α2-Macroglobulin null mice demonstrate increased resistance to endotoxin challenge (Umans, L., Serneels, L., Overbergh, L., Van Leuven, F., and Van den Berghe, H. (1995) J. Biol. Chem. 270, 19778–19785). We hypothesized that this phenotype might reflect the function of murine α2M (mα2M) as a neutralizer of transforming growth factor-β (TGF-β) and inducer of nitric oxide synthesis in vivo. When incubated with wild-type mouse plasma, TGF-β1 and TGF-β2 bound only to mα2M. Alternative TGF-β-binding proteins were not detected in plasma from α2M(−/−) mice. Wild-type mouse plasma, but not plasma from α2M(−/−) mice, inhibited TGF-β1 binding to TGF-β receptors on fibroblasts. Purified mα2M bound TGF-β1 and TGF-β2 with similar affinity; the Kd values were 28 ± 4 and 33 ± 4 nm, respectively. Murinoglobulin, the second murine α2-macroglobulin, bound both TGF-β isoforms with 30-fold lower affinity. mα2M counteracted the activities of TGF-β1 and TGF-β2 in an endothelial cell growth assay. mα2M also induced NO synthesis when incubated with RAW 264.7 cells, an activity which probably results from the neutralization of autocrine TGF-β activity. Human α2M induced NO synthesis comparably to mα2M; however, MUG had no effect. These studies demonstrate that the ability to neutralize TGF-β is a property of mα2M, which is not redundant in the murine α2-macroglobulin family or in murine plasma. mα2M is the only murine α2-macroglobulin that promotes NO synthesis. The absence of mα2M in α2M(−/−) mice, may allow TGF-β to more efficiently suppress excessive iNOS expression following endotoxin challenge.

Bacterial endotoxin is a lipopolysaccharide complex derived from the outer membranes of Gram-negative bacteria and a major mediator of the pathophysiologic changes referred to as septic shock (1, 2). Endotoxin triggers release of inflammatory cytokines, including tumor necrosis factor-α and interleukin-1β, from a number of cell types, including macrophages and endothelial cells (1, 3). These cytokines, in addition to endotoxin, induce increased cellular synthesis of nitric oxide (4–7). NO1 plays a significant role in mediating the profound vasodilation, hypotension, and hyporeactivity to pressor agents, which are characteristic of severe septic shock (2, 5–9).

NO is produced by three enzymes of the nitric oxide synthase (NOS) family (4, 10). Two constitutive forms of NOS (NOS-I, NOS-III) produce low levels of NO in response to agonists which increase intracellular calcium. The resulting NO is important in the regulation of normal homeostatic processes, including blood pressure and neuronal transmission (10). The inducible form of NOS (iNOS or NOS-II) produces high levels of NO, independently of intracellular calcium. The NO produced by iNOS functions in the immune response to pathogens and may also provide cytoxic activity toward cancer cells (10). Since iNOS activity is not calcium-dependent, regulation of iNOS expression is critical and many cytokines are involved in this process in various cell types (11).

Intravenous administration of endotoxin in experimental animals causes an initial, transient increase in NO that is probably due to stimulation of constitutive NOS followed by a sustained increase in NO produced by iNOS (8). Mice that are iNOS-deficient, due to gene disruption in embryonic stem cells, demonstrate resistance to doses of endotoxin that are lethal in wild-type animals (12, 13). iNOS(−/−) mice are also less effective at eliminating infections caused by Listeria monocytogenes or Leishmania major. Macrophages that are isolated from iNOS(−/−) mice are less effective killers of cancer cells in vitro (12).

Human α2-macroglobulin (hα2M) is a proteinase inhibitor and specific cytokine carrier that induces iNOS expression and NO synthesis in the murine macrophage-like cell line, RAW 264.7 (14). The mechanism of iNOS induction involves the neutralization of autocrine cytokine activity by hα2M. Since RAW 264.7 cells synthesize, secrete, and activate transforming growth factor-β (TGF-β) and because TGF-β-neutralizing antibody induces RAW 264.7 cell NO synthesis, similarly to hα2M, we proposed that neutralization of autocrine TGF-β is most likely responsible for the increase in iNOS expression in hα2M-

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1 The abbreviations used are: NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; hα2M, human α2-macroglobulin; mα2M, murine α2-macroglobulin; MUG, murinoglobulin; TGF-β, transforming growth factor-β; LRP, low density lipoprotein receptor-related protein; FBS, fetal bovine serum; BS5, bis(sulfosuccinimidyl) suberate; FBHE, fetal bovine heart endothelial; PAGE, polyacrylamide gel electrophoresis.
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35 kDa, as expected. For some experiments, moαM was radioiodinated with Iodo-Beads. The specific radioactivity was approximately 1.0 μCi/μg.

