α2-Macroglobulin Functions as a Cytokine Carrier to Induce Nitric Oxide Synthesis and Cause Nitric Oxide-dependent Cytotoxicity in the RAW 264.7 Macrophage Cell Line*

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Nitric oxide (NO) is an important mediator of macrophage activities. We studied the regulation of macrophage NO synthesis by α2-macroglobulin (α2M), a proteinase inhibitor and carrier of certain growth factors, including transforming growth factor-β (TGF-β). Native α2M and the α2M receptor-recognized derivative, α2M-methylamine (α2M-MA), increased nitrite generation by the RAW 264.7 murine macrophage cell line. The level of nitrite accumulation, which is an index of NO synthesis, was comparable to that observed with interferon-γ. Native α2M and α2M-MA also increased inducible nitric oxide synthase (iNOS) mRNA levels and substantially reduced the number of viable cells, as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium/succinyl dehydrogenase assay or trypan blue exclusion. At slightly higher α2M concentrations, [3H]thymidine incorporation was inhibited. All of these activities were counteracted nearly completely when the iNOS competitive inhibitor NG-monomethyl-arginine was included. By in situ nick translation, native α2M and α2M-MA increased the percentage of cells with detectable single strand chromatin nicks from 4 to 12 and 17%, respectively. This change suggested apoptosis; however, electron microscopy studies demonstrated variability in the morphology of injured cells. To determine the mechanism by which α2M increases macrophage NO synthesis, we studied proteolytic α2M derivatives that retain partial activity. A 600-kDa derivative that retains growth factor binding activity increased RAW 264.7 cell NO synthesis and iNOS mRNA levels comparable to native α2M and α2M-MA. The purified 18-kDa α2M receptor-binding fragment had no effect on NO synthesis or iNOS expression. Thus, the growth factor-carrier activity of α2M and not its receptor-binding activity is essential for NO synthesis regulation. A TGF-β-neutralizing antibody mimicked the activity of α2M, increasing RAW 264.7 cell NO synthesis and decreasing cellular viability. These studies demonstrate that α2M can regulate macrophage NO synthesis and profoundly affect cellular function without gaining entry into the cell and without binding specific plasma membrane receptors.

† The abbreviations used are: NOS, nitric oxide synthase; α2M, α2-macroglobulin; α2M-MA, α2M modified by methylamine; IFN-γ, interferon-γ; iNOS, inducible nitric oxide synthase; LRP, low density lipoprotein receptor-related protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromid; NMMMA, N'N'-monomethyl-L-arginine; PGAD, phosphoglyceraldehyde dehydrogenase; RBF, the 18-kDa receptor binding fragment of α2M-MA; TGF-β, transforming growth factor-β; FBHE, fetal bovine heart endothelial; SFM, serum-free medium; ISNT, in situ nick translation.
important role for αM in the regulation of cellular growth and physiology (37, 39, 40). αM exists in at least two well-described conformations. The native form expresses proteinase-inhibitory activity but is not recognized by cellular receptors (36). “Activated” αM is generated by reaction with proteinases or small primary amines that modify the αM thiol ester bonds. The structure of αM after reaction with proteinase or methylamine (αM-MA) is equivalent, allowing the use of αM-MA as a model of the activated αM conformation (41, 42). αM-MA and αM-proteinase complexes are recognized equivalently by αM-specific cellular receptors. One such receptor has been purified and characterized; this receptor is identical to low density lipoprotein receptor-related protein (LRP) (43–46).

