Basic fibroblast growth factor in cells derived from Dupuytren's contracture: Synthesis, presence, and implications for treatment of the disease

Dupuytren's contracture (DC) is associated with fibroblast and endothelial cell proliferation. We have identified a fibroblast and endothelial cell mitogen, basic fibroblast growth factor (FGF), in cells derived from this tissue and characterized the effects of this growth factor on DC cells. Northern blot analysis of DC cells reveals the presence of basic FGF mRNA species, and the DC cells coexpress multiple forms of basic FGF. Radioreceptor assays establish that the DC cells have high-affinity binding sites for basic FGF and proliferate in response to exogenous recombinant basic FGF. Furthermore, a conjugate between saporin (a ribosome-inactivating protein) and basic FGF, which is cytotoxic to cells possessing the basic FGF receptor, is also cytotoxic to DC cells. The possibility that basic FGF-saporin could be a potential therapeutic agent for prevention of recurrence of the disease after surgery is discussed. (J HAND SURG 1992;17A:324-32.)

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Dupuytren's contracture (DC) is a proliferative disease of the palmar aponeurosis that can interfere with normal hand movement and can even produce deformity.[1-3] The disease is characterized by the presence of lesions with nodules that contain type III collagen, fibroblasts, and myofibroblasts[4] that are thought to emanate from the perivascular cellular cuff of small blood vessels.[5, 6] One of the most unusual features of the disease is that the majority of the capillaries within a nodule are narrowed or in some cases occluded by proliferating endothelial cells.[7, 8] The current treatment for DC is surgical excision of the fibroblastic mass, and recurrence after surgery is common.[2, 9]

The proliferation of fibroblastoid and endothelial cells in the disease has suggested to us that basic fibroblast growth factor (FGF) may be involved in the cause of the disease. Basic FGF is an 18-kD protein that is mitogenic for a number of different cell types, including endothelial cells and fibroblasts.[10] For this reason, we have hypothesized that an increase in the synthesis of basic FGF or an increase in the level of the basic FGF receptor in cells of the palm could lead to the cellular proliferation that characterizes DC. In this article we show evidence that this growth factor can be associated with the cells derived from this tissue.

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Materials and methods

Basic FGF. Basic FGF was a gift from Drs. Paolo Sarmientos and Marco Soria, Farmitalia Carlo Erba, Milan, Italy. Basic FGF-saporin mitotoxin was pre-

