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ORIGINAL ARTICLE

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Oral therapy with proteolytic enzymes decreases excessive TGF-β levels in human blood

Abstract Therapy with oral proteolytic enzymes (OET) with combination drug products containing papain, bromelain, trypsin, and chymotrypsin has been shown to be beneficial in clinical settings such as radiotherapy-induced fibrosis, bleomycin pneumotoxicity and immunosuppression in cancer, all of which are nowadays known to be accompanied by excessive transforming growth factor-β (TGF-β) production. It has been demonstrated that proteolytic enzymes reduce TGF-β levels in serum by converting the protease inhibitor α2 macroglobulin (α2M) from the "slow" form into the "fast" form, whereby the "fast" form binds and inactivates TGF-β irreversibly. In this study we have investigated the effect of OET on the concentration of TGF-β1 in serum of patients with rheumatoid arthritis (RA) ($n = 38$), osteomyelofibrosis (OMF) ($n = 7$) and herpes zoster (HZ) ($n = 7$). Seventy-eight healthy volunteers served as controls. TGF-β1 levels in serum were assessed by enzyme-linked immunosorbent assay (ELISA). We have demonstrated that in healthy volunteers and in patients there exists a correlation between active and latent TGF-β1 in serum ($r = 0.8021$; $P < 0.0001$). Treatment with OET had no significant effect on TGF-β1 concentration in healthy volunteers or patients with a normal level of TGF-β1. In patients with elevated TGF-β1 concentration (> 50 ng/ml serum), OET reduced TGF-β1 in RA ($P < 0.005$), in OMF ($P < 0.05$) and in HZ ($P < 0.05$). *Conclusion*: These results support the concept that OET is beneficial in diseases characterized in part by TGF-β1 overproduction.

Key words Transforming growth factor-beta · Alpha2 macroglobulin · Proteolytic enzymes · Rheumatoid arthritis · Herpes zoster · Osteomyelofibrosis

Abbreviations α2M alpha2 macroglobulin · *ELISA* enzyme-linked immunosorbent assay · *HZ* herpes zoster · *OET* oral enzyme therapy · *OMF* osteomyelofibrosis · *RA* rheumatoid arthritis · *TAMs* tumor associated macrophages · *TGF-β* transforming growth factor-beta · *PE* Phlogenzym · *WM* Wobe-Mugos E · *NK* natural killer cells

Introduction

The multifunctional cytokine TGF-β which belongs to a family of polypeptide growth factors has pleiotropic effects on embryogenesis, cell growth, differentiation, tissue repair and remodeling [39]. Three distinct isoforms of the peptide (TGF β1–3) exist in mammalian species. TGF-β1 is the prototype of this family [3], and has the ability to stimulate the activity of its own promoter via AP-1 sites, an activity which leads to a positive autocrine loop after an initial stimulus [25]. Local overproduction of TGF-β results in an increase in the expression of adhesion molecules, in the production of extra cellular matrix proteins and in the proliferation of fibroblasts [40, 47, 51]. Thus profibrotic and immunosuppressive effects based on overproduction of TGF-β are involved in several clinical conditions, which are characterized by prolonged tissue repair or chronic inflammatory reactions e.g., fibrosis following irradiation or bleomycin therapy, and also severe burns [1]. Furthermore, an inhibition of the cytotoxicity of

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macrophages, granulocytes and natural killer- and LAK cells leads to immunosuppression, which is observed during certain bacterial and viral (HIV, HZ, hepatitis C) infections, and also in cancer patients [39, 52]. Tumor cells as well as tumor infiltrating effector cells (macrophages, T-cells) have been shown to produce high amounts of TGF-β [8, 58].

Several efforts have been made to develop therapeutic strategies in order to reduce overproduction of TGF-β and other growth factors. TGF-β antagonists such as decorin [20, 23, 55] or soluble TGF-β receptors [17] successfully inhibit the development of fibrosis and sclerosis in many different animal models; reviewed by [5, 33, 35].

The principle of OET was developed by Wolf and Ransberger [53]. Pharmaceutical preparations which contain trypsin, chymotrypsin, bromelain and papain show promising effects in the treatment of diseases which are obviously associated with TGF-β overproduction.

