Fatty acids modulate transforming growth factor-β activity and plasma clearance

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ABSTRACT

The activity and plasma clearance of transforming growth factor (TGF)-β are known to be regulated by activated α2-macroglobulin (α2M*). This has been implicated in pathophysiological processes, but no small molecule compounds have been reported to modulate TGF-β activity by affecting the interaction of TGF-β and α2M*. Here, we demonstrate that fatty acids are capable of inhibiting complex formation of TGF-β isoforms and α2M* as demonstrated by nondenaturing and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This is dependent on carbon chain length (C20, C18, C16, C14 > C12 > C10), degree of unsaturation (polyunsaturated > saturated), and TGF-β isoforms (TGF-β1 > TGF-β2 > TGF-β3). Arachidonic acid, which is one of the most potent inhibitors, is also capable of dissociating TGF-β-α2M* complexes, but higher concentrations are required. Arachidonic acid appears to inhibit TGF-β-α2M* complex formation by binding specifically to α2M* as demonstrated by gel filtration chromatography. Arachidonic acid reverses the inhibitory effect of α2M* on TGF-β binding, TGF-β-induced growth inhibition, and TGF-β-induced transcriptional activation in mink lung epithelial cells and affects plasma clearance of TGF-β-α2M* complexes in mice. These results show that fatty acids are effective modulators of TGF-β activity and plasma clearance and may be useful in treating human diseases through their effects on the interaction of TGF-β and α2M*.

Key words: arachidonic acid • myristic acid • TGF-β • activated α2-macroglobulin • TGF-β-α2M complexes

Transforming growth factor (TGF)-β is a family of 25-kDa structurally homologous dimeric proteins that show ~70% amino acid sequence homology (1, 2). It has a remarkably wide range of activities. It inhibits growth of epithelial cells, endothelial cells, and lymphocytes but stimulates growth of mesenchymal cells such as fibroblasts. It has chemotactic activity toward mesenchymal and inflammatory cells, regulates angiogenesis, stimulates transcriptional activation of extracellular matrix synthesis-related genes, and plays an important role in the process
of wound repair (1–4). Low levels of active TGF-β in plasma and tissues have been linked to atherosclerosis, malignancy, and certain forms of autoimmune diseases (1–4).

In mammalian species, there are three known members, TGF-β₁, TGF-β₂, and TGF-β₃ (1, 2). These isoforms exert similar biological activities in some cell systems but different activities in other systems (5–7). In the mink lung epithelial cell model system, all three isoforms bind to cell surface TGF-β receptors with similar affinity and show similar growth inhibitory activity (5–7). They are not equivalent in inhibiting growth of endothelial cells (5–7). In a wound healing model, TGF-β₃ reduces scarring whereas TGF-β₁ enhances it (8). The mechanisms by which these isoforms exert different biological activities are not well understood. However, several TGF-β binding molecules have been reported to be involved in determining the activities of TGF-β isoforms (9–13). Heparin and the highly sulfated liver heparan sulfate potentiate the biological activity of TGF-β₁, but not the other isoforms (9). The expression of the TGF-β type III receptor and an alternatively spliced TGF-β type II receptor is known to be required for responsiveness to TGF-β₂ in several cell types (10). α₂-Macroglobulin (α₂M) can be altered by proteases or primary amines to form so-called activated α₂M (α₂M*), which interacts differentially with these TGF-β isoforms and contributes to their differential activities in some experimental systems (11–14). Among the various TGF-β binding molecules, α₂M* is unique in its ability to bind TGF-β isoforms with distinct affinities and to affect their plasma clearance (15). α₂M* also forms complexes with other growth factors, cytokines, and hormones and modulates their biological activities in many experimental systems (16–18).

An active site in TGF-β₁ and TGF-β₂ responsible for high-affinity binding to α₂M* has been recently identified at Trp-52 (19). Synthetic peptides containing Trp-52 are capable of blocking complex formation of α₂M* and TGF-β isoforms. They also block the formation of complexes between α₂M* and other growth factors, cytokines, and hormones (19, unpublished results). It is hypothesized that α₂M* forms complexes with TGF-β isoforms and these diverse molecules by interacting with hydrophobic surface regions that involve nonhomologous sequences (19). If this hypothesis is correct, one might be able to identify small hydrophobic compounds that could inhibit the formation of complexes between α₂M* and TGF-β and other factors, thereby modulating their activities. We therefore screened many small hydrophobic compounds that are known to be present in plasma and tissue for their ability to inhibit complex formation of ¹²⁵I-TGF-β₁ and α₂M*. Among them, only fatty acids have this property, suggesting that they may be important modulators of the interaction between TGF-β and α₂M* in vivo.

Here, we show that specific fatty acids strongly inhibit complex formation between α₂M and TGF-β isoforms and are also capable of dissociating TGF-β-α₂M* complexes. We also demonstrate that fatty acids modulate ¹²⁵I-TGF-β activity in mink lung epithelial cells and affect the plasma clearance of TGF-β₁-α₂M* and TGF-β₂-α₂M* complexes in mice.

