

Transforming Growth Factor β 1 (TGF- β 1) Induced Neutrophil Recruitment to Synovial Tissues: Implications for TGF- β -driven Synovial Inflammation and Hyperplasia

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Summary

We have studied the consequences of introducing human recombinant transforming growth factor β 1 (hrTGF- β 1) into synovial tissue of the rat, to begin to better understand the significance of the fact that biologically active TGF- β is found in human arthritic synovial effusions. Within 4–6 h after the intra-articular injection of 1 μ g of hrTGF- β 1 into rat knee joints, extensive recruitment of polymorphonuclear leukocytes (PMNs) was observed. Cytochemistry and high resolution histological techniques were used to quantitate the influx of PMNs, which peaked 6 h post-injection. In a Boyden chamber assay, hrTGF- β 1 at 1–10 fg/ml elicited a chemotactic response from PMNs greater in magnitude than that evoked by FMLP, establishing that TGF- β 1 is an effective chemotactic agent for PMNs in vitro as well as in vivo. That PMNs may represent an important source of TGF- β in inflammatory infiltrates was strongly suggested by a demonstration that stored TGF- β 1 was secreted during phorbol myristate acetate-stimulated degranulation in vitro. Acid/ethanol extracts of human PMNs assayed by ELISA contained an average of 355 ng of TGF- β 1 per 10^9 cells potentially available for secretion during degranulation of PMNs.

^3H Thymidine incorporation in vivo and autoradiography of tissue sections revealed that widespread cell proliferation was triggered by TGF- β 1 injection. Synovial lining cells and cells located deep within the subsynovial connective tissue were identified as sources of at least some of the new cells that contribute to TGF- β 1-induced hyperplasia. Our results demonstrate that TGF- β is capable of exerting pathogenic effects on synovial tissue and that PMNs may represent a significant source of the TGF- β present in synovial effusions.

Significant levels of both active and latent transforming growth factor β (TGF- β)¹ are found in the synovial effusions associated with rheumatoid arthritis, osteoarthritis, and gout (1–3). TGF- β 1 mRNA is detectable in extracts of diseased synovial tissues, and immunological evidence for its presence in synovial tissues has been reported as well (4). These data, and many of its effects on pertinent cell types, have implicated TGF- β in the pathogenesis of these arthritides. Specifically, TGF- β exerts dramatic effects on extracellular matrix deposition by mesenchymal cells (5–8), induces chemo-

taxis of fibroblasts in vivo (9) and in vitro (10), promotes angiogenesis (9, 11), exerts complex effects on chondrocytes (12), and can recruit monocytes and modulate monocyte activation (13–15). However, TGF- β also exerts suppressive effects on cells of the immune system that could have beneficial effects on synovial pathogenesis (16–19). In fact, its presence in synovial effusions seems to explain a long recognized paradox: that of a diminished responsiveness to T cell mitogens exhibited by otherwise activated synovial T cells (2, 18, 20–22).

To begin to identify and define synovial tissue responses to TGF- β 1 in vivo, we investigated the consequences of injecting hrTGF- β 1 into normal synovial tissue. Our results demonstrate that through chemoattraction of neutrophils and

¹ Abbreviation used in this paper: hrTGF- β 1, human recombinant transforming growth factor β 1.

other mechanisms, TGF- β 1 has a significant potential to exert pathogenic effects in the synovium and surrounding tissues that could contribute to development of the synovial pannus in arthritis.

Materials and Methods

Cytokines and Reagents. Recombinant human TGF- β 1 (hrTGF- β 1) and 125 I-TGF- β were generously donated by Genentech, Inc. (So. San Francisco, CA). Endotoxin levels in this preparation of human recombinant TGF/ β 1 were determined to be 1.84×10^{-3} endotoxin U/ μ g by the limulus polyphemus gelation assay. Purified porcine TGF- β 1 and a neutralizing antiserum against TGF- β were from R + D Systems, Inc. (Minneapolis, MN). The preparations of hrTGF- β 1 used throughout this study showed virtually identical competing activity and binding parameters when compared with purified porcine TGF- β in receptor competition assays on AKR-2B fibroblasts (23). 125 I-TGF- β (a gift of Genentech, Inc.) was used in preliminary experiments; in confirming experiments, hrTGF- β 1 was iodinated (24) and analyzed by receptor competition assay (23) to check its bioactivity, and by SDS-PAGE and autoradiography to confirm its purity. [3 H]Thymidine (2.0 Ci/mmol) was from Dupont/New England Nuclear (Wilmington, DE). FMLP was purchased from Sigma Chemical Co. (St. Louis, MO).

Experimental Animals. 6-wk-old female Wistar Outbred rats (175 g) obtained from Harlan Sprague Dawley (Indianapolis, IN) were used for most intra-articular injection studies. C3H/OuJ mice, used in thymidine labeling experiments, were 6–12 mo old when used and were obtained from The Jackson Laboratory (Bar Harbor, ME). Animals were housed in the animal care facilities at the Department of Veterans Affairs Medical Center (Nashville, TN) and given food and water ad libitum. After study, all animals were killed by carbon dioxide narcosis.

Intra-articular Injection of TGF- β and Histological Techniques. Rats were anesthetized with a mixture of ketamine/xylazine (80 μ g/g ketamine; 12 μ g/g xylazine) sufficient to keep the animals unconscious for 3–4 h. Injections were performed with 31-gauge needle tubing attached to a mechanical syringe driver (Hamilton Co., Reno, NV). The knee joint was slightly flexed, and the needle was inserted through the infrapatellar fat pad into the joint cavity. Either 1 μ g of recombinant human TGF- β 1 in 25 μ l sterile saline, or 25 μ l sterile saline alone as a control, was injected into the intra-articular joint spaces. Three animals were killed for each time point examined (2, 4, 6, 8, 12, 24, 48, and 72 h, 6 and 17 d post-injection), and the tissue was fixed and decalcified, embedded in paraffin, sectioned, and examined for cellular infiltration, plasma exudation, and synovial membrane thickening or "hyperplasia". In each case, sagittal sections that included views of the joint space, the meniscal cartilage, tibial and femoral cartilage surfaces, the infrapatellar fat pad, and its lining (synovial membrane) were examined. In this manner, cytokine-induced changes in the typically loose connective tissue of the fat pad were not confused with the normally more fibrous and cellular perichondral regions of the synovium.