Evidence exists that OET reduces the extent of fibrosis after bleomycin treatment in patients with head and neck cancers [41]. Furthermore OET reduces symptoms in RA [34, 45] and in several animal models of collagen II-induced arthritis [15]. OET reduces side effects of radiotherapy in humans [31, 46]. In rats OET reduces development of nephrosclerosis and tubulointestinal fibrosis of Goldblatt hypertension model [43]. Furthermore OET reduces TGF-β overproduction in glomeruli after streptozotocin-induced diabetes mellitus, and also after 5/6 nephrectomy [36, 42]. In mice, OET reduces the development of fibrosis after bleomycin (S. Tamhankar et al., manuscript in preparation). In humans, OET reduces blister development in HZ [26].

In vitro and in vivo polyenzyme preparations and their constituents stimulate cytotoxic activity in granulocytes, monocytes [56] and NK cells [30] in vitro. In TAMs and in melanoma cell lines polyenzyme preparations reduce TGF-β1 production in vitro both at mRNA and protein levels [10, 57]. The polyenzyme preparations are readily adsorbed via the gastrointestinal tract [4, 6, 27]. In clinical studies with OET only few side effects have been found [12].

In the present study we demonstrate in patients with elevated TGF-β1 serum levels such as RA, OMF and HZ, that this cytokine was reduced after OET. In healthy volunteers OET had only a transient effect on serum TGF-β1 concentration.

Patients and methods

Subjects

In our studies 29 healthy volunteers, 87 patients with RA, 7 patients with OMF and 21 patients with HZ were treated with OET. Patients with OMF and HZ received two tablets t.i.d. of an enzyme combination drug (WM, Mucos Pharma, Geretsried, Germany) containing 100 mg papain, 40 mg trypsin and 40 mg chymotrypsin. Chemotherapy in patients with OMF was hydroxyurea 1–2 g/day p.o. Patients with RA, and healthy volunteers, received two tablets t.i.d. of an enzyme combination drug (PE, Mucos Pharma, Geretsried, Germany) containing 90 mg bromelain, 40 mg trypsin and 100 mg rutoside. Serum samples of a cohort of 78 healthy volunteers (mean age 42 $\pm$ 15 years) served as controls for determining TGF-β1 levels.

Blood sample collection

Blood was taken at the time of diagnosis, and at various time points during the study period. Ten milliliters of blood were collected between 8 and 10 a.m. from patients during the pre-study visits (baseline) and after enzyme application. The serum was separated by centrifugation, and samples were immediately frozen at −70 °C until use.

TGF-β1 determination

ELISA

For TGF-β1 determination, latent TG-Fβ was activated with 1N HCl. Twenty microliters of serum were diluted in 900 µl PBS/BSA with 40 µl HCl for 1 h. Following this incubation, acidified samples were neutralized to pH 7.0–7.5 with 40 µl 1N NaOH. To distinguish between latent and active TGF-β1 present in the sera, both acid treated and untreated samples were analyzed. All TGF-β1 concentrations given are the mean values of duplicates, performed in at least two independent TGF-β1 immunoassays.

A murine monoclonal antibody specific for human TGF-β1 (R&D Systems, MAB 240) was coated onto the 96-well polystyrene microtiter plates (Maxisorp, Nunc-Immuno Plate, Nalge Nunc Int.). One hundred microliters of standards (recombinant human TGF-β1, R&D Systems, 240-B) in the range of 63–4,000 pg/ml or serum samples were added to test wells. Plates were incubated for 2 h at room temperature, and than washed with phosphate buffer. One hundred microliters of the biotinylated detection antibody (chicken) specific for TGF-β1 (R&D Systems, BAF240) were added to each well, and plates were incubated over night at 4 °C. After three washes each well was incubated with 100 µl of strepatavidin HRP (Zymed Lab., San Francisco) for 20 min at room temperature, and after three washes 100 µl of substrate solution (R&D Systems, DY999) were added to each well, incubated for 10–15 min at room temperature, and then stopped with 50 µl of 5N H_2SO_4. The optical density was determined within 30 min, using a microplate reader (Anthos htII) set to 450 nm with wavelength correction set at 540 nm. Color development was proportional to the amount of TGF-β1 bound to the capture antibody.

Statistical analysis

Data are expressed as mean $\pm$ SD. For statistical evaluation, a Kruskal-Wallis test with Dunn's multiple comparison and a Wilcoxon matched pairs test were used.