MUG was recovered in the flow-through fractions of the Ni²⁺-affinity chromatography column and further purified by gel filtration on Ultragel AcA 34 and ion exchange chromatography on Q-Sepharose FF (22). SDS-PAGE analysis of purified MUG revealed a single band with an apparent mass of 180 kDa. MUG and moαM preparations contained less than 1% by weight of endotoxin, as determined by the limulus amebocyte lysate assay (Associates of Cape Cod Inc., Woods Hole, MA), and were diluted at least 10-fold when introduced into cell culture systems. In control experiments, we determined that endotoxin (Sigma), at concentrations of 1.0 ng/ml or less, does not induce NO synthesis by RAW 264.7 cells.

MUG is effectively removed from the culture media by dialysis and nearly all of the protein is covalently bound to the membrane as cross-linked complexes with α2M (11, 12). Furthermore, MUG was shown to be clear of proteinase activity in cell culture systems by the specific proteinase inhibitor test, as described previously (34–36). The α2M-TGF-β complex, as well as native α2M itself, is covalent and resistant to degradation by endopeptidases and other proteases. These properties are essential for the specific binding of MUG to α2M and for the prevention of nonspecific binding to other cell components.

Levels of MUG in the plasma of non-pregnant αM(−/−) mice are unchanged compared with wild-type animals (23), raising the question of whether MUG and moαM are redundant as TGF-β neutralizers or NO inducers. The goals of this study were to: (i) compare the binding and neutralization of TGF-β by proteins from wild-type and αM(−/−) mouse plasma; (ii) characterize the binding of TGF-β1 and TGF-β2 to purified moαM and MUG; and (iii) determine whether moαM and/or MUG promote macrophage NO synthesis. Our results demonstrate that moαM is unique among murine α-macroglobulins, and murine plasma proteins in general, since moαM is the only major protein that binds TGF-β and inhibits TGF-β-receptor interactions. Purified moαM neutralized TGF-β in endothelial cell growth assays and promoted macrophage NO synthesis, while purified MUG was inactive in both cell culture systems. These studies identify TGF-β neutralization as a non-redundant activity of moαM and suggest a mechanism for the endotoxin insensitive phenotype of αM(−/−) mice.

Materials and Methods

Reagents and Proteins—TGF-β1 was from R&D Systems (Minneapolis, MN). TGF-β2 was from Genzyme (Cambridge, MA). Trypsin was purchased from Worthington and active-site titrated according to the method of Chase and Shaw (26). Chloramine T, fetal bovine serum (FBS), and bovine serum albumin were from Sigma. BSA (sulfosuccinimidyl-3-MGS) and Iodo-Beads were from Pierce. Na2[125I]I was from Amersham. Trypsin-EDTA, Dulbecco’s modified Eagle’s medium, RPMI 1640, and Earle’s balanced salts solution were from Life Technologies, Inc.

moαM was purified by the method of Imber and Pizzo (27). moαM was isolated by Ni²⁺-affinity chromatography, followed by gel filtration on AcA 22 (28). Analysis of purified moαM by SDS-PAGE under reducing conditions revealed three bands, with apparent masses of 180, 165, and 129 kDa, as expected. For some experiments, moαM was radioiodinated with Iodo-Beads. The specific radioactivity was approximately 1.0 μCi/μg.

