Chemical modification of α_2-macroglobulin to generate derivatives that bind transforming growth factor-β with increased affinity

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Abstract α_2-Macroglobulin (α_2M) binds a number of cytokines, including transforming growth factor-β1 (TGF-β1) and TGF-β2. The affinity of these interactions depends on the α_2M conformation. In this investigation, we treated human α_2M with *cis*-dichlorodiammineplatinum (II) (*cis*-Pt), a crosslinking reagent that partially 'locks' the α_2M conformation, and then with methylamine to generate a preparation (α_2M-P/M) consisting of stable α_2M conformational intermediates. α_2M-P/M bound TGF-β1 and TGF-β2 with higher affinity than any other form of α_2M studied to date. The equilibrium dissociation constants were 14 and 2 nM for TGF-β1 and TGF-β2, respectively. α_2M-P/M, at 100 nM, neutralized the activity of TGF-β1 by about 75% in an endothelial cell proliferation assay. The equivalent concentration of native α_2M or methylamine-modified α_2M had no effect. These studies demonstrate that the potential of α_2M as a cytokine carrier and neutralizer may not be fully realized in either the native or completely activated conformations.

Key words: α_2-macroglobulin; Transforming growth factor-β; Cytokine; Endothelium

1. Introduction

α_2-Macroglobulin (α_2M) is a naturally occurring protein in the blood which functions not only as a proteinase inhibitor but also as a carrier of specific cytokines, including transforming growth factor-β1 (TGF-β1), platelet-derived growth factor-BB, and nerve growth factor-β [1,2]. The binding affinities of α_2M for different cytokines vary widely and are not always affected similarly when the α_2M is modified by proteinases [1–3]. Many cytokines do not bind to α_2M at all, including PDGF-AA, interferon-γ, colony stimulating factor-1, and ciliary neurotrophic factor [1,2,4,5]. Thus, α_2M may function to neutralize cytokines, with partial specificity, in normal homeostasis and in select disease states.

α_2M in the blood is almost entirely in the native conformation. This form of α_2M is fully functional as a proteinase inhibitor but not recognized by cellular receptors [6]. When treated with small primary amines, such as methylamine, native α_2M undergoes a major conformational change [7,8]. The resulting structure, which is referred to as activated α_2M, retains no proteinase inhibitory activity but is recognized by α_2M-specific receptors, primarily in the liver, and thus rapidly cleared from the circulation [6]. Many proteinases induce a conformational change in α_2M which is equivalent to that caused by methylamine [9,10]; however, before adopting the 'fully activated' conformation, the α_2M apparently transitions through a series of variably stable structural intermediates [11–15].

Very little is known about cytokine binding to α_2M conformational intermediates; however, these intermediates may be identified as a small sub-population within purified native α_2M preparations [16,17]. In the present investigation, we utilized an α_2M chemical modification protocol which has been previously characterized for its potential to stabilize α_2M conformational intermediates [12,18]. The protocol utilizes the reagent, *cis*-dichlorodiammineplatinum (II) (*cis*-Pt), as an amino acid side-chain crosslinker to partially lock the α_2M conformation prior to adding methylamine. Treatment of *cis*-Pt-modified α_2M with methylamine results in only partial reorganization of the α_2M structure, as determined by nondenaturing PAGE, electron microscopy, and receptor recognition experiments [12,13,18,19].

The studies presented here demonstrate that α_2M, modified with *cis*-Pt and methylamine, binds TGF-β1 and TGF-β2 with higher affinity than any other α_2M preparation studied to date. To confirm the results of our equilibrium cytokine-binding analyses, we performed endothelial cell proliferation assays. TGF-β1 inhibited endothelial cell growth as expected and α_2M-P/M neutralized this activity while native α_2M and α_2M-methylamine, at the equivalent concentration, were ineffective.

2. Materials and methods

2.1. Materials

Porcine TGF-β1, which is identical in sequence to human TGF-β1, was from R&D Systems (Minneapolis, MN, USA). Human TGF-β2 was from Genzyme (Cambridge, MA, USA). TGF-β1 and TGF-β2 were radioiodinated according to the method of Ruff and Rizzino [20]. Specific activities were 100–200 μCi/μg. Methylamine-HCl, chloramine-T, bovine serum albumin (BSA) and fetal bovine serum were from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA, and Earle's balanced salts solution were from GIBCO BRL (Gaithersburg, MD, USA). $Na^{125}I$ was from Amersham (Arlington Heights, IL, USA). Acidic fibroblast growth factor and basic fibroblast growth factor were from Promega (Madison, WI, USA). *Bis*(sulfosuccinimidyl) suberate (BS^3) and Iodobeads were from Pierce (Rockford, IL, USA). *Cis*-Pt was from Aldrich (Milwaukee, WI, USA).

