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Transforming growth factor-beta (TGF-β) expression and interaction with proteinase 3 (PR3) in anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis

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SUMMARY

TGF-β is a multifunctional cytokine modulating the onset and course of autoimmune diseases as shown in experimental models. The aim of this study was to investigate TGF-β expression in ANCA-associated vasculitis (AAV), and the possible interactions of this cytokine with lysosomal enzymes identified as ANCA autoantigens (e.g. PR3). This included TGF-β effects on the translocation of the lysosomal enzymes to the cell surface of polymorphonuclear neutrophils (PMN), and the presumed activation of non-bioactive, latent TGF-β by these enzymes. Patients with various types of systemic vasculitis (SV) were studied, including three different types of AAV (Wegener's granulomatosis (WG), Churg–Strauß syndrome (CSS) and microscopic polyangiitis (MPA)). Regardless of the type of assay applied, the TGF-β1 isoform was found to be overexpressed in SV, including AAV, and to correlate with disease activity as shown for WG. Mean TGF-β1 plasma levels in AAV patients ranged from 8·9 ng/ml (WG) to 13·3 ng/ml (CSS) (control 4·2 ng/ml; $P < 0{\cdot}01$), while TGF-β2 levels were not elevated. Flow cytometry analysis showed TGF-β1 to be a potent translocation factor for PR3 comparable to other neutrophil-activating factors such as IL-8. PR3 membrane expression on primed PMN increased by up to 51% after incubation with TGF-β1. PR3 itself was revealed as a potent activator of latent TGF-β, thus mediating bioeffects of this cytokine. These findings, together with other features of TGF-β such as induction of angiogenesis and its strong chemotactic capacity, indicate that TGF-β might serve as a proinflammatory factor in SV, especially in AAV.

Keywords transforming growth factor-beta vasculitis anti-neutrophil cytoplasmic antibodies proteinase 3 autoimmunity

INTRODUCTION

TGF-β is a cytokine with multiple actions on various cells and tissues [1,2]. Studies in patients with rheumatoid arthritis showed local overexpression of bioactive TGF-β to be responsible for immunosuppression of activated cells in synovial fluid cells [3]. In animal models of human autoimmune diseases, local or systemic administration of TGF-β can modulate onset and course of inflammatory processes [4,5].

Within the group of chronic inflammatory rheumatic diseases, a close association between the presence of autoantibodies against different lysosomal enzymes and systemic vasculitis (SV) has been found [6,7]. This group of rheumatic diseases is now termed ANCA-associated vasculitis (AAV). While the etiology of SV, including AAV, is unknown, overexpression of proinflammatory cytokines, including IL-1, IL-6 and tumour necrosis factor-alpha (TNF-α) [6,8–10], is detectable and attributed to clinical signs such as constitutional symptoms or a strong acute-phase response. In addition, the overexpression of cytokines is believed to be responsible for the accessibility of autoantigens such as PR3 to the corresponding ANCA, and is implicated in the pathogenic model of AAV [11,12]. Also, cytokines may be essential to initiate or enhance endothelial damage by priming or activating endothelial cells and polymorphonuclear neutrophils (PMN) [13]. Among AAV, Wegener's granulomatosis (WG) shows the highest prevalence and disease specificity for ANCA [11]. PR3, which is localized in azurophilic granules of PMN, has been identified as main target antigen for the C-ANCA (a subtype of ANCA defined by immunofluorescence staining pattern on alcohol-fixed PMN, detected by indirect immunofluorscence technique) [14].

Since no data concerning the expression of TGF-β were available in AAV such as WG, and autoantigens in AAV display

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proteolytic activities and were expected to be able to activate latent TGF-β, we conducted a study on TGF-β expression in different types of SV, and on the interaction of TGF-β with lysosomal enzymes. Additional properties implicating TGF-β in the pathogenesis of SV included its stimulation of angiogenesis and formation of granulomatous tissue [15,16]. TGF-β expression was investigated *in vitro* and in plasma closely mimicking *in vivo* conditions. Patients both with and without AAV were investigated. For comparison with another inflammatory disease, TGF-β expression was determined in rheumatoid arthritis (RA). Culture supernatants of peripheral blood mononuclear cells (PBMC) were studied for TGF-β content by a TGF-β bioassay using the CCL64 cell line. TGF-β1 and TGF-β2 isoform determinations in plasma specimens were done using a newly established ELISA [17]. In addition, TGF-β expression was studied at the mRNA level employing a semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Since other cytokines are capable of translocating antigens such as PR3 to the cell membrane [6,13], we investigated the effect of TGF-β on membrane expression of various lysosomal enzymes present in PMN.

Collectively, our data revealed marked elevations of TGF-β1 both at mRNA and protein levels. The observations were made in AAV patients, but also in other inflammatory diseases, including RA and other SV. In the given setting, TGF-β was found to be a potent translocation factor for PR3 on PMN, comparable with other PMN-activating factors (e.g. phorbol myristate acetate (PMA), IL-8). PR3 also activated latent TGF-β, which was comparable with previous reports on plasmin [18].

Apart from its potential pathogenic role in PSV itself, TGF-β-induced proteinase translocation has to be considered as a new proinflammatory feature of TGF-β. Furthermore, PR3 can activate latent TGF-β, which adds further significance to the overexpression of TGF-β in PSV itself.