Clearance of Exogenous TGF- β from the Intra-articular Space. An aliquot of 125 I-TGF- β 1 (50,000 cpm/ng) containing 90,000 cpm was combined with 1 μ g unlabeled TGF- β and injected as described above. At each time point, rats were killed and the entire knee joints removed and bisected, and associated radioactivity was quantitated with a gamma counter. Two rats were used for each time point, both knees were injected, and, except for certain time points examined by histological methods (see below), all were counted. Portions of liver, spleen, kidney were also removed for counting

to confirm that the TGF- β had entered the circulation and was being removed through the expected hepatic clearance pathway described by others (25). At certain time points, intact knee joints were fixed in 10% neutral buffered formalin, acid decalcified, embedded in paraffin, and sections subjected to an autoradiography protocol to determine the spatial distribution of 125 I-TGF- β 1 in the tissue. Exposure times ranged from 3 d for the earliest time points post-injection (10 min) to 3 wk for the latest (24 h).

Kinetics of PMN Recruitment by TGF- β 1 In Vivo. At the indicated times after intra-articular injection of 1 μ g hrTGF- β 1 in sterile PBS with 0.01% BSA (PBS/BSA), or sterile PBS/BSA in contralateral knees, knee joints were acetone fixed and embedded in glycol-methacrylate without decalcification. This technique greatly improves morphological details and allows dual nonspecific esterase cytochemistry to be performed to identify infiltrating inflammatory cells as either monocytes or PMNs. PMNs are positive for naphthol AS-D chloroacetate ester, staining bright red/purple, whereas monocytes, positive for α -naphthyl acetate esterase, stain a diffuse black/brown; thus, the two cell types are clearly distinguishable. Sections were routinely counterstained with 0.1% toluidine blue or, after esterase staining, with Mayer's hematoxylin. Sufficient morphological detail was achieved with these techniques to permit the direct quantitation of PMNs after esterase cytochemistry by counting cells at $\times 400$ in 50 microscopic fields, which was performed for the 1- μ g dose. The sections examined were chosen at random from a series representative of a 100- μ m region taken from the center of the joint in the sagittal plane of section. Control knees injected with PBS were examined by the same method to determine the number of PMNs present due to the injection trauma alone. This number of PMNs, which was always <20% of the number found in the TGF- β -injected knees, was subtracted in each case to arrive at the data shown. The values reported are the averages of numbers of PMNs determined for at least two rats at each time point. Similar analyses were performed 6 h post-injection with 10, 20, 40, 100, 250, and 4,000 ng, as well, to assess the dose responsiveness of PMN recruitment.

Neutrophil Chemotaxis Assay. PMNs were isolated from heparinized blood from normal human donors by gravity sedimentation for 30 min at room temperature after mixing with an equal volume of 3% dextran (229,000 mol wt) (Sigma Chemical Co.) in 0.9% NaCl. Sedimented cells were mixed with an equal volume of 0.9% NaCl and centrifuged at 200 g for 10 min at 4°C. The recovered cell pellet was exposed to 10 ml of a 0.2% NaCl solution for 20 s to lyse contaminating erythrocytes and then mixed with 10 ml of a 1.6% NaCl solution to make the mixture isotonic. RPMI 1640 was added to the cell suspension, which was then centrifuged as described above, and resuspended at a final concentration of 1.5×10^6 cell/ml in RPMI 1640.

The cell suspension was added to the upper compartments of modified blind-well Boyden-type chemotaxis chambers previously prepared to contain in their lower compartments RPMI 1640 with and without different concentrations of natural or recombinant TGF- β -1, or FMLP. The upper and lower compartments were separated by a polycarbonate filter with 2- μ m pores (Nucleopore, Pleasanton, CA). Loaded chambers were incubated for 40 min at 37°C. After incubation, filters were removed from the chambers, stained with hematoxylin, and mounted on glass slides. Chemotactic activity was quantitated by counting in 10 oil immersion fields, at $\times 1,000$ magnification, the number of PMNs that migrated and adhered to the lower surface of each filter. Samples were tested in quadruplicate, and results are expressed as the mean or mean \pm SEM of PMNs/OIF of the replicas for each sample tested.

PMA Stimulated Release of TGF- β from Isolated PMNs. Human

peripheral blood PMNs were isolated essentially as described above for the chemotaxis assay, except that whole blood was first centrifuged in Ficoll-Hypaque to remove mononuclear cells and platelets before the Dextran sedimentation step. The PMNs were finally suspended in Hank's salts (calcium- and magnesium-free) at $1-2 \times 10^7$ cells/ml. PMA was added to a final concentration of 100 ng/ml, and the cells and controls were incubated for 30 min at 37°C. Cells were then removed by centrifugation in a microfuge and aliquots of the supernate removed for TGF- β assay. Aliquots of the supernates were transiently acidified with HCl for 60 min at room temperature to activate any latent TGF- β present and then neutralized with NaOH before assay. The experiment shown is representative of three independent experiments with cells from different donors. Although TGF- β 1 activity was always detected in the cell-free supernates, basal rates of secretion (without PMA) were somewhat variable, presumably due to spontaneous degranulation as a consequence of the conditions required for isolation. Routinely, cytofuge preparations of the purified PMNs were Wright's stained; the predominant cell contaminant was the eosinophil (~5%) with no evidence of the presence of platelet aggregates or individual platelets (removed in the Ficoll-Hypaque step). Less than 1% monocytes/lymphocytes were found in these preparations.

Serum-free Colony Formation Assay for TGF- β . This assay was performed essentially as previously described (26), except that cell stocks were maintained on 5% FCS and the concentration of human high density lipoprotein was reduced from 100 to 40 mg/ml. Briefly, a hyperresponsive clone of a rat kidney fibroblast cell line, NRK-49F, is used as an indicator cell in a serum-free anchorage-independent growth assay. On the day of the assay, subconfluent stock cultures of NRK-49F cells were washed several times with serum-free basal medium for 1 h at 37°C to remove residual serum factors before trypsinization and suspension in soft-agar to initiate the assay. High sensitivity of TGF- β detection is achieved in this

assay (levels >10 pg/ml) with a serum-free medium comprised in part of insulin, transferrin, human high density lipoprotein, epidermal growth factor, and retinoic acid. Antibody neutralization of the TGF- β bioactivity was used for confirmation.

Extraction of Total TGF- β from PMNs and Analysis by ELISA. PMNs were prepared exactly as described above and then subjected to an acid-ethanol extraction procedure originally described for use with platelets (27, 28). The final precipitate containing the TGF- β was dissolved into 1 M acetic acid, dialysed against 10 mM acetic acid, and aliquots were analyzed in triplicate by ELISA using a recently described protocol and TGF- β 1-specific mAbs (29).

