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Detection of transforming growth factor-beta in rheumatoid arthritis synovial tissue: lack of effect on spontaneous cytokine production in joint cell cultures

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SUMMARY

The presence of transforming growth factor-beta (TGF-β) in inflammatory joint disease was investigated. Synovial fluid from patients with rheumatoid arthritis (RA) and patients with other non-autoimmune inflammatory joint diseases contained high levels of both active and latent TGF-β. Levels of active TGF-β did not correlate with drug regimen in either patient group or with the recovery period in the individuals with non-RA joint disease. Freshly isolated synovial cells from individuals with RA were shown by Northern blotting to express the mRNA for TGF-β1 and to secrete latent TGF-β protein which could be neutralized by antibodies to TGF-β1 and TGF-β2. Lipopolysaccharide-stimulated peripheral blood mononuclear cells from normal donors produced interleukin-1 (IL-1) and tumour necrosis factor-alpha (TNF-α) which was inhibited by pretreatment of these cells with recombinant TGF-β. Cytokine production was not inhibited if the addition of TGF-β was used after the inducing stimulus, suggesting that in activated cells cytokine production cannot be inhibited. This was confirmed by the observation that neither TGF-β1 or TGF-β2 inhibited spontaneous IL-1 or TNF-α production by rheumatoid synovial mononuclear cells in culture. These findings show that despite the presence of active TGF-β in RA synovial joints and the spontaneous production of latent (potentially active) TGF-β by RA cells in culture, additional TGF-β did not inhibit ongoing cytokine synthesis *in vitro*. This suggests that TGF-β may not inhibit cytokine production in the rheumatoid joint although it cannot be ruled out that *in vivo* TGF-β already has an immunosuppressive effect which cannot be further increased *in vitro* by exogenous protein.

Keywords rheumatoid arthritis transforming growth factor-beta tumour necrosis factor interleukin-1

INTRODUCTION

It is now generally accepted that many autoimmune diseases such as rheumatoid arthritis (RA) involve disordered immune regulation, in which abnormalities of lymphocytes, antigen-presenting cells and cytokine production contribute to the development of the disease (reviewed by Dayer & Demczuk, 1984; Feldmann, 1987). Cytokines contribute to the pathogenesis of disease by augmenting the inflammatory responses and by stimulating autoreactive T and B cells. However, the relative importance of different cytokines in contributing to the disease process is difficult to define, as these proteins act within a complex network. Thus the production of interleukin-1 (IL-1) can be induced by IL-1 itself (Ghezzi & Dinarello, 1988), more effectively by tumour necrosis factor-alpha (TNF-α) (Nawroth *et al.*, 1986), and also by granulocyte/macrophage colony-stimulating factor (GM-CSF) (Sisson & Dinarello, 1988). In addition, the interaction between cytokines may be either synergistic or antagonistic; for example, interferon-gamma (IFN-γ) synergizes with TNF-α (or lymphotoxin) in inducing MHC class II expression on pancreatic islet cells (Pujol-Borrell *et al.*, 1987) while inhibiting IL-4-induced class II expression on B cells (Mond *et al.*, 1985).

In order to determine which cytokines are important in contributing to the RA disease process we have recently examined cytokine gene expression in cells freshly isolated from RA synovial joint. In the absence of any exogenous stimulus these cells produce a number of cytokines in culture, including IL-1 (IL-1α, IL-1β), TNF-α and interleukin-6 (IL-6) (Buchan *et al.*, 1988a; Hirano *et al.*, 1988) and T cell products such as interleukin-2 (IL-2), IFN-γ and lymphotoxin (Buchan *et al.*, 1988b). In contrast to antigen- or mitogen-activated peripheral blood mononuclear cells, the production of these mediators persists for the 6-day span of the cultures, without extrinsic

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stimulation. By using neutralizing antibodies to different cytokines, we have demonstrated that TNF-α is the major stimulus driving the production of IL-1 in RA cultures,but not in osteoarthritic joint cell cultures (Brennan *et al.*, 1989a). These and other cytokines are considered to contribute to the persistence of the immune and inflammatory processes occurring in the joint by activating autoreactive T and B cells and by contributing directly to the cartilage and bone destruction (reviewed by Dayer & Demczuk, 1984). Understanding the mechanism(s) regulating the production of TNF-α may help explain the immunopathology of RA.

