Brief Definitive Report

ACTIVE AND LATENT FORMS OF TRANSFORMING GROWTH FACTOR β ACTIVITY IN SYNOVIAL EFFUSIONS

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Transforming growth factor β (TGF-β) is a multifunctional growth regulator with diverse biological effects, including promotion and inhibition of fibroblastic and epithelial cell growth, respectively (1, 2), chemotaxis of dermal fibroblasts and monocytes (3, 4), facilitation of extracellular matrix remodeling by fibroblastic cells (5–7), and effects on proliferation and function of T and B lymphocytes (8–10). These cellular responses appear pertinent to development and/or maintenance of the synovial pannus in inflammatory arthritis. Furthermore, subcutaneous injection of purified TGF-β into newborn mice results in a multicellular response over 48–72 h (11), which is histologically similar to the synovial pannus seen in rheumatoid arthritis (RA). These findings led us to examine synovial effusions for the presence of TGF-β.

Materials and Methods

Synovial Fluids. Synovial fluids from the knees of 16 patients seen in the Vanderbilt University Rheumatology Clinic were collected by needle aspiration into heparin-free plastic syringes. Diagnoses determined by a rheumatologist included nine patients with RA, four with osteoarthritis, two with gout, and one patient with avascular necrosis.

Most fluids were routinely centrifuged at 1,200 *g* for 10 min at 4°C to remove cells. Some fluids were subjected to a more rigorous collection procedure involving centrifugation in plastic tubes at 5,000 *g* in a Sorvall high-speed centrifuge for 20 min, in order to minimize the possibility of contamination with platelet products released in vitro. The cell- and platelet-free supernatants were then collected with a glass pipette that was left in the tube at 37°C. Clotted material that formed around the glass pipette was discarded. The fluids were stored at −70°C until assay.

Assays for TGF-β. A radioreceptor assay for TGF-β was performed using a previously described procedure (12). Briefly, AKR-2B fibroblasts were plated in six-well culture plates at 1–2 × 10^5 cells per well using McCoy's 5A medium with 5% FCS. The next day, cells were washed three times with PBS and incubated for 2 h in the presence of 0.25 ng of ^{125}I-TGF-β in 1.0 ml of binding medium, with or without further additions. Nonspecific binding was determined by the addition of 1 μg of 50% pure unlabeled TGF-β.

After a 2-h incubation at room temperature with rocking, the cells were washed, released from the plate with collagenase, and cell-bound ^{125}I-TGF-β was measured in a gamma counter. Results were expressed as nanograms per milliliter TGF-β calculated from a standard curve (see Fig. 1). The concentrations of TGF-β-competing activity in synovial fluids (Table I) were derived from triplicate determinations at a final dilution that would yield a 40–60% inhibition of binding.

Two cell lines were also used in soft agar assays for TGF-β. AKR-2B cells were assayed in the presence of serum as previously described (13). Base layers of 0.8% agar in McCoy's

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5A medium supplemented with 10% FCS were prepared in 35-mm culture dishes. Upper layers of 0.4% agar in McCoy's 5A medium contained 10% FCS, 7.5×10^3 cells, and the synovial fluid to be tested. The number of colonies >50 μm was quantitated after 7 d using an image analyzer (Omnicon FAS III; Bausch & Lomb, Inc., Rochester, NY).

Soft agar assays were also performed in 24-well plates with NRK-49F indicator cells in a highly sensitive serum-free assay (>2 pg TGF-β) (14). Supplemental growth factors (see Fig. 2 legend), human high density lipoprotein (HDL), retinoic acid, and the samples to be tested were added simultaneously to a 0.3 ml overlayer of basal medium immediately after plating the cells. To evaluate the ability of specific antisera to block colony formation induced by TGF-β or acid-treated synovial fluid, 20 μg of normal rabbit IgG or 20 μg IgG isolated from antisera against TGF-β (15) were added to the upper medium layer (simultaneously with other factors). After 10 d incubation at 37°C, the colonies were fixed and stained with 10% formaldehyde/0.01% crystal violet, counted with the aid of a dissecting microscope, and the efficiency of colony formation was expressed as the percentage of plated cells forming colonies >50 μm in diameter.

Acid Activation of Latent TGF-β. TGF-β is known to exist in two forms, an active form that interacts directly with cell surface receptors and a less well characterized "latent" form(s) that can be activated by acidification, proteolysis, and other biochemical methods (12). Six randomly selected fluids were acid treated by dialysis of a 1.0-ml aliquot in 1 M acetic acid (pH 3.6) for 18 h at 25°C. The resultant precipitate was removed by centrifugation for 5 min at 12,000 *g*. The supernatant was then dialyzed against 10 mM acetic acid and assayed for TGF-β. Equivalent aliquots of 10 mM acetic acid were shown not to affect the assays.