MATERIALS AND METHODS

Na¹²⁵I (17.4 Ci/mg), [5,6,8,9,11,12,14,15-³H] arachidonic acid (683 mCi/mg), [methyl-³H] thymidine (102 mCi/mg), chelate-Sepharose FF, and Sephacryl S-300 HR were purchased from Amersham Biosciences (Buckinghamshire, UK). TGF-β₁, TGF-β₂, and TGF-β₃ were obtained from Austral Biologicals (San Ramon, CA) and R&D Systems (Minneapolis, MN). Disuccinimidyl
suberate (DSS) was obtained from Pierce (Rockford, IL). Fatty acids (cis), fatty acid derivatives and analogs, and bovine serum albumin (which was not delipidated) were purchased from Sigma (St. Louis, MO). Mink lung epithelial cells (Mv1Lu) were grown and maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS). ICR mice were obtained from the Laboratory Animal Center, National Taiwan University College of Medicine (Taipei, Taiwan).

**Preparation of human $\alpha_2$M and $\alpha_2$M**

Human $\alpha_2$M was purified from pooled citrate-treated human plasma, using Zn$^{2+}$ chelate-Sepharose FF affinity chromatography followed by gel filtration on Sephacryl S-300 HR as described previously (20, 21). $\alpha_2$M ($\alpha_2$M*) activated by methylamine and plasmin was prepared as described previously (12, 22).

**Iodination of TGF-β**

TGF-β1, TGF-β2 and TGF-β3 (5 µg) were each iodinated with 2 mCi of Na$^{125}$I, using chloramine T according to the procedure of Huang et al. (12). The specific radioactivity of $^{125}$I-TGF-β1, $^{125}$I-TGF-β2, and $^{125}$I-TGF-β3 was 1–5 × 10$^5$ cpm/ng in each case.

**Complex formation of $^{125}$I-TGF-β and $\alpha_2$M**

The reaction mixture contained 10 µg of $\alpha_2$M*, ~1 nM of $^{125}$I-TGF-β1, $^{125}$I-TGF-β2, or $^{125}$I-TGF-β3 and various concentrations of fatty acids (dissolved in 100% ethanol) in 0.05 ml of 50 mM HEPES-NaOH buffer, pH 7.4. The final concentration of ethanol in the reaction mixture was 0.5%. These fatty acids and fatty acid derivatives were soluble under the experimental conditions. After 30 min at room temperature, the complex formation of $^{125}$I-TGF-β and $\alpha_2$M* was determined by 5% nondenaturing polyacrylamide gel electrophoresis (PAGE) or by 7.5% sodium dodecyl sulfate (SDS)-PAGE following cross-linking by 0.6 mM DSS. After electrophoresis, the gel was stained with Coomassie blue and analyzed by autoradiography. The $^{125}$I-TGF-β-$\alpha_2$M* complex that co-migrated with free $\alpha_2$M* was quantified using a PhosphoImager (Fujiphoto Film, Tokyo, Japan).

**Gel filtration of $^3$H-arachidonic acid-$\alpha_2$M complexes**

The reaction mixture contained 100 µM $^3$H-arachidonic acid with or without 10 µg of $\alpha_2$M*, which was activated by methylamine and plasmin as described previously (12, 22), or native $\alpha_2$M in 0.05 ml of 50 mM HEPES-NaOH buffer, pH 7.4. After 30 min at room temperature, the reaction mixtures were applied onto a column (0.7 × 40 cm) of Sephacryl S-300 HR pre-equilibrated with 50 mM sodium phosphate buffer, 150 mM NaCl, pH 7.0. The column was then eluted with the same phosphate buffer, and the fractional volume was ~ 1 ml, 20 µl of which was counted with a scintillation counter and another 20 µl of which was analyzed by SDS-PAGE followed by Coomassie blue staining (to locate fractions containing $\alpha_2$M* or native $\alpha_2$M). The $^3$H-arachidonic acid-$\alpha_2$M* complex co-chromatographed with $\alpha_2$M* or native $\alpha_2$M. $\alpha_2$M*, whether activated by methylamine or plasmin, did not show significant differences in its ability to bind $^3$H-arachidonic acid with respect to the stoichiometry of $^3$H-arachidonic acid and $\alpha_2$M* (~ 2:1) in the complex.
Binding of $^{125}$I-TGF-β$_2$ to Mv1Lu cells

Mv1Lu cells grown on 24-well clustered dishes were incubated with various concentrations (1.25, 2.5, 5, and 10 pM) of $^{125}$I-TGF-β$_2$ and α$_2$M* (0 or 200 µg/ml) in the presence and absence of 30 µM arachidonic acid and 10 µM TGF-β peptantagonist (19) in binding buffer (50 mM HEPES/NaOH, pH 7.4, 128 mM NaCl, 5 mM KCl, 5 mM MgSO$_4$, 1.2 mM CaCl$_2$) containing 2 mg/ml of bovine serum albumin (23). After 2.5 h at 0°C, the cells were washed with binding buffer, and the cell-associated radioactivity was determined. All experiments were carried out in quadruplicate.

[Methyl-$^3$H]-thymidine incorporation assay

Mv1Lu cells were plated at a cell density of $7.5 \times 10^4$ cells/well in DMEM containing 0.2% FCS in 48-well cluster dishes. After 4 h at 37°C (to allow cell adherence), cells were treated with various concentrations of TGF-β$_2$, α$_2$M* (0 or 200 µg/ml), and arachidonic acid (0, 0.1, or 1 uM). After 16 h at 37°C, cells were pulsed with 1 uCi/ml [methyl-$^3$H]-thymidine for 2 h. The [methyl-$^3$H]-thymidine incorporation into cellular DNA was carried out in triplicate as described previously (23).

