[CANCER RESEARCH 61, 1100–1106, February 1, 2001]

Macrophage Arginase Promotes Tumor Cell Growth and Suppresses Nitric Oxide-mediated Tumor Cytotoxicity[1]

Chiung-I Chang, James C. Liao, and Lih Kuo[2]

Department of Medical Physiology, Cardiovascular Research Institute, Texas A&M University System Health Science Center, College Station, Texas 77843-1114 [C-I. C., L. K.], and Department of Chemical Engineering, University of California, Los Angeles, California 90095-1592 [J. C. L.]

ABSTRACT

Macrophages use L-arginine to synthesize nitric oxide (NO) and polyamines through the inducible NO synthase (iNOS) and arginase, respectively. The released NO contributes to the tumoricidal activity of macrophages, whereas polyamines may promote the growth of tumor cells. Both the tumoricidal and growth-promoting activities from macrophages have been reported; however, the underlying mechanisms for switching between this dual function of macrophages remain unclear. Here, we test the hypothesis that arginase participates in the switching between the cytotoxic and growth-promoting activities of macrophages toward tumor cells. To alter arginase activity in macrophages, cells (murine macrophage cell line J774A.1) were transfected with the rat liver arginase gene or treated with an arginase inhibitor, L-norvaline. The effects of macrophage arginase activity on the growth-promoting and cytotoxic activities of macrophages toward breast tumor cells (ZR-75–1) were investigated in a coculture system. The results demonstrated that overexpression of arginase in macrophages enhanced L-ornithine and putrescine production and consequently promoted tumor cell proliferation. This proliferative effect was down-regulated by the arginase inhibitor L-norvaline. Furthermore, increases in arginase activity also attenuated NO production by the lipopolysaccharide-activated macrophages and thus reduced the cytotoxic effect on cocultured tumor cells. Inhibiting arginase activity by L-norvaline effectively reversed the suppression of NO-mediated tumor cytotoxicity. Together, these results suggest that arginase induction in macrophages can enhance tumor cell growth by providing them with polyamines and suppress tumor cytotoxicity by reducing NO production. It appears that L-arginine metabolism through the arginase and iNOS pathways in macrophages can have very different influences on the growth of nearby tumor cells depending on which pathway is prevailing.

INTRODUCTION

Macrophages are present in most tumor sites and are the most abundant filtrating cell type in tumors (1, 2). It has long been recognized that activated macrophages can distinguish tumor cells from their normal cellular counterparts and are capable of reducing tumor cell growth and achieving tumor cytotoxicity without the aid of specific antibodies (2–4). On the other hand, sufficient evidence has also accumulated to conclude that macrophages, under certain circumstances, can stimulate cancer growth (1, 5). Although the mechanism underlying this dual nature of macrophages remains unclear, recent studies have shown that the pathways by which macrophages metabolize L-arginine may influence their tumoricidal function (5, 6).

In macrophages, L-arginine is metabolized by iNOS to form NO and L-citrulline. The antitumor activity of macrophages is found to be attributable to the production of NO[3] (7). In addition to the iNOS pathway, macrophages also express arginase which converts L-arginine into L-ornithine and urea (8–11). The resultant L-ornithine can be subsequently used by ODC to form polyamines (*i.e.*, putrescine, spermidine, and spermine; 12), the essential nutrients required for the proliferation, differentiation, and neoplastic transformation of mammalian cells (13–16). Thus, it is likely that macrophages are capable of promoting tumor cell proliferation through the arginase pathway. Interestingly, in an animal tumor model, the increase in NOS activity was seen at the stage of tumor rejection, whereas the increase in arginase activity was observed during tumor growth (5). These results suggest that L-arginine metabolism in macrophages at the tumor site, through the iNOS or arginase pathways, may have either detrimental or beneficial influence on the tumor growth, depending on which of the two pathways is prevailing. This contention is supported by the findings that the tumoricidal activity of macrophages was increased when transfected with the iNOS gene (7, 17, 18). However, whether macrophage arginase can promote tumor proliferation and functionally counteract the NO-mediated cytotoxicity remains undetermined. In this regard, we investigated the role of macrophage arginase in tumor growth/cytotoxicity by overexpressing the arginase gene in macrophages. The effects of arginase overexpression on tumor cell proliferation and cytotoxicity were investigated in a coculture system.

MATERIALS AND METHODS

Materials. All chemicals and drugs, except where otherwise noted, were purchased from Sigma Chemical (St. Louis, MO).

Cell Cultures. The murine macrophage cell line J774A.1 and human breast adenocarcinoma cell line ZR-75–1 were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in 60-mm culture dishes with CDMEM supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 50 μg/ml gentamicin, and 10% FBS (Summit Biotechnology, Fort Collins, CO) for macrophages or 5% FBS for tumor cells. Cells were cultured at 37°C under a humidified 10% CO_2 atmosphere. Media were changed daily, and cells were passaged after confluence by trypsinization with DPBS containing 0.25% trypsin (Life Technologies, Inc., Rockville, MD) and 0.02% EDTA. To study the interactions between macrophages and tumor cells, a Falcon cell culture insert system (Fisher Scientific Co., Pittsburgh, PA) was used. In this coculture system, macrophages were grown in the cell culture inserts with a membrane of 1.0-μm pore size, and the tumor cells were grown in the bottom wells of the tissue culture plate.

Arginase Transfection. Macrophages transfected with the rat liver arginase (arginase I) gene were used to characterize the possible roles of macrophage arginase in tumor growth. The arginase I cDNA (Ref. 19; a gift from Dr. Masataka Mori, Department of Molecular Genetics, Kumamoto School of Medicine, Kumamoto, Japan) was cloned into the eukaryotic expression plasmid pEGFP (Invitrogen Corp., Carlsbad, CA). All plasmid DNA was prepared using a plasmid DNA preparation kit from Bio-Rad Laboratories Inc. (Hercules, CA).

Macrophages were transfected using a DEAE-dextran method as described by Rupprecht and Coleman (20) with modifications. Before transfection, macrophages were plated at a density of 5×10^5 cells per insert and incubated overnight with CDMEM containing 10% FBS. Nonadherent cells were re-

Received 6/12/00; accepted 11/27/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by National Heart, Lung, and Blood Institute Grants KO2-HL-03693 (Research Career Award) and HL-55524 (to L. K.).

[2] To whom requests for reprints should be addressed, at Department of Medical Physiology, Cardiovascular Research Institute, Texas A&M University System Health Science Center, College Station, TX 77843-1114. Fax: (979) 847-8635; E-mail: lkuo@tamu.edu.