Of the cytokines capable of negatively regulating many aspects of immune function, transforming growth factor-beta (TGF-β) has been the most extensively studied. TGF-β1 is the best characterized member of a family of structurally and functionally related polypeptides which have diverse effects on cell growth and differentiation (reviewed by Massague, 1987). TGF-β1 is produced by cells of the immune system, including macrophages (Assoian *et al.*, 1987), B lymphocytes (Kehrl *et al.*, 1986a), and T lymphocytes (Kehrl *et al.*, 1986b). Furthermore, exogenous TGF-β1 inhibits a number of immune functions including T cell (Kehrl *et al.*, 1986b) and B cell proliferation, immunoglobulin production (Shalaby & Ammann, 1988), natural killer (NK) and lymphokine-activated killer (LAK) cell generation (Rook *et al.*, 1986; Jin *et al.*, 1989), and cytokine production (TNF, IFN-γ and IL-1) by peripheral blood mononuclear cells (Espevik *et al.*, 1987; Chantry *et al.*, 1989). Several experimental systems have shown that TNF-α and TGF-β1 are mutually antagonistic (Ranges *et al.*, 1987).

In addition to its immunoregulatory properties, TGF-β1 exhibits both stimulatory and inhibitory functions in bone resorption and wound repair. For example, in some culture systems (neonatal calvariae) TGF-β1 stimulates bone resorption whereas in others (cultures of neonatal long bone), bone resorption was inhibited (Pfeilschifter, Seyedin & Mundy, 1988). Similarly, *in vitro* TGF-β1 inhibits adhesion of blood cells to endothelial cells (Gamble & Vadas, 1988), a process essential to all inflammatory responses, while *in vivo* it stimulates neutrophils, macrophages and fibroblasts to accumulate at the sites of wound healing and promotes collagen synthesis and deposition at these sites (Roberts *et al.*, 1986; Pierce *et al.*, 1989). Thus the relevance of TGF-β in the RA synovial joint tissue, where many of these processes are occurring, is likely to be complex.

The aim of this investigation was to determine whether TGF-β was present in the synovial fluid of patients with RA and other inflammatory joint diseases, and whether it was produced by freshly isolated synovial cells. Furthermore, we investigated the effect of exogenous TGF-β on the spontaneous cytokine expression in the rheumatoid cultures described above in order to gain a clearer understanding of the potential of inhibitory cytokines to regulate the inappropriate immune and inflammatory responses occurring in these cultures.

MATERIALS AND METHODS

Patients

Patients with classical RA (defined by the revised ARA criteria; Arnett *et al.*, 1988) and patients with other non-RA-related joint disease from the Rheumatology Unit at the Charing Cross Hospital were included in this study. Synovial fluid exudate samples were aspirated from six patients with RA and six patients with non-RA-related joint disease as part of their routine treatment. These were collected in heparinized bottles, centrifuged at 1000 rev/min for 10 min to pellet the cells, fluid was aspirated and stored at $-70°C$ until assayed for TGF-β activity. In synovial fluid samples where sufficient mononuclear cells were present for *in vitro* culture, these were isolated from the pellet by Ficoll/Hypaque centrifugation (specific density 1·077 g/ml). Mononuclear cells were also extracted from synovial membrane samples ($n=5$) (knee and hip joints) obtained from patients undergoing total joint replacement. Synovial membrane tissue was digested in RPMI 1640 (GIBCO) containing 5% fetal calf serum (FCS) (GIBCO), 5 mg/ml collagenase type IV (Sigma) and 0·15 mg/ml DNAse type I (Sigma) and incubated at 37°C for 2 h. After incubation the tissue was pipetted through a 200-μl nylon mesh into a sterile beaker. The cells were then washed three times in complete medium (RPMI 1640 supplemented with 10% FCS). Both synovial membrane (SM) and synovial fluid (SF) cells are a heterogeneous mixture representing the entire spectrum of infiltrating mononuclear cells and connective tissue cells found in the synovial joint with T cells and macrophages the most abundant (Brennan *et al.*, 1989a) and are referred to as synovial cells in this report. Peripheral blood mononuclear cells (PBMC) were separated from blood of healthy donors ($n=4$) and RA patients ($n=6$) by Ficoll/Hypaque centrifugation (specific density 1·077 g/ml) as described above.

SM or SF cells were cultured at 1×10^6 cells/ml in 2 ml of complete medium in 24-well plates (Falcon 3047) for periods up to 6 days without exogenous stimulus with/without 10 ng/ml of TGF-β1 and TGF-β2. In the initial experiments single well cultures were set up (experimental plus control) of five rheumatoid SM or SF mononuclear cell preparations, supernatants were harvested after 1, 3 and 6 days in culture. In later experiments, triplicate well cultures were set up of each experimental condition and control obtained from four rheumatoid SM or SF cell preparations and harvested at a single time-point, 2 days. For Northern blotting analysis and TGF-β bioactivity determination, two SM preparations (obtaining from two separate joint replacements operations from the same individual) were set up for two days at 1×10^6 cells in 50 ml of serum-free RPMI 1640 supplemented with Neutridoma (Boehringer-Ingelheim). PBMC from healthy donors and RA patients with cultured at 1×10^6 cells/ml in RPMI 1640 containing 10% FCS. TGF-β1 (10 ng/ml) was added at the times indicated (in Results) and the cultures stimulated with lipopolysaccharide (LPS) (10 μg/ml) for 24 h. Supernatants were stored at $-20°C$ and assayed for TNF-α and IL-1β by ELISA.