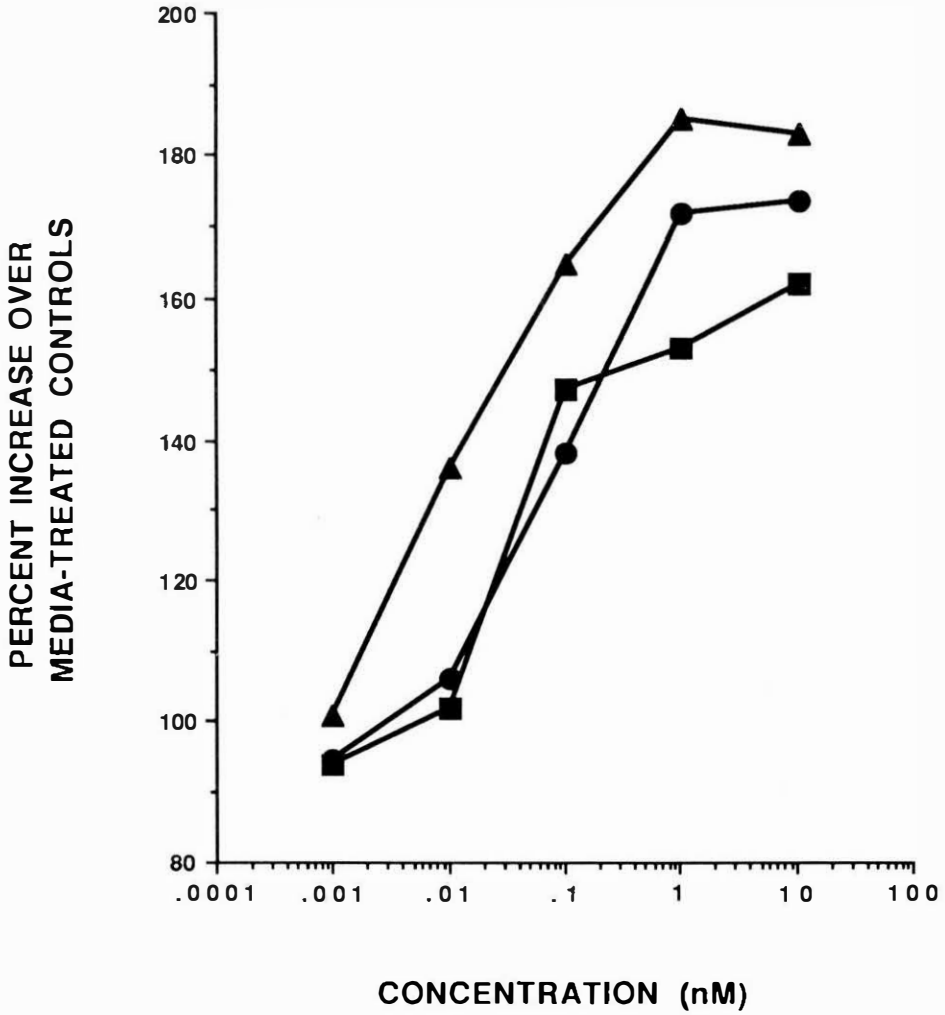

Fig. 1. Proliferative effects of basic FGF on Dupuytren's cells in culture. The growth factor stimulates a dose-dependent increase in the number of cells when counted after 3 days' incubation. The three Dupuytren's cell types responded in a similar manner with respect to sensitivity and maximal response. Cell types: ● = DC 2/5, ■ = DC 5/3, ▲ = DC 3/3.

pared as described previously.[11, 12] Briefly, this is a chemical conjugate that uses a free cysteine of basic FGF and saporin derivatized with N-succinimidyl-3(2-pyridyldithio)propionate (SPDP, Pharmacia, Uppsala, Sweden). The resulting disulfide-linked conjugate has approximately one molecule of basic FGF per molecule of saporin. Protein determinations were made with the BCA protein assay reagent (Pierce, Rockford, Ill.).

Cells. DC 5/3, 3/3, 2/5, and 6/3 cells were isolated from the lesions of four different patients who had undergone surgery for DC at Scripps Clinic and Research Foundation, La Jolla, Calif., and adapted for cell culture as described (Fox R, et al., manuscript in preparation). All four primary cell lines have fibroblastic morphology and contain collagen types I and III. The SK5-T12 cells are a primary culture of human foreskin fibroblasts. They were a gift of Dr. Russell Ross of the University of Washington in Seattle.

Cell proliferation assays. DC 2/5, DC 5/3 and DC 3/3 cells were plated at 10,000 cells per well of 24-well plates and allowed to attach overnight. Basic FGF in media (HEPES-buffered DMEM [GIBCO, Gaithersburg, Md.] with 10% FBS [HyClone, Logan, Utah]) was incubated at the indicated concentrations for 72