Luciferase assay

Mv1Lu cells that had been plated on 12-well clustered dishes at a cell density of $\sim 0.8–1.0 \times 10^5$ cells/plate were transfected with 4–6 µg of p3TP-Lux, using the calcium phosphate method (24). After 12 h, the transfected cells were washed with phosphate-buffered saline and allowed to grow in a medium containing 10% FCS for 12 h. The medium was then changed to DMEM with low serum concentration (0.2% FCS), and the cells were incubated for 4–6 h. The cells were then treated for 20 h with TGF-β$_2$ (0, 50, or 100 pM), α$_2$M* (0 or 200 µg/ml), and arachidonic acid (0, 12.5, or 25 µM) in the same low-serum medium. The cells were harvested and assayed for luciferase activity, using the Promega (Madison, WI) kit according to the manufacturer’s protocol. The luciferase activity was assayed in triplicate cell cultures and measured as arbitrary units.

Plasma clearance of $^{125}$I-TGF-β in the presence and absence of α$_2$M*

$^{125}$I-TGF-β$_1$ (1 nM) or $^{125}$I-TGF-β$_2$ (1 nM) was preincubated with α$_2$M* (10 µg/50 µl) in the presence and absence of 10 µM arachidonic acid at room temperature for 30 min before injection into the lateral tail veins of mice anesthetized with ketamine as described previously (19). Blood samples (~25 µl) were taken at 10 s and 1, 2, 3, 5, 10, 15, 20, 30, and 60 min from the retroorbital venous plexus, using heparinized hematocrit tubes. The radioactivity in the blood sample obtained at 10s was taken as 100%. Four mice were used for each group in the experiments. The Student’s t test was used for determining the statistically significant differences of data analyzed.

RESULTS

Fatty acids block complex formation of TGF-β$_1$ and α$_2$M

Saturated and unsaturated fatty acids are present in plasma and tissues (25, 26). We first examined the effects of various concentrations of saturated fatty acids on the formation of complexes between $^{125}$I-TGF-β$_1$ and α$_2$M*. $^{125}$I-TGF-β$_1$ (1 nM) was incubated with α$_2$M* (200 µg/ml) in the presence of various concentrations of n-caprylic acid (10 carbon atoms), lauric acid (12 carbon atoms), myristic

acid (14 carbon atoms), palmitic acid (16 carbon atoms), and stearic acid (18 carbon atoms). After 30 min at room temperature, the reaction mixture was analyzed by 5% nondenaturing PAGE and autoradiography, a standard method for determining complex formation of $^{125}$I-TGF-β and α₂M* (12). In this system, the complexes of α₂M* and various $^{125}$I-labeled interacting proteins co-migrate with α₂M* (which migrates slowly in the separating gel due to the large size of the molecule), whereas the free $^{125}$I-labeled proteins migrate at the dye front or do not migrate into the separating gel, depending on its acidity or basicity at the electrophoresis buffer pH. For example, $^{125}$I-TGF-β does not migrate into the separating gel due to its basicity under the electrophoretic conditions (pH 8.3). As shown in Figure 1A, these saturated fatty acids inhibited the formation of complexes between TGF-β₁ and α₂M* in a concentration-dependent manner, with IC₅₀s of 6.6 ± 0.9 (n=4), 8.5 ± 1.0 (n=4), 9.1 ± 0.5 (n=4), and 68 ± 10 (n=4) µM for stearic acid, palmitic acid, myristic acid, and lauric acid, respectively. n-Caprylic acid was a relatively weak inhibitor. At 100 µM, it inhibited ~20% of the complex formation between TGF-β₁ and α₂M*. Esterification consistently abolished the inhibitory activities of the fatty acids (data not shown). These results suggest that many saturated fatty acids are capable of inhibiting the complex formation between $^{125}$I-TGF-β₁ and α₂M* but require a minimum carbon chain length (≥14 carbon atoms) and the presence of a free carboxyl group for optimal activities.

As shown in Figure 1A, myristic acid, palmitic acid, and stearic acid, which contain 14, 16, and 18 carbon atoms, respectively, potently inhibited complex formation of $^{125}$I-TGF-β₁ and α₂M*. It was of interest to test various unsaturated fatty acids that have the same carbon chain length, because double bonds are known to shorten the molecular length of fatty acids and confer more rigid configurations. As shown in Figure 1B, arachidonic acid (20:4n6), oleic acid (18:1n9), γ-linolenic acid (18:3n6), linoleic acid (18:2n6), palmitoleic acid (16:1n7), and linolenic acid (18:3n3) inhibited complex formation of $^{125}$I-TGF-β₁ and α₂M* in a concentration-dependent manner, with IC₅₀s of 7.8 ± 1.4 (n=3), 5.2 ± 2.0 (n=3), 8.0 ± 2.0 (n=3), 7.2 ± 2.5 (n=3), 15.1 ± 2.0 (n=3), and 26 ± 3.1 (n=3) µM, respectively. The activities of most of these unsaturated fatty acids were similar to those of their saturated counterparts of identical chain length (arachidonic acid, linoleic acid, and γ-linolenic acid), but linolenic and palmitoleic acids were weaker than their saturated counterparts. Note that ω-6 fatty acids (arachidonic acid, γ-linolenic acid, and linoleic acid) were more potent than ω-3 fatty acids (e.g., linolenic acid). Because arachidonic acid was one of the most potent inhibitors among the fatty acids tested, we studied the structure and function relationship of arachidonic acid by examining the effects of arachidonic acid derivatives and analogs, including a nonmetabolic analog ETYA (8, 11, 14 eicosatrien-5-ynoic acid); arachidonic acid methyl ester; and its 20-, 15-, and 5-hydroxy derivatives on the formation of complexes between $^{125}$I-TGF-β₁ and α₂M*. As shown in Figure 2, ETYA (IC₅₀, 30 ± 3.0 µM) was not as effective as arachidonic acid in inhibiting complex formation of $^{125}$I-TGF-β and α₂M*, whereas arachidonic acid methyl ester was inactive. The hydroxy derivatives of arachidonic acid showed very weak activities (data not shown). The IC₅₀s of these derivatives were estimated to be >100 µM. These results indicate that replacement of the double bond with the triple bond, esterification of the carboxy group, and addition of a hydroxy group in the hydrocarbon chain all significantly diminish the ability of arachidonic acid to inhibit complex formation between TGF-β₁ and α₂M*. 
Fatty acids inhibit complex formation of TGF-β isoforms and α₂M*

