UvA-DARE (Digital Academic Repository)

Transforming factor-beta-induced collagen synthesis by human liver myofibroblasts is inhibited by alpha2-macroglobulin

Tiggelman, M.B.C.; Linthorst, C.; Boers, W.; Brand, H.S.; Chamuleau, R.A.F.M.

DOI

[10.1016/S0168-8278\(97\)80455-2](https://doi.org/10.1016/S0168-8278(97)80455-2)

Publication date

1997

Published in

Journal of hepatology

[Link to publication](#)

Citation for published version (APA):

Tiggelman, M. B. C., Linthorst, C., Boers, W., Brand, H. S., & Chamuleau, R. A. F. M. (1997). Transforming factor-beta-induced collagen synthesis by human liver myofibroblasts is inhibited by alpha2-macroglobulin. *Journal of hepatology*, 26, 1220-1228. [https://doi.org/10.1016/S0168-8278\(97\)80455-2](https://doi.org/10.1016/S0168-8278(97)80455-2)

General rights

It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations

If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: <https://uba.uva.nl/en/contact>, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.

Transforming growth factor- β -induced collagen synthesis by human liver myofibroblasts is inhibited by α_2 -macroglobulin

Anke M. B. C. Tiggeleman¹, Christiaan Linthorst¹, Willem Boers¹, Henk S. Brand² and Robert A. F. M. Chamuleau¹

¹Academic Medical Center, University of Amsterdam, J van Gool Laboratory for Experimental Internal Medicine and ²ACTA, Department of Oral Biochemistry, Amsterdam, The Netherlands

Background: Transforming growth factor- β (TGF β) plays a central role in the stimulation of matrix production during liver fibrosis. The action of TGF β in different systems has been shown to be influenced by α_2 -macroglobulin (α_2 M), a serum protein with strong protease-scavenging and cytokine-binding properties. **Aims:** In the present study, α_2 M derived from normal human plasma has been tested for its ability to modulate the TGF β -induced collagen production by human liver fat-storing cells (FSC), which had transformed into α -smooth muscle actin-expressing myofibroblasts in culture.

Methods: α_2 M has been tested after activation with methylamine (α_2 M-Me), an *in vitro* equivalent of protease activated α_2 M. The binding of ¹²⁵I-TGF β 1 to activated forms of α_2 M was demonstrated by rate electrophoresis. Collagen synthesis was examined in human liver myofibroblast cultures obtained from three different human livers by incorporation of ³H-proline into TCA-precipitable, specific collagenase degradable proteins. Uptake of α_2 M was studied by means of immunofluorescence.

Results: TGF β (1 ng/ml) significantly stimulated collagen synthesis of controls in the absence of TGF β . α_2 M-Me reduced this TGF β -induced collagen synthesis dose-dependently, reaching significant inhibition from 10 μ g/ml α_2 M-Me onward. Upon addition of 100 μ g/ml α_2 M-Me the effect of TGF β was reduced by 60% to $128 \pm 31\%$ (mean $\pm$ SD) of control values in the absence of TGF β . Human liver myofibroblasts endocytosed α_2 M-Me added to the cultures as detected by immunofluorescence. Accordingly, reduction of TGF β -activity by α_2 M-Me may be explained by receptor-mediated clearance of α_2 M-TGF β complexes by the cells.

Conclusions: TGF β -induced collagen formation by human liver myofibroblasts obtained from three different livers is reduced *in vitro* by activated α_2 M. From these results, we hypothesize that α_2 M may have an antifibrogenic effect *in vivo* by interference with TGF β -induced matrix synthesis during liver fibrosis.

Key words: α_2 -Macroglobulin; Collagen; Fat-storing cell; Fibrosis; Human; Liver; Myofibroblast; TGF β .

TRANSFORMING growth factor- β is considered to be the major pro-fibrogenic cytokine during liver fibrogenesis. Repeatedly, a positive correlation between the presence of TGF β and the degree of liver fibrosis has been reported in man (1–3) and rats (4,5). Moreover, TGF β has been shown to stimulate matrix pro-

duction by cultured human (6,7) and rat fat-storing cells (FSC) (8,9). FSC are generally viewed as the major producers of ECM in the liver (10–13). At sites of liver fibrosis and in culture the FSC undergo a gradual transition from a “fat-storing” phenotype characterized by presence of lipid droplets, to a myofibroblast-like phenotype characterized by reduction or absence of lipid droplets, appearance of the cytoskeleton filament α -smooth muscle actin and increased production of matrix components (10–13).

TGF β is secreted in a latent, biologically inactive form by several cell types, including Kupffer cells (14,15) and FSC (6,16–18). The primary mode of con-

Received 1 March 1996; revised 13 January; accepted 17 January 1997

Correspondence: Anke M. B. C. Tiggeleman, Academic Medical Center, University of Amsterdam, J van Gool Laboratory for Experimental Internal Medicine, G 2 room 133, PO Box 22700, 1100 DE Amsterdam, The Netherlands. Tel: 31 20 5664174. Fax: 31 20 6977192.

version of latent TGF β to its active form in the liver is unknown. Several hypotheses have been put forward, including acidification, proteolysis by plasmin, heparin-mediated dissociation from either α_2 -macroglobulin (α_2 M) or TGF β masking protein, or interactions between FSC and endothelial cells (14,19–22). Biologically active TGF β exerts its effects on the cell by binding to one of the three known specific TGF β membrane receptors (19,21,23). In addition to interaction with membrane receptors, however, TGF β can bind to other proteins, including several matrix molecules (e.g. decorin, biglycan) (14,19,24) and the serum protein α_2 M (14,19,21,25–27).