MATERIALS AND METHODS

Patients

Sixty-four patients and 101 healthy donors were included in this study. The diagnosis of patients selected included different types of ANCA-associated systemic vasculitides and other inflammatory rheumatic disease (RA). The group with non-granuloma-forming vasculitis consisted of patients with classical polyarteritis nodosa (PAN) and microscopic polyangiitis (MPA). Granuloma-forming AAV was seen in patients with WG and with Churg–Strauß syndrome (CSS). Each of the patients with primary vasculitides (WG, CSS, MPA) met the Chapel Hill Definition [19] and the criteria of the American College of Rheumatology (ACR). Secondary vasculitis was present in three of 15 RA patients. All patients were included with active, generalized disease and irrespective of current treatment.

Isolation of cells and culture conditions

For cytokine bioassay. Mononuclear cells (MNC) were separated from heparinized blood by Ficoll–Hypaque density gradient (1·077 g/ml) as described elsewhere [20,21]. Freshly isolated MNC (10^5 cells/well) were cultivated in 96-well flat-bottomed microtitre plates in serum-free RPMI 1640 medium supplemented with 1% glutamin plus penicillin (150 U/ml) and streptomycin (100 U/ml). The supernatants from the MNC were collected after 24 h culture.

For flow cytometry analysis. Human neutrophils were prepared from EDTA-blood of healthy donors using Ficoll–Paque centrifugation (400 ***g***, 35 min, 20°C). PMN-containing erythrocyte pellets were subsequently mixed with two volumes of polyvinylalcohol. After sedimentation for 20 min, the PMN-containing supernatant was collected. Contaminating erythrocytes were removed by hypotonic lysis. PMN purity was >98% as determined by Giemsa/Wright staining according to the manufacturer's instructions (Sigma, St Louis, MO). Cells were counted and examined at ×1000 magnification. PMN viability was >95% as determined by trypan blue exclusion.

Cytokine assays

TGF-β determination was performed by the growth inhibition assay of mink-lung cells (CCL64) and a newly established ELISA specific for TGF-β1 and TGF-β2, respectively. The sensitivity of the TGF-β bioassay was 300 pg/ml, while the TGF-β-ELISA was able to detect 10 pg/ml TGF-β. The samples (both culture supernatant and plasma) were tested at different dilutions with and without transient acidification to activate latent TGF-β [18,21,23]. The samples were acidified and neutralized after 30 min as described earlier [3]. Briefly, the samples were diluted 1 : 10 with PBS +50 mM HCl, and were neutralized with 5 M NaOH, 1 M HEPES pH 7·4, 200 mM EDTA.

TGF-β bioassay

The procedure for this bioassay is described in detail elsewhere [3]. Briefly, the CCL64 mink-lung cells were allowed to adhere in 96-well flat-bottomed microtitre plates using 5×10^3 cells/well in RPMI 1640 medium with 2% fetal calf serum (FCS). After a 4 h incubation period, samples or standards in RPMI 1640 medium were added and incubated for 72 h. The medium was removed and cells were fixed with 3% formalin for 10 min. After a washing step, cells were stained with 0·2% crystal violet in 10% methanol for 10 min [22]. Excess stain was removed and plates were rinsed in water. Cell-associated dye was eluted with 100 μl 0·5% SDS per well. Optical absorbance at 570 nm was determined with a microtitre plate reader. TGF-β levels were expressed in ng/ml based on a standard curve generated using human rTGF-β1 (Serva, Heidelberg, Germany). In addition, the isoform of TGF-β secreted by PBMC was determined by antibody neutralization as described elsewhere [3].

TGF-β-ELISA

Ninety-six-well microtitre plates were coated with a mouse MoAb directed against all three TGF-β isoforms (Genzyme, Cambridge, MA). After blocking with 1% bovine serum albumin (BSA), samples or standards were added to their respective wells. After repeated washings, a TGF-β1 isoform-specific polyclonal antibody was added (chicken; R&D Systems, Abingdon, UK). The immunoreactive TGF-β1 was detected using a chicken-specific antibody conjugate (rabbit anti-chicken IgG; Jackson ImmunoResearch, West Grove, PA) and TGF-β2 specificity was applied (rabbit anti-TGF-β2; R&D Systems). Goat anti-rabbit IgG conjugated with alkaline phophatase was added. Substrate buffer (1 mg/ml *p*-nitrophenylphosphate in 1 M diethanolamine pH 9·5) was added and substrate conversion was measured in an ELISA reader

(BioRad, Münich, Germany) as the difference in absorbance between 405 nm and 450 nm. For details refer to Szymkowiak *et al*. [17].

RT-PCR for TGF-β1

RNA isolation and reverse transcription. mRNA was isolated from 10^6 PBMC with RNAzol B (Cinna-Biotecx, Friendswood, TX) according to the manufacturer's protocol, and dissolved in DEPC H_2O. mRNA was reverse transcribed using 0·5 μg oligo (dT)12–18, 18 U RNAGuard (Pharmacia-LKB, Uppsala, Sweden), and 200 U MMLV reverse transcriptase (GIBCO-BRL, Eggenstein, Germany). cDNA was precipitated and resuspended in 20 μl DEPC H_2O.

PCR. The cDNA was used in a 30-cycle multiplex PCR (95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, and for 5 min for the last cycle). The reaction volume was 50 μl with 20 pmol of each primer, 200 nM dNTPs (Biotin-dUTP/TTP at a ratio of 1 : 10), 0·5 U of Taq-polymerase (GIBCO-BRL). Glyceraldehyde-3-phosphate-dehydrogenase (GA3PDH) served as internal control. For separation of DNA and visualization, 10 μl of the PCR reaction were applied to 6% acrylamidgel (19 : 1) and electrophoresis was performed at 3 W constant. Biotin-φX174 DNA-(Hinf I) fragments (GIBCO-BRL) were included as markers. The DNA was transferred to a Flash-Membrane (Stratagene, La Jolla, CA) using a semi-dry blotter. The DNA was cross-linked to the membrane by UV light. The Flash detection system (Stratagene) was used for the chemiluminescent reaction and the DNA was visualized on x-ray film (Kodak XAR). Intensity of the bands was evaluated by scanning of the x-ray film with a laser densitometer.