Cytokines

Recombinant TNF-α (specific activity, $1·2 \times 10^7$ U/mg, endotoxin content $<0·25$ EU/mg) and TGF-β1 (endotoxin content $<2·0$ EU/mg), were obtained from Genentech, South San Francisco, CA. TGF-β2 (endotoxin content $<2·0$ EU/mg) was provided by Sandoz (Basel). ^{125}I-TGF-β1 was prepared by the iodogen (Pierce). Briefly, 10 μg TGF-β1 were reacted with 1 mCi of Na ^{125}I iodine (Amersham) in the presence of 5 μg iodogen for 7 min. Iodinated protein was separated from free iodine by gel exclusion chromatography with a maximal protein recovery of 35%. The specific activity of ^{125}I-TGF-β1 was 129 μCi/μg with

no evidence of loss of biological activity as determined in the mink lung epithelial cell line (MV1Lu) bioassay.

Immunoassays

TNF-α protein was determined by assaying the supernatants in duplicate by ELISA as previously described (Prince, Harder & Saks, 1986) and IL-1 was detected using the IL-1β immunoassay kit (Cistron). Reagents used in the TNF-α assay were kindly provided by B. Reed (Genentech). Results are expressed as pg/ml of TNF-α or IL-1 derived from a standard curve using recombinant TNF-α protein (Genentech) and IL-1 (Cistron). The lower limit of sensitivity of the ELISA was 50 pg/ml both for TNF-α and IL-1.

RNA extraction and Northern blotting

Total RNA was prepared from SM cells (cultured for 2 days in serum-free media as described above) by guanidinium isothiocyanate lysis and centrifugation through 5·7 M caesium chloride as described previously (Buchan *et al.*, 1988a). RNA was quantified by absorbance at 260 nm and 10 μg RNA were electrophorosed through 1% agarose gels containing 6% formaldehyde, then photographed under u.v. illumination to ensure equal loading of RNA and integrity as judged by ribosomal RNA staining, and transferred onto nitrocellulose by capillary blotting. Filters were baked for 2 h at 80°C and prehybridised in a solution containing 50% formamide, 5 × SSC (1 × SSC = 150 mM NaCl, 15 mM tri-sodium citrate; pH 7·0), 7·5 × Denhardt's solution (1 × Denhardts = 0·05% Ficoll 400, 0·05% polyvinylpyrollidone, 0·05% bovine serum albumin, BSA), 50 mM phosphate buffer (pH 6·6), 0·5 mg/ml denatured salmon sperm DNA at 42°C for 4–16 h. The TGF-β cDNA insert (Derynck *et al.*, 1985) was resolved on a low-melting-point agarose gel, and labelled by random oligonucleotide priming (Feinberg & Vogelstein, 1986) using [α^{32}P]-dCTP (Amersham). Hybridization was for 16–24 h at 42°C and filters were washed twice in 2 × SSC, 0·1% SDS at room temperature, and twice in 0·2 × SSC, 0·1% SDS at 50°C and exposed to Fuji X ray film at −70°C with intensifying screens.

Preparation of synovial fluids and conditioned media

Synovial fluids were either diluted in binding buffer (DMEM/0·1% BSA) and used directly in the radioreceptor assay or were transiently acidified by sequential dialysis against 1 M acetic acid, 10 mM acetic acid and finally phosphate buffer, pH 7·4 (each round of dialysis was for 16 h at 4°C). Following transient acidification fluids were stored at −20°C until use. Serum-free conditioned media were also collected from two SM preparations cultured for 48 h as described above. PMSF was added to give a final concentration of 1 mM and conditioned media were stored at 4°C. Conditioned media were either used directly or transiently acidified by sequential acid dialysis, lyophilized and were then resuspended in 1 ml of serum-free RPMI 1640 (Partridge *et al.*, 1989). In control experiments, transiently acidified serum-free RPMI 1640 alone did not inhibit the binding of ^{125}I-TGF-β in the radioreceptor assay or inhibit the growth of the TGF-β-sensitive cell line MV 1Lu (data not shown).