αM may regulate cellular growth and physiology by at least two mechanisms, the first of which involves cytokine carrier activity. TGF-β1, TGF-β2, platelet-derived growth factor BB, nerve growth factor alpha, and interleukin-6 are among a growing list of cytokines reported to bind to αM (39, 40, 47). All of these cytokines, with the exception of TGF-β2, demonstrate higher affinity for the activated αM conformation (47, 48). Nevertheless, due to the large excess of native αM compared with activated αM in plasma and serum-supplemented cell culture medium, native αM is frequently responsible for the cytokine carrier activity observed in biological systems (39, 49–51). Many cytokines, including IFN-γ, do not bind αM with significant affinity (47). Therefore, αM may alter the balance of cytokines within the microenvironment surrounding responding cells. Binding of TGF-β isoforms to αM neutralizes the activity of the TGF-β toward various cells including endothelium (51), keratinocytes (52), and mink lung epithelium (49). The extent of TGF-β neutralization correlates with the affinity of the αM/growth factor interaction (39, 47, 51). The growth factor binding activities of tetrameric α-macroglobulins from different species, including human, mouse, and rat, are comparable (39).2

The second mechanism by which αM may regulate cellular function is independent of cytokine-carrier activity. Misra et al. (53, 54) proposed that mouse peritoneal macrophages express a second αM “signaling receptor.” The receptor recognizes only activated αM but is distinct from LRP. In response to activated αM, the second αM receptor initiates signal transduction responses, including rapid phosphatidylinositol 4,5-bisphosphate hydrolysis (53, 54). Activated αM has been shown to affect superoxide anion production (55) and prostaglandin E2 synthesis (56) in mouse peritoneal macrophages. The mechanism by which activated αM causes these changes has not been fully explored.

Macrophages and fibroblasts synthesize αM (36). Furthermore, with loss of vascular integrity, αM might be expected to reach high concentrations in the interstitial spaces (37). Therefore, we hypothesized that αM may be a significant regulator of macrophage activity at sites of infection or inflammation, in the intima of an injured artery, and in a vascularized tumor. The present study was undertaken to determine whether αM regulates macrophage NO production. Our results demonstrate that αM increases iNOS levels in RAW 264.7 cells, causing cellular death that is prevented by a specific competitive iNOS inhibitor. Regulation of iNOS was entirely due to the ability of αM to bind growth factors secreted by the macrophages. Furthermore, the activity of αM was mimicked by TGF-β-specific neutralizing antibody. Based on these studies, we propose that αM may be a component of an important macrophage autocrine regulatory pathway involved in controlling cellular NO production.

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tated using the Cell Proliferation Kit I marketed by Boehringer Mannheim. The assay detects succinyl dehydrogenase, a mitochondrial enzyme, only in living cells (62). The substrate for succinyl dehydrogenase is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). In our studies, 10^5 cells were plated in each well of a 96-well flat bottom tissue culture plate (Corning, NY). The culture medium was RPMI 1640, 10% FBS, 1% PS. After 24 h, the medium was replaced with SFM, and incubation was continued for an additional 24 h. Any of the following were then added: native α2M (280 nm), α2M-MA (0.14–280 nm), RBF (200 nm), the 600-kDa derivative (200 nm), or antibody 1D11.16 (50 μg/ml). After 24 h, viable cell number was determined by adding MTT directly into the cell culture wells, as described by the manufacturer. All incubation conditions were studied in quadruplicate. In some experiments, α2M and/or IFN-γ were added to cultures together with NMMA (1–10 mM).

RAW 264.7 DNA synthesis was assessed by [3H]thymidine incorporation. The cells were plated at 10^5/well in 24-well plates and cultured first in serum-supplemented medium for 24 h and then in SFM for an additional 24 h. At the beginning of the third day, α2M-MA was added and the cells were cultured for 24 h. [3H]thymidine (1 μCi/ml) was included during the last 6 h. Cells were then washed with Earle’s balanced salt solution, 10 mM HEPES, pH 7.4, and fixed in 10% trichloroacetic acid. Cell-associated radioactivity was recovered by incubation in 1.0 M NaOH for 12 h. The pH was neutralized with 1 M HCl. Cell extracts were then combined with Ready Safe scintillation fluid for counting in a Beckman scintillation counter.