α_2 M is the major proteinase inhibitor in man and rat, blocking a wide spectrum of proteinases by means of entrapment and covalent binding of the proteinase (27–29). Human α_2 M is a 720 kD glycoprotein consisting of four identical subunits. Each subunit contains a bait region that can be cleaved by a proteinase. Bait region cleavage results in an immediate and distinct conformational change of the α_2 M molecule, during which internal thiolesters are cleaved, resulting in “trapping” of the proteinase (25). *In vitro*, the conformational change after binding of proteinases can be mimicked by incubation of α_2 M with methylamine (30). Under nondenaturing conditions using rate electrophoresis, native α_2 M and α_2 M activated by methylamine or proteinases exhibit an obvious difference in mobility (31), referred to as the “slow” and “fast” form, respectively (32). In addition, partially activated α_2 M can be distinguished: a form intermediate between native and completely activated α_2 M due to the presence of one or two activated thiolesters, out of the four activable thiolesters (25). The conformational change induced by interaction of α_2 M with proteinases or with methylamine leads to expression of epitopes recognized by α_2 M receptors present on different cell types including fibroblasts and liver parenchymal cells (26,27,33,34).

In recent years, it has been established that activated α_2 M can bind different cytokines, including TGF β , tumor necrosis factor- α (TNF α), platelet-derived growth factor (PDGF), interleukin-1 β (IL-1 β), interleukin-6 (IL-6) and basic fibroblast growth factor (bFGF) (25–27,35–38). Binding of TGF β to α_2 M has been reported to result in reduction (39–43), promotion (44) or no alteration (40,44) of TGF β activity, probably depending on the type of target cell involved.

Several studies suggest the involvement of α_2 M in liver fibrosis. In patients suffering from liver fibrosis due to different causes, high serum levels of α_2 M have been measured (45–47). In addition, α_2 M was present in extracts and secretions from murine *Schistosoma*

mansoni liver granulomas (48), and we recently demonstrated its presence at sites of liver fibrosis in the rat (49). α_2 M was produced by nonparenchymal granuloma cells as demonstrated by the presence of α_2 M mRNA, and was taken up among others by liver parenchymal cells surrounding the granulomas as demonstrated by the presence of α_2 M protein in the absence of α_2 M mRNA in these cells (49). Since α_2 M is taken up only after its activation, these data suggest that α_2 M is activated at sites of fibroinflammatory lesions.

In the present study we investigate the effect of α_2 M on the capacity of TGF β to stimulate collagen synthesis by human liver fat-storing cells which had transformed into α -smooth muscle actin expressing myofibroblasts in culture.

We show that TGF β -induced collagen synthesis *in vitro* is significantly reduced by methylamine-activated α_2 M (α_2 M-Me). Furthermore, we demonstrate that human liver myofibroblasts endocytose α_2 M-Me in culture. This suggests that binding of TGF β to activated α_2 M may be followed by uptake of α_2 M-TGF β complexes by human liver myofibroblasts leading to reduced availability of extracellular TGF β .

Materials and Methods

TGF β

Highly purified natural human TGF β 1 (>95%) obtained from R&D Systems Europe Ltd (Abingdon, United Kingdom) was used in the collagen synthesis studies. Natural human TGF β 1 was activated according to the manufacturer's instructions, using 4 mM HCl.

Human recombinant 125 I-TGF β 1 (1283 Ci/mmol TGF β 1) purchased from Amersham (Slough, United Kingdom) was used in the binding studies. Lyophilized protein was dissolved in 17.5 mM acetic acid, according to the manufacturer's instructions.

α_2 M

α_2 M was purified from fresh, normal human EDTA-plasma according to the method of Sottrup-Jensen et al. (50) with slight modifications using Zn $^{2+}$ chelate affinity chromatography (51). The concentration of α_2 M in purified preparations was determined from the absorbance at 280 nm using an absorption coefficient (1.0%, 1 cm) of 8.93 (52).

Activated α_2 M (α_2 M-Me) was prepared by reacting native α_2 M (α_2 M) with 200 mM methylamine-HCl (Sigma Chemical Co., St. Louis, MO, USA) in 50 mM Tris-HCl, pH 8.0, for 2 h at 22°C (30). Excess methylamine was removed by dialysis against PBS.

α_2 M and α_2 M-Me preparations were checked for activation by rate electrophoresis according to Van Leuv-

en et al. (31). Native $\alpha_2\text{M}$ was judged to be at least 90% intact ("slow" form) on the basis of Phast Blue staining of the rate electrophoresis gels. Incubation of $\alpha_2\text{M}$ with methylamine rendered $\alpha_2\text{M-Me}$ greater than 80% in the electrophoretically "fast" form.

In the culture medium of human liver myofibroblasts, $\alpha_2\text{M}$ was measured with an ELISA using rabbit anti-human $\alpha_2\text{M}$ (A033, Dako A/S, Glostrup, Denmark) and sheep anti-human $\alpha_2\text{M}$ HRP (PP039, The Binding Site Limited, Birmingham, United Kingdom). The sensitivity of this assay is 11 ng/ml.