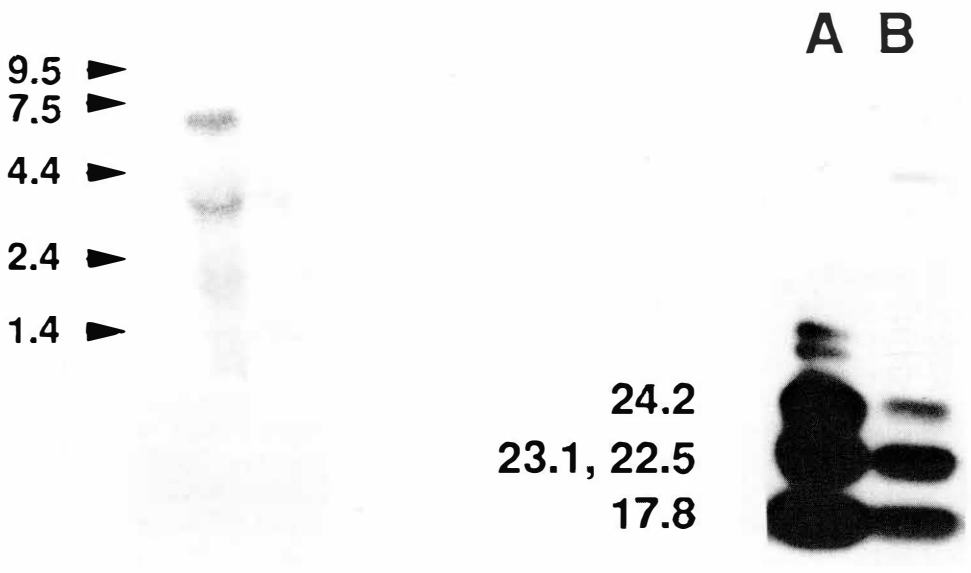

Fig. 2. A, Northern blot analysis of mRNA isolated from Dupuytren's cells in culture. The bands at 6.7 kb, 3.7 kb, and 2.3 kb establish that multiple forms of the mRNA for basic FGF are present in the cells and suggest that these cells actively synthesize the growth factor. **B,** Western blotting of the purified extract of Dupuytren's cells. Lane *A:* ZIP cell extract, which contains the four different species of basic FGF, used as a control.[41] Lane *B:* DC 3/3 cell extract. This figure shows that, as predicted by Fig. 2, **A,** basic FGF is present in the extracts of the Dupuytren's cells.

hours. Cells were treated with trypsin and counted with a Coulter particle counter.

Western blotting of heparin-binding extract of Dupuytren's cells. Analysis of heparin-Sepharose purified cell extracts were performed as previously described[20] with the following modifications: DC 3/3 and ZIP[41] cells were grown to near confluence. Cells (2×10^6) were lysed and incubated with heparin-Sepharose. Protein A-purified rabbit polyclonal antibody to the 1-24 peptide of basic FGF was used, diluted 1:500. Molecular weight markers were determined from expression of the four species in ZIP cells.

Receptor binding assays. Assays for Scatchard analysis were performed according to the procedure of Moscatelli[13] with minor modifications. Cells were washed one time with 2 M NaCl to remove low-affinity bound ligand. High-affinity receptor bound basic FGF was extracted by washing with 0.05% triton X-100 in water.

Cross-linking of basic FGF to the basic FGF receptor. Radiolabeled basic FGF was cross-linked to the cell surface receptor of DC 3/3, DC 2/5, DC 5/3, and baby hamster kidney (BHK) cells with the following modifications to the previously described procedure[14]: 1×10^6 cpm/15 ml of radiolabeled basic FGF was incubated with subconfluent cells in 10 cm plates. The radiolabeled cross-linked proteins were visualized by autoradiography after electrophoresis in 15% polyacrylamide SDS-PAGE (sodium dodecyl sulfate polyacrimide gel electrophoresis) gels.

Tyrosine kinase activation. Tyrosine kinase activation was performed with the use of antiphosphotyrosine antibodies as described by Pasquale et al.[15] Briefly, confluent DC 2/5, 3/3, and 5/3 cells were made quiescent by washing and incubation in serum-free media for 2 days. Basic FGF or control media were added, and after 5 minutes cells were harvested into sodium dodecyl sulfate sample buffer. The samples were electrophoresed in 7.5% polyacrylamide gels, and the proteins were transferred to nitrocellulose. Transfers were incubated with antiphosphotyrosine antibody and then with ^{125}I-labeled protein A. The transfers were autoradiographed overnight at $-70°$ C.

Northern blot analysis. Analysis was performed as described by Emoto et al.,[16] with some modifications. Briefly, cells were grown to confluence in four 10 cm tissue culture dishes. Cells were removed from the plates with trypsin and homogenized with a Polytron homogenizer (Brinkman, Westbury, N.Y.) in 4 M guanidine thiocyanate. RNA was pelleted by ultra-

centrifugation through 5.7 M CsCl, extracted with phenol/chloroform, and precipitated with ethanol. Samples of total RNA were denatured for 5 minutes in 50% formamide at 65° C and separated on 1% agarose gel containing 2% formaldehyde. RNA was transferred to Zeta-Probe blotting membrane (Bio-Rad Laboratories, Richmond, Calif.) by the capillary transfer method. Membranes were vacuum-dried and stained with methylene blue to verify transfer. A probe 460 bp in length, encoding human basic FGF, was used for hybridization. The probe was labeled with α-^{32}P-cytidine triphosphate by the Multiprime DNA labeling system (Amersham, Arlington Heights, Ill.). Blots were hybridized at 42° C and then washed in 2 × SSC 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0 at 50° C for 30 minutes and 0.1 × SSC for 15 minutes at 55° C and autoradiographed.