Determination of TGF-β levels

TGF-β levels were determined using the radioreceptor binding assay using the A549 cell line as previously described (Partridge *et al.*, 1989). Briefly, confluent monolayers of A549 cells in 24-well dishes were incubated with 150 pM of ^{125}I-TGF-β1 in the presence of various dilutions of the test supernatant or recombinant TGF-β1 for 2 h at 4°C. After incubation the cells were washed four times with ice cold HBSS (Gibco) containing 0·1% BSA. The cells were solubilized with 0·6 ml Triton X solution (20 mM HEPES, 2% Triton X 100, 10% glycerol, 0·01 BSA, pH 7·4) by a 20-min incubation at 37°C and counted on a gamma counter. Levels of TGF-β present in the test sample were determined by reference to a standard curve constructed using recombinant TGF-β1.

Levels of TGF-β biological activity were measured using the MV1Lu growth inhibition assay (Grubeck-Loebenstein *et al.*, 1989). MV1Lu cells were seeded at 1×10^4/well in 100 ml of DMEM 5% FCS in 96-well flat-bottomed microtitre plates and allowed to adhere overnight. Doubling dilutions of the conditioned media were added to give a final volume of 200 μl. Cultures were incubated 20 h at 37°C and ^{3}H-thymidine added for the last 4 h. Plates were freeze–thawed to ensure complete disruption of the cells and thymidine uptake determined by liquid scintillation counting. The specificity of the assay for TGF-β was confirmed by inclusion of a neutralising polyclonal rabbit against TGF-β1 (prepared by Dr E. Abney, as described in Chantry *et al.*, 1989) or TGF-β2 (provided by Dr B. Ryffel, Sandoz).

Table 1. Mean Levels of TGF-β in synovial fluids determined by radioreceptor assay

Disease group	Sample	Drug treatment	TGF-β (ng/ml) Untreated	Acid activated†
Rheumatoid arthritis (RA)	HI*	Prednisolone	2·6	45·0
	MC	NSAIDs	0·5	26·0
	PJ	Gold, NSAIDs	0·6	27·0
	TF*	Prednisolone	2·2	20·0
	GY	NSAIDs	3·0	46·0
	PP	Methotrexate	2·1	3·0
Non-RA joint disease	JF	None	10·0	13·0
	TB	None	21·0	35·0
	RT	NSAIDs	2·0	15·0
	RB	None	15·0	30·0
	SW	None	1·5	8·0
	PB	None treatment	9·0	28·0

* Synovial fluid mononuclear cells also cultured *in vitro* to determine effect of TGF-β on spontaneous cytokine production (see text).

† Samples were transiently acidified prior to assay by dialysis against 1 M acetic acid (see Materials and Methods).

Patients were either untreated or treated with non-steroidal anti-inflammatory drugs (NSAIDs), disease-modifying drugs such as methotrexate or gold, or steroids such as prednisolone.

RESULTS

Presence of TGF-β in synovial fluids

Synovial fluids from patients with RA ($n = 6$) and other inflammatory joint disease ($n = 6$) contained high levels of TGF-β as determined in the radioreceptor assay (Table 1). Active

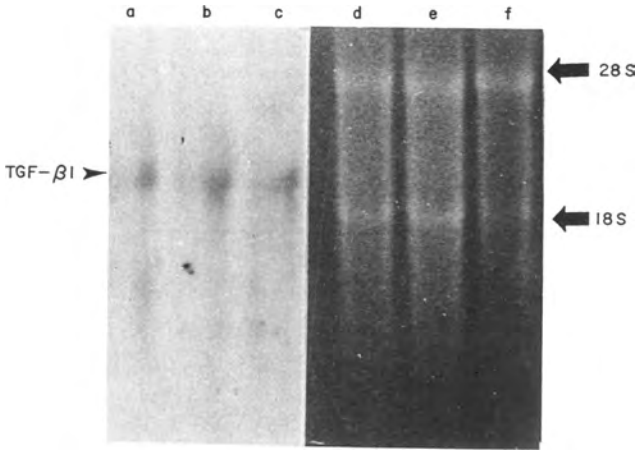

Fig. 1. Northern analysis of TGF-β1 by rheumatoid synovial membrane cells. Isolated mononuclear cells from synovial membrane preparations (ABa, ABb) were cultured 1×10^6/ml in the absence of exogenous stimulation for 2 days. RNA was harvested by guanidinium isothyocyanate lysis and separated on a denaturing agarose gel and photographed under u.v. illumination (lanes d, e, f). The nitrocellulose filter was probed using a cDNA for TGF-β1 as described in Materials and Methods and detects correctly sized mRNA in sample ABa (lane a) ABb (lane b) and control CESS cells (lane C).

