Fucoidan Modulates the Effect of Transforming Growth Factor (TGF)-β_1 on Fibroblast Proliferation and Wound Repopulation in *in Vitro* Models of Dermal Wound Repair

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Aberrant wound healing, either causing scarring or chronic wounds, is a significant cause of morbidity. There is therefore, considerable interest in agents which can modulate certain aspects of the wound healing process. Fucoidans, sulphated polyfucose polysaccharides which may be extracted from *Fucus* spp., have been shown to modulate the effects of a variety of growth factors through mechanisms thought to be similar to the action of heparin. We investigated the interaction between two commercial preparations of fucoidan and transforming growth factor (TGF)-β_1. These preparations of fucoidan, as well as heparin, inhibited fibroblast proliferation at concentrations from 0.01 to 100 mg/ml. The anti-proliferative effects of 1 ng/ml TGF-β_1 on dermal fibroblasts were abrogated by fucoidan preparation *F7* when used at concentrations over 1 mg/ml. In a three dimensional *in vitro* model of wound repair, the fibroblast populated collagen lattice or "dermal equivalent", TGF-β_1 reduced the rate of fibroblast repopulation of a wound defect created by punch biopsy. Addition of fucoidan to the model in the presence of TGF-β_1 increased the rate of fibroblast repopulation of the wound and at 10 mg/ml of fucoidan the number of cells which had migrated into the wounded defect was similar to that of control cultures. These data suggest that fucoidan has properties which may be beneficial in the treatment of wound healing.

Key words fucoidan; wound healing; fibroblast; transforming growth factor (TGF)-β

Cutaneous wound repair is a complex process which has evolved to achieve rapid restoration of skin integrity and protective function after injury.[1,2] Sometimes repair proceeds inappropriately leading to either chronic wounds where healing is pathologically slowed[3] or to scarring where there is an exuberant and unpredictable synthesis of the extracellular matrix.[4,5] These wound healing pathologies are a significant cause of morbidity and consequently there is substantial interest in agents which may modify the wound-healing process.

The fucoidans are a family of sulphated polyfucose polysaccharides. They have attracted considerable biotechnological research interest since the discovery that they possessed anti-coagulant activities similar to those of heparin.[6] They are also reported to possess other properties including antithrombotic, anti-inflammatory, anti-tumour, anti-adhesive, and anti-viral effects (see refs. 7, 8). Many of these effects are thought to be due to their interactions with growth factors such as basic fibroblast growth factor (bFGF),[9,10] and transforming growth factor-β (TGF-β).[11] Fucoidans may, therefore, be able to modulate growth factor-dependent pathways in the cell biology of tissue repair.

The experimental study of tissue repair is made very difficult by its inherent complexity, and in order to isolate individual components of the healing pathway a number of models have been developed. Cell monolayer model systems have been used extensively, but these do not provide a satisfactory representation of fibroblast biology *in vivo* because the cells are arranged closely juxtaposed in a two-dimensional plane with very little extracellular matrix present, whereas *in vivo* fibroblasts are dispersed within a three-dimensional matrix. Fibroblast-populated collagen lattice (FPCL) model systems[12] (sometime called "dermal equivalents") attempt to address some of these issues. Fibroblasts are cultured within a hydrated matrix of type I collagen which undergoes cell mediated reorganisation and contraction to result in a tissue-like structure which, superficially at least, appears to be similar to dermis. This structure has been used extensively to model different aspects of dermal wound repair (see refs. 13—15).

Repopulation of the wound space by fibroblasts from the surrounding dermal tissue is essential for wound repair and is achieved through a combination of fibroblast proliferation and migration.[16] Both processes are influenced by a variety of cytokines of which the TGF-β family are thought to be critical.[17] TGF-β_1 has previously been shown to interact with fucoidans[11] and we therefore speculated that the effects of TGF-β_1 on fibroblast biology within a wound model may be modulated by commercial preparations of fucoidans. We now show that two commercial preparations of fucoidan modulate the anti-proliferative effects of TGF-β_1 on dermal fibroblasts and that one of these preparations restores the rate of wound repopulation after inhibition by TGF-β_1.