Cytotoxicity of basic FGF-saporin. Cytotoxicity was determined by measurement of ^{3}H thymidine incorporation. Cells were plated at a density of 2000 cells/100 μl in 96-well plates and allowed to attach overnight. Test samples (100 μl) were added at the indicated concentrations the next morning and replaced with fresh samples 2 days later and 3 days later. Sixteen hours after the final addition, 20 μCi of ^{3}H thymidine was added and incubated for 3 to 8 hours. Cells were frozen, thawed, harvested on a cell harvester, and counted according to standard scintillation counter techniques. Untreated cell (control) ^{3}H thymidine incorporation for DC 2/5 cells was 2660 ± 213. Incorporation for cells treated at 10 nM (570 ng/ml) basic FGF-saporin was 65 ± 14. All values were standardized with incorporation by untreated wells as 100%.

Results

Responsiveness of cells derived from DC to basic FGF. Basic FGF stimulates DC cell proliferation in a dose-dependent manner (Fig. 1). All three cell types responded similarly, showing similar ED_{50}s (the dose at which 50% of the maximal response is seen) and similar maximal response (165% to 185%).

Basic FGF mRNA. For examination of the synthesis of basic FGF in Dupuytren's cells, total RNA was isolated and prepared for Northern blot analysis and hybridized with a human basic FGF cDNA probe. Fig. 2, *A* shows the autoradiogram from DC 6/3 cells, with a band of approximately 6.7 kb in length, a band of 3.7 kb and a third band of 2.3 kb, representing multiple forms of basic FGF MRNA. The three other DC cell types showed similar distribution of bands (data not shown). This is consistent with data from other laboratories analyzing RNA preparations for basic FGF message from human tissue extracts or cell cultures[17, 18] and indicates active synthesis of basic FGF by these cells.

Table I. Results of Scatchard analysis of ^{125}I-FGF binding to DC cells and a fibroblast culture

	Basic FGF binding	
Cell type	*Kd (pM)*	*High affinity sites/cell*
DC 2/5	41	10,800
DC 3/3	49	14,000
DC 5/3	71	10,400
DC 6/3	33	11,400
SK 5-T12	20	2,300

Scatchard plot analyses for binding of ^{125}I-basic FGF were performed as described by Moscatelli.[13]

Presence of basic FGF. We examined Dupuytren's cells for the presence of basic FGF by Western blotting. Cell extracts were partially purified with heparin-Sepharose[19] and analyzed with an antibody to basic FGF. Fig. 2, *B* shows the pattern of immunoreactive basic FGF detected. The results agree with those previously described for other cells in culture and reveal the presence of basic FGF in three of its molecular forms.[20, 21] Thus basic FGF is present in the DC cells.

Presence of the basic FGF receptor. We examined the cells in culture for the presence of the basic FGF receptor on the cell surface. Scatchard plot analysis revealed the presence of the high-affinity receptor. As seen in Table I, dissociation constants ranged between 33 and 71 pM, which is typical of the high-affinity receptor.[13] The number of high-affinity sites per cell was between 10,400 and 14,000 sites. While this number is high with respect to endothelial cells, which have been reported to contain between 2500 and 8000 receptors per cell, Moscatelli[13] has reported a chicken embryo fibroblast cell line to have 13,800 basic FGF receptors per cell and a bovine embryo skin fibroblast to have 3200 receptors. We have examined one primary culture of "normal" human foreskin fibroblasts (SK5-T12). These cells have 2300 receptors per cell (Table I). The number of receptors on the Dupuytren's cells thus appears to be in the high range for fibroblast cell types.

Cross-linking of basic FGF to the cell surface. When radiolabeled basic FGF is cross-linked to BHK or vascular endothelial cells, two bands of M_r 130 kDa and 150 kDa can be visualized by autoradiography of the electrophoresed cell extracts.[14, 22] We have used these methods to analyze the cross-linking of radiolabeled basic FGF to the surface of three DC cell lines.