TGF-β isoforms bind to α₂M* with different affinities: TGF-β₂ > TGF-β₁ (14). The active sites of TGF-β₁ and TGF-β₂ responsible for high-affinity binding to α₂M* are distinct from the low-affinity α₂M* binding site in TGF-β₁ (19). To determine whether fatty acids differentially affect the binding of TGF-β isoforms to α₂M*, we determined the effects of various concentrations of arachidonic acid and myristic acid on complex formation of ¹²⁵I-labeled TGF-β isoforms and α₂M*. Myristic acid and arachidonic acid were the most potent inhibitors of complex formation among the saturated and unsaturated fatty acids we tested. As shown in Figure 3A, myristic acid inhibited complex formation of α₂M* and ¹²⁵I-TGF-β₂ or TGF-β₃ much less than that of α₂M* and TGF-β₁. It inhibited 30% of the complex formation of α₂M* and TGF-β₂ and TGF-β₃ at 100 and >250 µM, respectively. Arachidonic acid, a polyunsaturated fatty acid, was a stronger inhibitor of complex formation of α₂M* and TGF-β₂/TGF-β₃. It inhibited 50% of the complex formation of α₂M* and ¹²⁵I-TGF-β₂ and ¹²⁵I-TGF-β₃ at ~50 and ~70 µM, respectively (Fig. 3A). The observation that myristic acid and arachidonic acid inhibited complex formation of ¹²⁵I-TGF-β₂ and α₂M* more weakly than they inhibited complex formation of ¹²⁵I-TGF-β₁ and α₂M* is consistent with binding affinity data. TGF-β₂ binds to α₂M* with higher affinity than TGF-β₁ (14). To further define the inhibitory effect of fatty acids on complex formation of TGF-β isoforms and α₂M*, the ¹²⁵I-TGF-β isoform-α₂M* complexes were cross-linked by a cross-linking agent (DSS) following incubation of ¹²⁵I-TGF-β isoforms and α₂M* in the presence of various concentrations of arachidonic acid. The cross-linked ¹²⁵I-TGF-β isoform-α₂M* complexes in the reaction mixtures were then analyzed by 7.5% SDS-PAGE and autoradiography. As shown in Figure 3B, arachidonic acid blocked complex formation of ¹²⁵I-TGF-β isoforms and α₂M* with effective concentrations comparable to those obtained by determining ¹²⁵I-TGF-β isoform-α₂M* complex formation with nondenaturing PAGE (Fig. 3A).

Fatty acids are capable of dissociating TGF-β-α₂M complexes

To determine whether fatty acids are capable of dissociating TGF-β-α₂M* complexes, we added various concentrations of arachidonic acid to a reaction mixture containing ¹²⁵I-TGF-β₁ or ¹²⁵I-TGF-β₂ and α₂M* that had been preincubated at room temperature for 30 min. After 30 min at room temperature, the ¹²⁵I-TGF-β isoform-α₂M* complexes in the reaction mixtures were analyzed by 5% non-denaturing PAGE. As shown in Figure 4, arachidonic acid was able to dissociate the ¹²⁵I-TGF-β₁-α₂M* and ¹²⁵I-TGF-β₂-α₂M* complexes with ED₅₀ₐ of >500 and ~250 µM, respectively. Note that arachidonic acid was more effective in dissociating the ¹²⁵I-TGF-β₂-α₂M* complex than the ¹²⁵I-TGF-β₁-α₂M* complex. This is in contrast to the observation that arachidonic acid inhibited complex formation of ¹²⁵I-TGF-β₁ and α₂M* more effectively than ¹²⁵I-TGF-β₂ and α₂M*. However, much lower concentrations of arachidonic acid were effective in inhibiting complex formation of ¹²⁵I-TGF-β₁ and α₂M* than were required to dissociate the ¹²⁵I-TGF-β₁-α₂M* complex. Myristic acid and other saturated fatty acids were inactive for dissociating the ¹²⁵I-TGF-β-α₂M* complexes at 250 µM (data not shown).