Results

Correlation between active and latent TGF-β1 in human serum: The conversion of "slow" form α2M into the "fast" form by OET and the subsequent clearing of TGF-β may alter the balance between latent and active TGF-β in enzyme-treated subjects. In such case it would be inappropriate to simply analyze the total TGF-β serum levels for comparison against untreated subjects. We therefore analyzed the correlation between active and latent TGF-β1 in human serum in patients before and after therapy with proteolytic enzymes, and in

healthy subjects by determining the concentration of total TGF-β1 (latent + active) after acidification procedure as well as the active form of TGF-β1. Figure 1 displays the correlation found between active and latent TGF-β1, which was found to be $r = 0.8021$; $P < 0.0001$.

TGF-β1 concentration in serum of healthy volunteers and of patients before enzyme therapy: We determined serum concentrations of TGF-β1 in 78 healthy volunteers, in patients with HZ ($n = 33$), with RA ($n = 87$) and with OMF ($n = 7$) before OET. Mean TGF-β1 concentrations in patients with RA ($P < 0.001$) and with OMF ($P < 0.01$) were found to be significantly elevated in comparison with controls (Fig. 2).

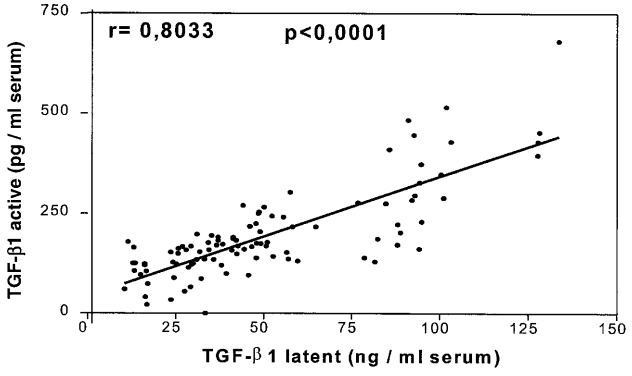

Fig. 1 Correlation between the concentration of latent TGF-β1 (total –active) and the active form of TGF-β1

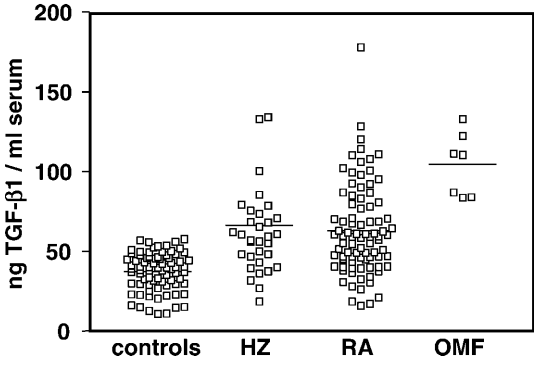

Fig. 2 Concentration of TGF-β1 in serum of healthy controls, in serum of patients with HZ, with RA and in serum of patients with OMF. TGF-β1 by ELISA in duplicates, results as ng TGF-β1/ml serum

TGF-β1 concentration in serum of healthy volunteers after enzyme treatment: In healthy volunteers (TGF-β1 < 50 ng/ml serum), TGFβ1 concentration was determined 4 h, 6 h and 24 h after PE administration (1×10 tablets). After 4 h and after 6 h, the concentration of TGF-β1 was significantly lower than at start; 24 h later no change to baseline was observed (Table 1).

TGF-β1 concentration in serum after enzyme therapy for a period of 28 days in volunteers with normal TGF-β1 level: Nine volunteers received PE (3×4 tablets per day) over a period of 4 weeks. Before and weekly during this treatment, blood was collected and prepared as described in Patients and methods. TGF-β1 level was unchanged during time of investigation (Table 2).

RA: The TGF-β1 concentration in 87 patients with RA before therapy was determined. TGF-β1 was significantly ($P < 0.001$) higher than in controls (Fig. 1). Thirty-eight patients of this group were treated by PE (3×2 tablets daily for 3 months). We examined TGFβ1 concentration in serum before and after 3 months (Fig. 2). Patients ($n = 23$) with high TGF-β1 concentration at start (> 50 ng/ml serum) were distinguished from patients ($n = 15$) with low TGF-β1 concentration (< 50 ng/ml serum). In patients > 50 ng TGF-β1/ml serum, after treatment with PE TG-β1 concentrations decreased from 80.11 ± 24.49 ng/ml serum to 67.84 ± 24.81 ng/ml serum ($P < 0.005$). No changes of TGF-β1 concentration were observed in the group with < 50 ng TGF-β1/ml serum (baseline: 39.03 ± 6.6; end: 41.48 ± 8.5, Fig. 3).