[3] The abbreviations used are: NO, nitric oxide; iNOS; inducible NO synthase; ODC, ornithine decarboxylase; DFMO, α-difluoromethylornithine; LPS, lipopolysaccharide; L-NMMA, N^G-monomethyl-L-arginine; IL, interleukin; CDMEM, complete DMEM; FBS, fetal bovine serum; DPBS, Dulbecco's PBS; LDH, lactate dehydrogenase.

moved by gently washing the monolayer twice with DPBS, and the adherent cells were used for transfection. A DEAE-dextran stock solution was prepared in DPBS at a concentration of 20 mg/ml and filter sterilized. The transfection medium was then prepared by adding 1.5 ml of DMEM containing 80 μg/ml DEAE-dextran to 1.5 ml of DMEM containing 2 μg DNA of the expression plasmid (pEGFP) or the expression plasmid containing the arginase gene (pEGFP-Arg). The transfection medium was gently mixed at room temperature for 2 min and was added to the insert. After incubating the cells for 90 min at 37°C, the transfection medium was replaced with CDMEM containing 10% FBS. Arginase activity and its protein expression were then evaluated in nontransfected (control) and transfected (*i.e.*, pEGFP and pEGFP-Arg) macrophages after a 24-, 36-, 48-, and 60-h incubation. In a separate set of experiments, cells were transfected with the *β-Gal* gene to determine transfection efficiency using a *β*-Gal staining kit (Invitrogen Corp.) following the manufacturer's instructions.

Arginase Activity Assay and Immunoblotting of Arginase. To prepare cell lysate for arginase activity assay and immunoblotting analysis, cells were first rinsed with ice-cold DPBS twice after each specified treatment and then scraped into 300 μl of lysis buffer containing 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.1 mM EGTA, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride. Finally, cells were lysed by sonication at the frequency of 20 KHz (Sonic & Materials, Inc., Danbury, Connecticut) for 30 s (10 s/cycle). Arginase activity in the cell lysates was measured as described previously (21, 22). In brief, cell lysate (50 μl) was added to 50 μl of Tris-HCl (50 mM; pH 7.5) containing 10 mM $MnCl_2$. Macrophage arginase was then activated by heating this mixture at 55–60°C for 10 min. The hydrolysis reaction of L-arginine by arginase was carried out by incubating the mixture containing activated arginase with 50 μl of L-arginine (0.5 M; pH 9.7) at 37°C for 1 h and was stopped by adding 400 μl of the acid solution mixture (H_2SO_4:H_3PO_4:H_2O = 1:3:7). For colorimetric determination of urea, α-isonitrosopropiophenone (25 μl, 9% in absolute ethanol) was then added, and the mixture was heated at 100°C for 45 min. After placing the samples in the dark for 10 min at room temperature, the urea concentration was determined spectrophotometrically by the absorbance at 550 nm measured with a microplate reader (Molecular Devices, Menlo Park, CA). The amount of the urea produced was used as an index for arginase activity.

Arginase protein was detected with the monoclonal antibody against human arginase I (Transduction Laboratories, Lexington, KY). The cross-reactivity of this antibody with rat arginase I has been demonstrated by the manufacturer. For immunoblotting of arginase I, cell lysates were prepared as described above, and equal amounts (25 μg) of proteins from each sample were subjected to a 10% SDS-PAGE. After electrophoresis, proteins were electrotransferred to nitrocellulose membranes (Bio-Rad Laboratories). The nitrocellulose membranes were incubated with the monoclonal primary antibody against arginase I and then with a horseradish peroxidase-conjugated antimouse IgG antibody. The proteins were visualized using the enhanced chemiluminescence detection kits (Amersham Pharmacia Biotech Inc., Piscataway, NJ).

Cell Proliferation Assay. Tumor cells were seeded at a density of 1.2×10^5 per well of a 6-well culture plate and incubated with CDMEM containing 0.5% FBS for 24 h. Inserts containing nontransfected macrophages or macrophages at 24 h after transfection (*i.e.*, pEGFP or pEGFP-Arg) were then introduced into the culture plate containing tumor cells. To carry out the experiments under a more physiological level of L-arginine, modified RPMI 1640 (Life Technologies, Inc.) containing 100 μM L-arginine, 4% FBS, 200 μM glutamine, 10 mM HEPES, and the antibiotics as described above was used for the coculture experiments. After a 36-h incubation, tumor cell proliferation was determined using a cell proliferation assay kit (CellTiter96 AQ_{ueous} one solution; Promega Corp., Madison, WI). In some experiments, the transfected macrophages were treated with L-norvaline (20 mM), a specific arginase inhibitor, at 6 h after transfection to verify the effect of arginase. At 24 h after transfection (*i.e.*, 18-h treatment of L-norvaline), the macrophages were used for coculture experiments. L-Norvaline was not added to the coculture medium to avoid any direct effects of L-norvaline on tumor cell growth. The macrophage arginase activity and tumor cell proliferation were determined after a 36-h incubation.

To study the direct effect of macrophage-derived polyamines on tumor cell growth, another series of experiments were performed using tumor cells pretreated with DFMO (1 mM; provided by Aventis Pharmaceuticals, formerly known as Hoechst Marion Roussel, Inc., Bridgewater, NJ), a suicide inhibitor of ODC, for 24 h to arrest polyamine synthesis. The contents of L-arginine, L-ornithine, and polyamines (*e.g.*, putrescine, spermine, and spermidine) in macrophages, tumor cells, and the culture media before and after coculture experiments were then measured with high-performance liquid chromatography.

Polyamines, L-Ornithine, and L-Arginine Measurement. The cell culture media were collected (1 ml) and the macrophages and tumor cells were harvested by scraping into 300 μl of DPBS. The cells were lysed with sonication at the frequency of 20 KHz for 30 s (10 s/cycle). Both the cell lysates and coculture media samples were centrifuged at 12,000 $\times$ *g* for 5 min to sediment cell debris and proteins, and the supernatant was passed through the ultra-free filtration units with 5,000 molecular weight cutoff (Millipore Corp., Bedford, MA). The polyamines and amino acids were separated by a C-18 reverse phase-packed column (model Hypersil AA-ODF; Hewlett Packard) and detected with an UV detector after precolumn derivitization of the hydrolyzed primary amino acids or polyamines with *o*-phthalaldehyde (OPA). The concentrations of polyamines, L-arginine, and L-ornithine in these supernatants were then quantified as their OPA derivatives by the AminoQuant System (Hewlett Packard, model HP 1090 L).

Cytotoxicity Assay. At 24 h after transfection, macrophages were treated with LPS (1 μg/ml) for 8 h to induce their cytotoxic activity. The activated macrophages were then cocultured with tumor cells in the RPMI medium containing 50 μM L-arginine. The activity of LDH released by the killed or dying tumor cells in the coculture medium was measured with a cytotoxicity assay kit (CytoTox96, Promega Corp.) and was used as an indicator for tumor cytotoxicity. The percentage cytotoxicity was obtained using the following formula provided by Promega Corp.

