine, the inhibitors of NO synthase L-NMMA and L-NAME both markedly decreased the production of angiogenic and chemotactic activity, suggesting that the enzyme NO synthase is critically involved in the process by which these activities are generated in Mϕ. Previous studies using human mononuclear cells have demonstrated NO production (23, 36, 37), and LPS stimulation of NO production by human Mϕ has recently been demonstrated (38). Under our conditions of Mϕ stimulation, using 5 μg of LPS per ml in MEM plus 0.5% FCS, human Mϕ clearly produced NO_2^- and NO_3^- at levels that are 5- to 8-fold higher than the level in the nonstimulated cells, using a pathway that was inhibited by the NO-synthase inhibitors L-NMMA and L-NAME (Fig. 2). These conditions were selected in our experiments because we used them previously to induce production of MDAA by human Mϕ (4, 26–28).

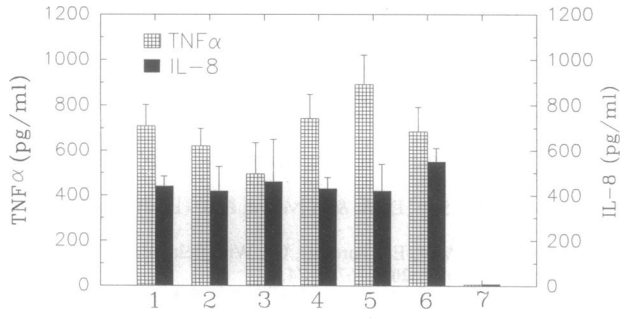

FIG. 3. Production of TNF-α and IL-8 by human Mϕ. Purified adherent Mϕ were incubated in the media indicated below for 48 hr. LPS (5 μg/ml) and L-NAME or L-NMMA (0.6 mM) were added to the incubation media as indicated. Media were harvested and assayed for TNF-α and IL-8 by ELISA. 1, LPS, no L-arginine; 2, LPS, L-arginine; 3, LPS, D-arginine; 4, LPS, L-arginine, L-NAME; 5, LPS, L-arginine, L-NMMA; 6, LPS, L-arginine, L-NAME, L-NMMA; 7, no LPS, L-arginine. Each sample was assayed in duplicate. Results shown are the mean ± SEM of three separate experiments.

NO is highly unstable, with a half-life in the order of seconds in the presence of oxygen under the standard culture conditions we have used (16–25). It is unlikely, therefore, that NO itself is the direct, active mediator of the Mϕ-derived angiogenic and chemotactic activity. A more stable product, generated through the pathway utilizing L-arginine via NO synthase, may be of major importance as a component of the Mϕ-derived angiogenic and chemotactic activity (39). On the other hand, this pathway may inactivate an inhibitor of angiogenesis present in the conditioned medium. We have shown previously that Mϕ produce cytokines such as TNF-α and IL-8 (32, 34) and that these cytokines are potently angiogenic in bioassays *in vivo* and exhibit chemoattractant activity for HUVECs and microvascular endothelial cells in assays *in vitro*. Using neutralizing antibodies, we have shown that these cytokines account for a major portion of the MDAA. In this study, we assayed by ELISA the production of TNF-α and IL-8 by human Mϕ under conditions where synthesis of NO was either promoted or inhibited, to determine if there was any dependence on this pathway for the induction of these cytokines. Induction of these cytokines was clearly dependent on the stimulation of Mϕ by LPS (Fig. 3). In the absence of LPS no detectable amounts of these cytokines were produced. However, no dependence on the presence of L-arginine or on the activity of NO synthase for the production of TNF-α or IL-8 in LPS-stimulated Mϕ was observed. The stimulation-dependent gene induction events involved in the increased transcription and translation of these cytokines are thus independent of the activity of the inducible enzyme NO synthase. Also, L-arginine is not required as an essential amino acid for their production.

In the experiments reported here, inhibition of the NO-synthase-dependent pathway abrogated the production of angiogenic activity, while the levels of expression of TNF-α and IL-8 were not significantly changed. This lack of correlation of expression of angiogenic activity with the expression of TNF-α and IL-8 poses an interesting problem. Several possibilities exist to explain these results. First, production of MDAA has been shown previously to be under the control of suppressor genes that are dominantly expressed in nonstimulated Mϕ (8, 40, 41). Although the mechanism of this suppression is not clear, it may be due at least in part to the production of angiogenesis inhibitors such as thrombospondin (35) and cytokine inhibitors such as TNF-α-binding proteins (42, 43) in the unstimulated cells. The generation of angiogenic activity would thus depend on a critical balance between the production of stimulators and inhibitors by the cells. It is possible that inhibition of NO synthase or deprivation of L-arginine may disrupt the balance between production of angiogenic cytokines such as TNF-α and IL-8 and the production of angiogenesis inhibitors, resulting in the maintenance of a nonangiogenic phenotype.

Alternatively, the angiogenic activity of cytokines may require either their direct modification by the NO-synthase pathway or the cooperation of cofactors that potentiate their angiogenic activity. The results of the TNF-α ELISA of the Mϕ-conditioned media presented in this study indicate that 1 ng of TNF-α per ml was the maximum attained in the unconcentrated conditioned media (Fig. 3). We have previously reported that in the corneal implant bioassay approximately 1–5 ng of TNF-α is required as the minimum dose incorporated in a 5-μl slow-release Hydron pellet implant to induce rat corneal angiogenesis (32). Five microliters of 20× concentrated medium used for a single Hydron implant contains a maximum of 0.1 ng of TNF-α, 0.1 to 0.02 the minimal amount previously shown to induce angiogenesis in the cornea. Thus, the contribution of TNF-α to the total angiogenic activity may be minor. The more likely possibilities, however, are either direct modification of the cytokines by the NO-synthase pathway, with a concomitant promotion of their activity, or synergism between TNF-α and other Mϕ products that are dependent on the L-arginine/NO-synthase effector mechanism. These mechanisms would explain the abrogation of angiogenic activity reported previously for studies using specific antibodies to TNF-α and IL-8 (31, 34). It has also been suggested that all the angiogenic cytokines and growth factors, including TNF-α and fibroblast growth factor (FGF), induce a low-level infiltration of macrophages when implanted in the corneal stroma that is not detectable as an overt inflammatory response (44). Thus, it may be that the angiogenic activity of cytokines such as TNF-α in the cornea may be mediated in part by these infiltrating induced macrophages. The L-arginine/NO-synthase effector mechanism of these infiltrating cells may be critical in the mechanism of angiogenesis induction. Again, it is possible that there is important synergy between the direct effects of the cytokines on the endothelium and their effects in inducing the influx of macrophages and the activation of their L-arginine/NO-synthase-dependent activity (45, 46).

Clearly, a critical reevaluation of the role of TNF-α and IL-8 in the induction of angiogenesis is required in the light of the L-arginine/NO-synthase component of the production of angiogenic activity by Mϕ. The data also support the idea that the modulation of the L-arginine-dependent NO-synthase pathway of Mϕ may have important consequences in inflammatory angiogenesis and fibroproliferative responses.

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