In Vivo Synovial Cell [3 H] Thymidine Labeling. Adult Ouj mice were subjected to the hfTGF- β 1 intra-articular injection protocol; a 31-gauge needle was used to minimize injection trauma. After 70 h, 60 μ l of [3 H] thymidine (2.0 Ci/mmol) was introduced by intraperitoneal injection. At 72 h post-injection, mice were killed, and the knee joints and a small sample of small intestine (to serve as a control to confirm that labeling of proliferating cells was achieved with each animal) were fixed in Carnoy's solution overnight, dehydrated, and embedded in glycol methacrylate. Sections were dipped in F.5 emulsion (Ilford Scientific Products, Mobberly Cheshire, UK), exposed for 3 wk, developed in Kodak D-19 developer, counterstained, and examined by light microscopy. Labeling index and mitotic index were determined by counting the total number of nuclei of resident tissue cells, the number of labeled nuclei, and the number of mitotic figures observed in 15 microscopic fields taken from the synovial membrane area and underlying tissue.

Results

Clearance of Injected TGF- β from the Tissue. First, the clearance time of TGF- β 1 injected into the joint space was determined. 1 μ g of unlabeled TGF- β 1 was added to 3-5 ng of 125 I-TGF- β 1 before injection. As shown in Fig. 1, the injected 125 I-TGF- β 1 was cleared from the joint space with a half-life of ~1 h. As expected from previous studies (25), after ~30 min, radioactivity was detectable in the liver, and to a lesser extent in the kidney and spleen. The amount of 125 I increased in these organs as a function of time for several hours (data not shown). The rapid clearance of TGF- β may be due in part to increased vascular permeability reflected by plasma exudate formation in the joint space, observed in histological studies performed simultaneously (see below). In spite of the rapid clearance of most of the injected TGF- β 1, significant amounts of TGF- β 1 may remain in the tissue many hours after its injection.

To determine the spatial distribution of the injected 125 I-TGF- β 1 at selected times post-injection, knee joints were fixed in formalin, processed for paraffin embedding, and replicate tissue sections were subjected to autoradiographic analysis. Since 1 μ g of unlabeled TGF- β 1 was added to the 125 I-TGF- β 1 before injection to duplicate the conditions used in the rest of the study, specific binding to cell receptors was obscured by "nonspecific" associations with other components of the tissue. At the earliest times examined (10-30 min), silver grains were primarily associated with the innermost surfaces of the synovial membrane and articular cartilage. At later times (4-24 h), the silver grains were preferentially distributed within the cartilage matrix and penetrated further towards the subchondral bone with time, probably due to

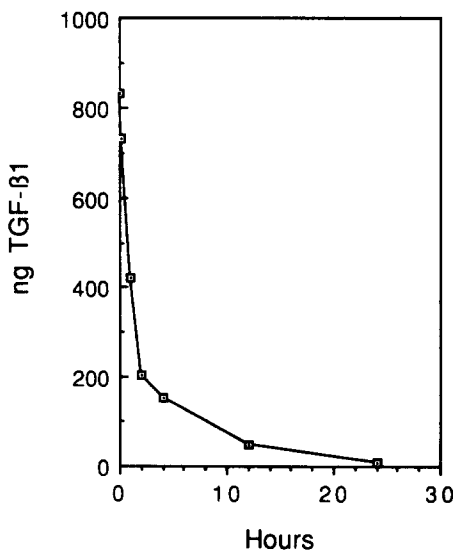


Figure 1. Kinetics of TGF- β 1 in the rat knee joint space. 125 I-hfTGF- β 1 (90,000 cpm with 1 μ g unlabeled hfTGF- β 1) was injected into the intra-articular space. At the times indicated, animals were killed, the knee joints removed, bisected transversely with care to retain any fluid present, and each half was placed in a tube for a determination of the total amount of 125 I-hfTGF- β 1 present in the tissue. Each point represents the average value obtained from at least three knee joints from two different rats.

electrostatic interactions between TGF- β 1 (pI = 9.8) (23, 30) and the overall negatively charged cartilage matrix (data not shown).

Neutrophil Recruitment Induced by TGF- β Intra-articular Injection. Within 2 h after injection, two striking morpho-

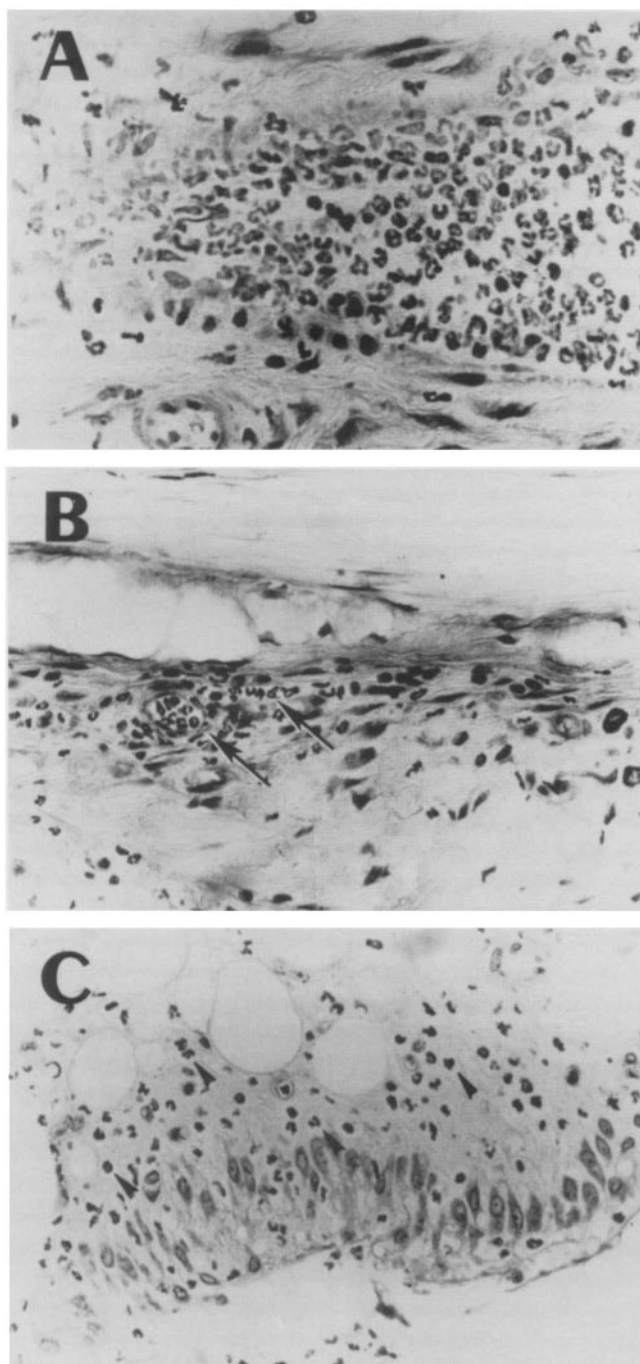


Figure 2. Influx of PMNs into vascular elements and the synovial membrane after intra-articular injection of hfTGF- β 1. 6 h after injection of 1 μ g of hfTGF- β 1, joints were processed for glycol-methacrylate embedding and histological analysis. Sections (3 μ m) were counter stained with toluidine blue. (A) PMNs in a large vascular element in the synovial fat pad; (B) microvascular elements (arrows) in synovial fat pad with PMNs emigrating into avascular space; (C) synovial membrane with PMNs (arrow heads) traversing into the joint space (original magnification, \sim 400).