2.2. Preparation of α_2M and α_2M derivatives

α_2M was purified from human plasma by the method of Imber and Pizzo [21]. The concentration of α_2M was determined by measuring the absorbance at 280 nm, using an $A_{1\%,1cm}$ of 8.93 [22]. All purified native α_2M preparations were screened for the presence of trace levels of partially activated forms by incubation with ^{125}I-TGF-β1 followed by nondenaturing PAGE, as previously described [17]. Any native α_2M preparations that showed TGF-β1-binding to α_2M species with increased mobility were discarded.

α_2M-MA was prepared by dialyzing native α_2M against 200 mM methylamine-HCl in 50 mM Tris-HCl, pH 8.2, for 12 h at 22°C, followed by extensive dialysis against 20 mM sodium phosphate,

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150 mM NaCl, pH 7.4 (PBS) at 4°C. Complete modification of native α_2M by methylamine was confirmed by loss of trypsin binding activity (>96%) [23] and by the characteristic increase in mobility by nondenaturing PAGE [24,25].

α_2M-P was prepared by reacting native α_2M with 1.6 mM *cis*-Pt for 6 h at 37°C. Unreacted *cis*-Pt was removed by extensive dialysis against PBS. α_2M-P/M was prepared by dialyzing α_2M-P against 200 mM methylamine-HCl in 50 mM Tris-HCl, pH 8.2, for 12 h at 22°C, followed by extensive dialysis against PBS.

2.3. Determination of apparent equilibrium dissociation constants

Equilibrium dissociation (K_d) constants were determined by the BS^3-rapid crosslinking method, as has been described by our laboratory [1,2,5,26]. Cytokine-binding to α_2M is modeled as a two-step reaction:

$$A + C \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AC \xrightarrow{k_2} AC^* \quad (1)$$

A is unbound α_2M, C is unbound cytokine, AC is reversibly associated (noncovalent) α_2M–cytokine complex, and AC^* is irreversibly associated (covalent) α_2M–cytokine complex, formed by thiol-disulfide exchange. For TGF-β1 and TGF-β2, reversible binding to α_2M occurs fairly rapidly and k_2 is sufficiently small so that it may be ignored in the determination of K_D values [2].

In the experiments presented here, various concentrations of α_2M-P or α_2M-P/M (0.002–2.5 μM) were incubated with ^{125}I-TGF-β1 or ^{125}I-TGF-β2 (1.0 nM) in PBS with 75 μM BSA, for 30 min at 37°C. The rapid-crosslinking agent, BS^3 (in H_2O), was then added at a final concentration of 5 mM for 1 min. For each α_2M concentration, an identical control incubation was treated with vehicle (H_2O) instead of BS^3. Crosslinking reactions were terminated instantaneously by acidification [4]. Samples were then denatured in 2.0% SDS for 30 min at 37°C, supplemented with Tris-HCl (100 mM) and glycerol (10%), and subjected to SDS-PAGE. The gels were sliced into 3 mm sections and the radioactivity content of each section was determined in a gamma counter. ^{125}I-Cytokine recovered in association with α_2M (AC_e) included BS^3-stabilized AC and AC^*. Free cytokine (in the gels) (C_e) included C plus AC which was not BS^3-stabilized. AC^* was quantitated independently by SDS-PAGE analysis of samples that were not BS^3-treated. Apparent K_d values were determined according to the following equation:

$$\frac{C_e}{AC_e} = \left(\frac{K_d}{z}\right)\left(\frac{1}{A}\right) + \left(\frac{1}{z} - 1\right) \quad (2)$$

z is the BS^3-crosslinking efficiency, a constant ($0 < z < 1$) for each cytokine and α_2M derivative which does not vary as a function of the α_2M concentration. AC_e is related to AC by the relationship: $[AC_e] = z[AC]$. Assumptions involved in the use of this method have been reviewed [1,2]. These include that the K_D value reflects a single cytokine-binding site per α_2M and that all of the α_2M in a given preparation binds cytokine with equal affinity.

