Transforming growth factor β 1 and interleukin 4 induced α smooth muscle actin expression and myofibroblast-like differentiation in human synovial fibroblasts in vitro: modulation by basic fibroblast growth factor

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Abstract

Objective—To discover if α smooth muscle actin expression and myofibroblastic differentiation are induced in synovial fibroblasts by cytokines found in the inflamed RA joint.

Methods—Immunofluorescent microscopy and western blotting were used to examine different cultures of human synovial fibroblasts for expression of α actin in the presence of the cytokines transforming growth factor β (TGF β 1), interleukin 1 α (IL1 α), IL4, IL6, tumour necrosis factor α (TNF α), and basic fibroblast growth factor (FGF).

Results—A small but significant population of cells ($14.4 \pm 12.9\%$) expressed α actin under standard culture conditions. Upon treatment with TGF β 1 there was a pronounced increase in the number of cells expressing α actin ($68.1 \pm 5.49\%$), accompanied by a change in morphology to a myofibroblast-like phenotype. Other cytokines found within the inflamed joint such as IL1, TNF α , IL6, and basic FGF failed to induce α actin expression. However, IL4, which is normally absent or only present at low concentrations in the RA joint had a similar effect to TGF β 1. It was also found that basic FGF inhibited the induction of α actin expression by TGF β 1 and IL4.

Conclusion—In the presence of TGF β 1 or IL4, fibroblasts derived from synovial tissue or synovial fluid are induced to differentiate into myofibroblast-like cells containing the α smooth muscle form of actin. This differentiation is inhibited by basic FGF. It is suggested that the balance between these particular cytokines may be important in the modulation of fibroblast behaviour, which could have significant effects on joint repair mechanisms and the generation of fibrous tissue within the rheumatoid joint.

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The formation of fibrotic, granulation tissue at the synovium-cartilage junction is a characteristic feature of the joints in patients with long-standing rheumatoid arthritis (RA), and may be seen as an attempt at tissue repair. Granula-

tion tissue is normally responsible for wound contraction and retractile phenomena, both in normal wound healing and in fibrotic diseases.^{1,2} In RA, articular cartilage may eventually be replaced completely by fibrous tissue, with consequent formation of fibrous adhesions that bind together opposing articular surfaces. This is a serious complication in the later stages of RA, and can cause first temporary, then permanent deformation of the joints. The particular factors driving the fibrosis in the rheumatoid joint are unknown, although cytokines such as transforming growth factor β (TGF β) and fibroblast growth factors (FGFs) are likely candidates because of their effects on cell proliferation and production of extracellular matrix (ECM) components.

In normal wound healing the contraction process is believed to be brought about by the generation of traction forces by specialised myofibroblast cells.³ These cells show several features of smooth muscle cells, including the expression of the cytoskeletal protein-smooth muscle actin. However they are derived from fibroblasts rather than smooth muscle cells.⁴⁻⁶ Recently it has been shown that TGF β 1 can induce α actin expression in fibroblasts, and differentiation to myofibroblasts.^{7,8} TGF β has been found at high concentrations in the synovial fluids of patients with RA,⁹ and expression of TGF β 1, β 2, and β 3 as well as latent TGF β 1 binding protein (LTBP) and TGF type II receptors has been found within cells of the synovial lining layer, particularly in specimens with pronounced fibrosis.¹⁰ In another study TGF β has been localised to cells of the synovial lining and the cartilage-pannus region.¹¹

TGF β is an important promoter of tissue repair by stimulating the formation of extracellular matrix through increased synthesis of ECM components such as collagen and fibronectin.¹² The increased ECM deposition is further enhanced by the inhibitory effects of TGF β on the synthesis and release of metalloproteinases such as collagenase (MMP1) and the accompanying stimulation of tissue inhibitors of metalloproteinases (TIMPs).¹³ Other effects of TGF β include suppression of T and B lymphocyte proliferation,^{14,15} down regulation of macrophage HLA-DR,¹⁶ and inhibition of IL1, interferon γ , and TNF α production by

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macrophages.^{17,18} Thus in the context of the RA joint, TGF β may help to suppress synovial inflammation but may also be involved in attempts to repair the damaged joint, which lead to excess fibrosis. The fibroblast-like cells of the synovium probably play an important part in this process as previous studies have shown that TGF β is associated with these cells in the synovium,¹¹ and antibodies to TGF β inhibit synovial fibroblast growth in serum free medium in vitro.^{19,20} This has led to the suggestion that TGF β may regulate RA synovial fibroblast growth through continuous autocrine production of this cytokine.