logic changes were apparent in the synovial tissues. These were the infiltration of PMNs into the tissue and the appearance of plasma exudate on the surfaces of the synovial membrane and articular cartilage, as well as within the joint space. As shown in Fig. 2 A and B, the magnitude of the PMN immigration was very dramatic at its peak with large numbers of PMNs observed in most vascular elements. Many PMNs were present in interstitial regions in the infrapatellar fat pad subjacent to the synovial membrane and were also observed migrating into and through the synovial membrane itself (Fig. 2 C). An exuberant plasma exudate infiltrated by PMNs formed within the joint space within 4–6 h, and at later times (after 8 h), monocytes were present as well (data not shown). PMNs and monocytes embedded in the material were presumably engaged in degradation of the fibrin-containing exudate (31). It is not yet clear what mediates the vascular permeability changes responsible for the exudate formation; TGF- β effects on endothelial cells consistent with induction of vascular permeability changes have not yet been reported. The large number of PMNs crossing the endothelial barrier may contribute to the permeability changes through release of other mediators. The amount of inflammation induced by injection trauma in the saline-injected control knees was much less than that seen with the TGF- β injection, although occasionally, bleeding into the joint occurred as evidenced by erythrocytes present in the joint space.

The responses described above were not consistently observed at lower doses of TGF/ β tested (10–100 ng), and were observed to be significantly reduced at 250 ng. The neutrophil recruitment observed with a 4- μ g dose was not significantly different from that seen with the 1- μ g dose. It is not clear why the PMN recruitment response appears to require a threshold dose of \sim 1 μ g of TGF/ β , although the capacity of tissue components to sequester TGF- β , especially the cartilage matrix, may be responsible.

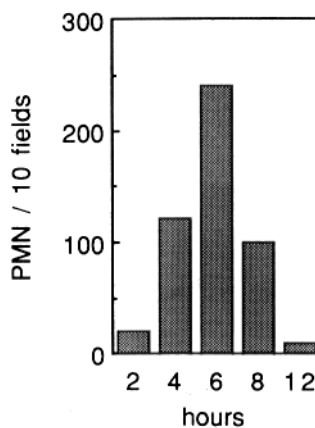


Figure 3. Kinetics and quantitation of neutrophil influx into the synovial membrane after hfTGF- β 1 injection. At the time indicated after injection of 1 μ g of hfTGF- β 1, or PBS into the contralateral knees as controls, knee joints were acetone fixed and embedded in glycol-methacrylate medium. Sections were subjected to an esterase cytochemical stain (naphthol AS-D chloroacetate esterase) to distinguish PMNs from monocytes. PMNs in the area of the synovial membrane were counted in 50 microscopic fields on three to five 3- μ m sections in the sagittal plane, randomly selected from the center

of the knee joints. Two rats were used for each time point, and the number of PMNs/10 fields in the PBS-injected knees was subtracted to obtain the values shown. PMNs present in the exudate in the joint space were not counted unless directly adherent to the synovial membrane.

Kinetics of PMN Recruitment into Synovial Tissue. Tissue sections prepared at various times after TGF- β 1 or saline intra-articular injections were subjected to a nonspecific esterase cytochemistry procedure and examined. Most infiltrating cells present at early times were PMNs with a gradual enrichment in monocytes seen later (8–12 h). To quantitate this response, PMNs were counted in 50 random microscopic fields ($\times 400$) at 2, 4, 6, 8, and 12 h after hfTGF- β 1 injection and corrected for the PMN recruitment that resulted from injection of PBS alone. Only PMNs present in the synovial membrane were counted; each field counted included the intimal lining layer of synoviocytes, similar to the field shown in Fig. 2 C. Although some microvascular elements were included in the count, most cells that were counted were observed traversing the synovial membrane interstitium. PMNs were also abundant throughout the infrapatellar fat pad, but these were not included in this quantitation. As seen in Fig. 3, the peak of PMN influx occurred ~ 6 h post-injection. Monocytes began

to appear in the tissue ~ 8 h post-injection, and at 12 h post-injection, more than half of the infiltrating cells stained positively for the monocyte marker, α -Naphthyl acetate esterase (data not shown).

Ultrastructural Characterization of the Synovial Membrane. The studies described above indicated that monocytic cells arrived later and persisted longer than PMNs in the tissue. Many appeared to remain present 4 d post-injection, primarily at the surface of the synovial membrane. At this time, the surface of the synovium was enriched with cells with membrane projections that could be resolved with the light microscope in semithin sections (data not shown). When examined by EM, these cells were found to be engaged in both pinocytotic (data not shown) and phagocytic activity, as illustrated in Fig. 4. The tightly interconnected nature of the cells normally associated with the surface of the synovial membrane was less apparent (32); intimal lining cells were loosely associated with the synovial surface. Many such cells had fea-