Arachidonic acid binds to α₂M* but not native α₂M

The mechanism by which arachidonic acid and other fatty acids inhibit complex formation of TGF-β isoforms and α₂M* was not known. We hypothesized that the mechanism involves specific binding to α₂M*. To test this, we determined the interaction of ³H-arachidonic acid and α₂M*, using gel
filtration. $^3$H-arachidonic acid was incubated with native $\alpha_2 M$ or $\alpha_2 M^*$ (which was activated by methylamine). After incubation at room temperature for 30 min, the reaction mixture was subjected to gel filtration chromatography on Sephacryl S-300 HR. The $^3$H-arachidonic acid radioactivity and concentrations of $\alpha_2 M^*$ or native $\alpha_2 M$ in the eluents were determined by scintillation counting and 5% SDS-PAGE followed by Coomassie blue staining, respectively. As shown in Figure 5, the reaction mixture containing $^3$H-arachidonic acid and $\alpha_2 M^*$ yielded one small and one large $^3$H-radioactivity peak after being subjected to gel filtration chromatography on Sephacryl S-300 HR. The small peak, which appeared in the flow-through fractions, contained the $^3$H-arachidonic acid-$\alpha_2 M^*$ complex and free $\alpha_2 M^*$, which was identified by Coomassie blue staining (Fig. 5, inset). The subsequent large peak, which appeared in the column bed volume fractions, was identified as free $^3$H-arachidonic acid. In contrast, the reaction mixture containing native $\alpha_2 M$ and $^3$H-arachidonic acid showed only the large peak, indicating no complex formation. Under the gel filtration conditions, the stoichiometry of the $^3$H-arachidonic acid and $\alpha_2 M^*$ complex was estimated to be ~2:1. $\alpha_2 M^*$, which was activated by plasmin, and was also found to form the $^3$H-arachidonic acid complex with the similar stoichiometry (data not shown). These results suggest that arachidonic acid is capable of forming complexes with $\alpha_2 M^*$ but not native $\alpha_2 M$. Arachidonic acid appears to block complex formation of TGF-β and $\alpha_2 M^*$ by specific binding to $\alpha_2 M^*$. The stoichiometry (~2:1) of the $^3$H-arachidonic acid-$\alpha_2 M^*$ complex is similar to that reported for the $^{125}$I-TGF-β$^1$-$\alpha_2 M^*$ complex (12). This supports the notion that arachidonic acid and TGF-β$^1$ bind to the same site in the $\alpha_2 M^*$ molecule.

**Fatty acids block the inhibitory effect of $\alpha_2 M^*$ on TGF-β binding to TGF-β receptors, TGF-β-induced growth inhibition, and TGF-β-induced transcriptional activation in Mv1Lu cells**

Fatty acids such as myristic acid and arachidonic acid are present in plasma and tissues, and their levels significantly increase during injury, inflammation, and fibrosis (25–28). The levels of TGF-β and $\alpha_2 M^*$ also dramatically increase. $\alpha_2 M^*$ is capable of inhibiting TGF-β activity by forming complexes with TGF-β and thus preventing it from binding to TGF-β receptors in the cells involved. Fatty acids may potentiate TGF-β activity by blocking complex formation of $\alpha_2 M^*$ and TGF-β under these conditions. To test this possibility, we determined the effects of arachidonic acid on $^{125}$I-TGF-β$^2$ binding (in the presence and absence of $\alpha_2 M^*$) to Mv1Lu cells. $\alpha_2 M^*$ is known to inhibit TGF-β$^2$ more strongly than TGF-β$^1$ binding to TGF-β receptors in cells (13). Various concentrations of $^{125}$I-TGF-β$^2$ were preincubated with 200 µg/ml of $\alpha_2 M^*$ in the presence or absence of 30 µM arachidonic acid for 30 min before the performance of binding assays in Mv1Lu cells. As shown in Figure 6A, $\alpha_2 M^*$ strongly inhibited $^{125}$I-TGF-β$^2$ binding to Mv1Lu cells. The residual $^{125}$I-TGF-β$^2$ binding associated with the cells after $\alpha_2 M^*$ inhibition was mainly due to nonspecific binding of $^{125}$I-TGF-β$^2$. In fact, $\alpha_2 M^*$ at 200 µg/ml completely inhibited the specific binding of $^{125}$I-TGF-β$^2$ to those epithelial cells as previously reported (13). The inhibition by $\alpha_2 M^*$ was completely reversed by 30 µM of arachidonic acid. To clarify the biological relevance of this observation, we determined the effect of arachidonic acid on the inhibitory effect of $\alpha_2 M^*$ on TGF-β$^2$-induced growth inhibition and transcriptional activation in Mv1Lu cells. $\alpha_2 M^*$ has been shown to be effective in blocking TGF-β$^2$-induced growth inhibition (13). As shown in Figure 6B, TGF-β$^2$ inhibited [methyl-$^3$H]-thymidine incorporation into DNA of Mv1Lu cells in a dose-dependent manner. In the presence of 200 µg/ml of $\alpha_2 M^*$, the dose-response curve of TGF-β$^2$ shifted to the right. In the absence of $\alpha_2 M^*$,
TGF-β2 (1 pM) inhibited ∼25% of [methyl-³H]-thymidine incorporation into DNA of these epithelial cells; this was completely abolished by the presence of α2M* in the medium. Addition of arachidonic acid at 0.5 and 1 µM reversed the inhibitory effect of α2M* on TGF-β2-induced growth inhibition as measured by [methyl-³H]-thymidine incorporation. One µM of arachidonic acid almost completely reversed the inhibitory effect of α2M* on growth inhibition induced by 1 pM of TGF-β2. In the absence of α2M*, arachidonic acid did not affect growth inhibition induced by TGF-β2 under the experimental conditions (data not shown).