RAW 264.7 cell viability was determined by trypan blue exclusion. Cells were plated at a density of 5 x 10^5/well in 6-well plates and cultured for 48 h. Native α2M (280 nm), α2M-MA (0.14–280 nm), the 600-kDa derivative (200 nm), and RBF (200 nm) were then added. After an additional 24-h incubation, the cells were released, either with trypsin/EDTA (Life Technologies, Inc.) or with a cell scraper, incubated with trypan blue, and counted using a hemocytometer. Since the monolayers were not washed prior to trypsin/EDTA treatment or scraping, adherent cells, and cells that detached during incubation with α2M were detected.

In Situ Nick Translation (ISNT) of RAW 264.7 Cell DNA—In ISNT, dATP is used instead of dCTP or dGTP that contains single-strand nicks (63, 64). The method detects programmed cell death but may also detect other forms of cell injury/death including what has been frequently referred to as necrotic death (65, 66). In our experiments, RAW 264.7 cells (5 x 10^5) were cultured on coverslips in 6-well plates for 24 h in SFM supplemented with native α2M (280 nm) or α2M-MA (280 nm). Cells were washed with Hank’s balanced salt solution, fixed with 3.7% formaldehyde in PBS, permeabilized with 0.5% Triton X-100, washed again, and air dried. Cells on coverslips were then incubated for 2 h at 37°C with 50 μM Tris-EDTA, 5 μM MgCl2, 10 mM mercaptoethanol, 0.005% bovine serum albumin, 0.02 mM dATP, 0.02 mM dCTP, 0.02 mM dGTP, 0.03 mM dTTP, 7 μM digoxigenin-11-dUTP, and DNA polymerase-1 (Boehringer Mannheim). After 1 h, the coverslips were washed twice with 20 μM EDTA and once with PBS, pH 7.4. The cells were then incubated with TBS and 5% nonfat dried milk for 1 h followed by anti-digoxigenin Fab antibody conjugated to alkaline phosphatase (Boehringer Mannheim) for 12 h (1:200 dilution). The next day, cells were washed and stained with the chromogen, 5-bromo-4-chloro-3-indolyl phosphate p-toluidine-nitro blue tetrazolium chloride. Cellular labeling was viewed and photographed using Nomarski differential interference contrast optics in an Olympus BH2 Microscope. In control experiments, no labeling was observed when the digoxigenin-11-dUTP, the anti-digoxigenin Fab antibody, or the DNA polymerase was omitted from the various steps of the procedure.

Electron Microscopy—RAW 264.7 cells were examined by transmission electron microscopy after treatment with α2M. Cells (5 x 10^5) were cultured in 6-well plates for 24 h. The medium was SFM supplemented with native α2M (280 nm) or α2M-MA (280 nm). Osmium tetroxide (2% w/v) dissolved in 0.1 M sodium cacodylate was slowly added to the medium to a final concentration of 1% and allowed to fix the cells for 10 min. The medium and fixative were then aspirated and replaced with 2% (w/v) glutaraldehyde, 2% (w/v) cacodylate, and 0.1 M sodium cacodylate to fix overnight at 4°C. The cells were removed from the wells with the aid of a rubber policeman and pelleted in an Eppendorf microcentrifuge (16,000 x g). The pellet was then processed by transfer sequentially into 70% acetone, anhydrous acetone, 1:1 acetone/ethanol, and pure resin. Finally, the pellet was transferred into a BEEM capsule filled with pure resin for polymerization at 60°C for 24 h. Thin sections were prepared, placed on 150 mesh nickel grids, stained with uranyl acetate lead citrate, and analyzed using a Zeiss 902 electron microscope.