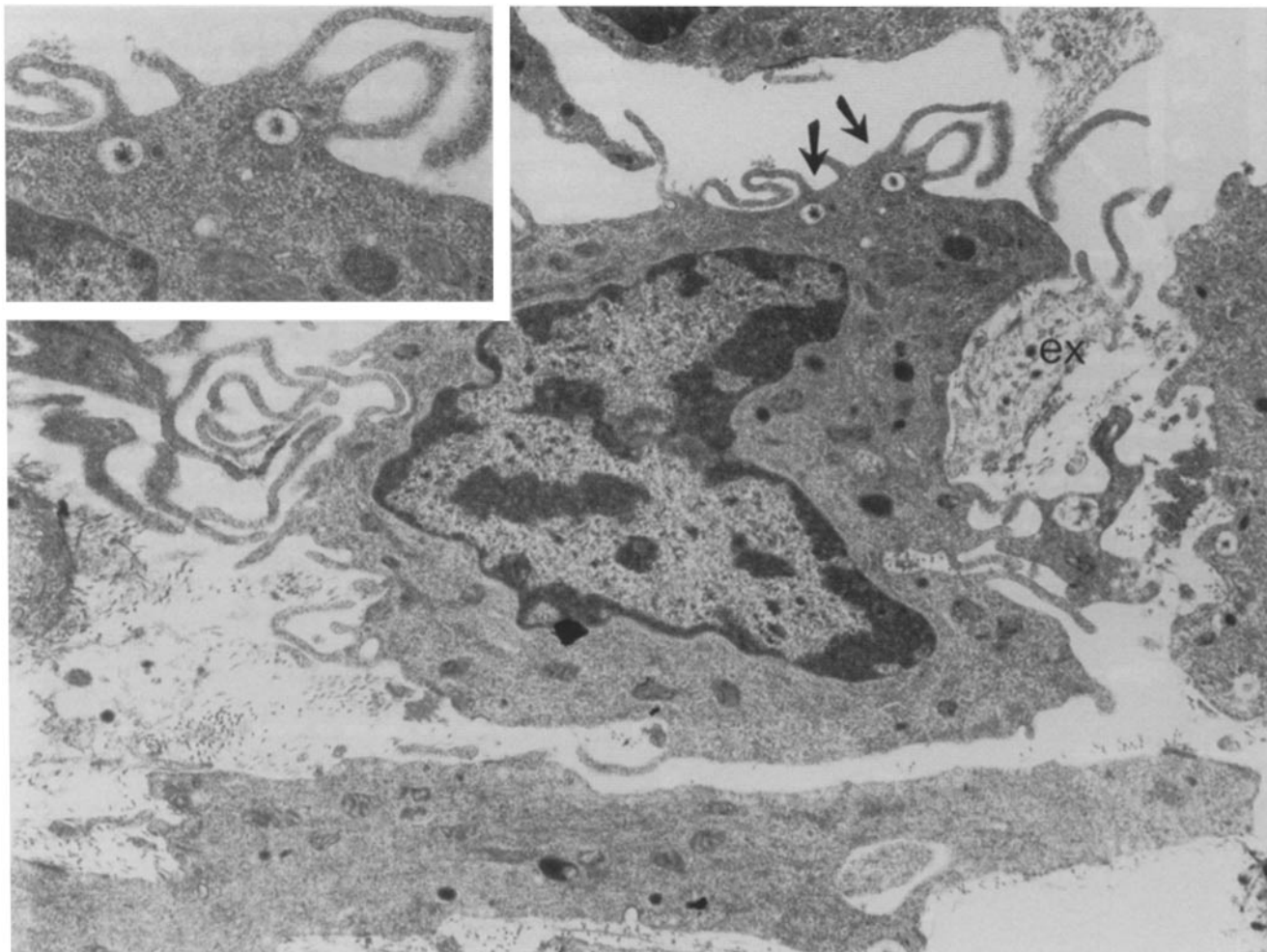


Figure 4. Ultra-structural appearance of the synovial membrane surface 4 d after intra-articular injection of TGF- β 1. Animals were killed at the time indicated, joint spaces were injected with 4% glutaraldehyde fixative in phosphate buffer, the infrapatellar fat pad was dissected, and small specimens were fixed an additional 6 h. The specimens were osmicated and embedded in EPON resin for ultrastructural analysis. Cells with the morphological appearance of monocytes and bearing numerous surface extensions were often observed at the surface, and appeared to be actively engaged in pinocytosis and phagocytosis. The majority of intracellular inclusions were empty pinocytotic vacuoles (data not shown), although some of the intracellular vesicles contained electron-dense material (arrows), as shown in the inset, presumably fibrin and other exudate components. Original magnification, 6,000; inset, 30,000.

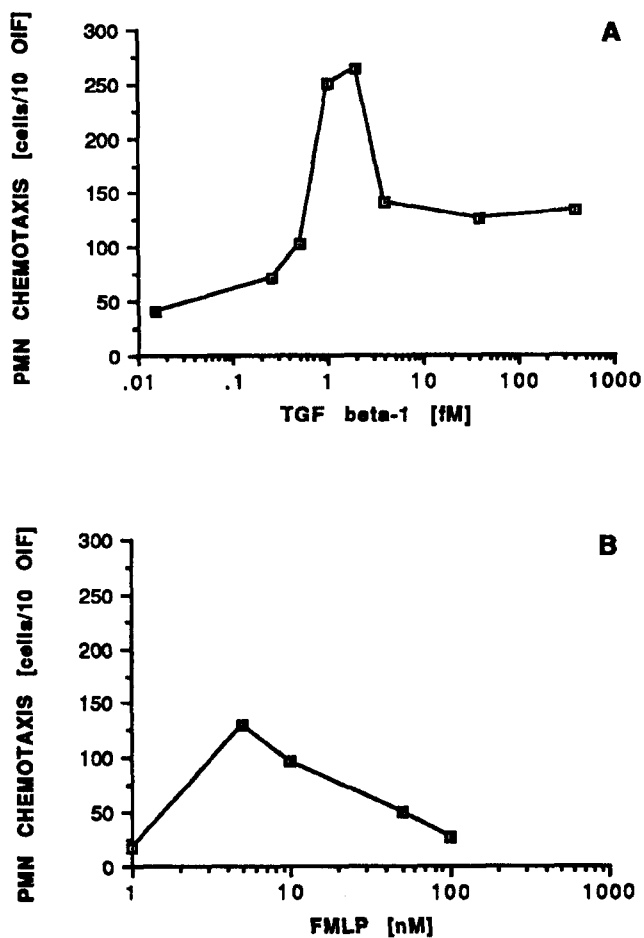


Figure 5. Direct chemotactic effects of TGF- β 1 on human peripheral blood PMNs and comparison with FMLP. Isolated human PMNs were subjected to a chemotaxis assay in Boyden chambers exactly as described in Materials and Methods. Quantitation was performed by counting cells that had migrated through a 2- μ m pore size Nucleopore filter in response to the factor present on the other side of the membrane at the indicated concentrations. The data are reported as number of cells per 10 oil immersion fields ($\times 1,000$). (A) TGF- β 1; (B) FMLP.

tures indicative of monocytes with cytoplasmic extensions and filopodia, a typical crescent-shaped nucleus and cytoplasmic heterophagic vacuoles/lysosomes, as shown in Fig. 4 (*arrows* and *inset*). The majority of the cells on the synovial membrane surface also displayed highly convoluted membrane projections and "empty" vacuoles, reflecting pinocytic activity of the cells. A striking similarity was noted between the ultrastructural appearance of cells populating the synovial membrane 4 d after TGF- β 1 injection to those reported in this location during the early stages of type II collagen-induced arthritis (32).

Direct Chemotactic Effects of TGF- β on Isolated PMNs. Since the data obtained *in vivo* indicated that TGF- β 1 rapidly recruited neutrophils to the site of injection, it was of interest to determine whether TGF- β had direct chemotactic effects on PMNs. The ability of native and recombinant TGF- β 1 to induce PMN chemotaxis was assessed by the Boyden chamber technique. Both hrTGF- β 1 and natural porcine TGF- β 1 stimulated migration of peripheral blood PMNs in a dose-dependent manner, as did FMLP (Fig. 5). The dose-response curves for natural TGF- β 1 (Fig. 5 A) and rhTGF- β 1 (data not shown) were essentially bell shaped, as has been shown for monocytes and fibroblasts, other cell types for which TGF- β is known to be chemotactic (10, 13). The ability of the purified porcine TGF- β 1 to induce a chemotactic response to PMNs was blocked with neutralizing antibodies (data not shown). A Zigmond-Hirsch checkerboard analysis revealed that TGF- β 1 affected the migration of PMNs only when present in higher concentration in the lower compartment of the chemotaxis chamber (Table 1), showing that it induces chemotactic migration and not simply chemokinesis (33).