One of the prominent biological activities of TGF-β is transcriptional activation of plasminogen activator inhibitor-1 (PAI-1) and fibronectin (1–4). We, therefore, determined the effect of fatty acids on the inhibition by α2M* of the expression of a TGF-β-responsive promoter construct p3TP-Lux in transfected Mv1Lu cells. The p3TP-Lux contains the PAI-1 promoter and three repeats of a phorbol-12-myristate-13-acetate (TPA)-responsive element (29). As shown in Figure 6C, α2M* (200 µg/ml) inhibited ∼25–30% of the luciferase activity induced by TGF-β2 (50 and 100 pM). This α2M* inhibition of the TGF-β2-induced luciferase activity was completely reversed by either 12.5 or 25 µM of arachidonic acid. In the control experiments, arachidonic acid (12.5 and 25 µM) did not influence the luciferase activity in cells treated with and without TGF-β2 in the absence of α2M* (data not shown). Together with the results described above, this suggests that fatty acids may be capable of modulating the biological activities of TGF-β2 under conditions in which α2M* is present.

**Fatty acids block α2M*-mediated plasma clearance of TGF-β1 and TGF-β2**

α2M* has been shown to be involved in plasma clearance of TGF-β1 and TGF-β2 (15). TGF-β1-α2M* and TGF-β2-α2M* complexes are cleared from plasma by the α2M* receptor in liver (30). Because fatty acids are able to block complex formation of TGF-β1,2 and α2M*, they might be able to affect the plasma clearance of TGF-β1,2 and α2M* complexes. To test this possibility, we preincubated ¹²⁵I-TGF-β1 or ¹²⁵I-TGF-β2 with α2M* in the presence or absence of 10 µM arachidonic acid at room temperature for 30 min, and then we injected this into mice via the tail vein according to published procedures (19). At several time intervals (10 s and 1, 2, 3, 5, 10, 15, 20, 30, and 60 min), ∼25 µl of blood were collected and counted by a γ-counter. As shown in Figure 7A and 7B, the estimated plasma clearance half times (t₁/₂s) of free ¹²⁵I-TGF-β1 (Fig. 7A) and ¹²⁵I-TGF-β2 (Fig. 7B) were 1.8 ± 0.2 (n=4) and 1.3 ± 0.3 (n=4) min, respectively. The t₁/₂s of ¹²⁵I-TGF-β1+α2M* and ¹²⁵I-TGF-β2+α2M* were 3.8 ± 0.2 (n=4) and 3.7 ± 0.1 (n=4) min, respectively. These t₁/₂s are consistent with published values of free ¹²⁵I-TGF-β1,2 and ¹²⁵I-TGF-β1,2-α2M* complexes, respectively (19). In the presence of arachidonic acid, the t₁/₂s of ¹²⁵I-TGF-β1+α2M* and ¹²⁵I-TGF-β2+α2M* were decreased to 1.9 ± 0.1 (n=4) and 1.8 ± 0.2 (n=4) min, respectively; these are essentially identical to the t₁/₂s of free ¹²⁵I-TGF-β1 and ¹²⁵I-TGF-β2 (Fig. 7A and 7B). In control experiments, arachidonic acid did not affect the plasma clearance of free ¹²⁵I-TGF-β1 and ¹²⁵I-TGF-β2 (data not shown). These results suggest that arachidonic acid may be capable of affecting the plasma clearance of TGF-β+α2M* by blocking complex formation.

**DISCUSSION**

TGF-β is a potent growth factor that has been the subject of intense study because of its role in diverse biological processes and its potential role in disease states. It exerts various biological...
activities with optimal concentrations in the range of ~ pM. Some of its activities are regulated at the transcriptional level, and others are regulated posttranscriptionally. Posttranslational control is also prominent and includes activation of latent TGF-β and modulation by TGF-β binding molecules such as α2M*, betaglycan, decorin, thrombospondin, fetuin, and latent TGF-β binding protein (11, 12, 31–36). The mechanisms of in vivo activation of latent TGF-β are not well understood, but it is generally believed that latent TGF-β is activated both by proteolysis at the cell surface and by acidic pH in endosomal compartments (34, 35). TGF-β binding molecules modulate TGF-β activities by inhibiting its binding to TGF-β receptors and/or by sequestering TGF-β molecules in the extracellular space. One such binding agent is α2M*, which affects TGF-β activities by forming a complex that does not bind to TGF-β receptors in cells. α2M* neutralizes TGF-β activities in many experimental systems (13, 16–18), but unlike other TGF-β modulators, α2M* is also involved in plasma clearance of TGF-β (15). α2M* is the major plasma binding protein for TGF-β, and the α2M* receptor in liver mediates plasma clearance of the TGF-β-α2M* complex (12, 15, 30).