The level of NO in the coculture medium was determined using a chemiluminescence assay as described in our previous study (21). In some experiments, a NOS inhibitor, L-NMMA (10 μM), was added together with LPS to confirm the specific effects of NO. In another series of studies, macrophages were treated with L-norvaline (20 mM) at 14 h after the transfection (*i.e.*, for an 18-h incubation before coculture) to examine the role of arginase in counteracting NO-mediated cytotoxicity.

Protein Determination. Proteins from each plate of cells were determined by bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL). Results from all of the experiments are expressed on the basis of cell proteins.

Statistical Analysis. Results are given as mean $\pm$ SE for at least three independent experiments performed in triplicate. The statistical significance of differences between groups was analyzed by one-way ANOVA followed by Fisher's protected least-significant differences test using StatView 4 (Abacus Concepts, Berkeley, CA). A $P < 0.05$ was considered to be statistically significant.

RESULTS

Transfection of the Arginase Gene Increases Arginase Expression and Activity. Arginase activities from macrophages transfected with pEGFP or pEGFP-Arg (at 24, 36, 48, and 60 h after transfection) as well as from nontransfected macrophages (control) were measured. Fig. 1*A* shows that nontransfected and pEGFP-transfected macrophages had low levels of arginase activity (*i.e.*, urea production) that did not change with time. On the other hand, macrophages transfected with pEGPF-Arg exhibited a time-dependent increase in arginase activity. The increase was observed at 24 h after transfection and reached the maximum (~5-fold increase) at 36 h after transfection. Arginase activity remained elevated for up to 48 h and then decreased at 60 h after transfection (Fig. 1*A*). Arginase protein was below the detectable range in both control and pEGFP-transfected macrophages, but its expression was significantly increased after transfection with pEGFP-Arg. The expression of arginase at 36 h after transfection of pEGFP-Arg is shown in Fig. 1*B*. A transfection efficiency assay carried out at 36 h after transfection indicated that about 14 $\pm$ 2% of cells were transfected. Because the arginase activity started to increase at 24 h after transfection, macrophages at this stage were used in the following coculture studies.

Arginase Activity and Tumor Cell Proliferation. The relationship between macrophage arginase activity and tumor cell prolifera-

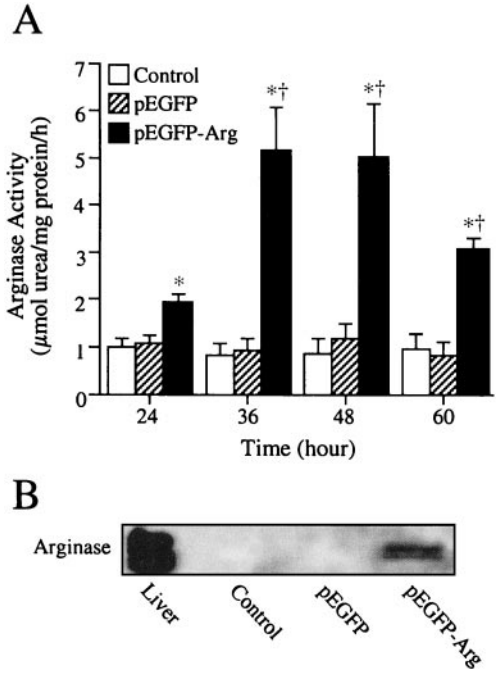

Fig. 1. Transfection of pEGFP-Arg increases enzyme activity and protein expression of arginase in macrophages. Cells were transfected with pEGFP or pEGFP-Arg using the DEAE-dextran method. *A*, arginase activities at different times after transfection were measured. In nontransfected cells (*Control*) or cells transfected with pEGFP, arginase activity remained low and did not change with time. Transfection of pEGFP-Arg increased arginase activity significantly at 24 h. The arginase activity reached a maximum at 36 h and began to decrease at 60 h after transfection. Values are means ± SE; $n = 6$. ∗, significantly different from control ($P < 0.05$); †, significantly different from pEGFP-Arg at 24 h ($P < 0.05$). *B*, Western blot analysis of arginase expression at 36 h after transfection. Arginase protein was not detectable in nontransfected (*control*) and pEGFP-transfected (*pEGFP*) cells, but was detected in macrophages transfected with pEGFP-Arg. Protein from rat liver cells was used as a positive control and each lane was loaded with an equal amount (25 μg) of protein.

tion was investigated after coculturing the cells for 36 h; the results are shown in Fig. 2. Macrophages transfected with pEGFP did not increase arginase activity and had no effect on tumor cell proliferation. On the other hand, transfection of pEGFP-Arg significantly increased arginase activity and enhanced proliferation of the tumor cells by 42% (Fig. 2*A*). Pretreating the pEGFP-Arg-transfected macrophages with an arginase inhibitor L-norvaline significantly inhibited arginase activity and concomitantly reduced the growth of cocultured tumor cells (Fig. 2*A*). These results indicate that overexpression of macrophage arginase enhances tumor cell proliferation. However, the observed overall tumor cell growth in this set of studies resulted from the contribution of both exogenously (*i.e.*, from macrophages)- and endogenously (*i.e.*, from tumor cells *per se*)-synthesized polyamines. To exclude the contribution of endogenously synthesized polyamines, tumor cells were pretreated with DFMO, and the effect of macrophage arginase on tumor cell growth was reexamined. As shown in Fig. 2*A*, overexpression of arginase in macrophages enhanced the growth of DFMO-treated tumor cells by 82%. L-Norvaline inhibited arginase activity in pEGFP-Arg-transfected macrophages (Fig. 2*B*) and also reduced their ability to promote tumor cell growth.

Intracellular and Extracellular Levels of L-Arginine, L-Ornithine, and Polyamines. To test our hypothesis that L-arginine metabolism plays a role in the macrophage-associated tumor proliferation, the amounts of L-arginine and its downstream metabolites, *i.e.*, L-ornithine and polyamines in macrophages, coculture media, and the cocultured tumor cells (pretreated with DFMO) were measured. Fig. 3*A* shows that L-arginine concentration in the medium was significantly reduced from 100 nmol/ml (Fig. 3*A*, *Medium only*) to 30 nmol/ml as tumor cells were cocultured with either nontransfected (Fig. 3*A*, *Control*) or pEGFP-transfected (Fig. 3*A*, *pEGFP*) macrophages. In the presence of pEGFP-Arg-transfected macrophages, L-arginine concentration in the medium was further reduced to 18 nmol/ml (Fig. 3*A*). This reduction was prevented by treating the pEGFP-Arg-transfected macrophages with L-norvaline. The intracellular level of L-arginine was not altered either by arginase transfection or by L-norvaline (Fig. 3*B*).