It was of interest to assess and compare the magnitude of PMN migration induced by TGF- β 1 and a standard chemoattractant, FMLP. Therefore, dose-response curves for both chemoattractants were determined in the same chemotaxis assay. FMLP induced maximal migration at a concentration of 5–10 nM, which was of a lesser magnitude than that observed with hrTGF- β 1 (Fig. 5). Similar results were obtained

Table 1. Effects of Varying TGF- β Concentrations on PMN Migration

TGF- β concentration in upper compartment	PMNs per 10 OIF at TGF- β concentration (fg/ml in lower compartment)				
	7.80	3.40	1.70	0.85	0
fg/ml					
7.80	50 \pm 12	30 \pm 6	33 \pm 4	33 \pm 6	37 \pm 6
3.40	42 \pm 2	34 \pm 4	31 \pm 4	14 \pm 5	29 \pm 6
1.70	81 \pm 9	65 \pm 8	65 \pm 9	44 \pm 10	38 \pm 11
0.85	92 \pm 9	87 \pm 13	54 \pm 4	35 \pm 5	34 \pm 5
0	26 \pm 3	168 \pm 17	113 \pm 7	85 \pm 4	66 \pm 12

Different concentrations of porcine TGF- β were added to the upper and lower compartments of chemotaxis chambers, and the number of neutrophils migrating to the lower surface of each filter was quantitated. The values represent the mean \pm SEM of triplicate determinations.

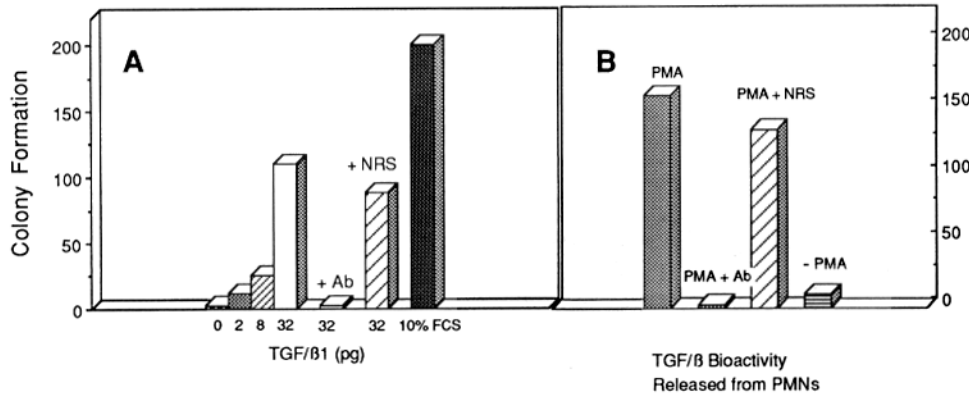


Figure 6. PMA-induced release of TGF- β from peripheral blood PMNs and antibody neutralization. Isolated PMNs were incubated for 30 min with 100 ng/ml PMA in Hank's medium, and cell-free supernates were analyzed for total TGF- β in a soft-agar colony formation assay. Response to 10% FCS represents colony formation by \sim 85% of the cells initially plated (\sim 200 colonies per four microscopic fields). (A) The dose response to the addition of 0, 2, 8, and 32 pg/ml hrTGF- β 1; 32 pg/ml hrTGF- β 1 plus 25 μ g anti-TGF- β IgG (+Ab); 32 pg/ml hrTGF- β 1 plus 25 μ g normal rabbit serum IgG (+NRS); 10% FCS. (B) The response to the addition of 100- μ l aliquots of cell-free supernates from PMA-treated PMNs either alone (PMA); with 25 μ g anti-TGF- β IgG, (PMA+Ab); or with 25 μ g normal rabbit serum IgG, (PMA+NRS). Also shown is addition of 100 μ l of supernatant from PMNs incubated without PMA, (-PMA).

when the experiment was repeated. These results indicate that not only is TGF- β 1 >100,000 times more potent than FMLP at inducing PMN chemotaxis, but that the magnitude of the chemotactic response is greater than that observed with FMLP, in the chemotaxis assay used in this study.

Release of TGF- β from PMNs by Degranulation. It is possible that PMNs may be a significant source of TGF- β in vivo (34, 35). To examine the question of whether or not TGF- β could be released from peripheral blood PMNs in conjunction with degranulation in vitro, we chose to use a well-characterized specific granule secretagogue, PMA (36, 37). TGF- β activity was assayed by a traditional bioassay, anchorage independent growth of NRK-49 cells (26). As shown in Fig. 6 B), TGF- β activity was released from freshly isolated PMNs incubated with 100 ng/ml PMA at 37°C for 30 min. There was little detectable activity released from unstimulated PMNs. Immunological neutralization of the activity secreted by PMA-stimulated PMNs, with an IgG fraction of an antiserum prepared against native TGF- β , confirmed that the colony formation was due to TGF- β (Fig. 6 B).

An additional immunologic method confirmed the identity of the TGF- β 1 present and was used to determine the quantity of TGF- β 1 contained in this novel storage site. Aliquots of acid/ethanol extracts of human peripheral blood PMNs were analyzed by a recently developed TGF- β 1-specific ELISA that uses two mAbs that recognize TGF- β 1. The mean level of TGF- β 1 determined by the extraction of three preparations of \sim 10⁹ PMNs was found to be 355 ng/10⁹ cells (SD = 44.4 ng). In addition, since the conditions chosen for the PMN-induced secretion of TGF- β from PMNs described in the above experiments were suboptimal for degranulation, PMNs were also stimulated with PMA in the presence of 5 μ g/ml cytochalasin B to optimize degranulation (38). In this case, PMNs released 44% of their total stored TGF- β 1

(measured by ELISA after in vitro acid activation of cell-free supernates).

TGF- β Induced Proliferative Effects in Synovial Tissue. In other studies, mitotic figures were observed in synovial tissue 72 h after initiating daily injections of TGF- β 1 (39). Therefore, we first compared the effects of our single injection protocol to those produced by multiple injections of TGF- β 1, monitoring the number and location of mitotic figures 72 h post-injection. Rats were given either a single injection of 1 μ g TGF- β 1, or daily injections of 1 μ g TGF- β 1 into the same knee joint. With either protocol, mitotic figures were occasionally observed 72 h post-injection, with no obvious differences detected in the frequency or location of the dividing cells (data not shown). In both cases, mitotic figures were seen in various regions of the synovial membrane, including cells close to the junction of the synovium with the articular cartilage, as illustrated in Fig. 7 A. With the administration of a single injection of TGF- β 1, however, there were markedly fewer inflammatory cells present in the tissue at 72 h compared with the number observed with the multiple injection protocol.