The exact molecular mechanisms by which α2M* forms complexes with TGF-β and many other factors that do not share amino acid sequence homology with TGF-β are not well defined. We hypothesize that α2M* forms complexes with TGF-β and these factors via noncovalent hydrophobic interactions with topologically diverse exposed molecular surfaces that do not have consistent amino acid motifs. Several lines of evidence support this hypothesis. These include the following three. First, TGF-β peptides containing the residue Trp-52 are potent inhibitors of complex formation between α2M* and TGF-β and other growth factors (19). Second, replacement of Trp-52 with alanine completely abolishes the inhibitory activity of the TGF-β peptides. However, replacement of the residue Trp-52 with hydrophobic amino acids such as phenylalanine and leucine leaves its inhibitory activity largely intact (19, unpublished results). Third, a hydrophobic small peptide whose amino acid sequence is derived from α2M* blocks complex formation of α2M* and both TGF-β and PDGF (37). Our findings that fatty acids are potent inhibitors of TGF-β-α2M* complex formation and that arachidonic acid binds to α2M* but not to native α2M further support this hypothesis. However, the inhibitory effect of fatty acids requires the presence of a free carboxyl group in addition to hydrophobicity at the binding site. It appears that α2M* contains high-affinity hydrophobic regions (pockets or cavities) that can specifically interact with hydrophobic subdomains of TGF-β and other factors. The hydrophobic subdomains of TGF-β located on the molecule surface likely include Trp-52 and other neighboring hydrophobic amino acid residues. The evidence presented here indicates that fatty acids with ≥14 carbon atoms and double bonds (e.g., arachidonic acid) bind to the proposed pocket or cavity in the α2M* molecule with high affinity.

Because low levels of active TGF-β in plasma and tissues have been implicated in the pathogenesis of atherosclerosis, autoimmune diseases, and malignancy (1–4), the identification of substances that can alter the TGF-β levels is currently being pursued by the pharmaceutical industry. Compounds that are capable of blocking and/or dissociating TGF-β-α2M* complexes, thereby affecting the levels of free TGF-β in plasma and tissues, have therapeutic potential as systemic or regionally delivered drugs for many common diseases. Here, we demonstrate that fatty acids are potent inhibitors of complex formation of TGF-β and α2M*. The IC50 of arachidonic acid and myristic acid (7.8 ± 1.4 and 9.1 ± 0.5 µM, respectively) are well below their critical micelle concentrations (20 µM and >1 mM, respectively) (27, 28). We also show that arachidonic acid is capable of modulating TGF-β binding and TGF-β activity in mink lung epithelial cells in the presence of 2
mg/ml bovine serum albumin (which was not delipidated) (Fig. 6A) and 0.2% FCS (Fig. 6B and 6C). This supports the physiological relevance of the observation that arachidonic acid modulates TGF-β activity in environments containing serum albumin. Human serum albumin plays an essential role in fatty acid transport. Its plasma concentration is ~0.6 mM, and the molar ratio of fatty acids to human serum albumin is ~0.5–2.0, depending on conditions (e.g., fasting) (38). The plasma concentrations of free fatty acids may be elevated and reach µM concentrations under certain pathophysiological conditions (injury, fasting, stress, heparin administration, diabetes, bacterial infection, and others) (38, 39). The IC₅₀s for the inhibition of TGF-β₁ binding to α₂M* are <10 µM for most of the fatty acids we examined. These concentrations can occur at sites of injury or inflammation. Fatty acids are known to be generated locally at considerably higher concentrations than the mean blood levels. In the interstitial space, where the albumin concentration is much lower than in blood, fatty acids may modulate TGF-β activity even more significantly than in plasma. Fatty acids (e.g., arachidonic acid) have also been found to block complex formation between α₂M* and nerve growth factor (NGF) and basic fibroblast growth factor (bFGF) in the laboratory (unpublished results). This suggests that exogenous fatty acids (e.g., polyunsaturated fatty acids, including those not found in natural products) can be designed to potentiate TGF-β and other growth factor/cytokine/hormone activities in order to treat human or animal diseases (16–18).

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complex formation between α₂-macroglobulin and growth factors, cytokines, and hormones. *J. Biol. Chem.* 276, 46212–46218