Primer sequences were: TGF-β1, 5′-CAG AAA TAC AGC AAC AAT TCC TGG-3′; 5′-TTG CAG TGT GTT ATC CGT GCT GTC-3′; GA3PDH, 5′-TCT GCC CCC TCT GCT GAT GCC CCC-3′; 5′-CCA TCA CGC CAC AGT TTC CCG GAG-3′.

Flow cytometric analysis of PMN surface expression of lysosomal proteins

Flow cytometric analysis was used to investigate the effect of rTGF-β1 on membrane expression of lysosomal proteins (PR3, human leucocyte elastase (HLE) and myeloperoxidase (MPO)) on PMN. MoAbs against PR3 (designated WGM2, IgG1) [24], HLE and MPO (both IgG1 isotype) (Dakopatts, Hamburg, Germany) were used to identify cell surface expression. As negative control an 'irrelevant' murine monoclonal IgG1 was used (Dianova, Hamburg, Germany). PMN (2×10^6/ml) from healthy donors were incubated for 30 min with TGF-β1 (1 ng/ml), PMA (1 μg/ml), or with buffer alone at 37°C. Incubations were stopped by ice-cold buffer and cell washing. All experiments were performed in duplicate. Before exposure to TGF-β1, PMA or buffer alone, PMN were incubated with TNF-α (150 pg/ml, 10 min), thus priming them to respond better to TGF-β1 and other cytokines, as established elsewhere [25]. Flow cytometry was performed as described elsewhere [25]. Briefly, PMN (10^6/ml) were incubated with the appropriate MoAb for 20 min. After washing, the cells were incubated with the fluorescein-labelled second antibody (DTAF-conjugated $F(ab)_2$ goat anti-mouse IgG; Dianova). After labelling, cells were washed and fixed with 1·5% paraformaldehyde. All procedures were performed on ice to minimize any activation of cells. Flow cytometric analysis of surface markers was done by Cytofluorograf System 50 (Ortho).

Effect of PR3 on activation of latent TGF-β

Enzymatically active PR3 was isolated from azurophilic granules of PMN as described by Leid *et al*. [26]. In brief, a Triton X-100 extract of azurophilic granules of PMN was subjected to cation exchange chromatography on a Biorex 70 column (BioRad). The PR3-containing fall-through fractions were concentrated by lyophilization and further purified by Superdex 75 fast performance liquid chromatography (FPLC; Pharmacia Biotech, Freiburg, Germany). The PR3-containing fractions were pooled and dialysed against PBS, and contained 150 μg/ml of PR3. PR3 concentration was determined in sandwich ELISA specific for PR3 together with a PR3 preparation with a known concentration. Proteolytic activity, as determined by cleavage of the chromogenic substrate tBOC-Ala-ONP (Sigma Chemie GmbH, Deisenhofen, Germany), was 632 mU/ml (by definition, 1 U of PR3 generates 1 μmol of *p*-nitrophenol per minute)

The proteolytic activation of latent TGF-β was performed as described [27]. Briefly, the PBMC culture supernatants pH 7·4 containing latent TGF-β1 were incubated with PR3 1 μg/ml, plasmin 55 U/ml (Sigma), HLE 1 μg/ml (Calbiochem, Bad Soden, Germany) and cathepsin G 1 μg/ml (Calbiochem) at 37°C for 2 h. Protease activity was then inhibited by the addition of PMSF and aprotinin to a final concentration of 2 mM and 0·55 U/ml. Complete activation of latent TGF-β was achieved by transient acidification as described above. The data were consistent in three independent experiments using pooled PBMC culture supernatants from WG patients.

Statistical analysis

Correlations were calculated according to Spearman and significance determined applying the Wilcoxon rank sum test.

RESULTS

TGF-β1 expression in SN of PBMC and in plasma

High levels of TGF-β were detected in granuloma-forming AAV (WG, CSS), in non-granuloma-forming AAV (MPA), and in primary SV (PAN). Table 1 shows the results of the bioassay

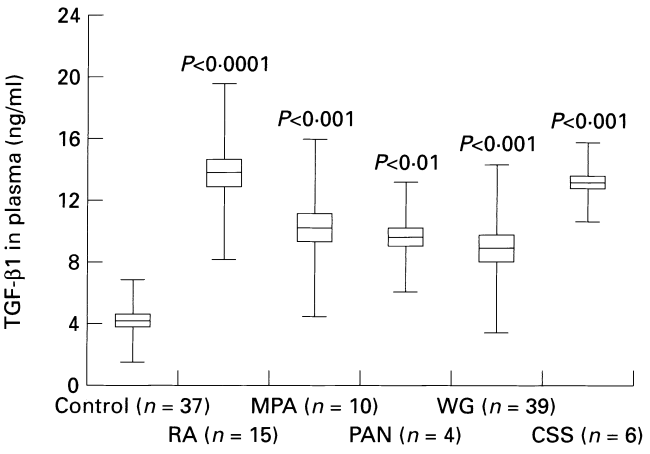

Fig. 1. Plasma levels of TGF-β1 in patients with rheumatoid arthritis (RA), microscopic polyangiitis (MPA), polyarteritis nodosa (PAN), Wegener's granulomatosis (WG) and Churg–Strauss syndrome (CSS) as measured by ELISA. Bars represent mean ± s.e.m., vertical lines mean ± s.d.