Formation of $\alpha_2\text{M-TGF}\beta$ complexes

$^{125}\text{I-TGF}\beta$ (20 nM) was incubated with $\alpha_2\text{M}$ or $\alpha_2\text{M-Me}$ (1.5 μM) for 2 h at 37°C. Complex formation was assessed by rate electrophoresis according to Van Leuven et al. (31). The gels were fixed in 45% methanol/10% acetic acid, stained with Phast Blue (Pharmacia LKB Biotechnology AB, Uppsala, Sweden), destained with 45% methanol/10% acetic acid, and dehydrated using a gel dryer. Binding of $^{125}\text{I-TGF}\beta$ to $\alpha_2\text{M}$ was detected by autoradiography at -70°C on preflashed Kodak X-Omat AR Imaging film (Eastman Kodak Company, Rochester, NY, USA).

Isolation of human liver myofibroblasts

Cells were isolated from tumor-free human liver tissue obtained during hepatectomy performed to remove a tumor from patients with no previous liver disease (VA5: male, 54 years old, VA6: female, 28 years old) and from a healthy donor liver unsuitable for transplantation because of the age of the donor (VA4: male, age over 55 years), as described previously (7). Briefly, after digestion of small pieces of liver tissue with pronase (Calbiochem, San Diego, CA, USA) and collagenase (Worthington Biochemical Corporation, Freehold, NJ, USA) cells were separated over gradients of Percoll (Pharmacia). FSC were recovered from the 5–15% interphase and were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco Life Technologies Ltd., Paisley, United Kingdom) supplemented with 1% antibiotic-antimycotic (Gibco), 2 mM glutamine (BDH Chemicals Ltd, Poole, United Kingdom) and 10% fetal calf serum (FCS) (heat inactivated, Gibco). Experiments were performed with FSC which had transformed in culture into myofibroblasts co-expressing vimentin and α -smooth muscle actin (VA4, VA5, VA6) as detected by immunofluorescence (7). VA4, VA5 and VA6 cells were obtained from three different human livers and were used between passages 2 and 9. The cytoskeletal phenotype of the cells did not change between passages 2 and 9.

Collagen synthesis

Cells were seeded (25×10^3 cells/0.5 ml DMEM supplemented with 1% antibiotic-antimycotic, 2 mM glutamine and 5% FCS per well) in 24-well plates (Falcon, Becton Dickinson, Lincoln Park, NJ, USA) and grown for 72 h to reach confluency. After 72 h wells were washed with PBS and incubated in 0.5 ml per well of proline-free DMEM (Gibco) supplemented with 1% antibiotic-antimycotic, 2 mM glutamine and 4 mg/ml human albumin in the presence of different concentrations of $\text{TGF}\beta$ and $\alpha_2\text{M-Me}$ for 48 h. $^3\text{H-proline}$ (1 $\mu\text{Ci}/0.5$ ml medium per well, specific activity 50 Ci/mmol, Amersham, International plc., Buckinghamshire, United Kingdom), fresh ascorbic acid (50 $\mu\text{g}/\text{ml}$, Merck, Darmstadt, Germany) and β -aminopropionitrile-fumarate (50 $\mu\text{g}/\text{ml}$, Serva) were added during the last 24 h. Experiments were performed in triplicate or quadruplicate wells of confluent cell cultures.

Collagen synthesis was assessed by incorporation of $^3\text{H-proline}$ into collagenase degradable protein according to the method of Peterkofsky et al. (53). Collagenase (collagenase type VII, Sigma) was chromatographically purified on a G200 gel filtration column (Pharmacia Biotech, Brussels, Belgium) and was checked for purity by failure of the purified collagenase to digest proteins labeled with $^3\text{H-tryptophan}$ (specific activity 27 Ci/mmol, Amersham) synthesized by human liver myofibroblasts in experiments similar to the collagen assay.

The relative collagen synthesis (RCS) was calculated with a formula (53) that takes into account the enrichment of proline in collagen compared with other proteins:

$$\% \text{ RCS} = \{(\text{dpm in CDP}) / ((\text{dpm in NCP} \times 5.4) + (\text{dpm in CDP}))\} \times 100\%$$

CDP represents Collagenase Degradable Protein (radioactivity in the sample treated with collagenase minus radioactivity in the sample not treated with collagenase, corrected with the dilution factor from the original sample), NCP represents Non Collagenous Protein (total radioactivity of the sample minus CDP).

Effects of $\text{TGF}\beta$ and $\alpha_2\text{M-Me}$ on the number of cells in confluent wells after 48 h of incubation were investigated by measuring the amount of protein present. After removal of the medium, cell-layers were rinsed three times with PBS and the amount of protein was determined in these cell-layers using the Bio-Rad DC Protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

Endocytosis of $\alpha_2\text{M}$ by human liver myofibroblasts

Uptake of $\alpha_2\text{M-Me}$ was investigated in nonconfluent cell cultures grown on glass coverslips in six well plates (Nunc, Inter Med Nunc, Roskilde, Denmark). Cells

were rinsed twice with PBS and were cultured in DMEM supplemented with 1% antibiotic-antimycotic, 2 mM glutamine and 4 mg/ml human albumin for 16 h. Cells were then rinsed twice with PBS and were incubated in the presence or absence of α_2M -Me (100 μ g/ml) in DMEM supplemented with 1% antibiotic-antimycotic, 2 mM glutamine, 4 mg/ml human albumin for 5, 15, 30 or 60 min. The cells on the coverslips were washed three times with PBS before and after fixation with cold methanol (10 min). Presence of α_2M was detected by means of immunofluorescence using rabbit anti-human α_2M (1:100) (A033, Dako A/S, Glostrup, Denmark) as first antibody and goat-anti-rabbit-FITC (1:100) (Sigma) as second antibody, as described previously (7). Control sections were incubated without the first or second antibody, respectively. These control sections never showed any staining.