MUG was recovered in the flow-through fractions of the Ni²⁺-affinity chromatography column and further purified by gel filtration on Ultragel AcA 34 and ion exchange chromatography on Q-Sepharose FF (22). SDS-PAGE analysis of purified MUG revealed a single band with an apparent mass of 180 kDa. MUG and moαM preparations contained less than 1% by weight of endotoxin, as determined by the limulus amebocyte lysate assay (Associates of Cape Cod Inc., Woods Hole, MA), and were diluted at least 10-fold when introduced into cell culture systems. In control experiments, we determined that endotoxin (Sigma), at concentrations of 1.0 ng/ml or less, does not induce NO synthesis by RAW 264.7 cells.
activity in each section. AC is the concentration of BS3-stabilized, noncovalent mαM- or mUG-TGF-β complex detected by PAGE (corrected for the presence of AC* by analysis of non-cross-linked samples). C is the concentration of free TGF-β detected by PAGE (including free TGF-β and AC which is not cross-linked by BS3). These values were plotted according to the following equation (34), which yields the KD and the BS3 cross-linking efficiency, z, which is a constant (0<z<1):

\[
\frac{C}{AC} = \frac{K_D}{z} + \frac{1}{\frac{1}{z} - 1}
\]

(2)

Apparent KD values, determined by this analysis, assume one TGF-β-binding site per a-macroglobulin. The KD values are “whole molecule” constants, accounting for both affinity and multiplicity of binding sites within a single binding protein, which is considered optimal for comparing two binding proteins. If there are two or four equivalent TGF-β-binding sites per a-macroglobulin, then the “isolated” site KD is 2- or 4-fold higher than the reported KD.

Endothelial Cell Growth Assays—Fetal bovine heart endothelial (FBHE) cells were maintained in complete medium, as described previously (36). For experiments, cells were harvested in trypsin-EDTA and plated at 2 X 10^4 cells/well in 24-well plates. After incubation for 15 h in complete medium, the cultures were washed and incubated with 10 µl TGF-β1 or TGF-β2, in the presence or absence of native hαM or native mαM (120 µl), in Dulbecco’s modified Eagle’s medium with 0.2% (vol/vol) FBS. Incubations proceeded for 30 h and then for an additional 18 h in the presence of [3H]thymidine (1 µCi/ml). [3H]Thymidine incorporation was determined as described previously (36). TGF-β1 and TGF-β2 are nearly equipotent inhibitors of FBHE growth when added in the presence of dilute FBS and in the absence of supplementary mαM (36, 38).

Nitric Oxide Synthesis Assay—RAW 264.7 cells were plated at 10^4 cells/well in 96-well plates and cultured, first in RPMI 1640 with 10% FBS for 24 h, and then in RPMI 1640 without FBS (SFM) for 24 h. Native hαM, native mαM, and MUG (each at 280 nM) were added separately to the cultures in SFM. After 24 h, conditioned culture medium samples were recovered. The stable NO oxidation product, nitrite, was measured in the conditioned medium samples, as described previously (14, 39). The concentration of the NO substrate, arginine, in RPMI 1640, is 1.2 mM. We have previously shown that the increase in cellular NO synthesis, induced by hαM, is inhibited by the NO inhibitor, N′-monomethyl-L-arginine (14).

RESULTS

TGF-β-binding Proteins in Murine Plasma—125I-TGF-β1 and 125I-TGF-β2 were incubated with plasma from wild-type or mαM(-/-) mice. BS3 was then added to covalently stabilize 125I-TGF-β-native a-macroglobulin complexes, which are known to be primarily noncovalent (34), and/or other TGF-β-plasma protein complexes (Fig. 1). As a control, both 125I-TGF-β isoforms were incubated with plasma from wild-type mice, labeling of all three of the 125I-TGF-β proteins in normal plasma was ineffective at inhibiting 125I-TGF-β protein complexes. The samples were then subjected to SDS-PAGE and autoradiography. The radiolabeled TGF-β preparations, that were not incubated with plasma, are labeled “control”. Panel B compares the mobilities of 125I-TGF-β2 incubated with wild-type plasma and 125I-mαM. Both samples were BS3-treated before electrophoresis.

with MUG or any other plasma protein, suggesting that mαM is the only significant TGF-β-binding protein in murine plasma. Another explanation for these data is that TGF-β1 and TGF-β2 bind to a plasma protein other than mαM, forming a complex that is not stabilized by the cross-linking reagent.