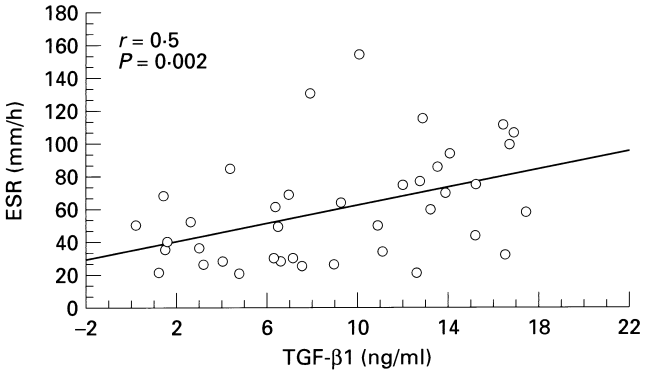

Fig. 2. Correlation of erythrocyte sedimentation rate (ESR) with TGF-β1 levels in plasma samples from patients with Wegener's granulomatosis (WG).

applied to supernatants of PBMC. TGF-β overexpression was most pronounced in supernatants from granuloma-forming AAV patients (WG, CSS). Neutralization experiments identified TGF-β1 as the dominant isoform. TGF-β1 levels in plasma specimens tended to be higher than in supernatants. Figure 1 shows the results of TGF-β1 determination in plasma. Vasculitis patients showed elevated TGF-β1 plasma levels of 8·9–13·3 ng/ml. Plasma TGF-β1 values correlated well with laboratory parameters, indicating disease activity (C-reactive protein, leucocyte counts). Figure 2 shows the correlation between the eythrocyte sedimentation rate (ESR) and TGF-β1 levels in patients with WG. Longitudinal studies for up to 12 months in four WG patients found a decrease in plasma TGF-β1 after substantial disease remission was achieved by cyclophosphamide and prednisolone therapy (data not shown). However, the values measured in patients with active RA (mean value 13·8 ng/ml) clearly indicate that high TGF-β1 levels are associated with inflammation in general and not with vasculitis *per se*. The values observed in RA with secondary systemic vasculitis were not significantly different when compared with other RA patients. TGF-β2 levels in plasma ranged about 3·6 ng/ml in controls, and were not found to be elevated in vasculitis patients nor in RA.

TGF-β1 bioactivity (mink lung assay) or immunoreactivity (ELISA) were only observed after transient acidification of samples. The values therefore represent the total TGF-β content, including newly activated TGF-β by transient acidification. The possibility of the discrepancies in supernatants and plasma being simply due to differences in the assays was excluded by demonstrating a reasonable correlation between TGF-β1 values in supernatants measured by bioassay or ELISA ($r = 0{\cdot}8$; $P < 0{\cdot}02$). A comparison between both assays in plasma specimens was not feasible, since plasma interfered with the bioassay, giving false low readings.

TGF-β mRNA expression

TGF-β mRNA expression was also studied in WG and CSS patients. As shown in Fig. 3 and Table 2, these patients had high readings for TGF-β1 compared with control individuals. A correlation with disease activity could be demonstrated, thus confirming the findings at the protein level.