MATERIALS AND METHODS

Fibroblast Isolation and Culture Dermal fibroblasts were isolated from human foreskin from a 2 year old donor with local ethical committee approval. The isolated cells were cultured as monolayers in fibroblast culture medium (FCM) consisting of 2 mM L-glutamine, 2 mM non-essential amino acids (NEAA), 1 U/ml penicillin/0.1 mg/ml streptomycin, 10% foetal calf serum (FCS), in Dulbecco's modified Eagle's Medium (DMEM) (all from Invitrogen, Paisley, U.K.), and used between passages 5 and 10.

Two commercial preparation of fucoidan, "Fucoidan fraction 7" supplied by Calbiochem, San Diego, California[18] termed *F7* here, and a cruder preparation of fucoidan from

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Fluka (Sigma-Aldrich Company Ltd. Poole Dorset, U.K.)[19] termed *FF*, were used. Heparin (Sigma-Aldrich) and the fucoidans were dissolved in FCM without FCS at the highest concentration to be used (100 mg/ml) and filter-sterilised through a 0.2 μm filter, FCS was added and further dilutions were made serially into complete FCM. TGF-β_1 (Sigma-Aldrich) was prepared as a stock solution according to manufacturer's instructions and used in all experiments at a final concentration of 1 ng/ml.

Determination of Cell Number Cultured fibroblasts were seeded into a 96-well culture dish at 1×10^3 per well and allowed to adhere overnight. Test medium was added to each well and the dishes were maintained as above for 3 d prior to analysis. Cell number was determined by the MTT assay: culture medium was aspirated and replaced with 100 μl of DMEM and 5 μl of MTT solution (5 mg/ml MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) in phosphate buffered saline (PBS)). Dishes were incubated for 4 h at 37 °C after which 100 μl of extraction buffer (10% w/v sodium dodecyl sulphate, SDS, Sigma-Aldrich) in 0.1 M HCl) was added to each well and incubated overnight and the absorption read at 570 nm on a microtitre plate reader. Results are expressed as relative cell number determined from three replicate wells $\pm$ S.E.M.

Collagen Extraction Rat-tail type I collagen was purified according to Bell *et al.*[12] with minor modifications by Rowling *et al.*[20] Briefly collagen was extracted from rat tail tendon by incubation in sterile 17 mM acetic acid with mechanical stirring for 48 h at 4 °C. Undissolved tendon pieces were removed by centrifugation at 17500 rev/min (30000$\times$***g***) at 4 °C in a SS-34 rotor of a Sorvall centrifuge for 60 min. Sterile 0.1 M NaOH was used to adjust the pH of the collagen solution to pH 7 causing precipitation of the collagen. The precipitated collagen was collected by centrifugation at 8000 rev/min (10000$\times$***g***) at 4 °C in a GSA rotor of a Sorvall centrifuge for 20 min. The type I collagen pellet was transferred to sterile 17 mM acetic acid where it re-dissolved upon stirring at 4 °C for 48 h.

Fibroblast Populated Collage Lattice Fabrication and Wounding FPCLs were fabricated according to Bell *et al.*[12] with minor modifications. The protocol for FPCL fabrication and wounding is shown in Fig. 1. Briefly, to fabricate FPCLs into one 12-well cluster tissue culture plate the following mixture was prepared on ice: 4ml of 2$\times$ DMEM (to make 100 ml combine 20 ml of 10$\times$ DMEM, 10 ml 7.5% $NaHCO_3$, 2 ml L-glutamine, 2 ml NEAA (all Invitrogen) with 66 ml sterile qH_2O), 1 ml FCS, 1 ml FCM containing 1×10^4 fibroblasts, 3 ml of 5 mg/ml collagen solution, and 1 ml NaOH. After mixing, 800 μl were pipetted into each well.