In contrast to the reduction of L-arginine in the coculture medium, the concentration of L-ornithine in the medium was generally increased, particularly in the presence of macrophages transfected with pEGFP-Arg (Fig. 4*A*). Intracellular L-ornithine was also significantly increased in the macrophages transfected with the arginase gene. Treating the transfected macrophages with L-norvaline not only re-

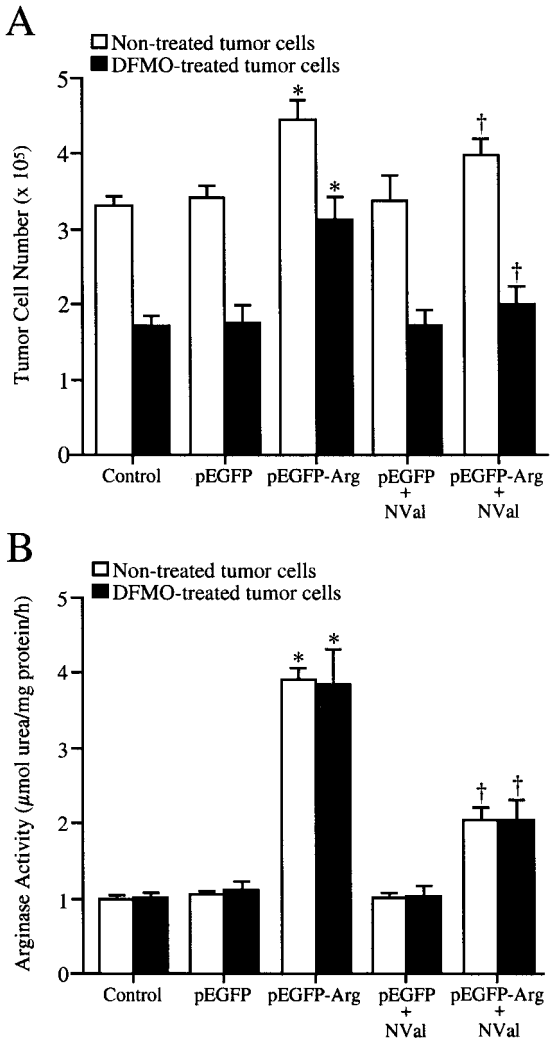

Fig. 2. Transfection of pEGFP-Arg in macrophages enhances the proliferation of tumor cells in the coculture system. Macrophages at 24 h after transfection with pEGFP or pEGFP-Arg and with or without pretreatment of L-norvaline (*NVal*, 20 mM; applied at 6 h after transfection for a total of 18-h treatment) were cocultured with non-DFMO-treated or DFMO-treated tumor cells for 36 h. *A*, total tumor cell number (□, non-DFMO-treated; ■, DFMO-treated) at the end of the coculture. *B*, arginase activity in macrophages cocultured with non-DFMO-treated tumor cells (□) and DFMO-treated tumor cells (■)). Transfection of pEGFP-Arg increased macrophage arginase activity and enhanced the proliferation of tumor cells. The increase in cell proliferation was 42% in non-DFMO-treated tumor cells and 82% in DFMO-treated tumor cells. In both nontreated tumor cells and DFMO-treated tumor cells, transfection of pEGFP did not affect macrophage arginase activity or tumor cell proliferation. Treating macrophages with NVal attenuated the increase in macrophage arginase activity and tumor cell proliferation. Values are means ± SE; $n = 6$. ∗, significantly different from pEGFP ($P < 0.05$); †, significantly different from pEGFP-Arg ($P < 0.05$).

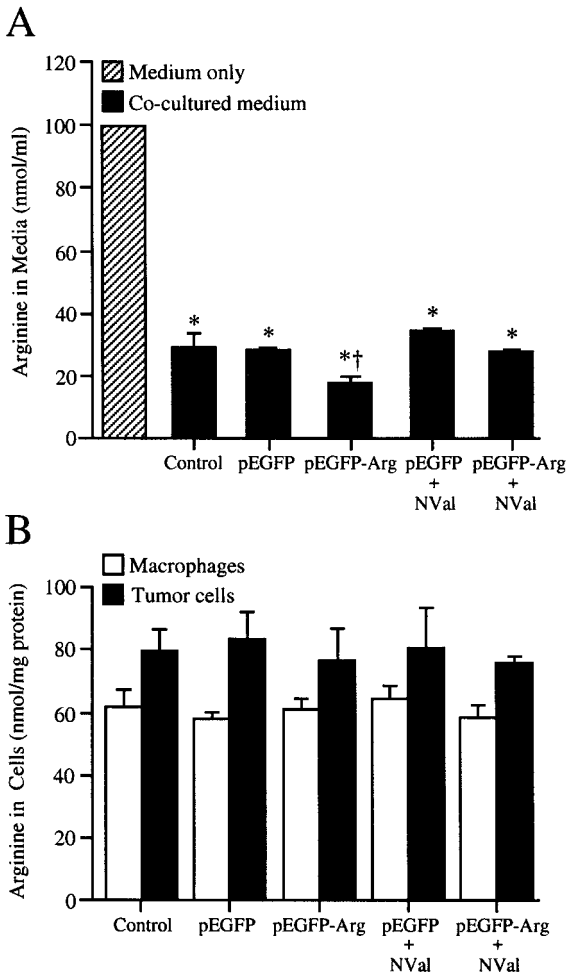

Fig. 3. Effects of pEGFP-Arg transfection in macrophages on L-arginine content in media, macrophages, and tumor cells. *A*, L-arginine concentration in the medium was significantly reduced in the coculture system. The decrease was more profound in the presence of pEGFP-Arg-transfected macrophages, and this reduction in L-arginine was attenuated by pretreating macrophages with L-norvaline (*NVal*, 20 mM). Values are means ± SE; $n = 3$. *, significantly different from "Medium only" ($P < 0.05$); †, significantly different from control and pEGFP-Arg + NVal ($P < 0.05$). *B*, L-arginine levels in macrophages and tumor cells remained the same regardless of treatments, and the level of L-arginine in the tumor cells was consistently higher than that in macrophages. Values are means ± SE; $n = 3$.

duced the L-ornithine in the media but also reduced the intracellular levels of L-ornithine in macrophages (Fig. 4*B*). The L-ornithine content in the tumor cells was consistently lower than that in the macrophages, but its level was significantly increased with the presence of the pEGFP-Arg-transfected macrophages (Fig. 4*B*). This increase was prevented by treating the macrophages with L-norvaline (Fig. 4*B*).