Affinity Labeling of TGF-β Receptors—As a second method for comparing the TGF-β binding activities of proteins in normal and αM-deficient mouse plasma, AKR-2B cells were affinity-labeled with 125I-TGF-β1 (Fig. 2). In the absence of plasma, the three well characterized TGF-β receptors in fibroblasts were identified, including ß1-glycan, a proteoglycan which migrates in a diffuse low-mobility band, the type II receptor, with an apparent mass of 75 kDa, and the type I receptor, with an apparent mass of 53 kDa (corrected for bound TGF-β). When 125I-TGF-β1 was added to cultures in the presence of plasma from wild-type mice, labeling of all three of the TGF-β receptors was decreased. By contrast, αM(-/-) mouse plasma was ineffective at inhibiting 125I-TGF-β1 binding to cellular receptors. These studies confirm the role of mαM in wild-type mouse plasma, as a major TGF-β-neutralizing protein. Other plasma proteins do not substitute for mαM in this capacity.

Binding of TGF-β1 and TGF-β2 to Purified mαM—The purified mαM preparations used in this study consisted entirely of protein in the native conformation as determined bydenaturing PAGE (Fig. 3). The mobility of the mαM was increased after reaction with trypsin, reflecting the transition to the activated conformation (31). Purified native mαM bound 125I-TGF-β1 or 125I-TGF-β2, as determined by nondenaturing PAGE and autoradiography. The autograph bands completely aligned with the Coomassie-stained native mαM bands, indicating that trace contamination of the mαM preparation with activated forms was not responsible for the observed TGF-β-binding. No attempt was made to quantitate the amount of 125I-TGF-β1 recovered in association with the mαM since recovery is influenced by
Using similar plots, separate experiments were averaged to generate the graphs shown in Fig. 2. Affinity labeling of TGF-β receptors in the presence of wild-type and α2M(−/−) mouse plasma. 125I-TGF-β1 was preincubated with wild-type (WT) plasma (10%), α2M(−/−) plasma (10%), or EHB. The samples were then transferred to confluent monolayers of AKR-2B fibroblasts and incubated for 4 h at 4°C. Cell extracts were analyzed by SDS-PAGE and autoradiography. The “Control” lane shows cells that were affinity labeled in the absence of plasma. The TGF-β receptor types are shown on the right.

Significant amounts of TGF-β was incubated with different concentrations of mα2M(−/−) and mα2M. Either lane a or 125I-TGF-β1 plasma (10%) was incubated with wild-type (WT) plasma (10%), α2M(−/−) plasma (10%), or EHB. The samples were then transferred to confluent monolayers of AKR-2B fibroblasts and incubated for 4 h at 4°C. Cell extracts were analyzed by SDS-PAGE and autoradiography. The “Control” lane shows cells that were affinity labeled in the absence of plasma. The TGF-β receptor types are shown on the right.

The results of six separate TGF-β equilibrium binding experiments were averaged to generate the graphs shown in Fig. 3. Using similar plots, separate K_D values were determined from each individual study and averaged to obtain the mean K_D values presented in Table I. The most important finding was the substantial difference in binding affinity of mα2M and MUG for the TGF-β isoforms. The K_D values for MUG binding to TGF-β1 and TGF-β2 were 30-fold higher than the corresponding constants for mα2M. Thus, MUG is not a significant TGF-β-binding protein capable of substituting for mα2M in α2M(−/−) mice, as was suggested by our experiments with α2M(−/−) mouse plasma.

A second unexpected finding was the high affinity of native mα2M for TGF-β1. Studies of bovine α2M (41) and hα2M (34, 41) have shown that these α-macroglobulins selectively bind TGF-β2 compared with TGF-β1. The K_D for the binding of TGF-β2 to native hα2M is 11 nM, compared with a K_D of 330 nM for the binding of TGF-β1 to native hα2M (34). The ability of native mα2M to function as an equally effective binding protein for TGF-β1 and TGF-β2 suggests a potentially enhanced role for mα2M in the regulation of TGF-β1 in this species.