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Figure 1. Inhibition of $^{125}$I-TGF-β₁ and α₂M* complex formation by saturated (A) and unsaturated (B) fatty acids. α₂M* was preincubated with various concentrations as indicated for saturated fatty acids (n-caprylic acid, lauric acid, myristic acid, palmitic acid, and stearic acid) and unsaturated fatty acids (oleic acid, palmitoleic acid, linolenic acid, γ-linolenic acid, linoleic acid, and arachidonic acid) for 30 min at room temperature and reacted with $^{125}$I-TGF-β₁. After 30 min at room temperature, the reaction mixtures were analyzed by 5% nondenaturing PAGE and autoradiography (a). The arrow indicates the location of the $^{125}$I-TGF-β₁-α₂M* complex, which was quantified by a PhosphoImager (b). Data are representative of four similar experiments.
Figure 2. Effects of arachidonic acid derivatives and analogs on $^{125}$I-TGF-$\beta_1$-$\alpha_2$M* complex formation. $\alpha_2$M* was preincubated with various concentrations as indicated for arachidonic acid (AA), arachidonic acid methyl ester (AA-O-Me), and analog (ETYA; 8, 11, 14 eicosatrien-5-ynoic acid) for 30 min at room temperature. $^{125}$I-TGF-$\beta_1$ was then added to the reaction mixture. After 30 min at room temperature, the reaction mixtures were analyzed by 5% non-denaturing PAGE and autoradiography (a). The arrow indicates the location of the $^{125}$I-TGF-$\beta_1$-$\alpha_2$M* complex, which was quantified by a PhosphoImager (b). Data are representative of four similar experiments.
Figure 3. Effects of myristic acid and arachidonic acid on formation of $^{125}$I-TGF-β isoform and α₂M* complexes identified on nondenaturing PAGE (A) and SDS-PAGE (B). α₂M* was preincubated with various concentrations as indicated for myristic acid and arachidonic acid for 30 min at room temperature and reacted with $^{125}$I-TGF-β₁, $^{125}$I-TGF-β₂, or $^{125}$I-TGF-β₃. After 30 min at room temperature, the reaction mixtures were analyzed by 5% nondenaturing PAGE (A) or 7.5% SDS-PAGE following cross-linking by DSS (B) and autoradiography (a). The arrow indicates the location of the $^{125}$I-TGF-β-α₂M* complex, which was quantified by a PhosphoImager (b). Data are representative of four similar experiments.
Figure 4. Dissociation of $^{125}$I-TGF-$\beta_1$-$\alpha_2$M* and $^{125}$I-TGF-$\beta_2$-$\alpha_2$M* complexes by arachidonic acid. $\alpha_2$M* was reacted separately with $^{125}$I-TGF-$\beta_1$ and $^{125}$I-TGF-$\beta_2$ for 30 min at room temperature. The reaction mixture was then treated with various concentrations of arachidonic acid as indicated. After 30 min at room temperature, the reaction mixtures were analyzed by 5% non-denaturing PAGE and autoradiography (a). The arrow indicates the location of the $^{125}$I-TGF-$\beta_1$-$\alpha_2$M* or $^{125}$I-TGF-$\beta_2$-$\alpha_2$M* complex, which was quantified by a Phospholimager (b). Data are representative of four similar experiments.
Figure 5. Gel filtration chromatography of $^3$H-arachidonic acid-$\alpha_2$M* complexes. $^3$H-Arachidonic acid ($^3$H-AA) was preincubated with and without $\alpha_2$M* (which had been activated by methylamine) or with native $\alpha_2$M. After 30 min at room temperature, the reaction mixture was applied onto a column (0.7 × 40 cm) of Sephacryl S-300 HR. The fractional volume was ~ 1 ml. The $^3$H-radioactivity in the fractions was determined by scintillation counting. $\alpha_2$M* and native $\alpha_2$M in the fractions were identified by Coomassie blue staining (inset). The arrow indicates the location of $\alpha_2$M*. Data are representative of three similar experiments.
Figure 6. Arachidonic acid reversal of the α₂M* inhibitory effect on $^{125}$I-TGF-β₂ binding to TGF-β receptors (A) and TGF-β₂-induced growth inhibition (B) and TGF-β₂-induced transcriptional activation (C) in Mv1Lu cells.  

A) α₂M* (200 µg/ml) was preincubated with arachidonic acid (AA) (0 or 30 µM) and various concentrations (0, 1.25, 2.5, 5, and 10 pM) of $^{125}$I-TGF-β₂ with and without TGF-β peptantagonist (30 µM) (19) in binding buffer (50 mM HEPES/NaOH, pH 7.4, 128 mM NaCl, 5 mM KCl, 5 mM MgSO₄, 1.2 mM CaCl₂) containing 2 mg/ml bovine serum albumin. After 30 min at room temperature, the $^{125}$I-TGF-β₂ solution was added to cells, and the $^{125}$I-TGF-β₂ binding was determined after 2.5 h at 0°C. The binding of $^{125}$I-TGF-β₂ obtained in the presence of α₂M* was mainly nonspecific binding because it was not further inhibited by the presence of TGF-β peptantagonist (19). Data are representative of four similar experiments.

B) Cells were treated with various concentrations of TGF-β₂ in the presence and absence of α₂M* (200 µg/ml) and arachidonic acid (AA) (0.5 and 1.0 µM) in DMEM containing 0.2% fetal calf serum. After 18 h at 37°C, the [methyl-$^3$H]-thymidine incorporation into cellular DNA of cells was determined. The [methyl-$^3$H]-thymidine incorporation in cells treated without TGF-β₂ and arachidonic acid was taken as 0% inhibition. Data are representative of four similar experiments.

C) Cells transiently transfected with the p3TP plasmid were treated with various concentrations of TGF-β₂ in the presence and absence of α₂M* (200 µg/ml) and arachidonic acid (AA) (12.5 and 25 µM) in DMEM containing 0.2% fetal calf serum. After 12 h at 37°C, the luciferase activity of the cell extracts was determined and expressed as arbitrary units (A.U.). Data were obtained from three different experiments; values are mean ±SD (*P<0.05 vs.luciferase activity of cells treated with α₂M* and TGF-β₂).
Figure 7. Plasma clearance of $^{125}$I-TGF-β$_1$ (A) or $^{125}$I-TGF-β$_2$ (B) treated with α$_2$M* in the presence and absence of arachidonic acid. $^{125}$I-TGF-β$_1$ (A) or $^{125}$I-TGF-β$_2$ (B) was incubated with α$_2$M* in the presence and absence of arachidonic acid (AA). After 30 min at room temperature, the $^{125}$I-TGF-β$_1$ or $^{125}$I-TGF-β$_2$ solution was injected into the tail veins of mice. Blood samples were collected at the time intervals indicated. The radioactivity in the blood sample collected 10 seconds after i.v. injection of the isotope solution was taken as 100%. Data are representative of four similar experiments.