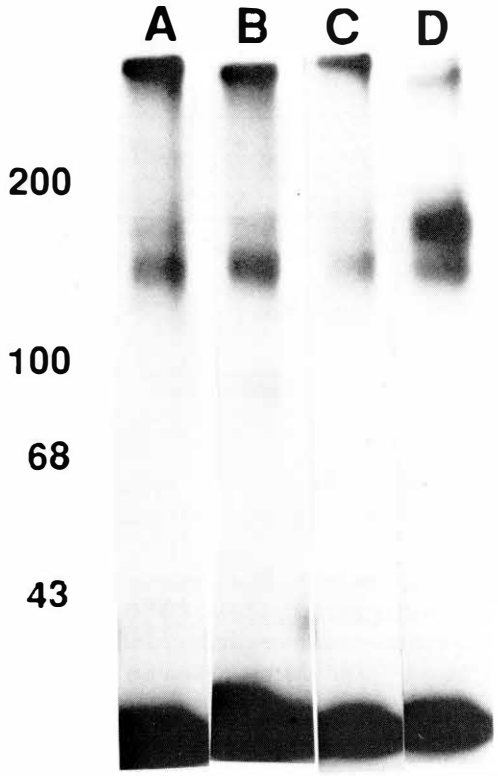

Fig. 3. Cross-linking of radiolabeled basic FGF to DC cells. Lane *A:* DC 2/5; lane *B:* DC 3/3; lane *C:* DC 5/3; lane *D:* BHK cells. Lane *D* shows the typical cross-linking pattern of iodinated basic FGF to its receptor. Cross-linking to the DC cells shows a similar pattern, indicating that a basic FGF receptor is present on the surface of DC cells and that the receptor size is similar to that of BHK cells. The position of molecular weight standards is indicated on the left (kDa $\times 10^{-3}$).

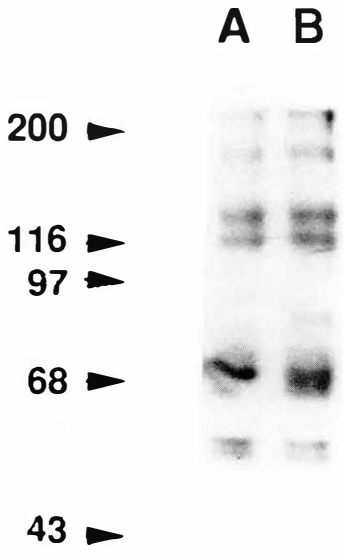

Fig. 4. Stimulation of tyrosine phosphorylation by basic FGF. Immunoblotting with antiphosphotyrosine antibody to DC 2/5 cells treated as follows: Lane *A*, control (no treatment). Lane *B*, cells treated with basic FGF. Molecular weight markers (kDa $\times 10^{-3}$) are shown on the left. The appearance of the band of phosphotyrosine in the basic FGF treated lane demonstrates that the basic FGF receptor in Dupuytren's cell is functional and thus active.

Fig. 3 shows the autoradiograms and compares the pattern observed with the DC cells with that of BHK cells (Fig. 3, *D*). All three DC cells (Fig. 3, *A* to *C*) exhibit a pattern similar to that seen with BHK and vascular endothelial cells.

Tyrosine kinase stimulation. Several laboratories have recently reported that the basic FGF receptor contains a tyrosine kinase domain.[23-25] Basic FGF stimulates phosphorylation on tyrosine of a 90 kDa protein, as detected by immunoblotting with antiphosphotyrosine antibodies.[15, 26] Immunoblotting analysis with antiphosphotyrosine antibodies[15] of total DC cell proteins after mitogenic stimulation by basic FGF was carried out. Fig. 4 shows the autoradiogram with DC 2/5 cells. There is a stimulation of tyrosine phosphorylation by basic FGF of a protein with a M_r of 85-90 kDa (lane *B*). DC 5/3 and 3/3 gave similar autoradiograms.

Basic FGF–Saporin treatment of cells. Three of the Dupuytren's cell cultures were incubated with a mitotoxin that has been shown to bind specifically to the basic FGF receptor and mediate cell death.[11, 12] The conjugate is cytotoxic to all three DC-derived cell types. A concentration of 1 nM (57 ng/ml) basic FGF-saporin reduced cell viability by 60% to 95%. A representative experiment showing the effect of the mitotoxin is presented in Fig. 5. The data show that DC cells are sensitive to the basic FGF-saporin conjugate in a dose-dependent manner. Saporin alone or a mixture of unconjugated saporin and basic FGF had little effect on cell viability.