The methods for preparing α_2M-P and α_2M-P/M involve the binding of an average of 17 mol platinum per mol of α_2M [12]. Since there is almost certainly heterogeneity in the extent of platinum binding and because the amino acids modified probably vary, α_2M-P and α_2M-P/M must be viewed as heterogeneous preparations in which different molecules may bind ^{125}I-TGF-β with different affinities. Each K_d value determined for α_2M-P or α_2M-P/M, by the BS^3-rapid crosslinking method, is a preparation-averaged constant, related to the different K_d values of n different α_2M species (A_i) by the following equation:

$$\frac{1}{K_d} = \frac{1}{K_{d_1}} - \frac{\Sigma_{i=2}^{n} A_i}{K_{d_1} A_T} + \frac{1}{K_{d_2}} - \frac{\Sigma_{i=1,3}^{n} A_i}{K_{d_2} A_T} + \frac{1}{K_{d_3}} - \frac{\Sigma_{i=1,2,4}^{n} A_i}{K_{d_3} A_T} + \ldots \quad (3)$$

A_T is the total concentration of all α_2M species in the preparation (sum of A_i). As an example, if an α_2M preparation consists of two species in equal proportion, which bind a given cytokine with K_d values of 100 and 500 nM, then the preparation-averaged K_d value would be 167 nM. When multiple species are present in an α_2M preparation, plots of C_e/AC_e against $1/[A_T]$ (according to Eq. 2) remain linear. Preparation-averaged K_d values are not affected by differences in the BS^3-crosslinking efficiencies (z values) amongst various α_2M species within a given preparation.

2.4. Inhibition of endothelial cell growth

FBHE cells were maintained in DMEM supplemented with 10% FBS, 20 ng/ml acidic fibroblast growth factor, and 80 ng/ml basic fibroblast growth factor. Cultures were passaged at subconfluence with trypsin-EDTA. FBHE cell proliferation assays were performed in dilute (0.2%) serum as previously described [26,27]. Briefly, FBHE cells were plated in 24-well culture plates (2×10^4 cells/well) and incubated in DMEM with 10% FBS for 15 h. After washing, fresh DMEM, supplemented with 0.2% FBS and TGF-β1, was added. Some cultures were simultaneously treated with native α_2M, α_2M-P, α_2M-P/M, or α_2M-MA. After incubation for 30 h, [^{3}H]thymidine was added for an additional 18 h. The cells were then harvested and radioactivity incorporation was measured in a scintillation counter.

3. Results

3.1. Nondenaturing PAGE analysis of the α_2M derivatives

Nondenaturing PAGE is commonly used to analyze α_2M conformation [24,25]. Fig. 1 shows a representative nondenaturing PAGE experiment in which the four α_2M preparations were compared. Native α_2M migrated in a single Coomassie-stained band. The mobility of methylamine-modified α_2M was increased compared with that of native α_2M reflecting the transition to the fully activated conformation. As expected, the mobility of α_2M-P/M was intermediate between native α_2M and α_2M-MA, reflecting partial conformational change [12,18]. The low mobility bands in the α_2M-P and α_2M-P/M preparations suggest some intermolecular α_2M-crosslinking, due to the high concentration of *cis*-Pt used.

3.2. Equilibrium binding of ^{125}I-cytokines to α_2M-P and α_2M-P/M

The BS^3-rapid crosslinking method was used to determine K_d values for the binding of ^{125}I-TGF-β1 and ^{125}I-TGF-β2 to α_2M-P and α_2M-P/M. In preliminary time-course experiments, noncovalent binding of each cytokine to the modified α_2M derivatives maximized within 15 min (results not shown). Fig. 2A shows a representative autoradiograph in which ^{125}I-TGF-β1 was incubated with various concentrations of α_2M-P for 30 min, and then with BS^3. The amount of ^{125}I-TGF-β1–α_2M-P complex detected was dependent on the α_2M-P concentration. In the control gel, which contained samples that were treated with vehicle instead of BS^3, the amount of ^{125}I-TGF-β1–α_2M-P complex was consistently less than 20% of