Translocation of lysosomal enzymes by TGF-β

Since various agents, including proinflammatory cytokines,

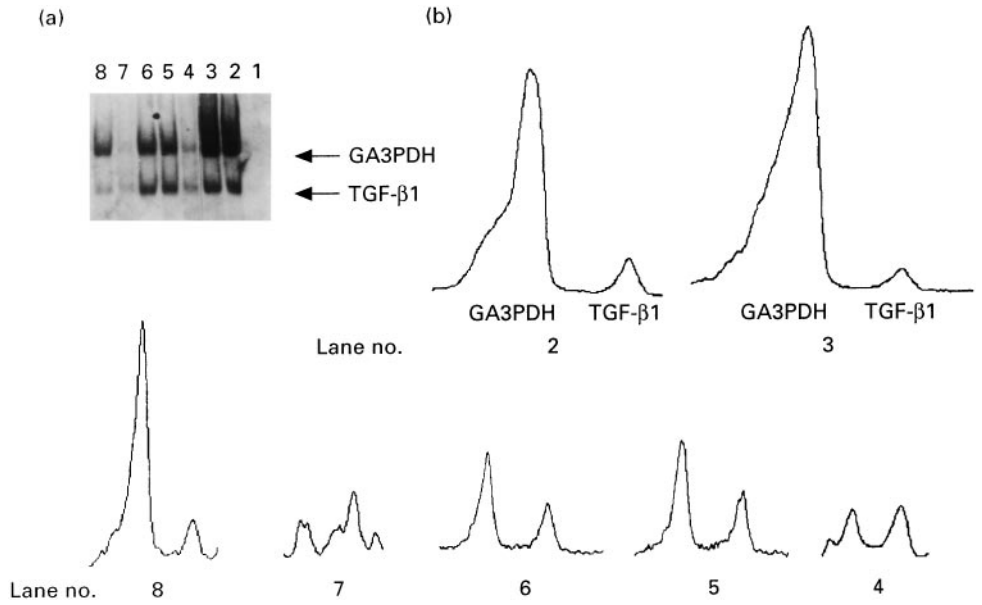

Fig. 3. (a) TGF-β1 mRNA expression in peripheral blood mononuclear cells (PBMC) from Wegener's granulomatosis (WG) and Churg–Strauss syndrome (CSS) patients. Lane 1, polymerase chain reaction (PCR) without cDNA as negative control; lanes 2 and 3, healthy blood donors which were used to get a standard relation between glyceraldehyde-3-phosphate-dehydrogenase (GA3PDH) and TGF-β1; lanes 5/6 and 7/8, two WG patients at different time points. Lane 4 shows a CSS patient. (b) The graphs indicate the individual intensities on the gel for both TGF-β and GA3PDH as scanned by laser densitometer. The increase in TGF-β expression was compared with the corresponding GA3PDH expression. See also Table 2. Assuming equal GA3PDH expression, elevated TGF-β mRNA levels were found: $\times$ 2·4 (no. 8), $\times$ 14·4 (no. 7), $\times$ 6·2 (no. 6), $\times$ 5·3 (no. 5), and $\times$ 15·4 (no. 4). In one WG patient (no. 8 $\rightleftharpoons$ no. 7), clinical deterioration was observed between the two time points. The corresponding supernatants of the PBMC fractions showed levels above 5 ng/ml TGF-β1 as measured by ELISA.

Table 1. TGF-β1 bioactivity in culture supernatants

Patients	TGF-β1 (ng/ml) (mean $\pm$ s.e.m.)	Cases/*P*
Control	0·7 $\pm$ 0·1	33/—
RA	2·5 $\pm$ 0·4	15/<0·002
MPA	3·8 $\pm$ 0·5	5/<0·002
PAN	1·3 $\pm$ 1·0	3/NA
WG	9·6 $\pm$ 1·5	36/<0·0001
CSS	5·8 $\pm$ 1·9	5/<0·01

RA, Rheumatoid arthritis; MPA, microscopic polyangiitis; PAN, polyarteritis nodosa, WG, Wegener's granulomatosis; CSS, Churg–Strauss syndrome; NA, not applicable.

Table 2. Relative increase of TGF-β1 mRNA expression

Patients	Relative TGF-β1 increase
2 + 3 (Controls)	1
4 (CSS)	15·4
5 (WG1)	5·3
6 (WG1)	6·2
7 (WG2)	14·4
8 (WG2)	2·4

Assuming equal glyceraldehyde-3-phosphate dehydrogenase (GA3PDH) expression, the ratio between GA3PDH and TGF-β1 was determined in controls and was set to 1. Elevated TGF-β1 mRNA levels were deduced from change in ratio between GA3PDH and TGF-β1 polymerase chain reaction (PCR) products in patients. Numbers 2 and 3 represent healthy blood donors. Number 4 shows Churg–Strauss syndrome (CSS). Number 5/6 (WG1) and number 7/8 (WG2) represent two Wegener's granulomatosis (WG) patients at different time points. In WG2, clinical improvement was observed between the two time points (number 7, active phase of disease *versus* number 8, remission phase).

have the potential to translocate lysosomal enzymes also serving as ANCA autoantigens, PMN were investigated for their expression of lysosomal enzymes after incubation with TGF-β. In contrast to the well known anti-inflammatory TGF-β profile, incubation of PMN with rTGF-β1 revealed an unexpected increase (up to 51%) in PR3 membrane expression (Fig. 4). This finding remained constant regardless of the individual TGF-β1 concentrations applied (from 0·1 to 10 ng/ml TGF-β1). This experimental approach included a TNF-α priming step which mimics *in vivo* conditions, since high TNF-α levels are present in active SV [8,9]. The omission of PMN priming by TNF-α resulted in a 60% reduction in PR3 translocation. The cytokine-induced translocation of enzymes in these experiments was restricted to PR3. Antibodies against other proteases present in PMN (e.g. HLE, MPO) did not show significant changes in their membrane expression. A similar observation was made using other PMN activators [25]. Our results in infectious diseases therefore do not reflect a specific PMN response to TGF-β.

Effect of PR3 on activation of latent TGF-β

Figure 5 shows a typical example of the activation experiments of latent TGF-β from pooled supernatants of four WG patients. This experiment was repeated three times using different samples from SN of other WG patients. The results were comparable in all experiments. PR3 showed the highest level of activation, reaching up to 51% of total activation achievable by transient acidification of the samples. PR3 was even more effective than plasmin (32%) in TGF-β activation. Two other lysosomal

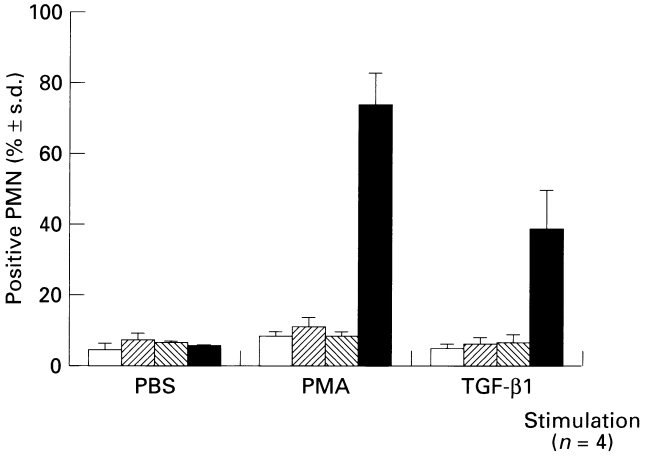

Fig. 4. Flow cytometric analysis of the membrane expression of granule proteins PR3 (■), elastase (HLE, ▧) and myeloperoxidase (MPO, ▨) on tumour necrosis factor-alpha (TNF-α)/phorbol myristate acetate (PMA)- and TNF-α/TGF-β-stimulated polymorphonuclear neutrophils (PMN). Results are shown as percentage of PMN positively stained for the respective enzyme. □, Control.