The levels of putrescine, spermidine, and spermine in the tumor cells were 6.0 ± 0.9, 2.1 ± 0.4, and 9.5 ± 0.8 nmol/mg protein, respectively. After treatment with DFMO, the intracellular polyamines were reduced below the detectable range. The spermine, spermidine, and putrescine concentration in the medium alone (*i.e.*, in the absence of cells) was barely detectable; however, putrescine level was significantly elevated in the coculture medium, particularly with the presence of pEGFP-Arg-transfected macrophages (Fig. 5*A*). Similarly, the intracellular putrescine contents were significantly increased in the arginase-transfected macrophages and the corresponding cocultured tumor cells (Fig. 5*B*). Again, pretreating the macrophages with L-norvaline abolished the effect of arginase overexpression (Fig. 5). On the other hand, spermine and spermidine were not detectable in either cell type regardless of treatments.

Effect of Arginase Gene Transfection on NO Production and Tumor Cytotoxicity. To examine whether overexpression of arginase in macrophages affects their NO production and ability to exert cytotoxic activity toward tumor cells, the pEGFP-Arg-transfected macrophages were activated by LPS, and the release of NO as well as tumor cytotoxicity were evaluated. Activation of macrophages with LPS did not affect the arginase activities in either the control or transfected macrophages (Fig. 6*A*). Inhibition of NO synthase (by L-NMMA) also had no effect on arginase activity. In terms of NO production, the nonactivated macrophages (*i.e.*, without LPS treatment) released only a negligible amount of NO (*i.e.*, nitrite measurement) in the cocultured medium (Fig. 6*B*). Under these conditions, the macrophages exerted about 8% cytotoxicity toward tumor cells. LPS elicited a high amount of NO production in the vector-transfected cells and produced a 3-fold increase in tumor cytotoxicity. The increased NO production and tumor cytotoxicity were attenuated by L-NMMA. Transfection of pEGFP-Arg into macrophages also attenuated the LPS-induced NO production as well as the tumor cytotoxicity, whereas L-norvaline partially reversed these inhibitory effects (Fig. 6*B*).

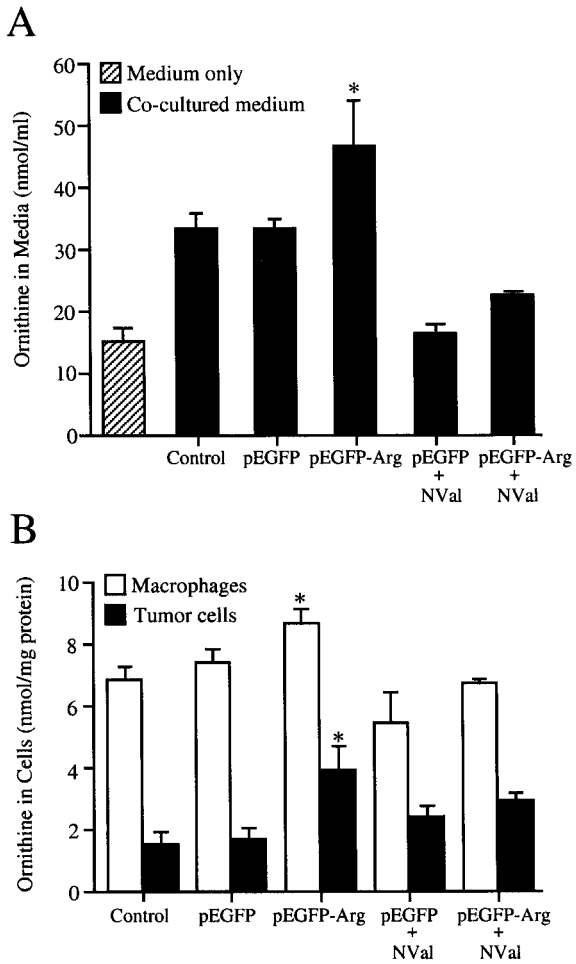

Fig. 4. Effects of pEGFP-Arg transfection in macrophages on L-ornithine content in media, macrophages, and tumor cells. *A*, L-ornithine concentration in the medium was significantly increased in the coculture system. Transfection of pEGFP-Arg further enhanced the L-ornithine content in coculture medium, whereas treating macrophages with L-norvaline (*NVal*, 20 mM) attenuated the effect of pEGFP-Arg transfection. Values are means ± SE; $n = 3$. *, significantly different from all other groups ($P < 0.05$). *B*, L-ornithine content in macrophages and tumor cells was increased by transfection of pEGFP-Arg in macrophages. Treating macrophages with L-norvaline (*NVal*, 20 mM) attenuated the increase of L-ornithine. Values are means ± SE; $n = 3$. *, significantly different from all other groups ($P < 0.05$).

DISCUSSION

Our results provide evidence that L-arginine metabolism by macrophages through the arginase pathway and iNOS pathway plays a significant role in the stimulation and inhibition of tumor growth. Whereas overexpression of arginase in macrophages enhanced tumor cell growth by providing polyamines, the induction of iNOS in macrophages triggered NO-mediated tumor cytotoxicity. Moreover, this NO-dependent tumoricidal effect was attenuated by arginase overexpression, which suggests that the competitive action between arginase and iNOS in macrophages contributes to the regulation of the nearby tumor growth.

In the present study, the correlation between tumor cell proliferation and macrophage arginase activity implicates the beneficial role of macrophage arginase in tumor growth, presumably through the production of L-ornithine and, thus, polyamines. Because arginase uses L-arginine as the substrate for L-ornithine synthesis, it is expected that the consumption of L-arginine would be increased in the presence of macrophages overexpressing arginase. Indeed, a significant decrease in the medium L-arginine in the coculture system was found, which indicated a high consumption rate of L-arginine by cultured cells, and this reduction in extracellular L-arginine was more pronounced in the presence of pEGFP-Arg-transfected macrophages. Treating the pEGFP-Arg-transfected macrophages with the arginase inhibitor L-norvaline prevented the reduction in L-arginine content in the medium, suggesting a direct result of arginase overexpression in this event. However, the levels of intracellular L-arginine remained unchanged in both macrophages and tumor cells regardless of different interventions, which implied that these cells mainly use exogenous L-arginine to maintain the homeostasis of an intracellular pool. This finding