Statistical analysis of the data

Data are expressed as mean $\pm$ SD when data within one experiment are presented or as mean $\pm$ SEM when data from separate subsequent experiments are shown. Statistical analyses were performed using the SPSS/PC+ Statistical Software Package, version 5.0 (SPSS Inc., Chicago, IL, USA).

ANOVA (two-way procedure) was used for comparison of differences in collagen synthesis in the presence

and absence of TGF β (Fig. 3). ANOVA (one-way procedure) was used for differences in the presence of different concentrations of TGF β (Fig. 2) or α_2M (Fig. 3), and for differences between the experimental groups in Table 1. The Scheffé procedure or Student's *t*-test was used for individual group comparison where appropriate. Levels of significance were set at $p < 0.05$. When significant, the results of the respective tests are included in the legends to the table and figures.

Results

TGF β binding to α_2M and α_2M -Me

To investigate whether the α_2M preparations used in the present study could bind TGF β , we incubated ^{125}I -TGF β with α_2M or α_2M -Me for 2 h at 37°C, and screened for binding on a rate electrophoresis followed by autoradiography (Fig. 1).

The native α_2M preparation seemed to contain some partially activated α_2M (35), representing α_2M with one or two activated thiolesters out of the four activable thiolesters. The α_2M -Me preparation was completely in the active form. TGF β bound to partially activated forms of α_2M present in the native preparation (lane b), and to completely activated α_2M in the α_2M -Me preparation (lane c). We have used α_2M -Me in the following experiments.

Fig. 1. Binding of ^{125}I -TGF β to α_2M and α_2M -Me as determined by rate electrophoresis (1), followed by autoradiography (2). ^{125}I -TGF β (20 nM) was incubated without α_2M or α_2M -Me (lane a), with α_2M (1.5 μ M) (lane b) or with α_2M -Me (1.5 μ M) (lane c) for 2 h at 37°C. An equal amount of α_2M was loaded in lanes b and c. The Phast Blue stained gel (1) and the autoradiograph (2) have been aligned so that mobilities of the bands may be directly compared. The "slow" form (s) corresponding to the conformationally unaltered state and the "fast" form (f) corresponding to the methylamine-treated, conformationally altered state are indicated. Note that ^{125}I -TGF β binds to partially activated forms of α_2M (found at sites intermediate between the "slow" and "fast" form) present in the α_2M preparation. Lane a shows that free ^{125}I -TGF β does not migrate at a position near α_2M .

Fig. 2. Effect of TGFβ on CDP and RCS in confluent cultures of human liver myofibroblasts. Confluent cells were cultured in proline-free DMEM containing 4 mg/ml human albumin with different concentrations of TGFβ for 48 h. ³H-proline, ascorbic acid and β-aminopropionitrile fumarate were added during the last 24 h of the culture. Combined data of three experiments with cell cultures obtained from three different human livers, each performed in quadruplicate wells, are presented. Data are expressed as percentage (mean ± SEM) of control in the absence of TGFβ (100%). ANOVA (one-way) for different concentrations of TGFβ: *p* < 0.05 both for CDP and RCS. Scheffé's comparison for differences *p* < 0.05 ^a vs 0, ^b vs 0.001, ^c vs 0.1 ng/ml TGFβ.

Effect of α₂M on TGFβ-induced collagen synthesis

Incubation of confluent cell cultures for 48 h with the highest concentrations of TGFβ and α₂M used in the collagen studies did not alter the total amount of cellular protein recovered from each well, indicating that the number of cells was not changed during this incubation period (data not shown). Therefore, we used confluent cell cultures to investigate the effects of TGFβ and α₂M on collagen production.

Myofibroblasts obtained from different human livers reacted comparably to TGFβ with respect to their absolute (CDP) and relative (RCS) collagen production. Addition of TGFβ in concentrations of 0.1 ng/ml and higher stimulated collagen synthesis significantly (Fig. 2), confirming previous results (6,7).

The effect of α₂M-Me was tested in the presence of 1 ng/ml TGFβ, a concentration of TGFβ that significantly stimulates collagen synthesis. α₂M-Me inhibited TGFβ-induced collagen production dose-dependently, reaching significant inhibition of the effect of TGFβ

from 10 μg/ml α₂M-Me (a 350-fold molar excess of α₂M-Me) onward (Fig. 3).

The effect of a high concentration of α₂M-Me (100 μg/ml) on TGFβ-induced collagen production was comparable in myofibroblast cell cultures obtained from different human livers (Table 1).

Using the native α₂M preparation that contained partially activated forms of α₂M, similar inhibition of TGFβ-induced collagen synthesis was observed (data not shown). This suggests that the partially activated α₂M present in the preparation binds and inactivates TGFβ sufficiently to reduce TGFβ-induced collagen synthesis, or that during culturing native α₂M becomes activated, binds and inhibits TGFβ.

α₂M-Me by itself sometimes slightly reduced basal collagen formation (Table 1, Fig. 3), probably by scavenging collagen stimulating factors secreted by the cells in culture.