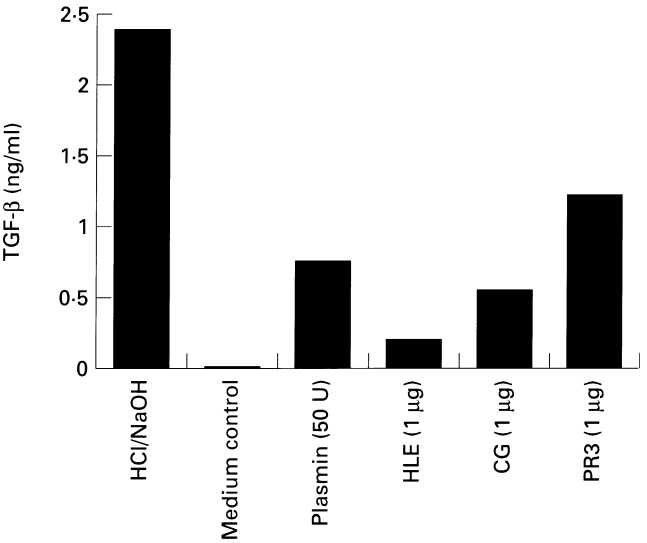

Fig. 5. TGF-β activation by lysosomal enzymes. The experiment was conducted as described in Materials and Methods. The plot shows the results from one experiment with SN from four individual Wegeners' granulomatosis (WG) patients. HCl/NaOH shows the complete activation of TGF-β1 by acidification. The control consists of untreated SN in PBS.

proteases, HLE and cathepsin G, showed a weak activation of latent TGF-β (8% and 23%, respectively).

DISCUSSION

The present study of cytokine expression in different types of vasculitic disorders revealed signs of moderate to strong overexpression of TGF-β1 both in supernatants of PBMC and in plasma. Supernatants of granuloma-forming AAV (WG, CSS) showed the highest TGF-β1 levels. TGF-β1 overexpression at the mRNA level indicates *de novo* synthesis of TGF-β1 and substantiates the results at the protein level. However, TGF-β1 overexpression is not a specific finding in vasculitis, as demonstrated by similar TGF-β1 overexpression in RA patients with and without SV. The obvious link between inflammation and TGF-β1 overexpression was clearly demonstrated by the positive correlation of TGF-β1 plasma levels with disease activity.

So far, TGF-β overexpression has only been described in autoimmune diseases, such as diabetes, in malignancies, and in infectious diseases [21,23]. Some pathogenic aspects described in these groups may, however, play a role in SV as well. First, TGF-β overexpression is associated with cellular and humoral immunodeficiency [21,23]. In SV, infectious disease shows a high prevalence, in which TGF-β may account, together with other factors including immunosuppression by therapy, for a high mortality rate [28]. Second, TGF-β overexpression has been shown to be a tumour-promoting factor [29]. Underlying events related to cancer induction are a decrease in lymphokine-activated cells and natural killer cells. In SV, a high rate of malignancy has been found as well [7,30]. In addition, the very marked expression of TGF-β in supernatants of PBMC from patients with granuloma-forming AAV suggests a pathogenic role for TGF-β in granuloma formation itself. From animal experiments it is well known that subcutaneous application of TGF-β induces granulomatous tissue formation and neovascularization [15,16]. High TGF-β levels have also been reported in pleural effusions from patients with tuberculosis [31]. However, TGF-β plasma levels in patients with tuberculosis or other granulomatous diseases have still to be determined.

The studies on the effects of TGF-β on translocation of lysosomal enzymes revealed a new proinflammatory feature of TGF-β by making the autoantigen PR3 accessible to the corresponding autoantibody (C-ANCA). The observed TGF-β overexpression may be included into the established hypothetical model for the development of AAV [6,32]. In this model, the interaction between autoantigen (e.g. PR3) and the corresponding antibody (e.g. C-ANCA) results in PMN degranulation, release of lysosomal proteases, generation of oxygen radicals, and subsequent endothelial cell injury. Concerning the preferential detection of PR3 on PMN by flow cytometry experiments, the findings may be explained by different binding properties for various lysosomal enzymes [25]. This study could also exclude major discrepancies due to different affinities of the antibodies employed. In addition, data from animal studies suggest that the production of proinflammatory cytokines, e.g. TNF-α and IL-1, is also induced by TGF-β, resulting in chronicity of the inflammatory process [1]. This, and the translocation of PR3 by TGF-β itself, strongly argues for a proinflammatory role of TGF-β, which is of major importance in AAV.

All TGF-β1 detected in SN or plasma of our patients was biologically inactive, which may raise doubts as to the physiological meaning of these findings. *In vitro* and *in vivo* studies, however, have clearly indicated constitutive TGF-β activation [2]. Factors activating latent TGF-β have been described, and it was expected that a wide range of granular proteases cleave the 'latency-associated peptide' [2]. This is especially likely at inflammatory sites. For the first time we report that PR3 is a potent activator of latent TGF-β. Further experiments have to clarify whether cell membrane-associated PR3 also activates TGF-β as shown for free PR3. The fact that free PR3 in circulation is physiologically inhibited by α_1-antitrypsin explains the observation that no bioactive TGF-β was found in our plasma samples.