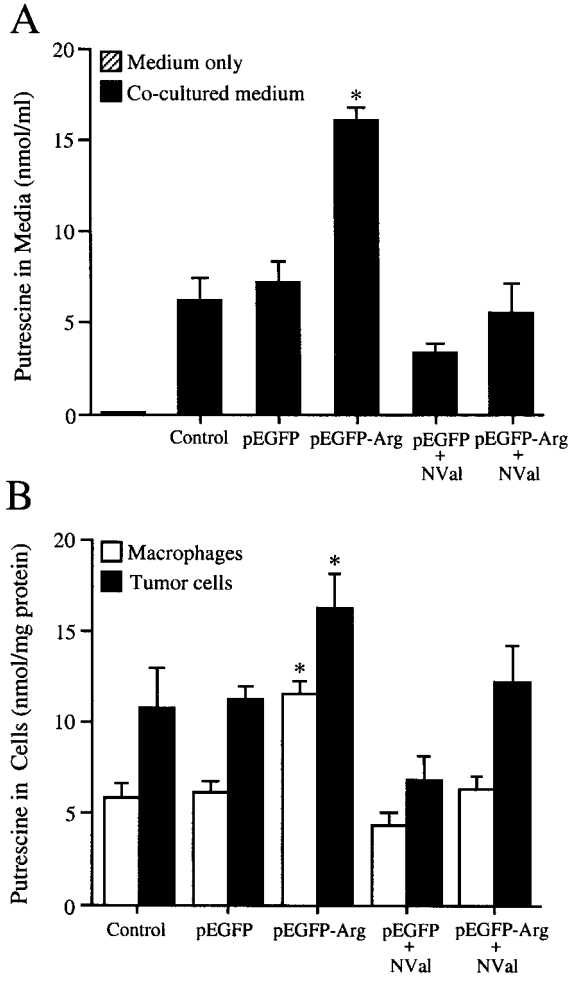

Fig. 5. Effects of pEGFP-Arg transfection in macrophages on putrescine content in media, macrophages, and tumor cells. *A*, putrescine concentration in the medium was significantly increased in the coculture system. Transfection of pEGFP-Arg increased putrescine content in coculture medium, whereas pretreating macrophages with L-norvaline (*NVal*, 20 mM) attenuated the increase. Values are means ± SE; $n = 3$. *, significantly different from all other groups ($P < 0.05$). *B*, putrescine content in macrophages and tumor cells was increased by transfection of pEGFP-Arg in macrophages. Treating macrophages with L-norvaline (*NVal*, 20 mM) attenuated the increase of L-ornithine. Values are means ± SE; $n = 3$. *, significantly different from all other groups ($P < 0.05$).

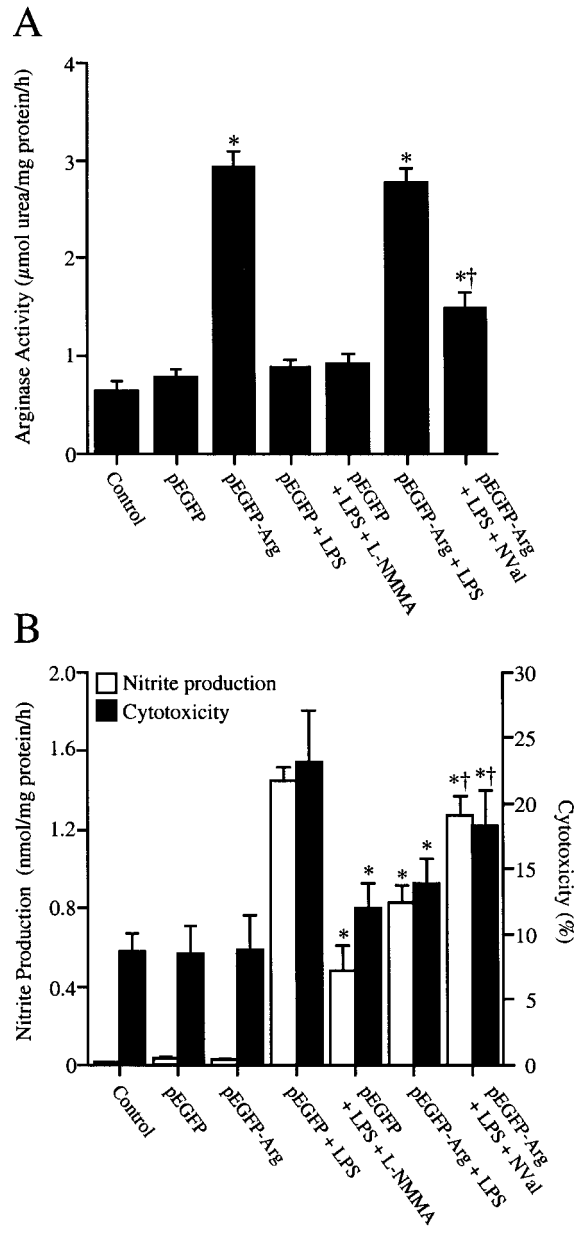

Fig. 6. Effects of arginase overexpression on macrophage NO production and tumor cytotoxicity. *A*, transfection of pEGFP-Arg significantly increased macrophage arginase activity. This increased arginase activity was not affected by LPS but was down-regulated by L-norvaline (*NVal*, 20 mM). L-NMMA also had no effect on arginase activity. Values are means ± SE; $n = 5$. *, significantly different from control ($P < 0.05$); †, significantly different from pEGFP-Arg + LPS ($P < 0.05$). *B*, the basal release of NO and tumor cytotoxicity exerted by resting macrophages was not affected by transfection. However, the release of NO and tumor cytotoxicity was enhanced in the presence of LPS-activated macrophages. These effects were attenuated by treating the macrophages with L-NMMA (10 μM) or by transfection of pEGFP-Arg. L-norvaline (*NVal*, 20 mM) partially restored the attenuated NO production and tumor cytotoxicity caused by pEGFP-Arg transfection. Values are means ± SE; $n = 5$. *, significantly different from pEGFP + LPS ($P < 0.05$); †, significantly different from pEGFP-Arg + LPS ($P < 0.05$).

agrees with a previous study by Hrabák *et al.* showing that macrophage arginase mainly uses extracellular L-arginine, although the level of L-arginine within these cells was not reported (23).

In contrast to L-arginine, the L-ornithine concentration in the coculture medium was elevated, particularly in the presence of macrophages transfected with the arginase gene. In addition, the L-ornithine level in both macrophages and tumor cells was also increased as a result of arginase overexpression in macrophages. Because the increase in L-ornithine level was abolished by pretreating macrophages with L-norvaline and the arginase activity in tumor cells was not altered during the coculture experiments (data not shown), we conclude that the increased L-ornithine in tumor cells is derived from the macrophage arginase. Interestingly, the L-ornithine level in macrophages was found to be consistently higher than that in the tumor cells, which may be attributable to the 3- to 4-fold higher arginase activity generally found in control macrophages than in tumor cells (data not shown). The low arginase activity and low L-ornithine level in tumor cells may also imply that they are heavily dependent on the exogenous supply of L-ornithine. As mentioned earlier, L-ornithine is converted to putrescine by a reaction catalyzed via ODC, thus the increased L-ornithine may contribute to the tumor cell growth through polyamine formation.