Detection of Active TGF-β in RAW 264.7 Cell-conditioned Medium—Active TGF-β was detected in conditioned medium from RAW 264.7 cells by measuring FBHE growth inhibition. Under the conditions of the assay, equivalent concentrations of TGF-β1 and TGF-β2 are equipotent (51). The RAW 264.7 cell culture medium (500 μl/well) was conditioned by incubation with 5 x 10^5 cells in 24-well plates for 24 h. The FBHE cells were plated at 2 x 10^4 cells/well in 24-well plates and cultured in Dulbecco modified Eagle’s medium with 10% FBS for 14 h. The FBHE cells were then washed and incubated with different dilutions (1:2, 1:20, 1:200) of the RAW 264.7 cell-conditioned medium, in the presence and absence of TGF-β-neutralizing antibody (10 μg/ml), or with standard concentrations of purified TGF-β2 in Dulbecco’s modified Eagle’s medium plus dilute (0.2%) FBS. After 30 h, [3H]thymidine was added for an additional 18 h. The cells were washed and then fixed in 10% trichloroacetic acid. FBHE-associated radioactivity was recovered by incubation in 1.0 M NaOH for 12 h at 22°C. The solutions were neutralized in pH with HCl and combined with Ready Safe scintillation fluid for counting in a Beckman scintillation counter.

RESULTS

Effects of α2M on NO Synthesis and iNOS mRNA Expression—RAW 264.7 cells that were exposed to native α2M or α2M-MA for 24 h synthesized significantly increased levels of NO, as determined by nitrite analysis (Fig. 1). The increase was comparable to that observed in cultures exposed to IFN-γ (10 ng/ml), a known inducer of macrophage iNOS (18, 67). The combination of IFN-γ and native α2M caused an additive NO synthesis response. IFN-γ and α2M-MA caused an NO synthesis response that was at least additive. The ability of the two α2M conformations to induce RAW 264.7 cell NO production was not significantly different in the absence of IFN-γ. In the presence of IFN-γ, NO synthesis in response to α2M-MA was significantly higher than that observed with native α2M (p < 0.05).

Native α2M and α2M-MA increased iNOS mRNA levels in RAW 264.7 cells, as determined by Northern blot analysis (Fig. 2). The increase in iNOS mRNA caused by α2M-MA was either equivalent to the increase caused by native α2M or slightly greater in six separate experiments. In the particular study shown in Fig. 2, nitrite levels were measured in the cultures from which mRNA was harvested. The nitrite levels, like the iNOS mRNA levels, were increased similarly by native α2M and α2M-MA.

The increase in iNOS mRNA caused by native α2M or α2M-MA was significantly less than the increase observed with IFN-γ (10 ng/ml) even though all three agents increased nitrite levels similarly (as shown in Figs. 1 and 2). Thus, while the increase in NO synthesis caused by α2M is at least partially explained by the increase in iNOS mRNA, a second contributing mechanism may be involved. Feasible secondary mechanisms
include an increased rate of iNOS mRNA translation and/or stabilization of the synthesized enzyme.

NO Synthesis and iNOS Expression in Cultures Treated with $\alpha_2$M Derivatives—In the next series of experiments, we sought to determine the mechanism by which $\alpha_2$M increases iNOS expression and NO synthesis in RAW 264.7 cells. Our experiments showing that native $\alpha_2$M and $\alpha_2$M-MA are comparably active in promoting NO synthesis provided some insight into the mechanism. For example, since native $\alpha_2$M does not bind LRP (36) or the second $\alpha_2$M signaling receptor (53, 54), the increase in iNOS mRNA and NO synthesis could not be due to an $\alpha_2$M receptor-mediated mechanism. If the activity of $\alpha_2$M was due to its ability to neutralize an extracellular proteinase that regulates RAW 264.7 cell NO synthesis, $\alpha_2$M-MA would be inactive since this form of $\alpha_2$M retains no proteinase inhibitory activity (36). Thus, the most likely mechanism involved the cytokine binding activity of $\alpha_2$M. We hypothesized that $\alpha_2$M binds one or more cytokines secreted by the RAW 264.7 cells, thereby interrupting an important autocrine regulatory loop controlling NO synthesis.