Despite the data presented here on TGF-β1 expression in AAV and its potential pathogenic effects, some questions remain open. Since the etiology of vasculitic disorders is unknown, the initial event inducing cytokines (including TGF-β) and producing autoantibodies to lysosomal enzymes has still to be determined. Many authors have postulated infectious agents based on the therapeutic effect of antibiotics, at least in limited forms of WG [7,34]. From the observation of Zhou *et al*. [35] that IL-6 induces production of TGF-β, it is not surprising that high levels of TGF-β are found in chronic inflammatory diseases, including SV and RA. The correlation with disease activity further supports the idea that TGF-β is non-specific activated within the cytokine cascade. Moreover, since our study was also limited to experiments investigating TGF-β1 production by PBMC, other contributors to TGF-β overexpression cannot be ruled out. PMN themselves may be candidates for the production of TGF-β, but their TGF-β production is reportedly low [36]. Cells in vasculitic lesions including endothelial cells may also express increased levels of TGF-β [37]. In addition, platelets contain very high levels of TGF-β1 and also contribute to the findings of elevated TGF-β1 levels in plasma. Prompted by the observation that C-ANCA may activate PMN [7], additional studies are ongoing to investigate direct cytokine induction by C-ANCA, including the induction of TGF-β. Other techniques, such as *in situ* hybridization for TGF-β in vasculitic lesions and in granulomas, will provide further insight into the process of inflammation leading to vasculitis, tissue necrosis, and granuloma formation.

Our results confirmed the proinflammatory type effects of TGF-β which have previously been described for this cytokine [4,38,41]. Regarding the effect of TGF-β on different cell types, no other report has implicated TGF-β in PMN activation. Since enzymes such as PR3 released by PMN can activate latent TGF-β, they may also contribute to elevated TGF-β levels, because TGF-β augments its own expression [2]. Further studies on TGF-β expression in autoimmune diseases, including AAV, are necessary for understanding their pathogenesis and to make possible new therapeutic interventions, as already anticipated in experimental studies using TGF-β antibodies or TGF-β-binding proteins such as decorin [42–44].

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REFERENCES

1 Wahl SM. Transforming growth factor β: the good, the bad, and the ugly. J Exp Med 1994; **180**:1587–90.

2 Kekow J, Wiedemann GJ. Transforming growth factor β: a cytokine with multiple actions in oncology and potential clinical applications. Int J Oncol 1995; **7**:177–82.

3 Lotz M, Kekow J, Carson DA. Transforming growth factor-β and cellular immune responses in synovial fluids. J Immunol 1990; **144**: 4189–94.

4 Allen JB, Manthey CL, Hand AR, Ohura K, Ellingsworth L, Wahl SM. Rapid onset synovial inflammation and hyperplasia induced by transforming growth factor β. J Exp Med 1990; **171**:231–47.

5 Brandes ME, Allen JB, Ogawa Y, Wahl SM. TGF-β1 suppresses leukocyte recruitment and synovial inflammation in experimental arthritis. J Clin Invest 1991; **87**:1108–13.

6 Gross WL, Schmitt WH, Csernok E. ANCA and associated diseases: Immuno-diagnostic and pathogenic aspects. Clin Exp Immunol 1993; **91**:1–12.

7 Gross WL, Csernok E, Helmchen U. Antineutrophil cytoplasmic autoantibodies, autoantigens, and systemic vasculitis. APMIS 1995; **103**:81–97.

8 Kekow J, Szymkowiak C, Gross WL. Involvement of cytokines in granuloma formation within primary systemic vasculitis. In: Romagnani S, Mosmann TR, Abbas AK, eds. New advances on cytokines. New York: Raven Press, 1992:341–8.

9 Deguchi Y, Shibata N, Kishimoto S. Enhanced expression of the tumor necrosis factor/cachectin gene in peripheral blood mononuclear cells from patients with systemic vasculitis. Clin Exp Immunol 1990; **81**:311–4.

10 Roux-Lombard P, Lin JM, Peter JB, Dayer JM. Elevated serum levels of TNF soluble receptors in patients with positive anti-neutrophil cytoplasmic antibodies. Br J Rheumatol 1994; **33**:428–31.

11 Gross WL, Csernok E. Immunodiagnostic and pathophysiologic aspects of antineutrophil cytoplasmic antibodies in vasculitis. Curr Opin Rheumatol 1995; **7**:11–19.

12 Kallenberg CGM, Cohen Tervaert JW, Van der Woude FJ, Goldschmeding R, van dem Borne AEGK, Weening JJ. Autoimmunity to lysosomal enzymes: new clues to vasculitis and glomerulonephritis. Immunol Today 1991; **12**:61–64.

13 Gross WL, Csernok E, Flesch BK. 'Classic' anti-neutrophil cytoplasmic autoantibodies (cANCA), 'Wegener's autoantigen' and their immunopathogenic role in Wegener's granulomatosis. Autoimmunity 1993; **6**:171–84.

14 Lüdemann J, Utecht B, Gross WL. Anti-neutrophil cytoplasm antibodies in Wegener's granulomatosis recognize an elastinolytic enzyme. J Exp Med 1990; **171**:357–62.

15 Wahl SM. The role of transforming growth factor-beta in inflammatory processes. Immunol Res 1991; **10**:249–54.

16 Roberts AB, Sporn MB, Assoian RK *et al*. Transforming growth factor type-beta: rapid induction of fibrosis and angiogenesis *in vivo* and stimulation of collagen formation *in vitro*. Proc Natl Acad Sci USA 1986; 83:4167–71.