The dishes were maintained at 37 °C in a 5% CO_2/95% air atmosphere for 1 h to allow collagen polymerisation. After polymerisation 2 ml of FCM was added to each well. Lattices were detached from the plastic substratum by ringing gently with a 23-gauge hypodermic needle and allowed to contract for 5—7 d until they reached approximately 10% of their starting diameter. FPCLs were wounded by 3 mm punch biopsy and the wounded FPCL transferred to an acellular collagen gel fabricated identically to the method for FPCLs above but substituting 1ml of FCM for the fibroblast suspension. Ten microliters of un-polymerised collagen was used to adhere the FPCL to the underlying collagen lattice. We have

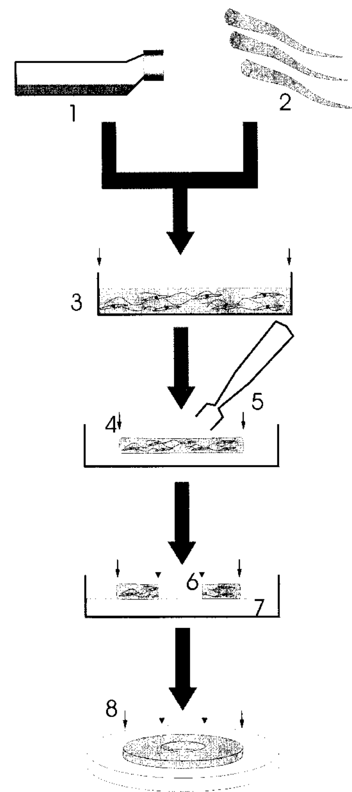

Fig. 1. Fabrication of the Wounded Fibroblast Populated Collagen Lattice

Cultured human dermal fibroblasts (1) were combined with type I collagen in nutrient medium (2) to form the fibroblast-populated collagen lattice (FPCL) (3) as described in Materials and Methods. The FPCL was allowed to contract until it had reached approximately 10—30% of the initial diameter (4). Arrows indicate the maximum diameter of the lattice. The lattices were wounded by 3 mm punch biopsy (5) which completely penetrated the lattice (6): arrowheads are used to identify the diameter of the wound. Immediately after wounding the lattice is transferred to an acellular collagen lattice (7) and 10 μl of collagen solution was applied to the collagen lattice prior to the FPCL being overlaid to act as a "glue" between the two lattices. The resulting wounded model, shown in three dimensions (8), consists of a ring of fibroblast-populated collagen lattice resting on a disk of acellular collagen.

found that 3 FPCLs which had initially been fabricated in 12 well dishes could be easily accommodated on one acellular lattice fabricated in one well of a standard 6-well dish.

Defect repopulation was then measured by counting the number of cells which had migrated from the cut edge of the lattice at 3 d post wounding. Results are expressed as a mean of cell counts in three identical photographic fields from three replicated wounds $\pm$ S.E.M.

RESULTS

Dermal Fibroblast Proliferation The effect of the two preparations of fucoidan and heparin on fibroblast proliferation in the monolayer system are shown in Fig. 2A. Proliferation was determined by the MTT assay after 3 d of incubation in the presence of fucoidan, heparin or control. There was a consistent trend of inhibition of cell growth for all preparations, typically causing an approximately 55% inhibition at 100 μg/ml.

The inhibition of fibroblast proliferation by TGF-β_1 at 1 ng/ml is well established[21] and prior experiments, not reported here, confirmed that the fibroblast cell line used in these experiments was responsive to TGF-β at 1 ng/ml typically causing inhibition of proliferation of 70%. Figure 2B