Results

A dose-response curve for the radioreceptor competition assay that was used to quantitate TGF-β activity in untreated and acid-activated synovial effusions is shown in Fig. 1. In all synovial fluid samples examined, dose-response curves were observed that were similar to that seen when purified unlabeled TGF-β was added as competitor. Thus, despite their complex composition, the synovial fluids appear to have capacity to compete directly for receptor binding, similarly to purified TGF-β.

The mean levels of active TGF-β in untreated synovial fluids (Table I) were 10.1 ng/ml for RA fluids and 3.8 ng/ml for osteoarthritis fluids, a statistically significant difference ($p < 0.005$). Two gout fluids showed a mean level of 8.0 ng/ml, and a noninflammatory fluid (white blood cells, <200) obtained from a patient with avascular necrosis showed no TGF-β activity detectable by the radioreceptor assay (<1

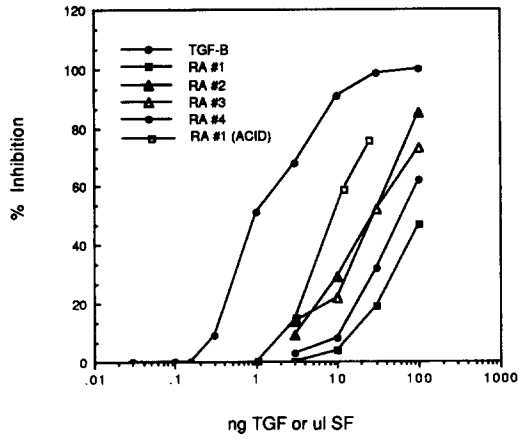

FIGURE 1. Competition of untreated and acid-activated synovial fluids and purified TGF-β in a receptor binding assay performed with cultured AKR-2B fibroblasts. Percent inhibition represents the reduction in specific binding, observed when cells were incubated for 2 h at 25°C, in the presence of ^{125}I-TGF-β (0.25 ng/ml) and the indicated amounts (ng) of unlabeled TGF-β (●) or aliquots (μl) of synovial fluid preparations. All illustrated synovial fluids were from patients with diagnoses of RA. One fluid, RA#1 (ACID), was also assayed after acid activation as described in Materials and Methods.

TABLE I

Mean Levels of TGF-β (ng/ml) in Synovial Effusions by Radioreceptor Assay

Diagnosis	Number of specimens	Untreated	Acid activation*
Rheumatoid arthritis	9	10.1‡	37.5
Osteoarthritis	4	3.8‡	12.0
Gout	2	8.0	21.0
Avascular necrosis	1	<1.0	0.8§

* Transient acidification before assay (Materials and Methods).
‡ $p < 0.005$ between these two groups.
§ Determined by NRK-49F colony formation assay.

ng/ml). Acid activation resulted in increased TGF-β activity in all six fluids tested, indicating the likelihood of a latent TGF-β pool.

To confirm the presence of the TGF-β-like activity using an additional assay, five fluids were examined for the capacity to promote soft-agar colony formation of AKR-2B and NRK-49F fibroblasts. Each of the fluids examined was found to promote soft-agar colony formation by the indicator cell lines in a dose-dependent manner (data not shown). Colony formation of NRK-49F cells induced by each of five fluids examined required exogenous epidermal growth factor in this serum-free assay, as has been previously described for TGF-β (14). The use of this extremely sensitive assay enabled recognition of 0.8 ng/ml TGF-β for the acid activated avascular necrosis fluid shown in Table I.

The TGF-β activity present in acid-activated synovial fluid, at a dose equivalent to 8 pg TGF-β, was completely abolished when 20 μg of anti-TGF-β IgG, but not an equivalent amount of normal rabbit IgG, was included in the assay (Fig. 2). Three synovial fluids from RA patients examined in this manner gave similar results.