Fig. 3. Effect of different concentrations of α₂M-Me on TGFβ-induced (1 ng/ml) collagen synthesis (CDP) in confluent cultures of myofibroblasts (VA4 and VA6) obtained from two different human livers. Confluent cells were cultured for 48 h in proline-free DMEM containing 4 mg/ml human albumin with described mediators. ³H-proline, ascorbic acid and β-aminopropionitrile fumarate were added during the last 24 h. Values represent mean ± SD of representative experiments performed in triplicate wells. Data are expressed as percentage of control values (100%) in the absence of TGFβ and α₂M-Me. ANOVA (two-way) for the effect of TGFβ in the presence of increasing concentrations of α₂M-Me (within VA4 and VA6: both *p* < 0.05). ANOVA (one-way) for the effect of α₂M-Me (within VA4 and VA6: both *p* < 0.05), α₂M-Me and TGFβ (within VA4 and VA6: both *p* < 0.05). Scheffé comparison for differences *p* < 0.05 ^a vs 0, ^b vs 0.1, ^c vs 1 μg/ml α₂M-Me. Student's *t*-test for differences for each concentration of α₂M-Me in the presence versus absence of TGFβ: **p* < 0.05.

TABLE 1

Effect of α_2 M-Me on TGF β -induced collagen production by three different human liver myofibroblast cultures

	VA4	VA5	VA6
TGF β	194 $\pm$ 10 ^{*,**}	196 $\pm$ 4 ^{*,**} .a	175 $\pm$ 7 ^{*,**} .a
TGF β + α_2 M-Me	141 $\pm$ 6	159 $\pm$ 2 ^{*,**}	85 $\pm$ 2
α_2 M-Me	102 $\pm$ 11	87 $\pm$ 1	54 $\pm$ 6

Confluent cultures of myofibroblasts (VA4, VA5, VA6) derived from different human livers were incubated with TGF β (1 ng/ml), α_2 M-Me (100 μ g/ml) or a combination of TGF β and α_2 M-Me for 48 h. Data are expressed as percentage (mean $\pm$ SD) of control values without TGF β and without α_2 M-Me (100%). Each experiment was performed in triplicate wells.

ANOVA (one-way) for effects of TGF β and α_2 M-Me (within VA4, VA5 and VA6: each p <0.05).

Scheffé comparison for differences within groups p <0.05 *vs Control,

^avs TGF β + α_2 M-Me, **vs α_2 M-Me.

Endocytosis of α_2 M by human liver myofibroblasts

Since reduction of cytokine activity by α_2 M is suggested to be caused by endocytosis and subsequent degradation of the α_2 M-cytokine complex, we tested whether the human liver myofibroblasts could endocytose α_2 M.

As shown in Fig. 4, α_2 M could be demonstrated by means of immunofluorescence in granules of the cells within 5 min after addition of α_2 M-Me (100 μ g/ml). Presence of α_2 M positive granules was also observed after 15, 30, and 60 min incubation. When no α_2 M-Me was added to the culture media, α_2 M was not observed within the cells, indicating that the human liver myofibroblasts did not produce α_2 M. The latter was confirmed by the absence of α_2 M in the culture medium of these cells as measured in an ELISA (data not shown).

Discussion

TGF β , which is considered to be a major stimulator of matrix production in liver fibrosis, has been shown in different studies to bind to α_2 M (36,37,39,41,44,54,55). The effect of binding of TGF β to α_2 M on the activity of TGF β is variable: α_2 M has been shown to have no influence (40,44), to reduce (39–42) or to enhance (43) the activity of TGF β on different cell cultures.

In the present study we demonstrate that TGF β -induced collagen synthesis by human liver myofibroblasts is significantly reduced by methylamine-activated α_2 M (α_2 M-Me), an *in vitro* equivalent of protease-activated α_2 M (30). The TGF β preparation used was a commercial highly purified preparation which may have contained small amounts of contamination and which may lead to secondary production of other mediators in the cell culture. In future experiments these possibilities will be examined by use of neutralizing antibodies.

In addition to effects on TGF β -induced collagen formation, α_2 M-Me by itself sometimes moderately re-

Fig. 4. Localization of α_2 M in granules of human liver myofibroblasts in nonconfluent cultures at 5 (A) and 15 min (B) after incubation with α_2 M-Me, or without α_2 M-Me (C) as detected by immunofluorescence. No fluorescence was observed when the primary or secondary antibody was omitted. (Magnification $\times$ 90).

duced basal collagen synthesis (see Table 1 and Fig. 3). This effect may partly contribute to the effects of α_2 M-Me on TGF β -induced collagen formation. The influence of α_2 M-Me on basal collagen synthesis may be explained in different ways. FSC have been shown to express TGF β mRNA (6,17) but to secrete TGF β predominantly in its latent form (16,18,56). If, however, under the present culture conditions some of the TGF β produced by the cells was activated, this may have contributed to the basal collagen production. α_2 M may interfere with this process by inhibition of the enzymes activating latent TGF β (14,19–21), or by the binding of activated TGF β . Alternatively, α_2 M-Me may interfere with other, not yet identified, mediators produced in culture which regulate basal collagen synthesis.

In general, binding of TGF β to α_2 M may prevent the interaction of TGF β with its receptor on the cell. Binding of TGF β to α_2 M, however, has been shown not to interfere with interaction of α_2 M with its receptor (37). Hence, α_2 M-TGF β complexes may bind to the α_2 M receptor and be subsequently cleared via uptake of the complexes by the cells. To test the capacity of human liver myofibroblasts to endocytose α_2 M-Me, the uptake of α_2 M-Me in the cells was studied by means of immunofluorescence. Human liver myofibroblasts appeared to be very active in endocytosing α_2 M-Me: α_2 M could be clearly demonstrated in granules of the cells within 5 min after addition of α_2 M-Me. This suggests that in addition to hepatocytes, which account for the majority of the clearance of α_2 M-proteinase complexes from the circulation (33), myofibroblasts may contribute to the clearance of α_2 M-proteinase complexes at sites of human liver fibrosis.