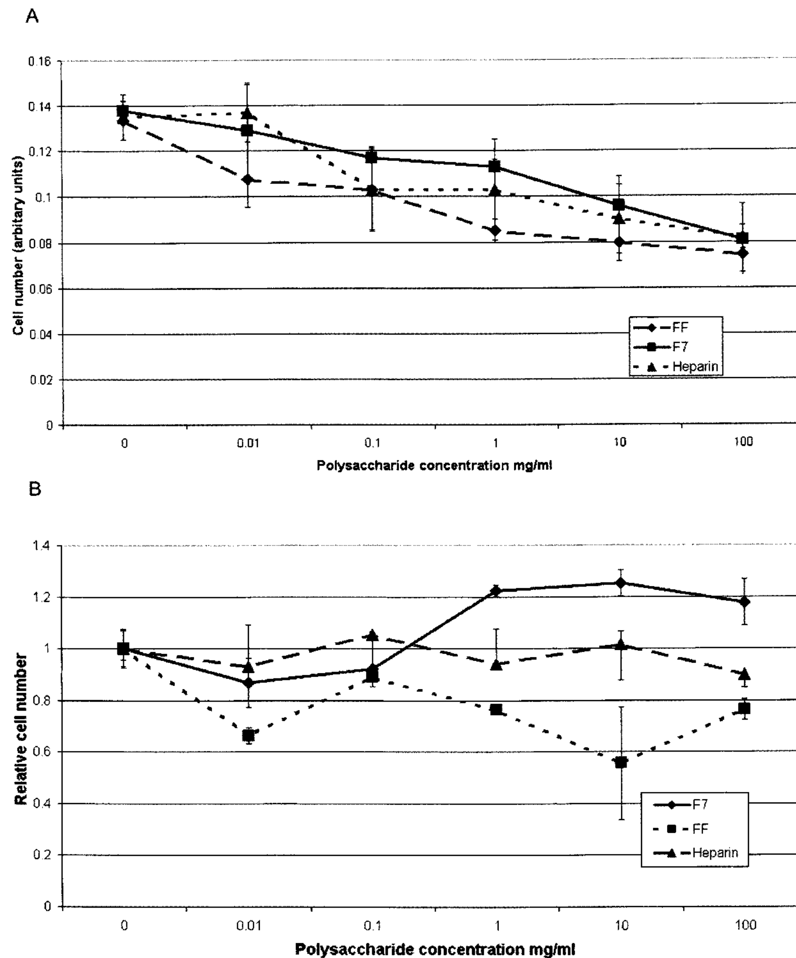

Fig. 2. Effect of Fucoidan on Fibroblast Proliferation and Interaction with TGF-β_1

Fibroblasts were cultured as monolayers as described in Materials and Methods and treated with one of two preparations of fucoidan or with heparin. A: All three polysaccharides caused a dose–response decrease in fibroblast number. B: All cultures were treated with 1 ng/ml TGF-β_1 and results are expressed as changes in relative fibroblast number, *i.e.* control experiments were used as a basis to calculate change in cell number. Addition of *F7* fucoidan to TGF-β_1 treated cultures appeared to reverse the decrease in cell number caused by TGF-β_1 whereas heparin did not alter fibroblast number over a range of concentrations. Cultures treated with fucoidan *FF* showed further decreases in cell number.

shows relative fibroblast cell number in response to treatment with fucoidan preparations or heparin in the presence of TGF-β_1. These data show that *FF* did not alter the effects of TGF-β_1 at any concentration, whereas the purified preparation *F7* caused a relative increase in cell number at concentrations over 1 mg/ml. No similar effect was observed with heparin which inhibited cell growth further at high concentrations.

***In Vitro* Wound Repopulation** The proliferation experiments strongly suggested that one of the commercial preparations of fucoidan, *F7*, interacted with TGF-β_1 to abrogate its anti-proliferative effects. We wanted to determine whether these effects could be observed in a more realistic model of wound repair, the wounded FPCL. We examined the effects of fucoidan on fibroblast repopulation of a wound defect in the 3D model system. Wounding was achieved by punch biopsy as shown in Fig. 1. Defect repopulation was then measured by counting the number of cells which had migrated from the cut edge of the lattice after 3 d.

After wounding, fibroblasts migrated from the cut edge of the wound into the defect. Figure 3 illustrates the appearance of the wound edges 3 d post wounding and shows that 1 ng/ml of TGF-β_1 reduces the number of fibroblasts within the wound defect (compare Fig. 3A with 3B), but addition of increasing concentrations of fucoidan (Figs. 3C—E) increases fibroblast number within the wound to approximately control levels.

Figure 4 shows the effect of fucoidan and heparin on defect repopulation of wounded FPCLs in the presence of TGF-β_1. The rate of wound repopulation is dramatically decreased by TGF-β_1 which is reversed by the addition of increasing concentrations of fucoidan to the wound model.