FBHE Proliferation Assays—TGF-β1 and TGF-β2 are nearly equipotent inhibitors of FBHE proliferation when the cells are cultured in dilute FBS (36, 38). α-Macroglobulins counteract the activity of TGF-β and promote FBHE growth to an extent that depends on the affinity of the TGF-β/α-macroglobulin interaction (36). Due to the unprecedented low K_D determined for the interaction of native mα2M with TGF-β1, FBHE studies were performed to compare the TGF-β neutralizing activities of native mα2M and native hα2M in a biological system. As shown in Table II, TGF-β1 and TGF-β2 (each at 10 pM) inhibited FBHE [3H]thymidine incorporation by 89 ± 2 and 80 ± 3%, respectively. mα2M and hα2M (120 nM) substantially inhibited the activity of TGF-β2. By contrast, only mα2M inhibited the activity of TGF-β1; hα2M was ineffective at the studied concentration. The ability of mα2M to antagonize the activities of both TGF-β1 and TGF-β2, in the FBHE growth assay, confirms the results of the equilibrium binding experiments and indicates that mα2M is unique among α-macroglobulins studied thus far.
in its capacity to neutralize TGF-β1.

**Regulation of Nitric Oxide Synthesis by Mα2M and MUG**

Induction of NO synthesis by hα2M, mα2M, and MUG was compared in RAW 264.7 cells (Fig. 6). Each α-macroglobulin was present at the same concentration (280 nM); however, only hα2M and mα2M significantly increased nitrite levels in the conditioned media. The responses elicited by mα2M and hα2M were equivalent. By contrast, MUG did not cause a detectable increase in NO synthesis. We have previously shown that hα2M, at 280 nM, induces a response comparable to that observed with 10 ng/ml interferon-γ (14), a well known stimulator of macrophage iNOS (42, 43). In further control experiments, the α-macroglobulin preparations were boiled for 20 min and then added to the RAW 264.7 cell cultures. The boiled preparations did not increase NO synthesis, eliminating the possibility that endotoxin contributed to the observed responses. These studies demonstrate that the induction of NO synthesis, like the ability to neutralize TGF-β, is an activity of mα2M which is non-redundant among murine α-macroglobulins.
DISCUSSION

TGFB-β functions within the context of autocrine pathways to regulate many of the properties of cells in culture, especially when the culture medium is not serum-supplemented. In colon carcinoma cells, autocrine TGFB-β increases integrin αv gene expression (44); in rat VSMCs, autocrine TGFB-β suppresses platelet-derived growth factor α-receptor expression (45) and in RAW 264.7 cells, autocrine TGFB-β suppresses iNOS expression (14). In each of these studies, the importance of autocrine TGFB-β was demonstrated using a pan-isof orm TGFB-β-neutralizing antibody (14, 45) or a constitutively expressed TGFB-α antisense cDNA construct (46). hα2M binds a number of cytokines (34); however, in the absence of exogenously added cytokines, many of the activities of hα2M in cell culture may be attributed to specific neutralization of TGFB-β (14, 45). Whether α-macroglobulins counteract TGFB-β activity in vivo is undetermined.

Gene knock-out experiments in mice have demonstrated that TGFB-β1 functions in vivo to provide homeostatic suppression of immune/inflammatory responses (47). Endotoxin challenge causes a form of inflammatory response which can be lethal. iNOS(−/−) mice are insensitive to endotoxin challenge (12), reflecting the important role played by iNOS in mediating endotoxin-associated shock (5–9). Since α2M(−/−) mice also demonstrate decreased sensitivity to endotoxin (23), we decided to examine the effects of the murine α-macroglobulins on TGFB-β activity and iNOS activity. Although normal murine plasma has two major members of the α-macroglobulin family, only hα2M binds TGFB-1 and TGFB-2 with significant affinity. Murine α2M, but not MUG, inhibits TGFB-β binding to its cellular receptors and the biological response of FBHE cells to TGFB-β. Furthermore, among the murine α-macroglobulins, only hα2M induces macrophage NO synthesis. Thus, while the proteinase-inhibitory specificities of hα2M and MUG are at least partially redundant, the growth factor-carrier activities of these two proteins are not. In α2M(−/−) mice, MUG is expressed at normal levels and, during pregnancy, at increased levels (23); however, as shown with in vitro experiments in Figs. 1 and 2, neither this protein nor any other protein in hα2M-deficient plasma substitutes for hα2M as a TGFB-β carrier and neutralizer. The studies described here suggest that hα2M, in wild-type mice, may counteract TGFB-β locally, at sites of inflammation, and thereby permit augmented iNOS expression in response to endotoxin. Further in vivo testing is warranted to test this hypothesis.