α_2 M could not be demonstrated in cells or supernatants of cell cultures that were not supplemented with α_2 M-Me, indicating that human liver myofibroblasts did not produce α_2 M in culture. This is in contrast to observations on cultures of human fibroblasts derived from skin and lung (57,58), and rat FSC (42,59,60). The difference in α_2 M production between human and rat FSC may be explained by species differences, as has also been observed for other features of FSC (61,62). Alternatively, differences in the state of activation of the human and rat FSC used may explain the variation in α_2 M production. All studies on rat FSC (42,59,60) were performed in primary cultures between 5 and 11 days after isolation of cells, whereas we used human liver myofibroblasts between passages 2 and 9.

The effects of TGF β on the ECM are not restricted to a direct stimulating effect on matrix synthesis. TGF β has been shown to enhance proliferation of human FSC indirectly by upregulation of the PDGF re-

ceptor (13). In addition, TGF β is able to decrease the production of proteases degrading the ECM such as collagenase, transin/stromelysin and plasminogen activator and to increase the synthesis of protease inhibitors like plasminogen activator inhibitor-1 (PAI-1) and tissue inhibitor of metalloproteinases (TIMP) (19,20,24). Therefore, *in vivo*, binding of α_2 M to TGF β could induce different effects.

Based on the present *in vitro* study, we hypothesize that α_2 M may induce antifibrogenic effects *in vivo* due to inhibition of TGF β -induced collagen synthesis.

Acknowledgements

The authors gratefully acknowledge Mrs. F.J.M. Habets (Department of Pulmonology, Academic Medical Center, Amsterdam) for performing the α_2 M ELISA, and Mr. C.J. Hersbach and Mr. C.E. Gravemeijer for photographic work.

References

1. Annoni G, Weiner FR, Zern MA. Increased transforming growth factor- β 1 gene expression in human liver disease. *J Hepatol* 1992; 14: 259–64.
2. Nagy P, Schaff Z, Lapis K. Immunohistochemical detection of transforming growth factor- β 1 in fibrotic liver diseases. *Hepatology* 1991; 14: 269–73.
3. Castilla A, Prieto J, Fausto N. Transforming growth factors β 1 and α in chronic liver disease. Effects of interferon alpha therapy. *N Engl J Med* 1991; 324: 933–40.
4. Czaja MJ, Weiner FR, Flanders KC, Giambrone MA, Wind R, Biempica L, Zern MA. *In vitro* and *in vivo* association of transforming growth factor- β 1 with hepatic fibrosis. *J Cell Biol* 1989; 108: 2477–82.
5. Armendariz-Borunda J, Seyer JM, Kang AH, Raghow R. Regulation of TGF β gene expression in rat liver intoxicated with carbon tetrachloride. *FASEB J* 1990; 4: 215–21.
6. Casini A, Pinzani M, Milani S, Grappone C, Galli G, Jezquel AM, Schuppan D, Rotella CM, Surrenti C. Regulation of extracellular matrix synthesis by transforming growth factor β 1 in human fat-storing cells. *Gastroenterology* 1993; 105: 245–53.
7. Tiggeleman AMBC, Boers W, Linthorst C, Sala M, Chamuleau RAFM. Collagen synthesis by human liver (myo)fibroblasts in culture: evidence for a regulatory role of IL-1 β , IL-4, TGF β and IFN γ . *J Hepatol* 1995; 23: 307–17.
8. Matsuoka M, Pham NT, Tsukamoto H. Differential effects of interleukin-1 α , tumor necrosis factor α , and transforming growth factor β 1 on cell proliferation and collagen formation by cultured fat-storing cells. *Liver* 1989; 9: 71–8.
9. Armendariz-Borunda J, Katayama K, Seyer JM. Transcriptional mechanisms of type I collagen gene expression are differentially regulated by interleukin-1 β , tumor necrosis factor α , and transforming growth factor β in Ito cells. *J Biol Chem* 1992; 267: 14316–21.
10. Ramadori G. The stellate cell (Ito-cell, fat-storing cell, lipocyte, perisinusoidal cell) of the liver. New insights into pathophysiology of an intriguing cell. *Virchows Archiv B Cell Pathol* 1991; 61: 147–58.