To test this hypothesis, two $\alpha_2$M derivatives with different activities were studied. The 600-kDa derivative retains growth factor binding activity but is not recognized by $\alpha_2$M receptors (58, 60). RBF does not bind growth factors (60) but interacts with LRP and the second $\alpha_2$M signaling receptor described by Misra et al. (53, 54). The RBF/second $\alpha_2$M signaling receptor interaction apparently causes the full spectrum of signal transduction responses observed with activated $\alpha_2$M. Fig. 3 shows that the 600-kDa derivative increased NO synthesis in RAW 264.7 cells. The extent of the response, in the presence and absence of IFN-γ, was comparable to that observed with $\alpha_2$M-MA. By contrast, RBF had no effect on NO synthesis. The 600-kDa derivative also increased the level of iNOS mRNA while RBF had little or no effect (Fig. 4). These results confirm that the ability of $\alpha_2$M to increase NO synthesis in RAW 264.7 cells results from $\alpha_2$M-cytokine interactions occurring in the medium and not from an $\alpha_2$M receptor interaction.

RAW 264.7 Cell Proliferation and Viability—Incubation with $\alpha_2$M-MA (280 nM) decreased the number of RAW 264.7 cells detected by MTT assay and this effect was time-dependent. At 2 and 6 h, cell number was decreased by less than 10% compared with SFM-treated control cultures; however, at 24 and 48 h, cell number in $\alpha_2$M-MA-treated cultures was decreased by 72 and 84%, respectively (results not shown). To assess the role of NO synthesis in causing RAW 264.7 cell death, cultures were treated with various forms of $\alpha_2$M for 24 h. All of the derivatives of $\alpha_2$M that increased NO synthesis, including native $\alpha_2$M, $\alpha_2$M-MA and the 600-kDa derivative, decreased the number of viable cells detected by MTT assay (Fig. 5). RBF did not reduce the number of viable cells. Therefore, cytokine carrier activity and/or NO synthesis was apparently responsible for the decrease in cell number caused by $\alpha_2$M.

To determine if the observed decrease in RAW 264.7 cell number was due to a change in the rate of proliferation or altered cellular viability, [3H]thymidine incorporation and trypan blue exclusion experiments were performed. Increasing concentrations of $\alpha_2$M-MA were added to the cultures. By MTT assay, an $\alpha_2$M-MA concentration-dependent decrease in the number of viable cells was apparent (Fig. 6). This decrease was accompanied by a significant increase in the fraction of cells that did not exclude trypan blue in cultures treated with 10–300 nM $\alpha_2$M-MA. [3H]Thymidine incorporation was also decreased by high concentrations of $\alpha_2$M-MA; however, unchanged or slightly increased [3H]thymidine incorporation was observed with 1.4–10 nM $\alpha_2$M-MA. Thus, $\alpha_2$M-MA increases the fraction of RAW 264.7 cells undergoing cell death and, at higher concentrations, decreases the fraction of cells progress-
Cultures were treated with native α2M, RBF, or the 600-kDa derivative for 24 h and studied by trypan blue exclusion. The a2M derivatives that decreased cell number by MTT assay (as shown in Fig. 5) also decreased the fraction of cells that excluded trypan blue (Table I). By contrast, RBF did not decrease trypan blue exclusion. Equivalent results were obtained whether the cells were released from the wells by scraping or with trypsin/EDTA.

NO as the Mediator of a2M-induced Cytotoxicity—Experiments were performed with the competitive iNOS active site inhibitor NMMA to determine whether NO synthesis was responsible for the observed loss of viability in a2M-treated RAW 264.7 cells. Fig. 7 shows that NMMA blocked NO synthesis and the resulting nitrite accumulation in cultures treated with a2M-MA (280 nM) or a2M-MA + IFN-γ (10 ng/ml). NMMA also prevented the decrease in cell number caused by native a2M, a2M-MA, or a2M-MA + IFN-γ, as determined by MTT assay. The protective activity of NMMA was almost complete at a concentration of 10 mM in experiments with native a2M and a2M-MA. NMMA was slightly less effective in counteracting the decrease in cell number caused by the combination of a2M-MA and IFN-γ. These studies demonstrate that the cytotoxic activity of a2M in RAW 264.7 cell cultures is entirely, or almost entirely, due to NO synthesis.