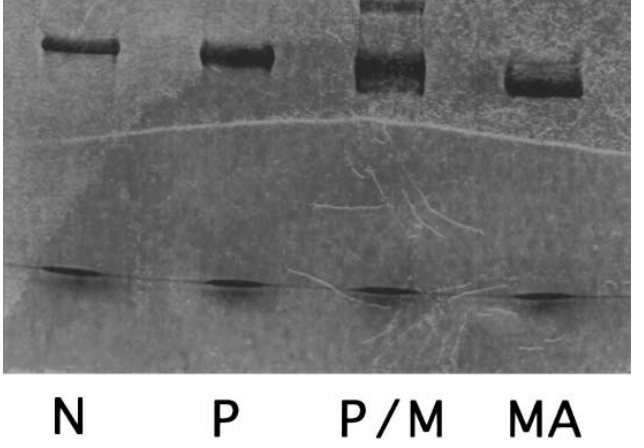
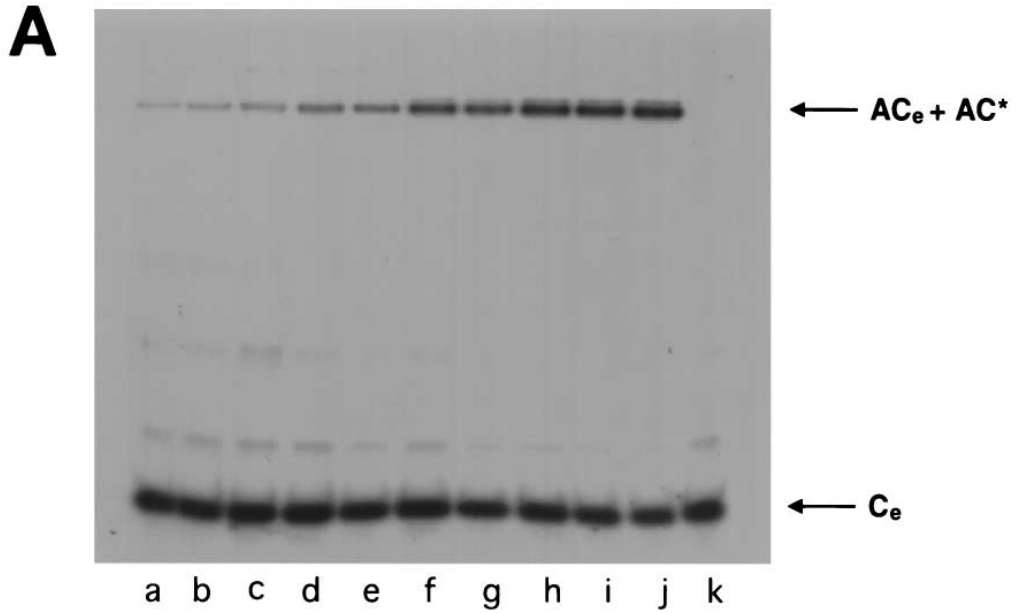


Fig. 1. Nondenaturing PAGE analysis of α_2M derivatives. Native α_2M (N), α_2M-P (P), α_2M-P/M (P/M) and α_2M-MA (MA) are shown. The gel was stained with Coomassie blue R-250.