11. Friedman SL. The cellular basis of hepatic fibrosis. *N Engl J Med* 1993; 328: 1828–35.
12. Milani S, Herbst H, Schuppan D, Grappone C, Heinrichs OE. Cellular sources of extracellular matrix proteins in normal and fibrotic liver. Studies of gene expression by *in situ* hybridization. *J Hepatol* 1995; 22: 71–6.
13. Pinzani M. Novel insights into the biology and physiology of the Ito cell. *Pharmacol Ther* 1995; 66: 387–412.
14. Bedossa P, Paradis V. Transforming growth factor- β (TGF- β): a key role in liver fibrogenesis. *J Hepatol* 1995; 22 (suppl 2): 37–42.
15. Meyer DH, Bachem MG, Gressner AM. Modulation of hepatic lipocyte proteoglycan synthesis and proliferation by Kupffer cell-derived transforming growth factors type β 1 and type α . *Biochem Biophys Res Commun* 1990; 171: 1122–9.
16. Meyer DH, Bachem MG, Gressner AM. Transformed fat-storing cells inhibit the proliferation of hepatocytes by secretion of transforming growth factor- β . *J Hepatol* 1990; 11: 86–91.
17. Weiner FR, Giambrone MA, Czaja MJ, Shah A, Annoni G, Takahashi S, Eghbali M, Zern MA. Ito-cell gene expression and collagen regulation. *Hepatology* 1990; 11: 111–7.
18. Bachem MG, Meyer D, Melchior R, Sell KM, Gressner AM. Activation of rat liver perisinusoidal lipocytes by transforming growth factors derived from myofibroblastlike cells. A potential mechanism of self perpetuation in liver fibrogenesis. *J Clin Invest* 1992; 89: 19–27.
19. Attisano L, Wrana JL, López-Casillas F, Massagué L. TGF- β receptors and actions. *Biochim Biophys Acta* 1994; 1222: 71–80.
20. Lawrence DA. Transforming growth factor- β : an overview. *Kidney Int* 1995; 47: S19–23.
21. Miyazono K, Ichijo H, Heldin CH. Transforming growth factors- β : latent forms, binding proteins and receptors. *Growth Factors* 1993; 8: 11–22.
22. De Bleser PJ, Jannes P, van Buul-Offers SC, Hoogerbrugge CM, van Schravendijk CFH, Niki T, Rogiers V, van den Brande JL, Wisse E, Geerts A. Insulinlike growth factor-II/mannose 6-phosphate receptor is expressed on CCl₄-exposed rat fat-storing cells and facilitates activation of latent transforming growth factor- β in cocultures with sinusoidal endothelial cells. *Hepatology* 1995; 21: 1429–37.
23. Kingsley DM. The TGF- β superfamily: new members, new receptors, and new genetic tests of function in different organisms. *Genes Dev* 1994; 8: 133–46.
24. Border WA, Ruoslahti E. Transforming growth factor- β in disease: the dark site of tissue repair. *J Clin Invest* 1992; 90: 1–7.
25. Borth W. α_2 -Macroglobulin, a multifunctional binding protein with targeting characteristics. *FASEB J* 1992; 6: 3345–53.
26. Petersen CM. α_2 -Macroglobulin and pregnancy zone protein. Serum levels, α_2 -macroglobulin receptors, cellular synthesis and aspects of function in relation to immunology. *Dan Med Bull* 1993; 40: 409–46.
27. Chu CT, Pizzo SV. α_2 -Macroglobulin, complement, and biologic defense: antigens, growth factors, microbial proteases, and receptor ligation. *Lab Invest* 1994; 71: 792–812.
28. Sottrup-Jensen L. α -Macroglobulins: structure, shape and mechanism of proteinase complex formation. *J Biol Chem* 1989; 264: 11539–42.
29. Sottrup-Jensen L. Role of internal thiol esters in the α -macroglobulin-proteinase binding mechanism. *Ann NY Acad Sci* 1994; 737: 172–87.
30. Gonias SL, Reynolds JA, Pizzo SV. Physical properties of human α_2 -macroglobulin following reaction with methylamine and trypsin. *Biochim Biophys Acta* 1982; 705: 306–14.
31. Van Leuven F, Cassiman JJ, Van den Berghe H. Functional modifications of α_2 -macroglobulin by primary amines. *J Biol Chem* 1981; 256: 9016–22.
32. Barret AJ, Brown MA, Sayers CA. The electrophoretically “slow” and “fast” forms of the α_2 -macroglobulin molecule. *Biochem J* 1979; 181: 401–18.
33. Davidsen O, Christensen EI, Gliemann J. The plasma clearance of human α_2 -macroglobulin-trypsin complex in the rat is mainly accounted for by uptake into hepatocytes. *Biochim Biophys Acta* 1985; 846: 85–92.
34. Moestrup SK, Gliemann J, Pallesen G. Distribution of the α_2 -macroglobulin receptor/low density lipoprotein receptor-related protein in human tissues. *Cell Tissue Res* 1992; 269: 375–82.
35. Borth W. α_2 -Macroglobulin. A multifunctional binding and targeting protein with possible roles in immunity and autoimmunity. *Ann NY Acad Sci* 1994; 737: 267–72.
36. LaMarre J, Wollenberg GK, Gonias SL, Hayes MA. Cytokine binding and clearance properties of proteinase-activated α_2 -macroglobulins. *Lab Invest* 1991; 65: 3–14.
37. LaMarre J, Hayes MA, Wollenberg GK, Hussaini I, Hall SW, Gonias SL. An α_2 -macroglobulin receptor-dependent mechanism for the plasma clearance of transforming growth factor- β 1 in mice. *J Clin Invest* 1991; 87: 39–44.
38. James K. Interactions between cytokines and α_2 -macroglobulin. *Immunol Today* 1990; 11: 163–9.
39. LaMarre J, Wollenberg GK, Gaudie J, Hayes MA. α_2 -Macroglobulin and serum preferentially counteract the mitoinhibitory effect of transforming growth factor- β 2 in rat hepatocytes. *Lab Invest* 1990; 62: 545–51.
40. Danielpour D, Sporn MB. Differential inhibition of transforming growth factor β 1 and β 2 activated by α_2 -macroglobulin. *J Biol Chem* 1990; 265: 6973–7.
41. Hall SW, LaMarre J, Marshall LB, Hayes MA, Gonias SL. Binding of transforming growth factor- β 1 to methylamine-modified α_2 -macroglobulin and to binary and ternary α_2 -macroglobulin-proteinase complexes. *Biochem J* 1992; 281: 569–75.
42. Bachem MG, Burschel G, Boers W, Tiggelman MBC, Sell KM, Kropf J, Gressner AM. Feed back regulation between α_2 -macroglobulin and TGF β 1. Its putative role in liver fibrogenesis. In: Knook DL, Wisse E, eds. *Cells of the Hepatic Sinusoid*, Vol. 4. Leiden: Kupffer Cell Foundation, 1993: 218–21.
43. Stouffer GA, LaMarre J, Gonias SL, Owens GK. Activated α_2 -macroglobulin and transforming growth factor- β 1 induce a synergistic smooth muscle cell proliferative response. *J Biol Chem* 1993; 268: 18340–4.
44. O'Connor-McCourt MD, Wakefield LM. Latent transforming growth factor- β in serum. A specific complex with α_2 -macroglobulin. *J Biol Chem* 1987; 262: 14090–9.
45. Hiramatsu S, Kojima J, Okada TT, Inai S, Ohmori K. The serum protein profile in chronic hepatitis, cirrhosis and liver cancer. *Acta Hepato-Gastroenterol* 1976; 23: 177–82.
46. Meliconi R, Parracino O, Facchini A, Morselli-Labate AM, Bortolotti F, Tremolada F, Martuzzi M, Miglio F, Gasbarrini G. Acute phase proteins in chronic and malignant liver diseases. *Liver* 1988; 8: 65–74.
47. Naveau S, Poynard T, Banattar C, Bedossa P, Chaput JC.