We wished to investigate whether TGF β was able to influence the differentiation of synovial fibroblasts by examining its effect on α actin expression and cell morphology in vitro. We also compared the effects of TGF β on synovial fibroblast differentiation with the effects of other cytokines found in the inflamed RA joint. In addition we examined the effect of IL4, which, like TGF β , is often associated with anti-inflammatory processes, but is generally absent or at very low values in the RA joint.²¹

Methods

CELL CULTURE

Synovial fibroblasts from patients with RA (n=4) and osteoarthritis (OA) (n=4) were obtained from synovium digested with collagenase, as described previously.²² Some synovial fibroblast cell cultures were also obtained from the adherent cell population of cells isolated from inflammatory joint fluids of patients undergoing diagnostic/therapeutic aspiration and injection of joints (n=2). Ethics committee approval was obtained for all procedures. Cells were routinely maintained in tissue culture flasks (Corning) containing Dulbecco's modified Eagles medium (DMEM) with 10% heat inactivated fetal calf serum, 2 mM L glutamine, penicillin (100 U/ml), streptomycin (100 U/ml), and fungizone (0.25 μ g/ml) (standard culture medium). Primary cultures of cells from synovium and synovial fluid contained adherent macrophages but these were removed by successive passaging (by trypsinisation) of cells until pure cultures of synovial fibroblasts were obtained. The purity of the fibroblast population was established by the absence of the monocyte/macrophage marker CD14. All experiments were carried out on cells between passage 5-15.

EFFECTS OF CYTOKINES ON α ACTIN EXPRESSION

Cells were cultured for various time periods (up to five days) with and without individual cytokines or combinations of cytokines. Experiments were carried out at least twice on each of the cell cultures available (n=10). The following cytokines and concentration ranges were used: TGF β 1 (1-5 ng/ml), IL1 α (0.1-5 ng/ml), IL4 (0.1-5 ng/ml), IL6 (1-10 ng/ml), TNF α (1-10 ng/ml), basic FGF (1-20 ng/ml). All cytokines were human recombinant proteins obtained from R & D Systems, UK.

IMMUNOLocalISATION OF α ACTIN

Synovial fibroblasts

Subconfluent and confluent synovial fibroblasts cultured on glass coverslips were fixed and permeabilised in ice cold methanol, washed in phosphate buffered saline (PBS), and incubated with monoclonal antibody to α actin (1:400, Sigma) for 30 minutes at room temperature. After washing in three changes of PBS the cells were incubated with fluorescein conjugated sheep antimouse antibodies (1:100, Sigma) for 30 minutes at room temperature, followed by extensive washing in PBS. Coverslips were mounted in PBS/glycerol (1:9) containing 25 mg/ml of 1,4-diazobicyclo-(2.2.2) octane (to prevent quenching of fluorescence). Cells were examined by fluorescence microscopy using a Nikon Optiphot-2 microscope with an epi-fluorescence attachment, and photographed using the Microflex UFX system. Ten fields per slide were examined and the numbers of cells expressing α actin was expressed as a percentage of the total population. Slides were examined by two independent observers who were blinded as to the particular experiment performed.

Synovium

Cryostat sections (5 μ m thickness) were cut from synovial tissues snap frozen in liquid nitrogen after collection from RA (n=5) and OA (n=5) patients undergoing knee replacement operations. Multiple pieces of synovium from each patient were selected randomly for histological and immunohistochemical studies. Standard histological examination with haematoxylin and eosin were carried out to investigate the degree of synovial thickening. Immunofluorescent staining for α actin was carried out as described above.