ISNT Experiments—RAW 264.7 cells, cultured on glass coverslips, were treated with native a2M (280 nM) or a2M-MA (280 nM) for 24 h. The cultures were then processed for ISNT and photomicrographed using Nomarski differential interference contrast optics. Fig. 8 shows representative fields for each treatment protocol. The number of cells remaining on the coverslip was decreased after treatment with native a2M or a2M-MA. Among the remaining cells, considerable heterogeneity in size was apparent; at least a few cells in each field were substantially enlarged compared with the control cultures. Occasional multinucleated cells were observed.

DNA fragmentation was detected in some cells of the control preparation by ISNT. Cells in the a2M and a2M-MA-treated cultures were labeled as well. The percentage of adherent cells that stained positively was increased after a2M or a2M-MA treatment (Table II), even though many of the cells that were injured or dead had already detached from the coverslips. DNA
was isolated from RAW 264.7 cells treated with native \( \alpha_2 \)M and \( \alpha_2 \)M-MA were 280 nM. Each value represents the average ± S.E. of three experiments, each with duplicate determinations. For a given determination, at least 50 separate high power fields were examined.

| Cells/high power field | \( \alpha_2 \)M-MA | \( \alpha_2 \)M |
|------------------------|------------------|----------------|
| Control                | 30 ± 3           | 7 ± 1          |
| Labeled cell/high power field | 1.1 ± 0.3       | 1.1 ± 0.3      |
| ISNT-labeled (%)       | 4                | 17             |

was isolated from RAW 264.7 cells treated with native \( \alpha_2 \)M or \( \alpha_2 \)M-MA for 24 h and analyzed by agarose gel electrophoresis. Interunucleosomal DNA fragmentation was not detected (data not shown). Detection of DNA ladders provides strong evidence for apoptosis; however, the absence of DNA ladders does not preclude apoptosis in part or all of a cell population (68, 69).

The TGF-\( \beta \)-Carrier Activity of \( \alpha_2 \)M and iNOS Production—Of the 13 growth factors studied by our laboratory to date, TGF-\( \beta \) binds to \( \alpha_2 \)M with the highest affinity (47, 51). Furthermore, TGF-\( \beta \) is the only growth factor that binds to native \( \alpha_2 \)M and \( \alpha_2 \)M-MA with equivalent affinity. Given the role of TGF-\( \beta \) isoforms as down-modulators of macrophage NO synthesis (21) and previous studies demonstrating TGF-\( \beta \) expression by macrophages (70–72), we decided to determine whether the observed increase in iNOS expression and NO synthesis could be explained by the TGF-\( \beta \)-carrier activity of \( \alpha_2 \)M. RAW 264.7 cells were treated for 24 h with antibody 1D11.16 (50 \( \mu \)g/ml), which neutralizes TGF-\( \beta \)1 and TGF-\( \beta \)2. As shown in Fig. 10, the antibody caused a significant increase in nitrite accumulation, similar to \( \alpha_2 \)M-MA. Furthermore, the antibody significantly decreased the number of viable cells detected by MTT assay. The IgG subclass-matched control antibody AB 6.01 (50 \( \mu \)g/ml) did not significantly affect nitrite levels relative to the control (results not shown). These studies demonstrate that the activities of \( \alpha_2 \)M described here can be fully mimicked by antibody that neutralizes TGF-\( \beta \) released by macrophages in culture.

Conditioned medium from RAW 264.7 cells was analyzed for TGF-\( \beta \) activity using the FBHE proliferation assay. The conditioned medium inhibited \( ^{3} \)H]thymidine incorporation by the FBHE cells, as expected for a TGF-\( \beta \)-containing vehicle. Antibody 1D11.16 neutralized the activity in the conditioned medium, confirming that the FBHE growth inhibition was due to TGF-\( \beta \). The concentration of TGF-\( \beta \) in the RAW 264.7 cell conditioned medium, as determined by comparison to purified TGF-\( \beta \), was 9 ± 4 pm (n = 4).