OMF: The serum concentrations of TGF-β1 in all patients ($n = 7$) with OMF was significantly elevated ($P < 0.001$). Additionally to chemotherapy they received WM for 1 year ($n = 6$) and/or 2 years ($n = 3$). The difference in TGF-β1 concentrations before and during treatment was significant ($P < 0.05$), (Fig. 4).

Table 1 TGF-β1 concentration (ng/ml) in serum of healthy volunteers after enzyme administration (n = 6)

	Start	4 h	6 h	24 h
TGF-β1 (ng/ml serum) (mean ± SD)	44.5 ± 5.2	35.92 ± 9.7	38.26 ± 4.8	43.87 ± 3.2
P value (Wilcoxon matched pairs test)		$P < 0.01$	$P < 0.01$	n.s.

Table 2 Serum concentration of TGF-β1 in healthy volunteers during PE treatment over 28 days

n = 9	Day 0	Day 15	Day 21	Day 28
TGF-β1 (ng/ml serum) (mean ± SD)	27.29 ± 11.24	32.4 ± 15.2	28.75 ± 12.3	26.8 ± 12.8
P value (Wilcoxon matched pairs test)		n.s.	n.s.	n.s.

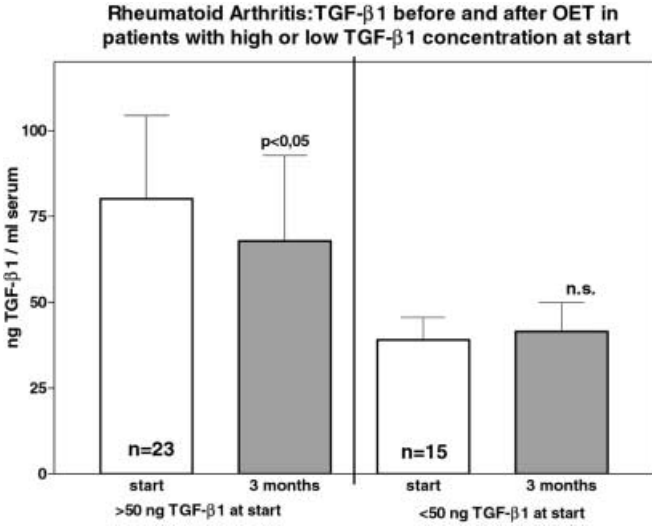

Fig. 3 Serum TGF-β1 concentration in patients with RA with elevated level (> 50 ng TGF-β1/ml serum; $n = 23$) and with normal level (< 50 ng/ml serum; $n = 15$) at baseline. Patients received PE daily for 3 months, 2 × 3 tablets per day. Results are expressed as mean ± SD. Significance was determined by Wilcoxon matched-pairs test

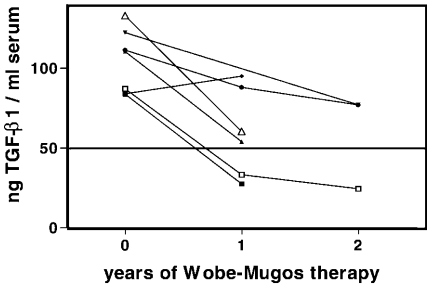

Fig. 4 Serum TGF-β1 concentration in patients with OMF. Patients received WM (2 × 2 tablets) daily for 1 or 2 years. Results are expressed as mean ± SD. Kruskal-Wallis test, Dunn's multiple comparison test

Table 3 TGF-β1 concentration in serum of patients with HZ before and after 2 weeks treatment with WM. Sera with high TGF-β1 (> 50 ng/ml serum) and normal (< 50 ng/ml) levels of TGF-β1 at start were separated into two groups

TGF-β1 (ng/ml serum)	Numbers	Start	After 2 weeks WM
< 50 ng	14	37.24 ± 7.9	39.46 ± 10 n.s.[a]
> 50 ng	7	55.50 ± 6.2	47.54 ± 5.7 $P < 0.05$[a]

[a] Wilcoxon matched pairs test

HZ: A group of 21 patients with HZ were treated with WM. Before and 2 weeks after treatment, levels of TG-Fβ1 were determined. Sera with high and with normal levels of TGF-β1 at start were identified for presentation in Table 3.

