Regulation of arginase isoforms I and II by IL-4 in cultured murine peritoneal macrophages

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Regulation of arginase isoforms I and II by IL-4 in cultured murine peritoneal macrophages. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R237–R242, 1999.—Macrophages can express two arginase isoforms with distinct subcellular localization (cytosolic AI and mitochondrial AII). These isoforms are products of different genes and are capable of differential induction. Experiments were performed to identify the specific arginase isoforms induced by interleukin (IL)-4, a Th2 cytokine shown by others to increase arginase activity in macrophages, and serum. Results indicate IL-4, in concert with serum, increases AI, but not AII, mRNA in cultured murine macrophages. Moreover, they show serum to induce both arginase isoforms and to be required for maximal AI induction by IL-4. Together with the enhanced expression of AI, IL-4 induced the expression of the cationic amino acid transporter MCAT-2 and increased L-arginine transport into the cells. Present results confirm, then, specificity in the ability of macrophage arginase isoforms to be induced by different stimuli. Moreover, they suggest that a decrease in intracellular L-arginine concentration resulting from its consumption by arginase may be repaired by concurrent increases in L-arginine influx into the cell.

L-arginine; cytokines; amino acid transport; interleukin-4

TWO DISTINCT ISOFORMS OF arginase (L-arginine-urea-hydrolase, EC 3.5.3.1) have been shown to be expressed in macrophages (12, 15, 28, 34). These isoforms are products of different genes and localize to different subcellular compartments. Arginase I (AI) is identical to the liver-type arginase and is present in the cytosol, whereas arginase II (AII) is identical to the kidney-type arginase and is contained in mitochondria.

Macrophage arginase activity can be increased by a variety of agents, including two Th2 cytokines, interleukin (IL)-4 and IL-10 (6, 7, 19, cAMP (11), transforming growth factor-β (4), prostaglandin E2 (6), lipoplysaccharide (LPS) (7, 25), serum (11), and hypoxia/anoxia (1). The arginase isoform(s) induced by these agents, with the exception of LPS, which results in increases in both isoforms (10, 15, 28), and hypoxia, which increases only AI (15), have not yet been identified.

The functional relevance of the expression of two distinct isoforms of arginase in macrophages remains unexplained. Arginase was originally described as a secretory product of macrophages that could deplete extracellular L-arginine and, thus, make this amino acid limiting for the proliferation and survival of lymphocytes, tumor cells, or microorganisms (8, 20). More recent work has shown arginase not to be actively secreted from macrophages but to exit the cells and consume extracellular L-arginine only after the cells’ lytic demise (2, 26). It follows that the primary function of macrophage arginase may not be that of an extracellular cytotoxic effector.

It has been proposed, in this connection, that a physiological role of intracellular arginase in macrophages could be to reduce the availability of L-arginine to inducible nitric oxide synthase (iNOS), the cytosolic enzyme primarily responsible for the production of NO in these cells (reviewed in Ref. 18). Moreover, it has been shown that certain Th2 cytokines, specifically IL-4 and IL-10, markedly downregulate NO production in macrophages and proposed that they do so by the dual mechanism of suppressing the induction of iNOS by proinflammatory mediators like interferon (IFN)–γ and enhancing arginase expression (17). The increased arginase activity in the cells would presumably reduce intracellular L-arginine accessible to iNOS.

In considering alternative functions for intracellular arginase that take into consideration the different subcellular locations of its isoforms, it is tempting to hypothesize that each arginase isozyme may be spatially associated with enzymes that metabolize the products of the arginase reaction, ornithine and urea. Because urea is not metabolizable by mammalian cells, ornithine is the only arginase product that can be subjected to further metabolism. In this regard, ornithine amino transferase (OAT), the enzyme that initiates the conversion of ornithine to glutamate and proline, is located within mitochondria (12). Flux of arginine through AII could then directly provide substrate for OAT and result in enhanced production of glutamate and proline. Similarly, ornithine dehydrogenase (ODC), which is located within the cytosol, utilizes ornithine in the production of polyamines. Indeed, evidence for the simultaneous induction of arginase and ODC has been presented (27). Thus the modulation of intracellular arginase and, most specifically, the differential induction of its cytosolic or mitochondrial isoforms could serve as flux-generating steps in regulating biosynthetic processes involving L-arginine metabolites.

To begin to better define the potential biological roles of arginase in macrophages, it is important to establish whether its isoforms can be differentially regulated by different stimulatory agents. In this regard, work from this laboratory demonstrated that hypoxic culture of...
macrophages specifically increases AI without changes in AII (15). Experiments reported here investigated the effects of an alternative inducer of arginase activity, IL-4, and demonstrated that, like hypoxia, IL-4 exclusively increases AI expression. In addition, results to be shown indicate homologous or heterologous serum induces both arginase isoforms and provide evidence for the enhanced expression of the L-arginine transporter MCAT-2 and for the stimulation of L-arginine uptake into macrophages cultured with IL-4.