**DISCUSSION**

The growth factor-carrier activity of \( \alpha_2 \)M was originally identified in studies of whole plasma. O'Connor-McCourt and Wakefield (73) demonstrated that nearly all of the TGF-\( \beta \)1 in plasma is associated with \( \alpha_2 \)M and Huang et al. (74) showed that TGF-\( \beta \)1 is inactive while bound to \( \alpha_2 \)M. Based on these early studies, \( \alpha_2 \)M-TGF-\( \beta \) complex was initially referred to as latent TGF-\( \beta \), a term subsequently reserved for the precursor form of TGF-\( \beta \) secreted by cells in complex with a latent TGF-\( \beta \) binding protein (75). More recent studies have shown that TGF-\( \beta \)1 (39, 50), platelet-derived growth factor-BB (39), and
nerve growth factor-β bind to native α2M when injected intravenously in mice. After an initial rapid clearance phase, the complex with α2M becomes the primary form of each growth factor present in the plasma. The α2M-growth factor complex is relatively stable in the blood, presumably forming a pool of slowly releasable activity since complexes of growth factors with native α2M remain primarily noncovalent and reversible.

These animal model and whole plasma experiments demonstrate that α2M-growth factor interactions are physiologically significant in vivo.

In this study, we demonstrated that α2M is an important regulator of RAW 264.7 cell NO synthesis. The underlying mechanism depends on the growth factor-carrier activity of α2M. Since the experiments were performed in serum-free medium and an alternative source of cytokines was not provided, the critical interaction must have involved α2M and one or more cytokines secreted by the RAW 264.7 cells. For a number of reasons, we examined the possible role of the TGF-β superfamily in the α2M/NO-regulatory system. First, TGF-β isoforms inhibit macrophage NO synthesis (21). Furthermore, it is known that monocytes-macrophages synthesize TGF-β (70–72), a result confirmed for RAW 264.7 cells in this study. Since TGF-β decreases iNOS mRNA translation and destabilizes the protein (22), a neutralizing interaction of α2M with RAW 264.7 cell TGF-β is consistent with our observation that α2M elevates NO synthesis disproportionately with iNOS mRNA. Finally, we have shown that the TGF-β-neutralizing activity of α2M in FBHE cultures closely correlates with the binding affinity (1/KD) of the α2M/TGF-β interaction (51). We assume that the same principle holds for macrophage cultures. Since native α2M was as active, or nearly as active, as α2M-MA in the regulation of NO synthesis, the involvement of TGF-β2 is suggested; TGF-β2 is the only growth factor studied to date that binds native α2M and α2M-MA with equal affinity.

To test the hypothesis that TGF-β-binding accounts for the NO synthesis induced by α2M, we studied a TGF-β-neutralizing antibody. The antibody completely mimicked the activity of α2M, increasing cellular NO synthesis and decreasing cellular viability. Although the spectrum of cytokines that interact with α2M in the macrophage culture medium may be complex, the antibody studies allow us to conclude that TGF-β binding is sufficient to account for the activities of α2M observed here. Furthermore, these antibody studies identify an important autocrine regulatory loop for the RAW 264.7 macrophage cell line.

**Fig. 9.** Representative electron micrographs of RAW 264.7 cells from control cultures (panel A) and from cultures incubated for 24 h with 280 nM α2M-MA (panel B). Most of the cells in the untreated cultures had centrally located, round nuclei with prominent nucleoli and uniform morphology. Many of the cells treated with α2M-MA displayed various morphologic changes suggesting injury/death. The arrow marks a cell with changes suggestive of apoptosis. The arrowhead marks a cell with predominantly cytoplasmic changes, such as dilated organelles and discontinuity of the plasma membrane. An enlarged cell is marked with the asterisk. The bar represents 2.45 μm.