- Alpha-2-macroglobulin and hepatic fibrosis. Diagnostic interest. *Dig Dis Sci* 1994; 39: 2426–32.
48. Truden JL, Boros DL. Detection of α_2 -macroglobulin, α_1 -protease inhibitor, and neutral protease-antiprotease complexes within liver granulomas of *Schistosoma mansoni*-infected mice. *Am J Pathol* 1988; 130: 281–8.
49. Tiggelman AMBC, Boers W, Moorman AFM, De Boer PAJ, Van der Loos CM, Rotmans JP, Chamuleau RAFM. Localisation of α_2 -macroglobulin protein and mRNA in rat liver fibrosis: evidence for the synthesis of α_2 -macroglobulin within *Schistosoma mansoni* egg-granulomas. *Hepatology* 1996; 23: 1260–7.
50. Sottrup-Jensen L, Petersen TE, Magnusson S. A thiol-ester in α_2 -macroglobulin cleaved during proteinase complex formation. *FEBS Lett* 1980; 121: 275–9.
51. Lonberg-Holm K, Reed DL, Roberts RC, Hebert RR, Hillman MC, Kutney RM. Three high molecular weight protease inhibitors of rat plasma. Isolation, characterization, and acute phase changes. *J Biol Chem* 1987; 262: 438–45.
52. Hall PK, Roberts RC. Physical and chemical properties of human plasma α_2 -macroglobulin. *Biochem J* 1978; 171: 27–38.
53. Peterkofsky B, Chojkier M, Bateman J. Determination of collagen synthesis in tissue and cell culture systems. In: Furthmayer H, ed. *Immunocytochemistry of the Extracellular Matrix*. Vol 2. Boca Raton: CRC, 1981: 19–47.
54. Huang SS, O'Grady P, Huang JS. Human transforming growth factor β - α_2 -macroglobulin complex is a latent form of transforming growth factor β . *J Biol Chem* 1988; 263: 1535–41.
55. Crookston KP, Webb DJ, LaMarre J, Gonias SL. Binding of platelet-derived growth factor-BB and transforming growth factor- β 1 to α_2 -macroglobulin *in vitro* and *in vivo*: comparison of receptor-recognized and non-recognized α_2 -macroglobulin conformations. *Biochem J* 1993; 293: 443–50.
56. Marra F, Bonewald LF, Park-Snyder S, Park I-S, Woodruff KA, Abboud HE. Characterization and regulation of the latent transforming growth factor- β complex secreted by vascular pericytes. *J Cell Physiol* 1996; 166: 537–46.
57. Brissenden JE, Cox DW. α_2 -Macroglobulin production by cultured human fibroblasts. *Somat Cell Genet* 1982; 8: 289–305.
58. Mosher DF. Synthesis of α_2 -macroglobulin by cultured adherent cells. *Ann NY Acad Sci* 1983; 421: 327–31.
59. Andus T, Ramadori G, Heinrich PC, Knittel T, Meyer zum Büschenfelde KH. Cultured Ito cells of rat liver express the α_2 -macroglobulin gene. *Eur J Biochem* 1987; 168: 641–6.
60. Ramadori G, Knittel T, Schwögler S, Bieber F, Rieder H, Meyer zum Büschenfelde KH. Dexamethasone modulates α_2 -macroglobulin and apolipoprotein E gene expression in cultured rat liver fat-storing (Ito) cells. *Hepatology* 1991; 14: 875–82.
61. Tiggelman AMBC, Boers W, Linthorst C, Brand HS, Sala M, Chamuleau RAFM. Interleukin-6 production by human liver (myo)fibroblasts in culture. Evidence for a regulatory role of LPS, IL-1 β , and TNF α . *J Hepatol* 1995; 23: 295–306.
62. Rosenbaum J, Blazewski S. Regulation of Ito cell proliferation by soluble factors. *J Hepatol* 1995; 22 (suppl 2): 65–70.