To determine the full extent of the proliferative response in the tissue triggered by TGF- β 1 injection, intra-articular injection experiments were repeated in mice that also received a 2-h pulse of [³H]thymidine 70–72 h after the single TGF- β 1 injection. The proliferative response was found to be much more widespread when analyzed by this method. As shown in Fig. 7 B, resident synovial membrane cells and another cell type of uncertain identity scattered throughout the subjacent synovial areolar tissue were labeled with [³H]thymidine. Another interesting aspect revealed by [³H]thymidine labeling was that many tendon cells also proliferated 72 h after TGF- β 1 injection; this effect was observed to occur both near and quite far from the actual site of injection (data not

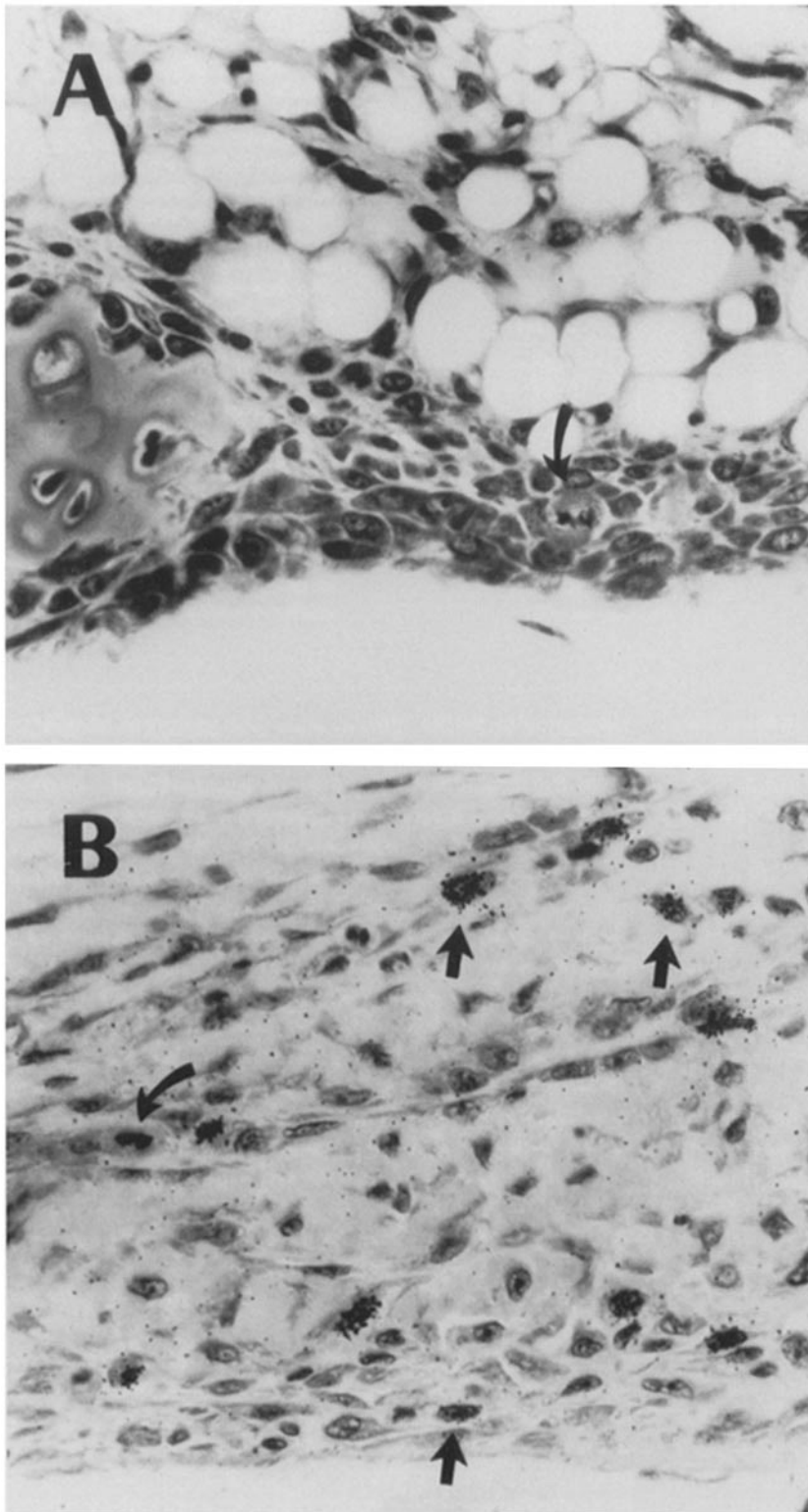


Figure 7. Proliferative response of synovial cells 72 h after TGF- β 1 intra-articular injection in the knee joint space. Rats (A) or mice (B) received 1 μ g TGF- β 1 by intra-articular injection or saline control of equal volume in the contralateral knee joint space. After 70 h, the mice received 60 μ Ci [3 H]thymidine injected intraperitoneally. After death the knee joints were fixed and embedded in glycol-methacrylate medium, and 3- μ m sections were subjected to autoradiography with F.5 emulsion (Ilford) and developed in Kodak D-19. Exposure time was 3 wk. (A) Mitotic figure (arrow) observed in the vicinity of the cartilage/synovium junction in the rat knee; (B) mitotic figure (unlabeled) and examples of labeled cells (up arrows) in more fibrous region of the synovium of the mouse (original magnification, 400).

shown). The labeling index was determined to be from 5 to 15% for tendon cells and from 9 to 11% for cells in the vicinity of the synovial membrane, whereas the mitotic index (mitotic figures) was from 0.1 to 0.5% for synovial membrane cells. There was no evidence of proliferation of en-

dothelial, smooth muscle, or striated muscle cells seen at this time point after TGF- β 1 injection.

Control (PBS-injected) contralateral knees that displayed no histological signs of injection trauma showed no labeling of cells, nor were mitotic figures ever seen. The basal rate

of synovial cell proliferation in mice that did not receive intra-articular injections of any type was also undetectable by [³H]thymidine pulse labeling. Interestingly, in one control knee in which signs of injection trauma were apparent (RBC, fibrin, inflammatory cells), there also was a proliferative response, suggesting that the same mediators are present after traumatic tissue injury as are triggered by TGF- β 1 injection. Thus, the increased number of cells observed in the synovial membrane 4–6 d post-injection (40) were in part due to the proliferation of both resident synovial cells and another unidentified cell type located somewhat remotely from the synovial membrane surface.