Since TGF-β is normally stored in platelets and released upon platelet lysis, it appeared of value to examine whether any portion of the observed TGF-β activity in routinely collected cell-free fluids may have been derived from in vitro platelet lysis. Therefore, replicate aliquots of two randomly chosen synovial fluids were pre-

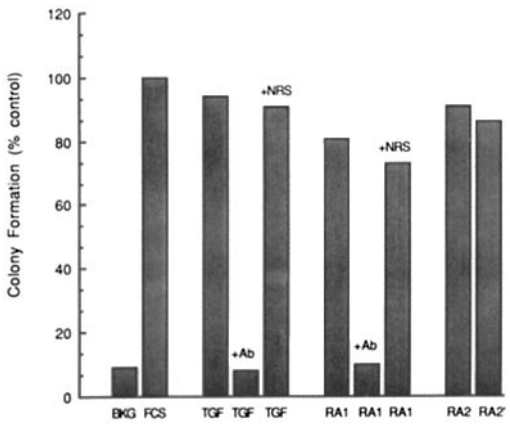

FIGURE 2. Soft-agar colony formation by acid activated and untreated synovial fluids and inhibition by antibody against TGF-β. NRK-49F cells were plated in soft-agar in a serum-free medium supplemented with insulin (10 μg/ml), transferrin (5 μg/ml), HDL (100 μg/ml), retinoic acid (100 ng/ml), and epidermal growth factor (50 ng/ml) and additions were made as shown in the Figure. 10% FCS induced 86% of the cells to form colonies in this experiment. The background level (BKG) without additions was 9% of FCS control. The response to 10 pg TGF-β and an aliquot (1 μl) of acid-activated synovial fluid equivalent to 8 pg TGF-β (RA1) were completely blocked by 20 μg/ml of anti TGF-β IgG (+Ab) but not by 20 μg/ml of IgG from normal rabbit serum (+NRS). The responses to aliquots of another (RA2) acid-activated and untreated (RA2') fluid are also illustrated.

TABLE II

Comparison of Low Speed and High Speed Centrifugation of Synovial Fluids

	Percent inhibition in radioreceptor assay	
Sample	Low speed	High speed
1	15.2	10.9
2	34.8	33.6

50 μl of each sample added to assay. Results are mean values of triplicate determinations.

pared under routine and more stringent conditions designed to eliminate platelet contamination (see Materials and Methods). There was essentially no difference in the amount of TGF-β detected after either method of preparation for the two fluids examined (Table II).

Discussion

The presence of TGF-β-like activity in inflammatory synovial fluids suggests that this multifunctional mediator may participate in the initiation or maintenance of synovitis, or may appear as the consequence of a tissue response to the presence of other cytokines. It is possible that the competition observed in the radioreceptor assay may result in part from TGF-β binding proteins present in whole synovial fluids. However, the demonstration of activity in soft-agar colony formation, of immunological neutralization of biological activity, and activation of latent forms, would strongly suggest that TGF-β activity is present in synovial fluid.

Several cellular responses known to be mediated by TGF-β might play a role in the development and/or maintenance of inflammatory synovitis, including: (*a*) the capacity to stimulate chemotaxis and, therefore, local "accumulation" of fibroblasts (3); (*b*) promotion of collagen, proteoglycan, and fibronectin production by fibroblastic cells (5–7), which could contribute to elevated levels of atypical isoforms of fibronectin that have been reported to accumulate in RA joint fluids (16, 17); (*c*) chemotaxis of monocytes (4); and (*d*) antiproliferative or immunosuppressive effects on T cells (8–10), which may account in part for the reduced in vitro response of isolated synovial fluid lymphocytes to proliferation stimuli (18).

The TGF-β found in synovial fluid may be delivered from T lymphocytes, B lymphocytes, and/or activated macrophages, all of which have been shown to produce TGF-β (8, 9, 19), platelets lysed at sites of microvascular injury, and synovial cells themselves (20). Activation of latent TGF-β-like activity can be effected by proteases such as plasmin and cathepsin D (12). If present in the synovial pannus, such "activating" proteases could increase levels of active TGF-β locally. Thus, alterations in the levels of such proteases and/or their inhibitors in the synovial pannus could act in concert with latent TGF-β production by endogenous or infiltrating cell types in sustaining the complex cellular activities observed in this lesion. Further investigation into the role of TGF-β in the development of synovitis may provide important clues to the etiopathogenesis of the inflammatory arthritides.

Summary

We have evaluated the possible involvement of TGF-β in rheumatoid arthritis by assay of 16 cell-free synovial fluids for the presence of its active and "latent" forms.

Evidence has been obtained for TGF-β-like activity in synovial effusions by four criteria: (*a*) TGF-β receptor competition, (*b*) soft-agar colony formation of AKR-2B and NRK-49F indicator cells, (*c*) immunological neutralization of the biological activity, and (*d*) biochemical activation of a latent form.

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