Discussion

Oral therapy with proteolytic enzymes has been used since 1954, but the mode of action is not yet entirely clarified. This therapy has been proven to be beneficial in clinical situations in which TGF-β1 overproduction has been described. These are fibrosis induced by irradiation or by bleomycin treatment, glomerulosclerosis and immunosuppression in cancer or chronic inflammations [24, 29, 54].

Studies [22] have demonstrated that protease inhibitors, and especially α2M, interfere with the TGF-β1 dependent immunosuppression. Harthun et al. [21] described a reduction of TGF-β1 activity in supernatant of TGF-β1 producing cell lines through the induction of the "fast" form of α2M. TGF-β1 binds to the activated form of α2M. A subsequent phagocytosis by cells expressing the α2M receptors (LRP) eliminates the complex of activated α2M + TGF-β1 [28]. Activated α2M can function as a selective neutralizer for cytokines, and thereby promotes the activation of NK, LAK and tumor-specific CTL responses [21]. In this present study, we determined the TGF-β1 concentration in the serum of healthy volunteers and of patients with a variety of diseases with presumable TGF-β1 overproduction.

In RA local TGFβ1 overproduction by synovial cells as well as elevated TGF-β1 serum concentration in later stages has been observed [14, 16]. TGF-β1 induces the expression of the proinflammatory cytokine IL-6 [13, 49], and seems also to be a key factor in progression and in immunosuppression in RA patients [18]. Reduction of elevated TGF-β1 concentration after enzyme therapy was associated with the beneficial clinical effect of this therapy [34].

In OMF, which is a result of chronic myeloproliferative disorders, high TGF-β1 concentrations are correlated with disease progression [32, 38]. The observed significant reduction of TGF-β1 in these patients could explain the observed prolongation of the stable phase after enzyme therapy (D. Holomanova, manuscript in preparation).

Our results show for the first time that OET decreases significantly TGF-β1 levels in serum of patients with elevated TGF-β1 concentrations (> 50 ng/ml serum) at start of treatment.

Our findings are in agreement with investigations by Sebekova [42], who described that OET reduces TGF-β1at mRNA and protein levels in rat glomeruli, as demonstrated in streptozotocin-induced diabetes mellitus and after 5/6 nephrectomy.

In in vitro experiments, we have demonstrated that the enzyme preparations, and also their constituents, reduce TGF-β1 production at mRNA and protein levels in TAMs and in melanoma cell lines [18, 57] .

There have been several attempts to suppress TGF-β1 overproduction, such as interferon-alpha [7, 11] or TGF-β antibodies. These applied drugs can suppress experimentally induced glomerulonephritis [50] and reduce

scar formation during wound healing in rats [44]. Soluble TGF-β-receptors (TGF-β RII) administration by intra-tracheal injection reduces bleomycin-induced lung fibrosis in mice [51].

The blockade of TGF-β signaling by adenovirus-mediated dominant-negative type II TGF-β receptor in the rat liver prevents liver dysfunction in dimethylnitrosamine-induced liver fibrosis [37].

Fetuin, as a TGF-β Type II receptor mimic, antagonizes TGF-β activity [9].

Decorin, a natural inhibitor for TGF-β, provides its activity by interfering with TGF-β1 binding to its cell receptor [20, 23].

The role of α2M in reducing TGF-β has been examined by Tiggelman et al. [48]. These investigators reduced TGF-β-stimulated collagen synthesis in liver myofibroblasts by introducing the "fast" form of α2M. Activated α2M neutralizes TGF-β, produced by breast-cancer cells, and thereby promotes the activation of NK, LAK and tumor-specific T-cell response [21] by interleukin-2. Reduction of TGF-β overproduction reduces TGF-β synthesis [47]. It has been described by Hall et al. [19] that proteases react with α2M in blood, and convert the "slow" form into the "fast" form. After this reaction, α2M can recognize the receptor LRP on hepatocytes, endothelial cells, fibroblasts etc. which is responsible for the rapid plasma clearance of transformed α2M, whereas the "slow" form of α2M shows no affinity for LRP [2]. α2M in the "fast" form (α2M + enzymes) binds TGF-β. TGF-β bound to the "fast" form of α2M cannot bind to its cell receptor and this complex is phagocytized very quickly.

We hypothesize that this mechanism contributes to the effects of OET.

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