It should be noted that in the present study, the tumor cells were pretreated with DFMO, a suicide inhibitor for ODC that prevents the conversion of L-ornithine to putrescine. Therefore, these tumor cells were incapable of using L-ornithine for polyamine synthesis. This contention was supported by our study that showed DFMO completely blocked ODC activity in tumor cells as indicated by the depletion of endogenous polyamines. In this regard, it is believed that the observed increased tumor growth in the present study is a result of the increased availability of exogenous polyamines provided by the macrophages overexpressing arginase. Although three main polyamines, *i.e.,* putrescine, spermidine, and spermine are found in mammalian cells, only putrescine was detected in macrophages in our study. This result is consistent with the findings of Boutard *et al.* that macrophages produced mainly putrescine (6). The putrescine content in the coculture medium and in the macrophages as well as the tumor cells was significantly increased when the pEGFP-Arg-transfected macrophages were present. Interestingly, the amount of putrescine in the tumor cells was generally higher than that in macrophages, which suggests that these tumor cells are capable of taking up putrescine from the extracellular source (16, 24). The ability of tumor cells to use exogenous polyamines may also explain why ODC inhibitor DFMO can potently deplete polyamines and thus inhibit cells growth in most cell types in a mono-cell type culture system, but was ineffective in animal models (25, 26) or in a coculture system as shown in the present study. Although we did not measure the transport of putrescine in tumor cells, our results indicate that arginase overexpression leads to a higher production of putrescine in macrophages. It appears that the putrescine released into the coculture medium is subsequently taken up by the tumor cells for their growth because blockade of arginase activity in macrophages prevented the increase of putrescine in both the coculture medium and tumor cells and consequently attenuated tumor cell proliferation.

In addition to arginase, the other main L-arginine-consuming enzyme iNOS is expressed in activated macrophages, and the generated NO is known to mediate the cytotoxic effects of macrophages (7, 27–29). Our results show that a high amount of NO production in macrophages, after LPS-activation, was accompanied with high tumor cytotoxicity. Interestingly, the nonactivated macrophages also exhibited some degrees of tumor cytotoxicity (~8%). This basal cytotoxic effect is independent of NO because the release of NO from resting macrophages is negligible, and this basal cytotoxicity is insensitive to a NOS inhibitor L-NMMA (data not shown). It is likely that the release of reactive oxygen species and proteases by macrophages (2, 3) in response to "irritating" products released from tumor cells (1) accounts for this basal cytotoxicity. Nevertheless, the increased tumoricidal effect of LPS-activated macrophages was abolished by L-NMMA, indicating the important role of NO in tumoricidal process. Overexpression of arginase in macrophages resulted in a reduction of NO production and also attenuated the tumor cytotoxicity. These effects were reversed by the arginase inhibitor L-norvaline, which suggests the direct role of arginase in regulating NO production and thus the tumoricidal activity of activated macrophages. The down-regulation of NO production by arginase might be a result of substrate competition, *e.g.,* reduction of L-arginine availability to iNOS by the up-regulated arginase (21). We have previously shown that the effect of arginase on NO production is more pronounced when the extracellular supply of L-arginine is limited (21). Therefore, in the present study, the reduced L-arginine content in the medium, caused by arginase overexpression, might further enhance the counteraction of arginase on the tumoricidal function of macrophages. Although a decreased extracellular level of L-arginine was commonly observed in wounds and during septic shock (30–32), the L-arginine level at the sites of tumors has not yet been determined. It is expected that the change in L-arginine availability will significantly influence the biological function of macrophages.

Together, our results suggest that the tumoricidal and growth-promoting activities of macrophages are regulated by the L-arginine metabolism through the iNOS and arginase pathways. Because both arginase and iNOS pathways have been shown to be up-regulated under certain pathophysiological conditions (5, 6, 33–35), macrophages can therefore have very different functions in tumor growth, depending on which of the two pathways is prevailing. However, it is not yet known what factor, or combination of factors, is responsible for the regulation of iNOS or arginase activities in macrophages at tumor sites. Nevertheless, a recent study has shown that transforming growth factor–β can attenuate the macrophage-mediated tumor cytolysis by down-regulating iNOS activity, although enhancing arginase activity in macrophages (6). In addition, several cytokines such as IL-4, IL-10 (33, 35), and IL-13 (36) have been reported to induce arginase activation in macrophages. However, the involvement of these cytokines in the regulation of tumor cytotoxicity/growth remains to be elucidated. Furthermore, it has been shown that arginase activity can be inhibited by N^G-hydroxy-L-arginine, an intermediate of the NOS pathway (37). The cross-regulation between the NOS and arginase pathways further amplifies the complexity of the regulatory mechanism of L-arginine metabolism toward NO and polyamines. Understanding the regulation of iNOS and arginase activation and their reciprocal interaction may provide novel information for controlling tumor growth.

ACKNOWLEDGMENTS

We thank Dr. Travis W. Hein for helpful discussions and critical evaluation of the manuscript.

REFERENCES

1. Mantovani, A., Bottazzi, B., Colotta, F., Sozzani, S., and Ruco, L. The origin and function of tumor-associated macrophages. Immunol. Today, *13:* 265–270, 1992.
2. Seljelid, R., and Busund, L. T. The biology of macrophages: II. Inflammation and tumors. Eur. J. Haematol., *52:* 1–12, 1993.
3. Duerksen-Hughes, P. J., and Gooding, L. R. Macrophage-mediated cytotoxicity. *In:* M. V. Sitkovsky and P. A. Henkart (eds.), Cytotoxic Cells, pp. 439–467. Boston: Birkhäuser, 1993.
4. Seljelid, R., and Eskeland, T. The biology of macrophages: I. General principles and properties. Eur. J. Haematol., *51:* 267–275, 1993.