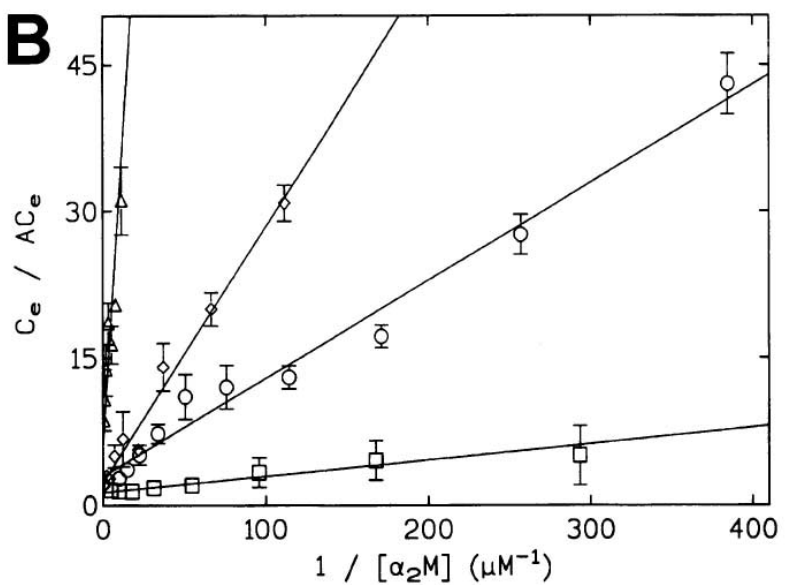


Fig. 2. Equilibrium binding of ^{125}I-TGF-β1 to α_2M-P and α_2M-P/M. ^{125}I-TGF-β1 was incubated with α_2M-P for 30 min at 37°C. The samples were pulse-exposed to 5 mM BS^3 and subjected to SDS-PAGE and autoradiography. The autoradiograph of a representative gel is shown in (A). The concentrations of α_2M-P were 2 nM (lane a), 4 nM (lane b), 6 nM (lane c), 9 nM (lane d), 13 nM (lane e), 20 nM (lane f), 30 nM (lane g), 45 nM (lane h), 67 nM (lane i), 0.1 μM (lane j), and 0 nM (lane k). The terms, AC_e, AC*, and C_e are defined in the text. B: Results of BS^3-rapid crosslinking studies analyzing the binding of TGF-β1 to native α_2M (△), α_2M-P (○), α_2M-P/M (□) and α_2M-MA (◇). The results of four separate experiments were averaged and plotted according to Eq. 2 in the text.

that detected with BS^3 (not shown). Thus, *AC** represented only a small fraction of the covalent α_2M-P–TGF-β1 complex recovered after BS^3 treatment. Similar results were obtained when α_2M-P/M was substituted for α_2M-P and when TGF-β2 was studied.

Fig. 2B shows plots of C_e/AC_e against $1/A_T$ for the binding of ^{125}I-TGF-β1 to native α_2M, α_2M-P, α_2M-P/M and α_2M-MA. The presented graphs were generated from the results of at least four separate experiments. Individual experiments, with TGF-β1 and TGF-β2, were analyzed using similar plots,

Table 1
Equilibrium dissociation constants for ^{125}I-TGF-β-binding to α_2M

Cytokine	α_2M-P (nM)	α_2M-P/M (nM)	Native α_2M (nM)	α_2M-MA (nM)
TGF-β1	36 ± 2	14 ± 4	320 ± 65	82 ± 6
TGF-β2		2 ± 1	14 ± 3	15 ± 2

Results were determined using the BS^3-rapid crosslinking method. Each value represents the mean ± SE (n = 4). The K_d values for the binding of each cytokine to native α_2M and α_2M-MA have been presented previously [1,2].

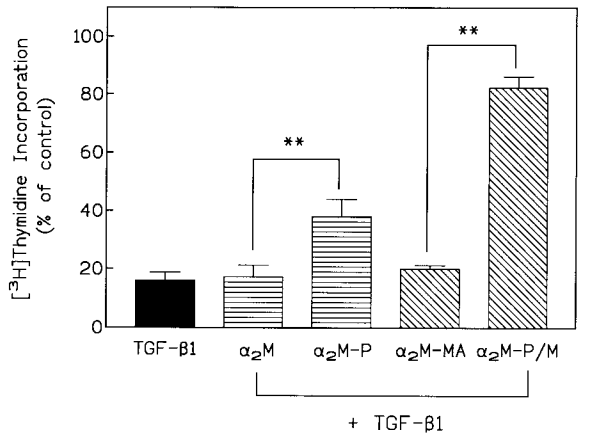


Fig. 3. Effects of α_2M on TGF-β1 activity in an endothelial cell proliferation assay. FBHE cells were incubated with 10 pM TGF-β1 in the presence and absence of 100 nM native α_2M, α_2M-P, α_2M-MA, or α_2M-P/M. The culture medium contained 0.2% FBS. After 30 h, 1 μCi/ml [^{3}H]thymidine was added to the cultures for an additional 18 h. [^{3}H]Thymidine incorporation was then determined and expressed as a percentage of that observed in control cultures, which were not exposed to α_2M or TGF-β1. **Statistically significant differences ($P < 0.005$).

which were all apparently linear, as expected. K_d values from individual experiments were averaged to obtain the constants presented in Table 1. Binding constants for the interaction of TGF-β1 and TGF-β2 with native α_2M and α_2M-MA have been presented previously [2].