17 Szymkowiak C, Mons I, Gross WL, Kekow J. Determination of transforming growth factor-beta 2 in human blood samples by ELISA. J Immunol Methods 1995; **184**:263–71.

18 Lyons RM, Gentry LE, Purchio AF, Moses HL. Mechanism of activation of latent recombinant transforming growth factor beta 1 by plasmin. J Cell Biol 1990; **110**:1361–7.

19 Jennette JC, Falk RJ, Andrassy K *et al*. Nomenclature of systemic vasculitides. Arthritis Rheum 1994; 37:187–92.

20 Lotz M, Tsoukas CD, Robinson CA, Dinarello CA, Carson DA, Vaughan JH. Basis for defective responses of rheumatoid arthritis synovial fluid lymphocytes to anti-CD3 (T3) antibodies. J Clin Invest 1986; **78**:713–21.

21 Kekow J, Wachsman W, MacCutchan JA, Cronin M, Carson DA, Lotz M. Transforming growth factor β and noncytopathic mechanisms of immunodeficiency in human immunodeficiency virus infection. Proc Natl Acad Sci USA 1990; **87**:8321–5.

22 Absher M, Baldor, L, Kelley J. A rapid colorimetric bioassay for transforming growth factor beta (TGF-beta) using a microwell plate reader. J Immunol Methods 1991; **138**:301–3.

23 Kekow J, Wachsman W, Mc Cutchan JA *et al*. TGF-β and suppression of humoral immune responses in HIV infection. J Clin Invest 1991; 87:1010–6.

24 Csernok E, Lüdemann J, Gross WL, Bainton DF. Ultrastructural localization of proteinase 3, the target antigen of anti-cytoplasmic antibodies, circulating in Wegener's granulomatosis. Am J Pathol 1990; **137**:1113–20.

25 Csernok E, Ernst M, Schmitt W, Bainton DF, Gross WL. Activated neutrophils express proteinase 3 on their plasma membrane *in vitro* and *in vivo*. Clin Exp Immunol 1994; 95:244–50.

26 Leid RW, Ballieux BE, van der Heijden I *et al*. Cleavage and activation of human C1 inhibitor by the human leukocyte proteinase, proteinase 3. Eur J Immunol 1993; 23:2939–44.

27 Lyons RM, Keski-Oja J, Moses HL. Proteolytic activation of latent transforming growth factor-beta from fibroblast-conditioned medium. J Cell Biol 1988; **106**:1659–65.

28 Reinhold-Keller E, Kekow J, Schnabel A *et al*. Influence of disease manifestation and antineutrophil cytoplasmic antibody titer on the response to pulse cyclophosphamide therapy in patients with Wegener's granulomatosis. Arthritis Rheum 1994; 37:919–24.

29 Torre Amione G, Beauchamp RD, Koeppen H *et al*. A highly immunogenic tumor transfected with a murine transforming growth factor type beta 1 cDNA escapes immune surveillance. Proc Natl Acad Sci USA 1990; 87:1486–90.

30 Tatsis E, Reinhold-Keller E, Csernok E, Gross WL. Wegener's granulomatosis and malignancy: is there an association? Arthritis Rheum 1994; **37**:S408.

31 Maeda J, Ueki N, Ohkawa T *et al*. Local production and localization of transforming growth factor-beta in tuberculous pleurisy. Clin Exp Immunol 1993; 92:32–38.

32 Gross WL, Hauschild S, Mistry N. The clinical relevance of ANCA in vasculitis. Clin Exp Immunol 1994; **93**:7–11.

33 Samuel SK, Hurta RAR, Kondaiah P *et al*. Autocrine induction of tumor protease production and invasion by a metallothionein-regulated TGF-β1 (Ser223, 225). EMBO 1992; 11:1599–605.

34 Gross WL. New developments in the treatment of systemic vasculitis. Curr Opin Rheumatol 1994; **6**:11–19.

35 Zhou D, Munster A, Winchurch RA. Pathologic concentrations of interleukin 6 inhibit T cell responses via induction of activation of TGF-β. FASEB 1991; **5**:2582–5.

36 Cassatella MA. The production of cytokines by polymorphonuclear neutrophils. Immunol Today 1995; **16**:21–26.

37 Schultz-Cherry S, Murphy-Ullrich JE. Thrombospondin causes activation of latent transforming growth factor-β secreted by endothelial cells by a novel mechanism. J Cell Biol 1993; **122**:923–32.

38 Wahl SM. Transforming growth factor beta (TGF-beta) in inflammation: a cause and a cure. J Clin Immunol 1992; **12**:61–74.

39 Roberts AB, Sporn MB. Transforming growth factor beta. Adv Cancer Res 1988; **51**:107–45.

40 Border WA, Noble NA. Transforming growth factor β in tissue fibrosis. N Engl J Med 1994; **331**:1286–92.

41 McCartney-Francis NL, Wahl SM. Transforming growth factor beta: a matter of life and death. J Leukoc Biol 1994; **55**:401–9.

42 Wahl SM, Allen JB, Costa GL, Wong HL, Dasch JR. Reversal of acute and chronic synovial inflammation by anti-transforming growth factor β. J Exp Med 1993; **177**:225–30.

43 Border WA, Noble NA, Yamamoto T *et al*. Natural inhibitor of transforming growth factor-β protects against scarring in experimental kidney disease. Nature 1992; 360:361–4.

44 Border WA, Okuda S, Languino LR, Sporn MB, Ruoslahti E. Suppression of experimental glomerulonephritis by antiserum against transforming growth factor β1. Nature 1990; **346**:371–4.