MATERIALS AND METHODS

Animals. Male B6D2F1 mice (Taconic, Germantown, NY) were used in all experiments. Animals were certified free of common rodent pathogens by the suppliers and were housed in an isolation environment on their arrival at the laboratory. The animals were monitored by Brown University/Rhode Island Hospital (Providence, RI) veterinary personnel.

Cell culture. Peritoneal macrophages were obtained from mice by peritoneal lavage. Cells were incubated in culture medium [RPMI 1640 (Life Technologies, Grand Island, NY) with 5 × 10−5 M 2-mercaptoethanol, 10 mM MOPS and antibiotics] at a density of 1.5 × 106 cells in 1% CO2, for the times indicated in the legends of Figs. 1–5. Culture medium contained <3 pg/mL endotoxin as detected by a chromogenic assay (QCL1000; Wittaker M. A. Bioproducts, Walkersville, MD). Culture medium was supplemented with different concentrations of FCS (HyClone Laboratories, Logan, UT) normal mouse serum, and/or recombinant mouse IL-4 (Pharmingen, San Diego, CA) as indicated.

RNA isolation. Total RNA was isolated from 2 × 107 cells using the reagent Ultraspec (Biotex, Houston, TX) according to manufacturer’s recommendations.

Probes for Northern blot analysis. The probe used to detect AI was described previously (15). For AI, a plasmid (pGEM-RAI1-1) containing a 248-bp fragment of rat AII cDNA shown to detect AII mRNA in RAW 264.7 cells and murine macrophages was employed (10, 15). This plasmid was a gift from Drs. Tomomi Gotoh and Masataka Mori (Kumamoto University School of Medicine, Kumamoto, Japan). For MCAT-2, a pBluescript plasmid (clone 20.5.1) containing cDNA to MCAT-2 was a gift from Dr. Carol MacLeod (University of California at San Diego, La Jolla, CA). Bal1 digestion of the plasmid yielded a 2-kb cDNA insert that recognizes two transcripts of ~4.5 and 8.5 kb (9, 16). For 18S ribosomal RNA, a pUCB30 plasmid containing the mouse 18S ribosomal cDNA (21) was obtained from American Type Culture Collection (Rockville, MD). Sph1 and BamH1 digestion of the plasmid yielded a 752-bp cDNA insert that detects 18S ribosomal RNA in rat and mouse macrophages (15). Measurement of 18S ribosomal RNA was used as a control for variations in the amount of RNA loaded in each lane.

Northern blot analysis. Total RNA was fractionated by 1% agarose-0.66% formaldehyde gel electrophoresis (31), transferred to a nylon membrane, and immobilized by ultraviolet cross-linking. AI, AII, mCAT-2, and 18S ribosomal RNA were detected with the cDNA probes described above, radiolabeled with [3P]dCTP by random priming (Pharmacia). The membrane was hybridized as described previously (15). AI, AII, mCAT-2, and 18S ribosomal RNA were quantitated by densitometry using a Protein Databases desktop densitometer and Quantity 1D gel analysis software (PDI, Huntington Station, NY).

Western blot analysis. Postnuclear supernatants of cell lysates (30 µg of protein/lane) were size fractionated in 15% SDS-PAGE and transferred to a nitrocellulose membrane (1).

AI was detected with a rabbit antibody against human hepatic AI shown previously to detect AI in rat macrophages (15, 28) and murine macrophages (15) as described (15). Protein was determined with the bichinchonic acid protein assay (Pierce Chemical, Rockford, IL). Densitometric analysis of immunoreactive bands was performed as described above.

Arginase activity. Arginase activity was measured in cell lysates as described in (6) with minor modifications. Briefly, 12.5 µl of cell lysates were added to 12.5 µl of 25 mM Tris-HCl, pH 7.4, with 5 mM MnCl2. The assay was initiated by the addition of 25 µl of 0.5 M L-arginine (pH 9.7) and incubated at 37°C for 60 min. Ten microliters of the above reaction was mixed with 200 µl of blood urea nitrogen (rate) reagent (Sigma Chemical, St. Louis, MO), and the urea concentration was measured at 340 nm every 20 s over a 3-min period.