Discussion

We have observed and characterized a very rapid and dramatic PMN recruitment mediated by TGF- β 1 *in vivo* that precedes, and is therefore likely to play a central role in, TGF- β 1-induced synovial hyperplasia. The precise mechanism(s) by which TGF- β recruits PMNs to synovial tissue *in vivo* is not entirely clear; however, the ability of TGF- β 1 to stimulate a chemotactic response by peripheral blood PMNs *in vitro* identifies one possible component. The remarkable potency of TGF- β 1 in eliciting this response (>100,000 times more potent than FMLP) must qualify TGF- β 1 as the most potent chemotactic agent for PMNs known to date. Although the *in vivo* PMN recruitment we observed was rapid and extensive, little data from other experimental systems help in understanding the mechanics of this response. In fact, *in vitro* studies with cultured human umbilical cord endothelial cells and isolated peripheral blood PMNs would suggest that TGF- β would be inhibitory to PMN–endothelial cell interactions, through its ability to decrease PMN adhesiveness to endothelial cells (albeit only modestly) (41). It may be important to note, however, that this effect was observed after 6–24 h of exposure of endothelial cells to TGF- β ; it is possible that this phenomenon contributes to attenuation of the PMN recruitment at later times after TGF- β injection. It is not known whether TGF- β can induce expression of other neutrophil chemotactic factors, such as IL-8. The involvement of resident mast cells in the tissue remains a possibility (42), although TGF- β does not stimulate release of mediators from bone marrow–derived mast cells (43).

TGF- β receptors on isolated peripheral blood PMNs have not yet been characterized in detail. In preliminary experiments, we have observed specific binding of ¹²⁵I-TGF- β 1 to \sim 8,000 receptors/cell. The TGF- β 1 binding characteristics displayed by PMN receptors were similar to that seen with many other cell types; cell surface binding and saturation occurred in the same concentration range as observed for TGF- β binding to most other cell types, from \sim 0.1 to 10.0 ng/ml (Fava, R.A., unpublished results). This concentration range is several orders of magnitude greater than the doses at which the chemotactic response was observed, and it therefore is unclear whether the chemotactic response is mediated through the types of receptors usually described for TGF- β 1 binding (44–46). This difference in receptor-binding characteristics and the dose required for a chemotactic response is not un-

precedented; others have reported similar discrepancies between the very low concentrations of TGF- β required to elicit a chemotactic response by monocytes (peak activity at 0.02 pM) and an apparent affinity of cell surface receptors that require 40 pM TGF- β for saturation (13). Unfortunately, it is impossible to radioactively label TGF- β 1 to a sufficiently high specific activity to perform the equilibrium-binding experiments necessary to demonstrate the existence of receptors with the affinity constants expected for a biological response in the fg/milliliter range.

In other studies, immunoreactivity for TGF- β in inflammatory neutrophils has been observed (35), and it also has been shown that human peripheral blood PMNs contain mRNA for TGF- β , and that the protein is detected in culture medium after PMNs are incubated in culture dishes for 24 h (34). These data suggest that PMNs have the capacity to phagocytose, synthesize, and/or secrete TGF- β . One possibility is that the TGF- β found in the culture medium had been previously synthesized and may have been released from storage granules during PMN degranulation that occurs during attachment and contact with the culture surface. Recently, we have obtained evidence by immuno-localization techniques at the ultrastructural level that TGF- β is in fact detectable within granules of neutrophilic myelocytes in bone marrow, the precursors of mature peripheral blood PMNs (47; and unpublished results). Our ability to demonstrate that TGF- β was secreted from PMNs stimulated with a known specific granule secretagogue PMA supports the notion that PMNs may be an important source of stored TGF- β *in vivo*. Our quantitation of acid/ethanol extracts of peripheral blood PMNs reveals that PMNs contain \sim 1/20 the amount of TGF- β stored in platelets, if compared on a wet weight basis (48). That TGF- β may be delivered via this novel mechanism whenever there exists a signal to trigger PMN degranulation *in vivo* has broad implications in wound healing and chronic immune-driven inflammation. The identification of PMNs as a potential source of presynthesized TGF- β *in vivo* may also explain a recently reported paradox concerning the high levels of TGF- β observed in synovial effusions, even though very little mRNA for TGF- β has been detected by *in situ* hybridization of isolated synovial tissue cells *in vitro* (49).

Recently, Allen et al. (39) reported that multiple intra-articular injections of 1 μ g TGF- β 1, at 24-h intervals, had the capacity to induce a synovial hyperplasia similar to that described in the present study. The evidence for cell proliferation consisted primarily of mitotic figures that were observed 72 h after the initiation of the TGF- β 1 injections. We also observed a round of replication at this time, however, with the important difference that only a single injection of TGF- β 1 was used in our studies. With a single injection of TGF- β 1, recruitment of inflammatory cells occurred as a transient phenomenon with relatively few monocytic cells observed in the synovial tissue at the time of cell division. This situation differs significantly from the results of Allen et al. (39), who have proposed that contact, or at least close proximity of fibroblastic cells with monocytes, mediates the TGF- β 1-induced proliferation of synovial cells, possibly through the induction and secretion of macrophage-derived mediators such

as IL-1. We attribute the paucity of monocytes observed in the subsynovial tissue at the time of cell proliferation in our study to the single-injection protocol that we used. We propose that daily injections of TGF- β leads to a superimposition of monocyte recruitment over the proliferative response and that this repeated monocyte recruitment is not required for the TGF- β induction of synovial cell proliferation seen 72 h post-injection. With regard to the importance of IL-1 production by monocytes in the proliferative response, it is important to note that although TGF- β increases levels of mRNA for IL-1 in monocytes (13, 49a), the mRNA may not be translated, as little IL-1 bioactivity is secreted by TGF- β -stimulated PBMC (49a). Moreover, TGF- β has been shown to significantly reduce the levels of IL-1R in some cell types (50). Thus, the importance of IL-1 as a secondary mediator in TGF- β -induced synovial cell proliferation remains to be proven.

While the kinetics of the TGF- β 1-induced proliferative response in synovial tissue, and the induction of mRNA for certain cytokines (IL-1 and TNF) (39), is consistent with the involvement of other cytokines and secondary mediators in this response, several known aspects of TGF- β biology may bear on the likelihood of the continuation of its presence in the synovium after its initial introduction. Among these are the ability of TGF- β to stimulate its own production in mesenchymal and other cells (autocrine) (51–53), its secretion from activated monocytes (paracrine) (54), and, as we show here, its recruitment of PMNs that contain and can release TGF- β (autorecruitment). All of these potential contributions must be considered to completely define the molecular mechanisms involved in the proliferative stimulus initiated by exposure of the tissue to TGF- β 1.

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