TGF-β was present in all of the fluids analysed and levels were higher in the non-RA group ($9{\cdot}75 \pm 7{\cdot}5$ ng/ml *versus* the RA group $1{\cdot}8 \pm 1{\cdot}04$ ng/ml; $P < 0{\cdot}029$). Levels of latent TGF-β were slightly higher in the RA group ($27{\cdot}83 \pm 16$ ng/ml *versus* $24{\cdot}66 \pm 13{\cdot}5$ ng/ml; not significant). Levels of active TGF-β did not appear to be influenced by the drug treatment with the RA group (Table 1), nor did disease activity or recovery period relate to levels of TGF-β in either patient group (data not shown). However, due to the small sample size of the groups used in this study it is not clear whether these differences are of significance *in vivo* or whether they are influenced by drug treatment. Synovial fluid samples were also assayed for TFG-β using the MV1Lu bioassay. Although the fluids caused marked inhibition of thymidine uptake, this was not reversed by neutralizing antibodies to TGF-β1 and TGF-β2 (presumably due to other specific/non-specific inhibitory factors in the fluid environment), and hence this assay could not be used for determination of TGF-β levels in synovial fluid.

Production of TGF-β1 and TGF-β2 mRNA by synovial joint cells

Figure 1 illustrates the Northern blot obtained by hybridization of cDNA for TGF-β1. The results show hybridization of correctly sized mRNA for TGF-β1 in two SM cell preparations (samples ABa, ABb) cultured for 2 days. TGF-β2 mRNA could not be detected in these samples (data not shown), but this probably reflects the low mRNA copy number for this mRNA, as TGF-β2 protein was detected in the supernatants of the same cultures (see below). Although TGF-β1 is produced by a range of cell types in RA, it is likely to be produced chiefly by activated T lymphocytes and/or the macrophages which constitute the major cell type in these cultures (data not shown).

Detection of TGF-β1 and TFG-β2 bioactivity

In order to determine whether the TGF-β mRNA was translated into functional protein, serum-free conditioned media from 2-day SM cultures (samples ABa, ABb) were assayed for TGF-β. Consistent with previous studies on cultured cells, active TGF-β was not detected in either of the conditioned media tested using either radioreceptor assay or MV1Lu growth inhibition assay (Table 2). However, following transient acidification TGF-β could be readily detected in both samples and these levels were comparable when measured by radioreceptor assay and by bioassay.

Table 2. Production of TFG-β by synovial mononuclear cells in culture.

	Rate of TGFβ secretion (ng/ml per 10^6 per 24 h)	
Sample	Active	Latent
ABa*	<0·01	$2{\cdot}50 \pm 0{\cdot}09$ (3·1)
ABb	<0·01	$3{\cdot}33 \pm 0{\cdot}02$ (4·2)

Levels of TGFβ before (active) or after acid activation (latent) were determined using the MV1Lu growth inhibition assay. Figures in parentheses show the levels of TGF-β in the same sample assayed by the radioreceptor assay.

* Mononuclear cells were isolated from the synovial membrane of RA patient AB as the result of two separate joint replacements, placed in culture for 2 days in serum free media as described in the text.

In order to verify that the growth inhibition detected in the MV1Lu growth inhibition assay was indeed due to TGF-β, conditioned media were pre-incubated with rabbit polyclonal antibodies to TGF-β1 or TGF-β2. As shown in Fig. 2a, both TGF-β1 and TGF-β2 were equipotent inhibitors of MV1Lu growth. Pre-incubation of recombinant TGF-β1 or TGF-β2 with these antisera demonstrated that these antibodies recognized predominantly the antigen to which they were raised. However, both antisera also partially neutralized the other species of TGF-β (reflecting the high degree of conservation in the sequences of these molecules, Fig. 2b and 2c). The growth inhibitory effects of the synovial cell conditioned media could be completely neutralized by pre-incubation with antisera to TGF-β1 and TGF-β2 (Fig. 2d and 2e) but as unaffected by the control antisera. Comparison of the relative neutralizing effects of each antisera alone suggested that both TGF-β1 and TGF-β2 was presented in the conditioned media from synovial membrane sample ABa (Fig. 2d) but to be predominantly TGF-β1 in sample ABb (Fig. 2e).

Effect of TGF-β1 on TNF-α and IL-1 production in RA synovial cells

The results from initial experiments in which TNF-α production was determined in five RA SM or SF cell cultures in the presence or absence of 10 ng/ml TGFβ1 over a period of 6 days are shown in Fig. 3. The levels of TNFα in the day 1 supernatants ranged from 82 pg/ml (TF) to 2000 pg/ml (JN), and declined slowly in culture. Despite high levels of TNF-α protein in some cultures no difference was observed in the single well cultures in the presence of TGF-β1. To establish that the effect of TGF-β had no significant effect on cytokine production, triplicate cultures