WESTERN BLOTTING

Cells were cultured in 60 mm³ culture dishes in the presence or absence of cytokines. Whole cell extracts for polyacrylamide gel electrophoresis (SDS-PAGE) were prepared by adding hot sample buffer to the cells and removing with a rubber policeman. As cells proliferated at different rates in different cytokines it was necessary to add different volumes of sample buffer to the cultures to ensure that an equivalent number of cells were compared on the western blots. The cell numbers for each treatment were calculated from equivalent cultures run in parallel, from which cells were removed by trypsinisation and counted using a haemocytometer. Samples were loaded onto 15% discontinuous polyacrylamide gels and electrophoresed for one hour at 200 V in a mini-gel system (Bio-Rad). Proteins were transferred electrophoretically onto nitrocellulose membrane, and after blocking with 3% gelatin were probed with monoclonal antismooth muscle actin (1:2000, Sigma). Reactive protein bands were detected using biotinylated antimouse antibodies (1:5000, Dako), followed by avidin conjugated to horseradish peroxidase (1:10 000, Dako) and development with an enhanced chemiluminescence technique

Figure 1 Representative photographs to show immunofluorescent staining of α actin in cultures of synovial fibroblasts. (A) In control cultures the majority of cells show no α actin expression and have an elongated, spindle shaped appearance. Occasional cells possess positively stained α actin filaments (arrow). (B) In RA synovial fibroblasts incubated with TGF β 1 (5 ng/ml) for three days the majority of cells are stained for α actin. (C) OA synovial fibroblasts incubated with IL4 (1 ng/ml) for three days. Expression of α actin is characterised by the appearance of numerous bundles of α actin filament (stress fibres), and is accompanied by a change to a flattened myofibroblast type morphology. (D) RA synovial fibroblasts incubated with TGF β 1 (5 ng/ml) in the presence of basic FGF (10 ng/ml) show no increase in the expression of α actin, and remain mainly spindle shaped, or take on a dendritic morphology. (Bar in (A), (B), (D) represents 4 μ m, bar in (C) represents 1 μ m).

(ECL, Amersham International). A quantitative estimate of the amount of α actin expression under different conditions was obtained by examination of band density.

Results

IMMUNOLocalisation of α SMOOTH MUSCLE ACTIN IN SYNOVIAL FIBROBLASTS

Immunofluorescent microscopy of different cultures of human synovial fibroblasts ($n=10$) under standard culture conditions revealed a small but significant population of cells ($14.4 \pm 2.9\%$) expressing α actin (fig 1A). Some background staining of the cytoplasm was seen in most cells but only those cells demonstrating filamentous staining were counted as α actin positive. Although the basal level of expression varied between cell cultures from individual patients it did not seem to be related to whether the patients suffered from RA or OA. For any individual patient, a similar degree of expression was found in growing (subconfluent) and quiescent (confluent) cell cultures.

Upon treatment with TGF β 1 (5 ng/ml) there was a pronounced increase in the number of cells expressing α actin ($68.1 \pm 5.49\%$, $n=10$) within two to three days. This was accompanied by a change in morphology from a typical spindle shape to a flattened, irregular shape, characteristic of a myofibroblast-like phenotype (fig 1B). The expression of α smooth muscle actin was characterised by the appearance of abundant stress fibres within the cytoplasm of the cells. The percentage of cells staining for α actin was maximal at 5 ng/ml with decreasing numbers at 2.5 ng/ml ($50.4 \pm 2.55\%$) and 1.25 ng/ml ($31.8 \pm 1.68\%$). Below 1 ng/ml there was little difference to control cultures.

Other cytokines found within the inflamed joint such as IL1, TNF α , IL6, and basic FGF failed to induce α actin expression at any concentration. Various combinations of these cytokines also had no effect. However, we found that IL4, which is normally absent or found at low concentrations in the RA joint, also caused a significant increase in the expression of α actin by synovial fibroblasts (from OA and RA patients), as well as inducing a similar change in morphology to that of TGF β 1 (fig 1C). Maximal numbers of cells expressing α actin were obtained after three days treatment with 1 ng/ml of IL4. The changed morphology and expression of α actin was maintained in cultures for up to seven days in the presence of TGF β or IL4. Cultures were not examined beyond this time.

We investigated whether any cytokines inhibited the TGF β 1 or IL4 induced α actin expression and morphology changes. TNF α , IL1 α , and IL6 had no effect, but basic FGF inhibited the increased expression of α actin induced by TGF β 1 and IL4. Basic FGF also prevented the TGF β 1 and IL4 induced switching of synovial fibroblasts to a myofibroblast-like morphology (fig 1D). Complete inhibition of the TGF β 1 and IL4 induced effects were obtained with 10 ng/ml of basic FGF. Partial inhibition was seen with lower concentrations of FGF down to 1 ng/ml.