Arginase transport. Arginase transport was measured in cells as described in Ref. 32 with slight modifications. Briefly, 8 × 105 cells were cultured overnight in culture medium containing 1% FCS with or without 20 U/ml IL-4. After 18 h, cells were washed with PBS and incubated with RPMI 1640 without arginine for 2 h. Medium was removed, and cells were incubated with 1 ml of Hanks’ balanced salt solution containing 1 µCi [L-(3-H)]arginine (NEC Life Science Products, Boston, MA) and different concentrations of unlabeled arginine up to 1 mM for 30 s. L-Arginine transport has been found to be linear over this time period. Transport was terminated with four washes of cold PBS, and cells were lysed with 20% TCA. L-[3H]arginine-associated radioactivity in cell lysates was measured by liquid scintillation counting in an LKB 1218 RackBeta (Wallach, Gaithersburg, MD). Kinetic transport parameters were calculated using Lineweaver-Burke transformation on Enzyme Kinetics (Trinity Software, Compton, NY) software.

Data presentation. Data shown are representative of at least two independent experiments. Quantitative results are means ± SD from quadruplicate samples in a representative experiment. Statistical analysis was by ANOVA or unpaired t-test, as indicated in Fig. 4 legend or in RESULTS.

RESULTS

IL-4 increases arginase activity in cultured murine macrophages. Initial experiments to investigate the induction of arginase in murine peritoneal macrophages by IL-4 included this cytokine in culture media at 20 U/ml based on reports of Modolell et al. (17) and Corraliza et al. (6) that this concentration yielded maximal arginase induction in cultured murine bone-marrow derived macrophages. At variance with the culture conditions used by those authors, however, FCS was present in these cultures at 1%, rather than 10%. This concentration was chosen because Jakway et al. (11) described the induction of arginase by serum when included in cultures at concentrations ranging from 3 to 30%. Figure 1 shows results from these experiments and demonstrates a threefold higher arginase activity in lysates from cells cultured for 18 h with recombinant murine IL-4 and 1% FCS than in cells cultured with serum alone (P < 0.05, unpaired t-test).

Induction of AI and AII mRNA in cultured murine macrophages. To explore the potential differential regulation of arginase isoforms by IL-4, AI and AII mRNAs were determined by Northern blot analysis in freshly isolated macrophages and in cells cultured for up to
18 h in media containing 1% FCS with or without 20 U/ml IL-4 (Fig. 2). AI mRNA was detected, albeit at a low level, in freshly isolated cells. Culture with 1% FCS increased AI mRNA by more than sevenfold after 2 h, and it was still detectable above levels found in freshly isolated cells at 4 and 18 h.

Addition of IL-4 to the cultures markedly enhanced the induction of AI at all time points, with AI mRNA in these cells being greater than that in freshly isolated cells by more than 20-fold at 2 h and greater than that in cells cultured with 1% FCS by more than 3-fold at 2 h, 11-fold at 4 h, and 3-fold at 18 h (Fig. 2).

AII mRNA was not detected in freshly harvested cells even after prolonged exposure of the film to the membrane. Similar to findings with AI, culture with serum resulted in the induction of AII by 2 h, with persistence of detectable AII mRNA in the cells for up to 18 h.

Contrasting with AI, however, IL-4 did not further increase AII message in the cells (Fig. 2).

Western blot analysis of cell lysates from freshly isolated macrophages and macrophages cultured in the presence or absence of IL-4 indicated immunoreactive AI antigen to be barely detectable in freshly harvested cells and in those cultured up to 4 h (not shown). Cells cultured overnight contained readily detectable AI antigen, with approximately three times more protein being present in those cultured with IL-4 (20 U/ml) and 1% serum than in those cultured with 1% serum alone (Fig. 3).

Effect of serum and IL-4 on arginase activity in lysates from cultured murine macrophages. To further explore the interaction of serum and IL-4 in the induction of arginase in macrophages, cells were cultured overnight in the presence of different concentrations of serum and/or cytokine before their lysis and analysis for arginase activity. Results shown in Fig. 4 demon-
strate that, different from the findings of Jakway et al. (11), FCS increased arginase activity in the cells when present in cultures at 1% by volume. Normal murine serum had arginase-enhancing activity identical to that of FCS (not shown). No detectable increase in arginase activity was found when IL-4 was added to the cultures by itself in concentrations up to 50 U/ml. IL-4 and serum appeared to synergistically induce arginase activity when serum was present at 1 or 5%. Addition of serum at 10 or 20% resulted in arginase activities greater than 10 µmol·min⁻¹·mg protein⁻¹, with no additional activity induced by adding IL-4 at up to 50 U/ml (not shown).