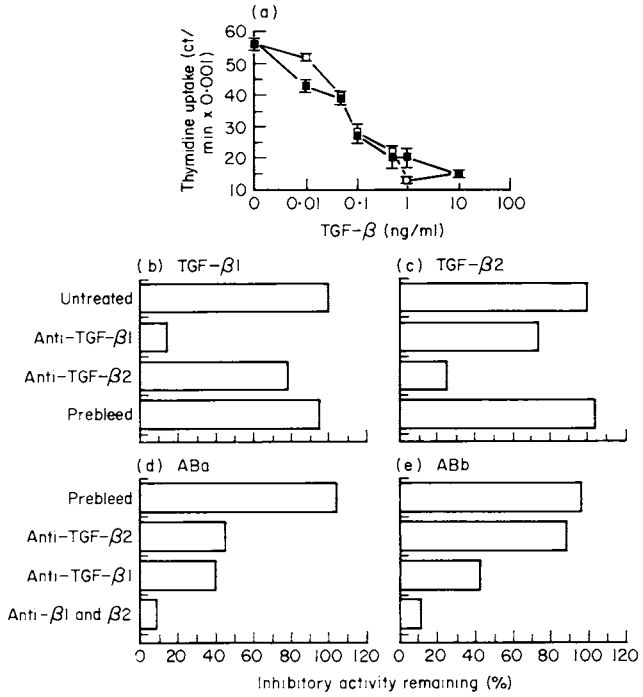

Fig. 2. Determination of TGF-β1 and TGF-β2 levels in synovial cell culture supernatants. Specificity of the MV 1LU bioassay for TGFβ. The MV 1LU cell line is inhibited equally by TGF-β1 (□) and TGF-β2 (■) (a); polyclonal antibodies raised against TGF-β1 and TGF-β2 specifically neutralize their specific antigens (b and c); conditioned media from synovial membrane cell preparations (ABa and ABb) were pre-incubated with antibodies to TGF-β1 or TGF-β2 prior to addition to the bioassay (d and e). Data are presented as the percentage maximall inhibitory activity.

from four further RA synovial cultures were set up and the effect of TGF-β1 and TGF-β2 determined after harvesting the cultures after a single time-point (day 2). The results (Table 3) show that (in this relatively small sample group) neither spontaneous TNF-α production nor IL-1 production (where detectable by ELISA) was inhibited in the presence of either TGF-β1 or TGF-β2. In view of our previous finding that TGF-β inhibited IL-1 and TNF production by PBMC induced by LPS it was suprising that TGF-β failed to inhibit cytokine production by synovial cells which were not exogenously stimulated.

Effect of TGF-β1 on TNF-α and IL-1 production in PBMC from healthy donors

The effect of TGF-β1 on cytokine production by normal ($n=4$) PBMC is shown in Table 4. Unstimulated PBMC produce negligible levels of IL-1 or TNF-α. Following stimulation with LPS these cells produce high levels of TNF-α (440–860 pg/ml) and IL-1 (210–900 pg/ml). Treatment with TGF-β prior to LPS stimulation resulted in inhibition of TNF-α (> 70%) and IL-1 (> 80%) production. However, if the addition of TGF-β was at the same time as LPS, or delayed until 1 or 2 h after, no inhibition was observed.

DISCUSSION

Our results demonstrate the presence of abundant TGF-β in the SF of patients with inflammatory joint disease and the production of TGF-β mRNA and protein by mononuclear cells