Our analysis of NO synthesis was performed using RAW 264.7 cells. Although this is a passaged cell line, RAW 264.7 cells demonstrate excellent conservation of differentiated macrophage properties and can be primed/activated for tumour-cidal and bacteriocidal activity, similarly to primary cultures of macrophages (48, 49). Human macrophages, like the RAW 264.7 cells, secrete TGFB-β (50) which functions as a potent inhibitor of human macrophage activation (51, 52). Thus, RAW 264.7 cells provide an accurate and reproducible model for studying the responses of normal macrophages.

Danielpour and Sporn (41) performed experiments with serum and α2M, purified from human and bovine plasma, to demonstrate selective binding of TGFB-β2, compared with TGFB-β1. The Kd forTGFB-β1 binding to native hα2M is 30-fold higher than the Kd forTGFB-β2-native hα2M interaction (34). Thus, the ability of α2M to bind TGFB-1 and TGFB-2 with equal affinity is novel, compared with other characterized α-macroglobulins. In addition to hα2M and hα2M, we have also studied the binding of hα2M to rat α2M, the constitutively synthesized homologue of hα2M, and to rat α2M, an acute-phase reactant (53). Based on these studies, we can rank order the α-macroglobulins according to their affinities for TGFB-β1 as follows: hα2M > rat α2M > rat α1M > hα1M ≈ MUG.

In our analysis of TGFB-β interactions with α2M and MUG, we focused on the native forms of each protein. Activated α-macroglobulins are rapidly taken up by cells that express LRP (17, 31). As a result, levels of activated α-macroglobulins are negligible in the plasma under most circumstances. In the pericellular spaces, higher concentrations of activated α-macroglobulins may accumulate unless the tissue includes cells that express LRP. One concern regarding our NO synthesis experiments was whether the RAW 264.7 cells might secrete sufficient levels of proteinases to convert the α-macroglobulins into activated forms during the 24-h incubation. We previously demonstrated that this does not occur in cultures of vascular smooth muscle cells (45). To test for proteinase secretion, we incubated radiolabeled native hα2M with the RAW 264.7 cells for 24 h. The hα2M was then subjected to nondenaturing PAGE. We also measured cell-associated radioactivity and the amount of trichloroacetic acid-soluble radioactivity in the medium. None of our tests revealed detectable conversion of hα2M into the activated conformation (results not shown). Thus, the induction of NO synthesis by native α2M and hα2M does not occur subsequent to proteinase modification of these proteins.

When challenged with bleomycin, the lungs of α2M(−/−) mice were not substantially affected while the lungs of wild-type mice developed inflammatory infiltrates and early connective tissue deposition (23). TGFB-β, which is secreted by alveolar macrophages and activated locally, has been implicated in the regulation of this process (54). It is intriguing to speculate that the α2M(−/−) mice are bleomycin tolerant due to enhanced function of TGFB-β as a general anti-inflammatory; however, TGFB-β may actually promote certain aspects of the bleomycin response, such as the deposition of extracellular matrix proteins (55). Understanding the relationship between TGFB-β and α2M, the development of immune cell infiltrates, and fibrosis, will require further investigation.

In addition to its ability to neutralize TGFB-β locally, α2M serves as a major carrier of TGFB-β in the plasma, delaying TGFB-β clearance and providing a potential pool of reversibly bound active factor (56–58). The unique and non-redundant activity of α2M as a TGFB-β carrier in murine plasma suggests that TGFB-β plasma pharmacokinetics may be altered in α2M(−/−) mice, in addition to TGFB-β activity. In our cell culture systems, we have shown that α2M induces iNOS activity in macrophages while MUG does not. These in vitro results provide a model for explaining some of the phenotypic properties of the α2M(−/−) mouse.