DISCUSSION

Wound healing is a highly complex yet elegantly co-ordinated process. Medical science would like to be able to intervene when pathology disturbs normal healing. Like many biological phenomena the study of wound healing has benefited from the use of *in vivo* and *in vitro* models which have attempted to identify, isolate and simplify some of the process associated with tissue repair. The fibroblast populated collagen lattice system is a useful model to isolate and study aspects of cutaneous wound healing. Further enhancements such as the addition of other cell types allow the model to be used to study other processes such as re-epithe-

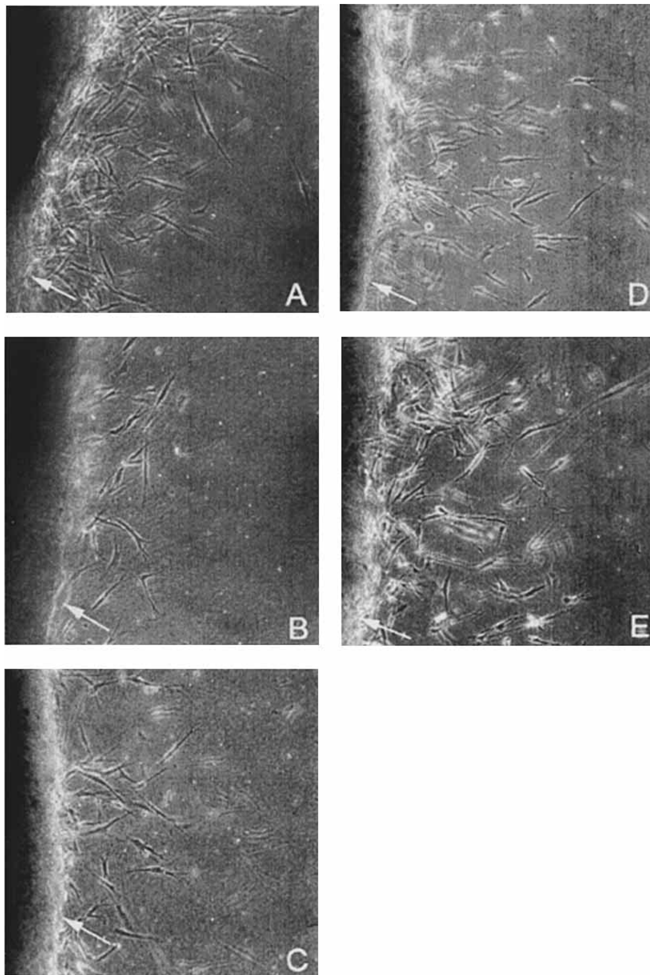

Fig. 3. Effect of Fucoidan on Fibroblast Repopulation of a Wounded Collagen Lattice

FPCLs were fabricated, allowed to contract for 7 d and wounded by complete penetration. The wounded FPCLs were placed onto an acellular collagen lattice and wound repopulation followed. A: Control, B: 1 ng/ml TGF-β_1, C: 1 ng/ml TGF-β_1 plus 0.1 mg/ml fucoidan, D: 1 ng/ml TGF-β_1 plus 1 mg/ml fucoidan, E: 1 ng/ml TGF-β_1 plus 10 mg/ml fucoidan. TGF-β_1 decreased the rate of defect re-population; compare B to A. Fucoidan appeared to abrogate the effects of TGF-β_1 as increasing concentrations of fucoidan restored the rate of defect repopulation to control values; compare C—E to A. All ×400 phase-contrast photomicroscopy. White arrows mark the wound edges of the wounded FPCL.

lialization[22] and angiogenesis.[23] We have used the FPCL model system to examine the rate of fibroblast repopulation of a wound space. Within this FPCL model there are low rates of fibroblast proliferation prior to wounding.[24,25] Wounding activates cell proliferation and migration from the collagen matrix into the wounded defect *via* migration across the underlying collagen lattice (see Fig. 1).[26—28] Cytokines, such as TGF-β_1, can be added to the model and their biological effects followed.