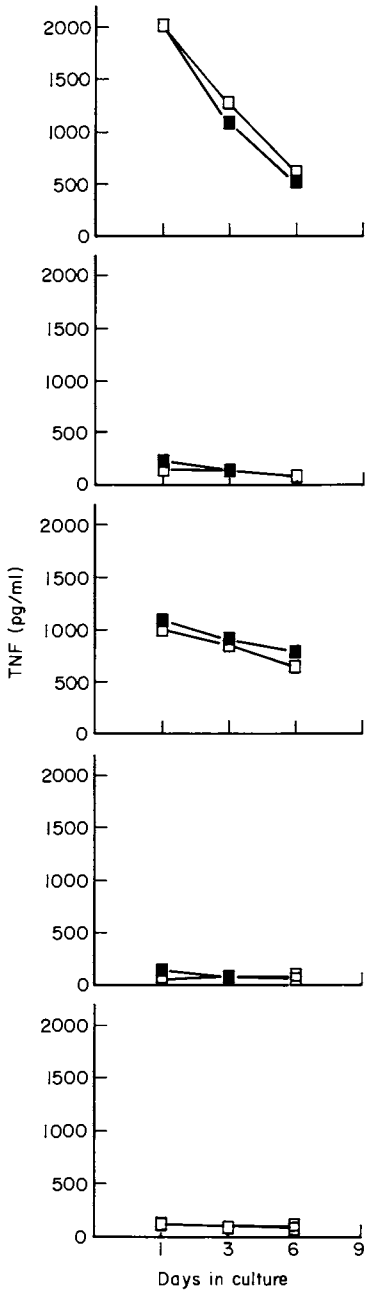

Fig. 3. TNF-α production by RA synovial cells in culture: effect of TGF-β. Synovial membrane (JN, AH, JR) or synovial fluid (TF, HI) mononuclear cells were incubated at 1×10^6 cells/ml in 2 ml RPMI 1640/10% FCS with (■) and without (□) 10 ng/ml TGF-β1. Supernatants were assayed after 1, 3, 6 days in culture for TNF-α by ELISA as described in Materials and Methods. Samples, from top: JN, TF, JR, AH, HI.

cultured from the synovial joint of patients with RA. Although TGF-β is secreted by cells in a latent form which does not bind to the receptor and is therefore biologically in active (Wakefield *et al.*, 1987), all the synovial fluids examined contained high levels of active TGF-β (range 0·5–21 ng/ml). The mechanism(s) whereby latent TGF-β is activated *in vivo* is currently unknown, although deglycosylation of the precursor and the action of proteases have been suggested (Lyons, Keski-Oja & Moses, 1988; Miyazono & Heldin, 1989). It is likely that the TGF-β detected in these fluids in the absence of acid activation

Table 3. TNF-α or IL-1 production by RA synovial cells is not inhibited by TGF-β1 or TGF-β2 after 2 days in culture

RA sample	Treatment	TNF-α (pg/ml)	IL-1 (pg/ml)
ABa (SM)	Control	500±86	78·3±3
	+TGF-β1	450±15	75·6±4
	+TGF-β2	453±70	84·4±15
ABb (SM)	Control	393±32	<50
	+TGF-β1	367±80	<50
	+TGF-β2	423±75	<50
RB (SF)	Control	330±61	60±13
	+TGF-β1	486±25	70±14
	+TGF-β2	430±30	50±20
AM (SF)	Control	160±20	<50
	+TGF-β1	140±30	<50
	+TGF-β2	155±20	<50

Synovial membrane (SM) or synovial fluid (SF) mononuclear cells were cultured *in vitro* with or without 10 ng/ml TGF-β1 or TGF-β2. Supernatants were harvested after 2 days in culture and assayed for TNF-α and IL-1 by ELISA.

Table 4. Effect of TGF-β on TNF-α and IL-1β production by normal PBMC

	Time off addition of TGF-β1 relative to LPS (h)					
	−16	0	1	2	+	−
TNF-α (pg/ml)	70	390	420	450	440	<50
IL-1β (pg/ml)	<50	100	620	575	630	<50
TNF-α (pg/ml)	<50	100	880	890	860	70
IL-1β (pg/ml)	80	200	490	510	500	<50
TNF-α (pg/ml)	120	600	630	650	640	60
IL-1β (pg/ml)	100	800	820	910	900	<60
TNF-α (pg/ml)	125	520	530	560	550	80
IL-1β (pg/ml)	<60	120	180	170	210	70

PBMC from four healthy donors were stimulated with LPS (10 μg/ml) at time 0 for 24 h and the supernatants assayed for TNF-α and IL-1β by ELISA. TGF-β1 (10 ng/ml) was added at the times indicated (−16, 16 h before the addition of TGF-β1). Positive control cultures (+) were stimulated with LPS alone and (−) were left unstimulated for the culture period.

represents material that has been activated by proteases present in the SF. These findings are in accord with those of Fava *et al.* (1989), who recently demonstrated the presence of TGF-β in the SF of patients with inflammatory joint disease.

Since TGF-β is produced by a wide variety of cell types (Sporn & Roberts, 1988) the TGF-β present in the SF could be produced by the synovial mononuclear cells, released from bone at sites of resorption since bone is a major source of TGF-β *in vivo*, or from platelets lysed at sites of microvascular injury. We therefore concentrated on production of TGF-β by isolated RA synovial cells, and found that cultured RA mononuclear cell samples produced latent TGF-β as determined by both the radioreceptor assay and the MV1Lu growth inhibition assay. There are currently three mammalian TGF-βs which have been cloned; TGF-β1, TGF-β2, and TGF-β3. Using neutralizing antisera which recognized predominantly TGF-β1 or TGF-β2 it was possible to show that both TGF-β1 and TGF-β2 were produced by RA synovial joint cells in culture. Northern blotting of mRNA from these cells demonstrated the presence of low but detectable TGF-β1 mRNA. TGF-β2 mRNA could not detected in these samples (data not shown), although the neutralizing antibody data suggested that TGF-β2 or a highly related (serologically cross-reactive) species of TGF-β (such as TGF-β3) was present in the supernatants from these cells. The most likely explanation for this discrepancy is that low levels of TGF-β2 mRNA were present but below the level of sensitivity of Northern blotting. Comparison of the 5′ untranslated regions of the TGF-β1 and TGF-β2 regions supports this view (Derynck *et al.*, 1985; de Martin *et al.*, 1987). The 'start' AUG codon in TGF-β1 is buried in a GC-rich region suggesting that the TGF-β1 mRNA may be inefficiently translated, this is not the case for TGF-β2 mRNA, and so a direct comparison of the relative abundance of mRNA *versus* protein for these two gene products is not possible.