As shown in Table 1, α_2M-P/M and α_2M-P bound TGF-β1 with higher affinity than α_2M-MA. The K_d for the binding of TGF-β1 to α_2M-P/M (14 nM) was decreased by a factor of 23 compared with the K_d for TGF-β1 binding to native α_2M (320 nM). The K_d for the binding of TGF-β2 to α_2M-P/M (2 nM) is the lowest binding constant reported for any cytokine and any α_2M derivative studied to date.

3.3. α_2M-P and α_2M-P/M counteract the activity of TGF-β in cell culture

TGF-β inhibits FBHE proliferation in cell culture and this activity is counteracted by α_2M [26,27]. Experiments with a variety of human α_2M derivatives and α-macroglobulins from different species have shown that the fraction of TGF-β activity neutralized directly correlates with the affinity of the α-macroglobulin/TGF-β interaction [3,26,27]. Since the K_d values determined for cytokine binding to α_2M-P and α_2M-P/M were preparation-averaged values, we wished to confirm that these constants accurately predict the cytokine-binding and/or -neutralizing activities of the α_2M preparations. Fig. 3 shows the results of FBHE proliferation experiments in which 10 pM TGF-β1 inhibited [^{3}H]thymidine incorporation by an average of 84%. A fairly low concentration of each α_2M derivative (100 nM) was added to different FBHE cultures such that high-affinity TGF-β–α_2M interactions would be selectively detected. As expected, native α_2M did not significantly affect [^{3}H]thymidine incorporation while α_2M-MA had only a small effect which was not statistically significant at the $P < 0.05$ level. By contrast, α_2M-P substantially reversed the growth inhibition caused by TGF-β1 and α_2M-P/M was even more effective. The 'double-stars' in Fig. 3 indicate statistically significant differences at the $P < 0.005$ level. The results of these FBHE growth inhibition studies confirm that the K_d values, determined by the BS^3-rapid crosslinking method, accurately predict the TGF-β-neutralizing activities of the modified α_2M preparations, even though these preparations are heterogeneous.

4. Discussion

Native α_2M functions as a physiologically significant carrier of TGF-β in the blood [1]. Circulating α_2M–TGF-β complexes are mostly noncovalent and reversible [28,29]. Thus, α_2M may provide a stable pool of slowly releasable cytokine activity, in the plasma, under normal homeostatic conditions. At sites of inflammation or other pathological processes that occur in tissue, concentrations of TGF-β isoforms may be in rapid flux and α_2M may serve to buffer cells against the full impact of changing TGF-β activity. Cell culture experiments, in which cells are exposed to a bolus of cytokine or allowed to respond to autocrine-secreted cytokines, probably model the microenvironment of tissues more closely than plasma. In these in vitro systems, α_2M has been shown to regulate cellular growth and gene expression by binding TGF-β [26,27,30,31].

The binding affinity of α_2M for TGF-β isoforms and other cytokines is dependent on the conformational state of the α_2M [1]. While most previous studies have been conducted using native α_2M or fully activated, methylamine-modified α_2M, the studies presented here demonstrate that neither of these well-studied α_2M conformations have optimized cytokine binding activity. α_2M-P and α_2M-P/M bound TGF-β1 and TGF-β2 with higher affinity than previously studied forms of α_2M. The same derivatives also neutralized the activity of TGF-β1 in FBHE proliferation assays, confirming the validity of the K_d values, determined by the BS^3-rapid crosslinking method. Thus, these chemically modified α_2M derivatives represent superior TGF-β-binding agents.

Based on hydrodynamic and electron microscopy studies, we previously proposed that α_2M-P/M may model intermediates which occur transiently during α_2M conformational change in vivo [12,13,18,19]. However, electron microscopy images of α_2M-P already show some changes in structure compared with native α_2M [12]. Thus, the model of *cis*-Pt as an α_2M 'conformational lock' may be oversimplified. The increased TGF-β-binding affinity of α_2M-P, compared with native α_2M, supports the hypothesis that *cis*-Pt alone induces some changes in the structure of α_2M that are yet to be defined.

In conclusion, we have shown that the TGF-β-binding activity of α_2M can be modified by reagents which alter the conformation of the molecule. The newly described derivatives may be useful as TGF-β-activity modifiers in vitro and in vivo. Whether the same α_2M modification protocol will enhance binding of other cytokines remains to be determined.