IL-4 increases MCAT-2 mRNA and L-arginine transport into cultured murine peritoneal macrophages. Following previous observations of the concurrent induction of AI and the L-arginine transporter MCAT-2 in macrophages by hypoxia (15), experiments investigated the effects of 1%FCS ± 20 U/ml IL-4 on MCAT-2 mRNA and on L-arginine transport into the cells. Figure 5 shows that MCAT-2 mRNA was increased by at least twofold after 4 h and 18 h culture by the addition of 20 U/ml IL-4 to the cultures. As previously shown in hypoxic cultures (15), the 8.5-kb MCAT-2 transcript was expressed to a higher extent than the 4.5-kb transcript. In agreement with these findings, L-arginine transport into cells was enhanced by the addition of IL-4 to the cultures (Vmax: 205.6 pmol·min⁻¹·10⁶ cells⁻¹ in cultures without IL-4 vs. 374.8 pmol·min⁻¹·10⁶ cells⁻¹ in cultures with IL-4, P < 0.05, unpaired t-test) without changes in Km (296.2 µM in cultures without IL-4 vs. 288.5 µM in cultures with IL-4, P > 0.05, unpaired t-test).

**DISCUSSION**

IL-4 has been shown to exert a variety of effects on macrophages. These effects include the ability of the cytokine to induce the expression of major histocompatibility complex class II antigen (29), the low-affinity receptor for IgE (35), integrin αvβ3 (14), 15-lipoxygenase (5), and IL-1 receptor antagonist (22). In regard to its capacity to regulate enzymes of L-arginine metabolism in these cells, IL-4 has been shown to decrease the stability of the message for iNOS and to enhance arginase activity in the cells (3). Present results demonstrate IL-4 specifically induces the expression of the AI isoform of macrophage arginase but not that of AII and that arginase induction in these cells is facilitated by the presence of serum. The nil AII response to IL-4 is not due to lack of inducibility of this gene, because LPS has been shown to induce both AI and AII in macrophages (15).

The mechanisms by which IL-4 induces gene transcription have been characterized. IL-4 binds to the IL-4 receptor complex, triggering the phosphorylation of Jak kinases (for review, see Ref. 30). This response, in turn, leads to the phosphorylation of two different signaling proteins: IL-4-induced phosphotyrosine substrate/insulin receptor substrate 2, and signal transducer and activator of transcription 6 (STAT 6) (30). Once activated, STAT 6 translocates to the nucleus and binds to specific DNA sequences on IL-4-responsive genes. Examination of the published promoter region for rat liver AI reveals the presence of six STAT 6 recognition sites (13). Experiments using reporter constructs are currently under way to determine the responsiveness of these sites to IL-4 treatment.

In regard to the effect of serum on the induction of arginase, results shown are similar to those reported by Jakway et al. (11), with the exception of demonstrating a requirement for lower serum concentrations in the induction of arginase than those originally published. The requirements for serum for enhanced responses to IL-4 are not unique to arginase. Indeed, IL-4 requires serum to downregulate CD14 expression on human monocytes (24) and to increase tissue plasminogen activator in smooth muscle cells (33). Although the serum component(s) required for induction of arginase by IL-4 are not known, it has been shown that platelet-derived growth factor (PDGF)-BB activates Jak 1 and STAT 6 (23). Addition of recombinant PDGF-BB, however, failed to reproduce the effects of serum in the induction of arginase when added to cells alone or in combination with IL-4 (results not shown).

It has been repeatedly proposed that arginase regulates NO production by iNOS in macrophages through the intracellular depletion of the shared substrate, L-arginine (reviewed in Ref. 18). This proposal does not appear sound, first, because of the large kinetic advantage conferred to iNOS by its micromolar Km for L-arginine (18) compared with the millimolar Km of arginase for the same substrate (18). Indeed, no arginase activity, as defined by the ability of intact cells to metabolize L-arginine to urea and ornithine, is detected in rat peritoneal macrophages stimulated in culture with IFN-γ and LPS. Most L-arginine consumption by the cells under these culture conditions is mediated through iNOS (1). Arginase activity in the cells is readily detectable, however, when iNOS is inhibited with N⁰-monomethyl-L-arginine or L-N-iminoethyl-L-ornithine (1). These findings support the conclusion
that it is iNOS, not arginase, that obtains preferential access to L-arginine for its metabolism when both isoforms are simultaneously expressed in macrophages. Moreover, current findings demonstrate that IL-4, in concert with serum, not only induces AI expression but also increases steady-state message for MCAT-2 and augments L-arginine transport into the cells. Thus any decrease in intracellular L-arginine concentration brought about by its metabolism through arginase (or iNOS) may be readily repaired by enhanced amino acid transport into the cell. It is relevant to keep in mind, in this regard, that the two stimuli so far identified that specifically increase AI expression, IL-4 plus serum and hypoxia (15), also promote MCAT-2 expression. It may be that the product of the MCAT-2 gene and AI are somehow associated and that extracellular L-arginine, funneled into AI by the MCAT-2 protein, is the preferred substrate pool for this arginase isoform.

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