5. Mills, C. D., Shearer, J., Evans, R., and Caldwell, M. D. Macrophage arginine metabolism and the inhibition or stimulation of cancer. J. Immunol., *149:* 2709–2714, 1992.
6. Boutard, V., Havouis, R., Fouqueray, B., Philippe, C., Moulinoux, J. P., and Baud, L. Transforming growth factor-β stimulates arginase activity in macrophages: implications for the regulation of macrophage cytotoxicity. J. Immunol., *155:* 2077–2084, 1995.
7. Lala, P. K. Significance of nitric oxide in carcinogenesis, tumor progression and cancer therapy. Cancer Metastasis Rev., *17:* 1–6, 1998.
8. Gotoh, T., Sonoki, T., Nagasaki, A., Terada, K., Takiguchi, M., and Mori, M. Molecular cloning of cDNA for nonhepatic mitochondrial arginase (arginase II) and comparison of its induction with nitric oxide synthase in a murine macrophage-like cell line. FEBS Lett., *395:* 119–122, 1996.
9. Chen, P. C., and Broome, J. D. Mouse macrophage arginase (40777). Proc. Soc. Exp. Biol. Med., *163:* 354–359, 1980.
10. Ryan, J. L., Yohe, W. B., and Morrison, D. C. Stimulation of peritoneal cell arginase by bacterial lipopolysaccharides. Am. J. Pathol., *99:* 451–461, 1980.
11. Sonoki, T., Nagasaki, A., Gotoh, T., Takiguchi, M., Takeya, M., Matsuzaki, H., and Mori, M. Coinduction of nitric-oxide synthase and arginase I in cultured rat peritoneal macrophages and rat tissues *in vitro* by lipopolysaccharide. J. Biol. Chem., *272:* 3689–3693, 1997.
12. Pegg, A. E., Shantz, L. M., and Coleman, C. S. Ornithine decarboxylase: structure, function and translational regulation. Biochem. Soc. Trans., *22:* 846–852, 1994.
13. Auvinen, M., Passinen, A., Andersson, L. C., and Hölttä, E. Ornithine decarboxylase activity is critical for cell transformation. Nature (Lond.), *360:* 355–358, 1992.
14. Davidson, N. E., Hahm, H. A., McCloskey, D. E., Woster, P. M., and Casero, R. A., Jr. Clinical aspects of cell death in breast cancer: the polyamine pathway as a new target for treatment. Endocr. Relat. Cancer, *6:* 69–73, 1999.
15. Janne, J., Alhonen, L., and Leinonen, P. Polyamines: from molecular biology to clinical applications. Ann. Med., *23:* 241–259, 1991.
16. Marton, L. J., and Pegg, A. E. Polyamines as targets for therapeutic intervention. Annu. Rev. Pharmol. Toxicol., *35:* 55–91, 1995.
17. Lorsbach, R. B., Murphy, W. J., Lowenstein, C. J., Snyder, S. H., and Russell, S. W. Expression of the nitric oxide synthase gene in mouse macrophages activated for tumor cell killing: molecular basis for the synergy between interferon-γ and lipopolysaccharide. J. Biol. Chem., *268:* 1908–1913, 1993.
18. Xie, K., and Fidler, I. J. Therapy of cancer metastasis by activation of the inducible nitric oxide synthase. Cancer Metastasis Rev., *17:* 55–75, 1998.
19. Kawamoto, S., Amaya, Y., Murakami, K., Tokunaga, F., Iwanaga, S., Kobayashi, K., Saheki, T., Kimura, S., and Mori, M. Complete nucleotide sequence of cDNA and deduced amino acid sequence of rat liver arginase. J. Biol. Chem., *262:* 6280–6283, 1987.
20. Rupprecht, A. P., and Coleman, D. L. Transfection of adherent murine peritoneal macrophages with a reporter gene using DEAE-dextran. J. Immunol. Methods, *144:* 157–163, 1991.
21. Chang, C. I., Liao, J. C., and Kuo, L. Arginase modulates nitric oxide production in activated macrophages. Am. J. Physiol. Heart Circ. Physiol., *274:* H342–H348, 1998.
22. Corraliza, I. M., Campo, M. L., Soler, G., and Modolell, M. Determination of arginase activity in macrophages: a micromethod. J. Immunol. Methods, *174:* 231–235, 1994.
23. Hrabák, A., Idei, M., and Temesi, á. Arginine supply for nitric oxide synthesis and arginase is mainly exogenous in elicited murine and rat macrophages. Life Sci., *55:* 797–805, 1994.
24. Grillo, M. A., and Colombatto, S. Polyamine transport in cells. Biochem. Soc. Trans., *22:* 894–898, 1994.
25. Ask, A., Persson, L., Oredsson, S. M., and Heby, O. Synergistic antileukemic effect of two polyamine synthesis inhibitors: host survival and cell-cyclic kinetic analysis. Int. J. Cancer, *37:* 465–470, 1986.
26. Persson, L., Holm, I., Ask, A., and Heby, O. Curative effect of DL-2-difluoromethylornithine on mice bearing mutant L1210 leukemia cells deficient in polyamine uptake. Cancer Res., *48:* 4807–4811, 1988.
27. Hibbs, J. B., Jr., Taintor, R. R., Vavrin, Z., and Rachlin, E. M. Nitric oxide: a cytotoxic activated macrophage effector molecule. Biochem. Biophys. Res. Comm., *157:* 87–94, 1988.
28. Xie, Q. W., and Nathan, C. The high-output nitric oxide pathway: role and regulation. J. Leukocyte Biol., *56:* 576–582, 1994.
29. Albina, J. E., and Reichner, J. S. Role of nitric oxide in mediation of macrophage cytotoxicity and apoptosis. Cancer Metastasis Rev., *17:* 39–53, 1998.
30. Albina, J. E., Mills, C. D., Henry, W. L., Jr., and Caldwell, M. D. Temporal expression of different pathways of L-arginine metabolism in healing wounds. J. Immunol., *144:* 3877–3880, 1990.
31. García-Martínez, C., Llovera, M., López-Soriano, F. J., and Argilés, J. M. The effects of endotoxin administration on blood amino acid concentrations: similarities with sepsis. Cell. Mol. Biol., *39:* 537–542, 1993.
32. Freund, H., Atamian, S., Holroyde, J., and Fischer, J. E. Plasma amino acids as predictors of the severity and outcome of sepsis. Ann. Surg., *190:* 571–576, 1979.
33. Corraliza, I. M., Soler, G., Eichmann, K., and Modolell, M. Arginase induction by suppressors of nitric oxide synthesis (IL-4, IL-10 and PGE_2) in murine bone-marrow-derived macrophages. Biochem. Biophys. Res. Comm., *206:* 667–673, 1995.
34. Szabó, C., and Thiemermann, C. Regulation of the expression of the inducible isoform of nitric oxide synthase. Adv. Pharmacol., *34:* 113–153, 1995.
35. Waddington, S. N., Tam, F. W. K., Cook, H. T., and Cattell, V. Arginase activity is modulated by IL-4 and HOArg in nephritic glomeruli and mesangial cells. Am. J. Physiol. Renal Physiol., *274:* F473–F480, 1998.
36. Chang, C. I., Zoghi, B., Liao, J. C., and Kuo, L. The involvement of tyrosine kinases, cyclic AMP/protein kinase A and p38 mitogen-activated protein kinase in IL-13-mediated arginase I induction in macrophages: its implications in IL-13-inhibited NO production. J. Immunol., *165:* 2134–2141, 2000.
37. Buga, G. M., Singh, R., Pervin, S., Rogers, N. E., Schmitz, D. A., Jenkinson, C. P., Cederbaum, S. D., and Ignarro, L. J. Arginase activity in endothelial cells: inhibition by N^G-hydroxy-L-arginine during high-output NO production. Am. J. Physiol. Heart Circ. Physiol., *271:* H1988–H1998, 1996.