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References

[1] Gonias, S.L., LaMarre, J., Crookston, K.P., Webb, D.J., Wolf, B.B., Lopes, M.B.S., Moses, H.L. and Hayes, M.A. (1994) Ann. NY Acad. Sci. 737, 273–290.

[2] Crookston, K.P., Webb, D.J., Wolf, B.B. and Gonias, S.L. (1994) J. Biol. Chem. 269, 1533–1540.
[3] Webb, D.J., Weaver, A.M., Atkins-Brady, T.A. and Gonias, S.L. (1996) Biochem. J. 320, 551–555.
[4] Bonner, J.C. and Osornio-Vargas, A.-R. (1995) J. Biol. Chem. 270, 16236–16242.
[5] Wolf, B.B. and Gonias, S.L. (1994) Biochemistry 33, 11270–11277.
[6] S.V. Pizzo, S.L. Gonias, in: P.M. Conn (Ed.), The Receptors. Vol. 1. Academic Press, Orlando, FL, 1984, pp. 178–221.
[7] Sottrup-Jensen, L., Petersen, T.E. and Magnusson, S. (1980) FEBS Lett. 121, 275–279.
[8] Howard, J.B. (1981) Proc. Natl. Acad. Sci. USA 78, 2235–2239.
[9] Gonias, S.L., Reynolds, J.A. and Pizzo, S.V. (1982) Biochim. Biophys. Acta 705, 306–314.
[10] Björk, I. and Fish, W.W. (1982) Biochem. J. 207, 347–356.
[11] Strickland, D.K., Steiner, J.P., Migliorini, M. and Battey, F.D. (1988) Biochemistry 27, 1458–1466.
[12] Gonias, S.L. and Figler, N.L. (1989) J. Biol. Chem. 264, 9565–9570.
[13] Marshall, L.B., Figler, N.L. and Gonias, S.L. (1992) J. Biol. Chem. 267, 6347–6352.
[14] Steiner, J.P., Bhattacharya, P. and Strickland, D.K. (1985) Biochemistry 24, 2993–3001.
[15] Roche, P.A. and Pizzo, S.V. (1988) Arch. Biochem. Biophys. 267, 285–293.
[16] Borth, W. (1994) Ann NY Acad. Sci. 737, 267–272.
[17] LaMarre, J., Hayes, M.A., Wollenberg, G.K., Hussaini, I., Hall, S.W. and Gonias, S.L. (1991) J. Clin. Invest. 78, 39–44.
[18] Gonias, S.L. and Pizzo, S.V. (1981) J. Biol. Chem. 256, 12478–12484.
[19] Gonias, S.L. and Pizzo, S.V. (1984) Ann. NY Acad. Sci. 421, 457–471.
[20] Ruff, E. and Rizzino, A. (1986) Biochem. Biophys. Res. Commun. 138, 714–719.
[21] Imber, M.J. and Pizzo, S.V. (1981) J. Biol. Chem. 256, 8134–8139.
[22] Hall, P.K. and Roberts, R.C. (1978) Biochem. J. 173, 27–38.
[23] Ganrot, P.O. (1967) Clin. Chim. Acta 16, 328–330.
[24] Barrett, A.J., Brown, M.A. and Sayers, C.A. (1979) Biochem. J. 181, 401–418.
[25] Van Leuven, F., Cassiman, J.-J. and Van den Berghe, H. (1981) J. Biol. Chem. 256, 9016–9022.
[26] 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.
[27] Burmester, J.K., Qian, S.W., Roberts, A.B., Huang, A., Amatayakul-Chantler, S., Suardet, L., Odartchenko, N., Madri, J.A. and Sporn, M.B. (1993) Proc. Natl. Acad. Sci. USA 90, 8628–8632.
[28] Philip, A. and O'Connor-McCourt, M. (1991) J. Biol. Chem. 266, 22290–22296.
[29] Crookston, K.P., Webb, D.J., LaMarre, J. and Gonias, S.L. (1993) Biochem. J. 293, 443–450.
[30] Lysiak, J.J., Hussaini, I.M., Webb, D.J., Glass II, W.J., Allietta, M. and Gonias, S.L. (1995) J. Biol. Chem. 270, 21919–21927.
[31] Weaver, A.M., Owens, G.K. and Gonias, S.L. (1995) J. Biol. Chem. 270, 30741–30748.