We also examined whether the effects of TGF β 1 and bFGF on synovial fibroblasts were reversible. Removal of TGF β 1 from the medium after three days treatment led to a gradual reduction of α actin staining in the majority of cells, although even after seven days the levels of expression were still higher than those seen in control cultures. The loss of α actin expression was accompanied by a reversion to a spindle shaped morphology over this time period. Similarly a loss of α actin expression was noted when bFGF (10 ng/ml) was added to synovial fibroblasts after three days pre-treatment with TGF β 1, although in this case there was evidence of disruption of the α actin cytoskeleton (fig 2) as well as a reduction in the number of α actin positive filaments in individual cells. The disruption of the α actin cytoskeleton was often accompanied by the cells taking on a smaller, more dendritic morphology. The effects of bFGF were

Figure 2 Disruption of the α actin cytoskeleton in cells pre-treated with TGF β 1 (5 ng/ml) for three days before addition of bFGF (10 ng/ml) for three days. Note that many cells have also taken on a smaller, more dendritic morphology. (Bar represents 2 μ m).

Figure 3 (A) Immunofluorescent staining of α actin in a cryostat section of RA synovium. Little or no staining is present in the lining layer (arrow) but intense staining is found around blood vessels, where smooth muscle cells are situated. (B) α Actin expression in a cryostat section of RA synovium showing a wider zone of staining around the blood vessels. The lining layer is mostly negative (arrow). (Bar represents 8 μ m).

reversible as removal of bFGF from cultures treated with TGF β 1 led to increased expression of α actin, and myofibroblast-like differentiation.

IMMUNOLocalISATION OF α SMOOTH MUSCLE ACTIN IN SYNOVIUM

Synovium from patients with RA or OA demonstrated little or no staining of cells within the lining layer or the underlying extracellular matrix, regardless of the amount of hyperplasia present. The degree of thickening of the synovial lining layer varied between specimens, with synovial hyperplasia clearly evident in three of five RA patients and in one of five OA patients. Bright staining was found around the numerous blood vessels within the synovium, probably corresponding to the perivascular smooth muscle cells in these regions (fig 3A). This staining pattern was found in both RA and OA specimens although it was generally more extensive in those RA tissues where there was an increase in the vascularity of the synovium. In most specimens staining was confined to a zone immediately around the blood vessels, although in one particular RA patient there was a wider area of staining around the vessels, possibly related to proliferation of smooth muscle cells (fig 3B).

WESTERN BLOTting OF SYNOVIAL FIBROBLASTS

The immunofluorescence results were confirmed by western blotting. The effects of TGF β and IL4 were similar on OA and RA synovial fibroblast cultures, and representative blots are shown in figure 4. Expression of α actin was considerably increased by TGF β 1

Figure 4 Western blots to show α actin expression in synovial fibroblasts with and without cytokine treatment. (A) OA synovial fibroblasts after three days in control medium (lane 1), IL4 (lane 2), TGF β (lane 3). (B) RA synovial fibroblasts after three days in control medium (lane 1), TGF β 1 (lane 2), basic FGF (lane 3), TGF β 1 and bFGF (lane 4). Cytokines were used at the following concentrations; IL4, 1 ng/ml, TGF β , 5 ng/ml, bFGF, 10 ng/ml. Molecular weight markers are shown in kilodaltons.

and IL4, although the maximal expression obtained with IL4 was never as great as with TGF β 1 (fig 4A and B).

Cells incubated with TGF β 1 or IL4 in the presence of basic FGF showed little or no increase in α actin expression (fig 4B). Basic FGF alone had no effect on the background level of α actin expression of cells cultured under standard conditions. IL1 α , TNF α , and IL6 also had no effect on α actin expression, and showed no inhibition of TGF β 1 or IL4 induced expression (data not shown).

Discussion

We have shown that fibroblasts derived from synovial tissue are capable of differentiating in vitro into myofibroblast-like cells containing the α smooth muscle form of actin, and that this differentiation can be triggered by TGF β 1 or IL4. Fibroblasts from other sources have been shown to demonstrate a myofibroblast phenotype under the influence of TGF β 1, but as far as we are aware this is the first report to show the induction of α actin expression and myofibroblast-like differentiation by IL4 alone.