The fucoidans, as isolated, seem to be a mixture of L-linked fucose polysaccharides sulphated to different extents.[8,29] Initial interest focused on the anti-coagulant activity with the aim of identifying a non-animal alternative to heparin (see refs. 30—34). However, as well as their anticoagulant activity, fucoidans have also been shown to interact and modulate the activities of a range of growth factors and cytokines through mechanisms which are thought to be similar to those of glycosaminoglycans such as heparin and chondroitin sulphate.[8] McCaffrey *et al.* showed that fucoidan as well as heparin can bind TGF-β_1 and protect it from proteolytic degradation.[11,35] Given the importance of TGF-β in wound healing we attempted to assess whether fucoidan and heparin could interact with TGF-β_1 to modulate its effects on wound repopulation.

Both preparations of fucoidan and heparin exhibited anti-proliferative effects on dermal fibroblasts in a dose–response manner up to 100 mg/ml. This is in agreement with work done by Ferro *et al.*[36] and similar to results observed using other cell systems such as smooth muscle cells.[37] Addition of TGF-β_1 to fibroblast monolayers dramatically reduced fibroblast proliferation. One of the commercial preparations of fucoidan which we used, *F7*, reversed the effect TGF-β_1 on cell proliferation and caused a relative increase in fibroblast cell number whereas the other one, fucoidan *FF*, a different preparation, did not exhibit similar properties.

The apparent abrogation of the effect TGF-β_1 seen with fucoidan *F7* compared to its anti-proliferative properties in the absence of TGF-β_1 led us to investigate whether this effect was reproduced in the three dimensional model. Fucoidan *F7* appeared to reverse the effect of TGF-β_1 on fibroblast repopulation of the wounded defect whereas heparin

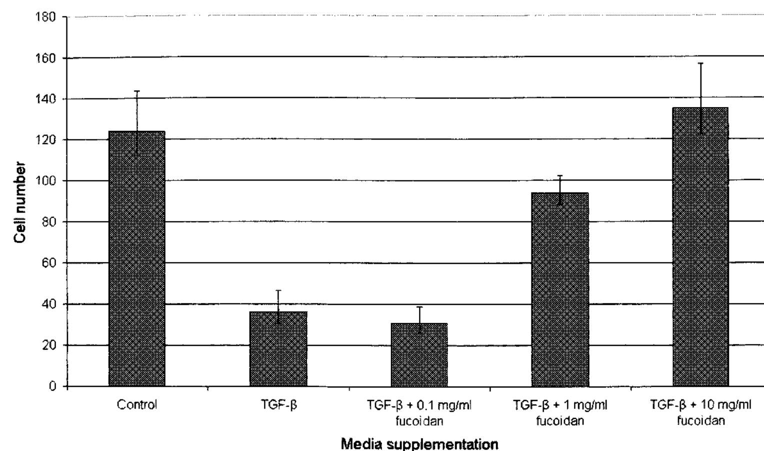

Fig. 4. Fibroblast Repopulation of the Wounded Defect

The number of fibroblasts within the wounded defect was determined by photo microscopy. FPCLs were treated with fucoidan in the presence/absence of 1 ng/ml TGF-β_1, untreated cultures acted as controls. TGF-β_1 decreases the rate of fibroblast repopulation of the wounded FPCL this is restored by increasing amounts of a partially purified fucoidan fraction, *F7*.

did not. In some respects these results are paradoxical. The binding of TGF-β_1 to heparin (or similar molecules) has been shown to potentiate the biological effects of TGF-β_1 by maintaining it in an active form.[38] whereas another study has shown that a heparin mimic can inhibit the fibrotic effects of TGF-β_1.[39]

Interpretation of our data is further complicated by work of Schmidt *et al.*[40] which showed that a heparin-like molecule can induce increased expression of TGF-β in coronary smooth muscle cells which was associated with the expected inhibition of cell proliferation. Our study differs from those discussed here in a number of respects, not least the fact that our preparation of fucoidan is commercial and therefore very heterogenous and contains polymers with potentially differing properties. Secondly we are assessing cell biology within a three-dimensional environment rather than monolayers of cells. Currently our data do not confirm that fucoidan can bind and abrogate the effects of TGF-β and there is obviously scope for other mechanisms to be involved but which we have not assessed. Nevertheless it does suggest that fucoidan can act within the wound environment to modulate fibroblast biology in a potentially clinically useful manner.

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