REFERENCES

1. Raether, C. R. H., Ulevitch, R. J., Wright, S. D., Sibley, C. H., Ding, A., and Nathan, C. F. (1991) FASEB J. 5, 2652–2660
2. Parrillo, J. E. (1993) N. Engl. J. Med. 328, 1471–1477
3. Burrell, R. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 786–792
4. Nathan, C. F. (1991) FASEB J. 5, 2652–2660
5. Wolfe, T. A., and Dasta, J. F. (1995) JAMA 273, 13725–13728
6. Concha, F. R., Assreuy, J., Moss, D. W., Rees, D., Leal, L. M. C., Moncada, S., Muller, W., Moncada, S., and Liew, F. Y. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 3629–3632
7. Cunha, F. Q., Assreuy, J., Moss, D. W., Rees, D., Leal, L. M. C., Moncada, S., Carrier, M., O'Donnell, C. A., and Liew, F. Y. (1994) Immunology 81, 211–215
8. Petroz, A., Lamb, G., Greene, S. M., Moncada, S., Bennett, D., and Vallance, P. (1994) Cardiovasc. Res. 28, 34–39
9. Szabo, C., Mitchell, J. A., Thiemermann, C., and Vane, J. R. (1993) Br. J. Pharmacol. 109, 796–797
10. Nathan, C., and Xie, Q.-W. (1994) Cell 78, 915–918
11. De Vera, M. E., Shapiro, R. A., Nussler, A. K., Mudgett, J. S., Simmons, R. L., Morris, S. M., Billiar, T. R., and Geller, D. A. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 1054–1059
12. MacMicking, J. D., Nathan, C., Hom, G., Chartnain, N., Fletcher, D. S., Trumbauer, M., Stevens, K., Xie, Q.-W., Sokol, K., Hutchinson, N., Chinn, H., and Mudgett, J. S. (1996) Cell 84, 641–650
13. Wei, X. Q., Charles, I. G., Smith, A., Ure, J., Feng, G.-J., Huang, F. P., Xu, D., Muller, W., Moncada, S., and Liew, F. Y. (1996) Nature 382, 408–411
14. Lysliak, J. J., Hussaini, I. M., Webb, D. J., Glass, W. F., II, Alletta, M., and
Induction of Nitric Oxide Synthesis by Murine α-Macroglobulins