Paradoxically we were able to demonstrate little or no α actin expression in the lining layer of RA synovial tissue. This cannot be explained solely by the absence of IL4 from RA synovium and synovial fluid as TGF β has generally been shown to be present at sufficiently high concentrations in the fluid, and has been localised to fibroblast-like cells in synovium.¹¹ The lack of staining in some synovia could possibly be explained by low numbers of fibroblasts in the synovial lining layer. Often the majority of cells (70–90%) present in the lining layer will be macrophage-like cells, although estimates based on the percentage of CD68 positive cells may be open to question because this marker is now known to recognise synovial fibroblast cells as well as macrophages.²³ The percentage of fibroblasts present in the RA synovial lining may vary considerably both between areas of an individual synovium and between synovia from different patients. We might have expected to see, however, some areas of strong staining based on examination of a number of

sections from various areas of each synovium. Another possible explanation for the lack of staining may be provided from our *in vitro* experiments in which we have demonstrated that basic FGF can inhibit TGF β 1 induced expression of α actin and conversion of synovial fibroblasts to myofibroblast-like cells. As basic FGF may also be found at high concentrations in the RA joint this could explain why we were able to demonstrate little expression of α smooth muscle actin in the synovial lining layer of RA synovium. The inhibitory action of bFGF on TGF β induced α smooth muscle actin expression has been reported previously in a study on quiescent human breast gland fibroblasts,⁸ and other antagonistic effects have been shown on TGF β 1 mediated matrix production in porcine vascular smooth muscle and skin fibroblasts.²⁴ Thus the potential of TGF β to induce fibrosis may be significantly modified by the local cytokine environment, and in particular the amount of bFGF present. Extracellular bFGF has been shown to bind to the extracellular matrix and cell surface because of its high affinity for heparan sulphate proteoglycan,²⁵ and this binding seems to be essential for the proper presentation of FGF to its receptors. As TGF β enhances the synthesis and incorporation of proteoglycan into the ECM²⁶ it seems probable that this in turn may regulate the amount of bFGF present in the local environment. In the case of the RA synovium the levels of these particular cytokines may change at different stages of the disease depending on the amount of ongoing inflammation.

It has been shown that both RA and normal synovial fibroblasts express TGF β and native bFGF when cultured in serum free medium, although only RA synovial cells in culture express higher molecular weight isoforms of bFGF.²⁰ It was suggested that endogenous TGF β 1 regulates synovial fibroblast growth *in vitro* through an external autocrine loop, but a similar mechanism seems unlikely for bFGF as neutralising antibodies to bFGF were without effect on cell growth (although it was pointed out that bFGF sequestered within the ECM may have been unavailable to the antibodies). We have found that under serum free conditions the percentage of synovial fibroblasts expressing α actin can vary between 1–30% in cultures from different RA patients (unpublished findings). In some serum free cultures the percentage of cells expressing α actin was reduced compared with standard culture medium while in other cultures there was no change. These variations may reflect differences in the amount of endogenous TGF β being produced in cells from different patients. Also, it is possible that bFGF expressed within the cells can cause internal activation of FGF receptors and have further modulating effects on TGF β induced α actin expression and myofibroblast differentiation. Our data also show that conversion of synovial fibroblasts to the myofibroblast phenotype is reversible in culture, although a certain number of α actin expressing cells are always

present. This confirms previous findings by Desmoulière *et al.*²⁷ who showed that any population of cultured fibroblasts always contains a subpopulation of α actin positive cells, even after cloning, suggesting that expression of α actin is a constitutive feature of fibroblasts *in vitro*, where serum and hence TGF β are present.

Our study suggests that the anti-inflammatory properties of TGF β and IL4 in the inflamed joint need to be considered in the context of their ability to promote differentiation of the synovial fibroblast population. In normal wound healing the induction of the myofibroblast phenotype from granulation tissue fibroblasts seems to be an essential feature of the repair process. These myofibroblasts temporarily acquire some smooth muscle features (such as α actin expression), which disappear after closure of the wound. It has been suggested that this disappearance is caused by apoptosis of such cells.²⁸ Within the inflamed joint the proliferation of synovial fibroblasts and the formation of granulation tissue may be an attempt at repair of the damaged joint, although the early stages of fibroblast activation and growth are more likely to be associated with joint destruction. The possible consequences of TGF β or IL4 inducing conversion of synovial fibroblasts to a myofibroblast-like phenotype are unknown, although a change in the differentiation state of these cells may affect their behaviour in terms of production of cytokines, metalloproteinases, etc. Clearly further studies are needed to examine the properties of this differentiated phenotype.

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