**Fig. 10.** TGF-β-neutralizing antibody mimics the activities of α2M. RAW 264.7 cells were treated for 24 h with either α2M-MA (280 nM) or TGF-β-neutralizing antibody (50 μg/ml). After 24 h, nitrite levels were measured in the culture media (panel A). The number of viable cells was assessed by MTT assay (panel B). The bars represent the mean ± S.E. of two separate experiments with quadruplicate determination/experiment.

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Apparently, TGF-β synthesized by the cells themselves functions to suppress iNOS expression. By interrupting this autocrine pathway, extracellular mediators, such as α2M or antibody, can profoundly affect cellular phenotype and function without gaining entry into the cell and without binding to plasma membrane receptors.

As a result of the shift in available cytokines in the culture medium, α2M-treated RAW 264.7 cells underwent cell death. One possible explanation for this result is that α2M withdraws a growth factor(s) that is otherwise available to the macrophages and necessary for continued growth. Alternatively, cell death might be mediated directly by NO, synthesized at cytotoxic levels in the α2M-treated cultures. These two mechanisms are similar since both involve α2M interacting with macrophage-secreted cytokines. Furthermore, the mechanisms are not mutually exclusive. To isolate the role of NO synthesis in cell death, among other changes in cellular function that may result from altered cytokine availability, we performed experiments with a specific, competitive INOS inhibitor, MMMA. Inhibition of iNOS substantially reversed the loss of cellular viability in α2M-treated cultures, demonstrating that NO is completely, or nearly completely, responsible for death of the macrophage cell line under these experimental conditions.

It has been reported that macrophages, which are induced to secrete high levels of NO by treatment with IFN-γ and lipopolysaccharide, undergo apoptosis (33–35). Therefore, we performed ISNT and agarose gel electrophoresis experiments to examine the changes occurring in α2M-treated RAW 264.7 cells. The ISNT experiments revealed evidence of single-stranded DNA fragmentation, at the single-cell level, in an increased percentage of the cells treated with native α2M or α2M-MA. The DNA-agarose gel electrophoresis experiments were negative. ISNT is more sensitive than agarose gel electrophoresis in detecting DNA fragmentation, especially when the change is limited to a subpopulation of the cells under examination (65). However, labeling of cells by ISNT may detect modes of cell death other than apoptosis. Therefore, we further explored the morphologic changes occurring in α2M-treated RAW 264.7 cells by electron microscopy. These studies showed a heterogeneous pattern of ultrastructural changes in each α2M-treated preparation. While some cells showed morphologic changes suggesting apoptotic cell death, other injured and dying cells did not. One possible explanation for this result is that the cells are at various stages of dying; however, we believe that dissonance in our cultures does not entirely explain the difference in our results and the previously reported studies (33–35). A second explanation is that the various agents used to induce NO synthesis (α2M, IFN-γ, and lipopolysaccharide) have different effects on the manner in which the macrophage responds to high levels of NO.

Since NO has been implicated in many normal physiologic and pathophysiologic processes, studies on the regulation of NO synthesis have broad relevance. The experiments performed here identify α2M as a regulator of macrophage NO synthesis for the first time. Additional work will be necessary to determine whether our model cell culture system is representative of monocytes and macrophages in vivo. Our results implicating α2M in the regulation of NO synthesis are unique compared with many previous studies that focused on the biological activities of α2M, since, in our system, native α2M was active in addition to the activated form (α2M-MA). Due to the efficiency of the LRP clearance mechanism, concentrations of activated α2M are very low in the blood (36, 39, 40). Higher levels of activated α2M may accumulate in interstitial spaces and in non-vascular body fluids when elevated levels of active proteinase are available locally (37); however, LRP may limit concentrations of activated α2M in these microenvironments as well. By contrast, the native form of α2M is not subject to receptor-mediated clearance and therefore is stable in the presence of LRP-expressing cells, including macrophages, smooth muscle cells and fibroblasts. Thus, native α2M, due to its growth factor binding activity and high concentration, may be the most significant form of the protein to consider as a potential regulator of NO synthesis in vivo.
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