Gonias, S. L. (1985) J. Biol. Chem. 260, 21919–21927
15. Barrett, A. J., and Starkey, P. M. (1973) Biochem. J. 133, 799–724
16. Gonias, S. L., Reynolds, J. A., and Pizzo, S. V. (1982) Biochim. Biophys. Acta 705, 306–314
17. Pizzo, S. V., and Gonias, S. L. (1984) in The Receptors (Conn, P. M., ed) Vol. 1, pp. 177–221, Academic Press, Inc., New York
18. Overbergh, L., Torrekens, S., Van Leuven, F., and Van den Berghe, H. (1991) J. Biol. Chem. 266, 16903–16910
19. Van Leuven, F., Torrekens, S., Overbergh, L., Lorent, K., De Strooper, B., and Van den Berghe, H. (1992) Eur. J. Biochem. 210, 319–327
20. Overbergh, L., Hilliker, C., Lorent, K., Van Leuven, F., and Van den Berghe, H. (1994) Genomics 22, 530–539
21. Saito, A., and Sinohara, H. (1985) J. Biol. Chem. 260, 775–781
22. Abe, K., Yamamoto, K., and Sinohara, H. (1989) J. Biochem. 106, 564–568
23. Umans, L., Serneels, L., Overbergh, L., Lorent, K., Van Leuven, F., and Van den Berghe, H. (1995) J. Biol. Chem. 270, 19778–19785
24. Vodovotz, Y., Bogdan, C., Paik, J., Xie, Q.-W., and Nathan, C. F. (1993) J. Exp. Med. 178, 605–613
25. Perrella, M. A., Yoshizumi, M., Fen, Z., Tsai, J.-C., Hsieh, C.-M., Kourembanas, S., and Lee, M.-E. (1994) J. Biol. Chem. 269, 14595–14600
26. Chase, T., Jr., and Shaw, E. (1967) Biochem. Biophys. Res. Commun. 29, 508–514
27. Imber, M. J., and Pizzo, S. V. (1981) J. Biol. Chem. 256, 8134–8139
28. Anonick, P. K., Vetter, W. H., and Gonias, S. L. (1989) Biochem. J. 264, 745–752
29. Ruff, E., and Rizzino, A. (1986) Biochem. Biophys. Res. Commun. 138, 714–719
30. Van Leuven, F., Cassiman, J.-J., and Van den Berghe, H. (1981) J. Biol. Chem. 256, 9016–9022
31. Gonias, S. L., Balber, A. E., Hubbard, W. J., and Pizzo, S. V. (1983) Biochem. J. 209, 99–105
32. Massague, J. (1987) Methods Enzymol. 146, 174–195
33. LaMarre, J., Vasudevan, J., and Gonias, S. L. (1994) Biochem. J. 302, 199–205
34. Crookston, K. P., Webb, D. J., Wolf, B. B., and Gonias, S. L. (1994) J. Biol. Chem. 269, 1533–1540
35. Wolf, B. B., and Gonias, S. L. (1994) Biochemistry 33, 11270–11276
36. Webb, D. J., Atkins, T. L., Crookston, K. P., Burmester, J. K., Qian, S. W., and Gonias, S. L. (1994) J. Biol. Chem. 269, 30402–30406
37. Crookston, K. P., and Gonias, S. L. (1994) Biochem. Biophys. Res. Commun. 200, 1578–1583
38. Burmester, J. K., Qian, S. W., Roberts, A. B., Huang, A., Amatyakul-Chantler, S., Sueredt, L., Odartchenko, N., Madri, J. A., and Sporn, M. B. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 8628–8632
39. Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., and Tannenbaum, S. (1989) Anal. Biochem. 180, 131–138
40. Gonias, S. L., LaMarre, J., Crookston, K. P., Webb, D. J., Wolf, B. B., Lopes, M. B., Moses, H. L., and Hayes, M. A. (1994) Ann. N. Y. Acad. Sci. 737, 273–290
41. Danielpour D., and Sporn, M. B. (1990) J. Biol. Chem. 265, 6973–6977
42. Lorschach, R. B., Murphy, W. J., Lowenstein, C. J., Snyder, S. H., and Russell, S. W. (1993) J. Biol. Chem. 268, 1908–1913
43. Xie, Q.-W., Cho, H. J., Calaycay, J., Munford, R. A., Swiderek, K. M., Lee, T. D., Ding, A., Troop, T., and Nathan, C. F. (1992) Science 256, 225–228
44. Wang, D., Zhou, G., Birkenmeier, T. M., Gong, J., Sun, L., and Brattain, M. G. (1995) J. Biol. Chem. 270, 14154–14159
45. Weaver, A. M., Owens, G. K., and Gonias, S. L. (1995) J. Biol. Chem. 270, 30734–30748
46. Wu, S., Theodorescu, D., Kerbel, R. S., Wilson, J. K. V., Mulder, K. M., Humphrey, L. E., and Brattain, M. G. (1992) J. Cell Bio. 116, 187–196
47. Kulkarni, A. B., Hub, C.-G., Becker, D., Geiser, A., Lyght, M., Flanders, K. C., Roberts, A. B., Sporn, M. B., Ward, J. M., and Karlsson, S. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 770–774
48. Haak-Frendscho, M., Wynn, T. A., Czuprynski, C. J., and Paulnock, D. (1990) Clin. Exp. Immunol. 82, 404–410
49. Lambert, L. E., and Paulnock, D. M. (1989) Cell. Immunol. 120, 401–418
50. Assion, R. K. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 6920–6924
51. Bermudez, L. E. (1993) J. Immunol. 150, 1838–1845
52. Randow, F., Syrbu, U., Meisel, C., Krausch, D., Zuckermann, H., Plater, C., and Volk, H.-D. (1995) J. Exp. Med. 181, 1887–1892
53. Webb, D. J., Crookston, K. P., Figler, N. F., LaMarre, J., and Gonias, S. L. (1995) Biochem. J. 312, 579–586
54. Denholm, E. M., and Rollins, S. M. (1993) Amer. J. Physiol. 264, L36–L42
55. Giri, S. N., Hyde, D. M., and Hollinger, K. M. (1993) Science 260, 775–781
56. O'Connor-McCourt, M., and Wakefield, L. M. (1987) J. Biol. Chem. 262, 187–196
57. Ruff, E., and Rizzino, A. (1986) Biochem. Biophys. Res. Commun. 138, 714–719
58. Onoue, M., and Sporn, M. B. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 770–774
59. Lau, K.-S., and Sporn, M. B. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 699–603