Having detected quantities of potentially immunosuppressive molecules in RA joints (TGF-β1 and TGF-β2), we examined the effects of exogenously added TGF-β on the spontaneous production of cytokines (TNF-α and IL-1) by RA mononuclear cells. We and others have previously demonstrated the ability of TGF-β to inhibit IL-1 and TNF-α production by PBMC on isolated monocytes (Chantry *et al.*, 1989; Espevik *et al.*, 1987). As monocytes appear to be the major producers of IL-1 and TNF-α in the rheumatoid joint (Field *et al.*, manuscript in preparation) it was surprising that TGF-β1 inhibit TNF-α production by these cells in any of the RA samples examined. It cannot be ruled out that endogenous TGF-β is already exerting a immunosuppressive effect on cytokine production, which cannot be increased by adding exogenous TGF-β1 or TGF-β2; however, the lack of any detectable active TGF-β in the RA conditioned medium argues against this. Furthermore it is conceivable that the lack of inhibitory effect of TGF-β is due to a loss of TGF-β receptors by the synovial mononuclear cells but we believe this is to be unlikely as the receptor for TGF-β has been found on all normal and transformed cell types thus far examined with the exception of a retinoblastoma cell lines, (Kimichi *et al.*, 1989). However, we have shown reduced sensitivity to TGF-β in a disease such as non-toxic goitre (Grubeck-Lobenstein *et al.*, 1989) and so receptor expression or function may be under regulation. Although TGF-β1 and TGF-β2 bind to the same receptor, differences in binding efficiencies and biological effects between these mediators have been reported. TGF-β1 is a more potent inhibitor of superoxide production by monocytes than is TGF-β2 (Tsunawaki *et al.*, 1988), whereas TGF-β2 but not TGF-β1 induces mesoderm induction in *Xenopus* embryos (Rosa *et al.*, 1988). It was therefore important to compare the effects of TGF-β2 with TGF-β1 on spontaneous cytokine production. TGF-β2 also did not inhibit TNF-α or IL-1 production by the synovial cells. In addition, as previously shown (Chantry *et al.*, 1989), pretreatment of normal PBMC with TGF-β inhibited cytokine production, but this inhibition of TNF-α and IL-1 production was unaffected if the addition of TGF-β was delayed until after that of the inducing stimulus (in this case LPS). Similar effects

were also found with TGF-β2 in normal PBMC (data not shown). These finding suggest that TGF-β does not inhibit cytokine production in already activated cells, and thus the RA synovial cells which were previously activated *in vivo* were refractory to the inhibitory effects of this cytokine. It is possible that TNF-α production by RA joint cell cultures can be inhibited by TGF-β in a minority of RA patients, but was not apparent due to the relatively small number of samples tested. Furthermore, as IL-1 production by the RA joint cell cultures had to be measured using an immunoassay as opposed to the more sensitive bioassay (TGF-β is inhibitory to mouse thymocyte proliferation) inhibition of IL-1 by TGF-β cannot be ruled out.

The apparent lack of inhibition of RA joint cell TNF-α production by TGF-β raises a number of important points concerning the role of TGF-β in chronic inflammation. Although the major proportion of TGF-β released from activated cells is in the latent form, one would expect a substantial amount to be activated in the rheumatoid synovial joint, whose inflammatory environment is rich in neutral proteases, cathepsins and other enzymes (Fava *et al.*, 1989). The finding that active TGF-β is present in SF supports this notion. In light of this, and the observation that substantial amounts of TNF and IL-1 are produced spontaneously by RA synovial cells, a major role for TGF-β by itself in regulating the production of these macrophage mediators in rheumatoid arthritis is unlikely but cannot be ruled out totally. Further, it has been found that despite an abundance of activated T cells in the synovial joint, cytokines such as IL-2, lymphotoxin and IFN-γ are readily detectable at the mRNA but not protein level (Firestein *et al.*, 1988; Brennan *et al.*, 1989b). Since TGF-β appears to act at a post-transcriptional level to regulate cytokine production by PBMC (Chantry *et al.*, 1989), it is conceivable that TGF-β may be involved in the selective down regulation of T cell products in this disease.

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