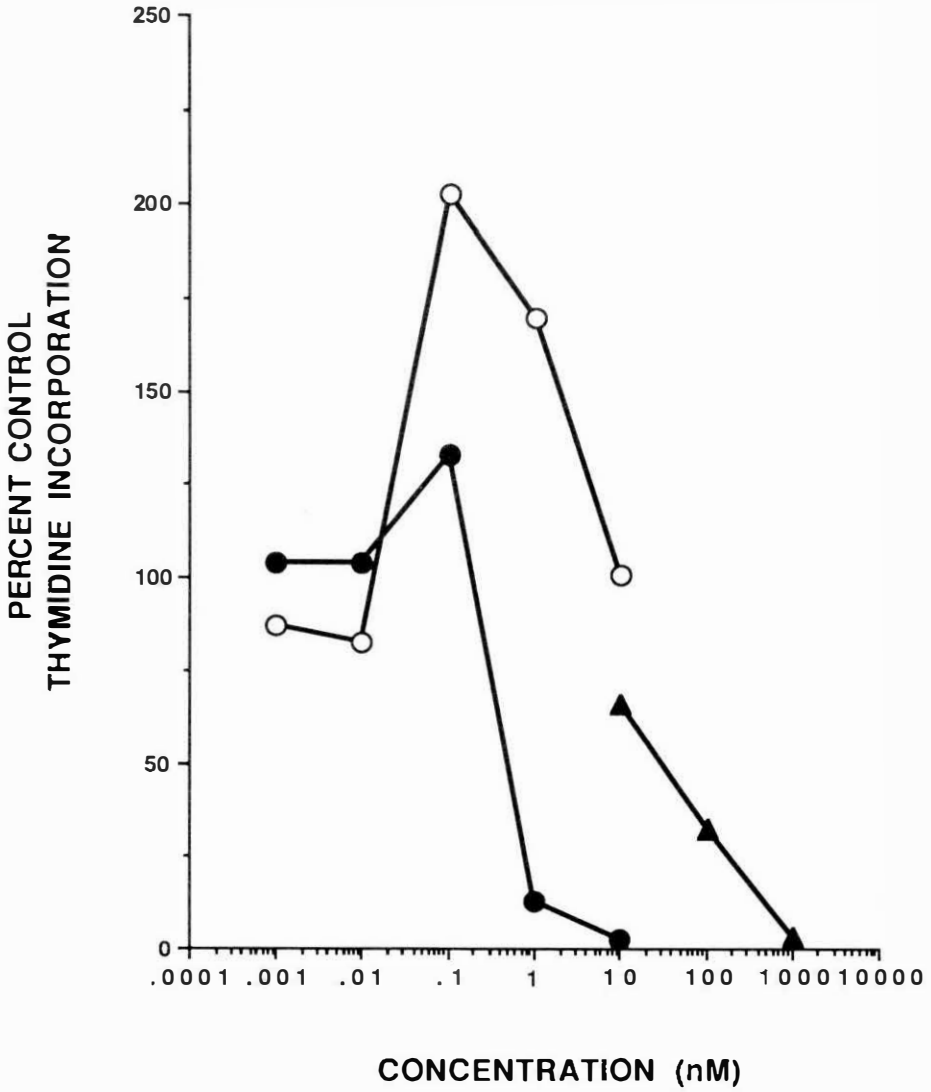

Fig. 5. Cytotoxicity assay of basic FGF-saporin conjugate on DC 2/5 cells. ● = Basic FGF-SAP; ○ = equimolar mixture of basic FGF and saporin; ▲ = saporin alone. Saporin targeted to the cells by basic FGF is cytotoxic to Dupuytren's cells, while saporin alone is not. Thus the basic FGF receptor can be used to eliminate Dupuytren's cells.

Discussion

The occlusion of the capillary lumen by vascular endothelial cells and the proliferation of (myo)fibroblasts in DC suggest the presence and the active role of growth factors that act on endothelial and fibroblastoid cells. It is interesting that there is a high incidence of DC in persons with diabetes who have vision-threatening retinopathy, a disease that has been associated with basic FGF.[27-30] Because of these associations, we have looked at the presence, synthesis, and effect of basic FGF in cells isolated from tissue excised from the mass of proliferating fibroblasts that make up the lesions in Dupuytren's disease.[2] Because these cells have some of the properties of the (myo)fibroblasts that are thought to be responsible for the contraction in DC,[2-4, 31-32] we reasoned that their responsiveness to growth factors might provide insight into the pathogenesis of the disease. Previous studies by Vande Berg et al.[4, 33] have shown that fibroblast cultures from granulating wounds and from DC show enough similarities to the endogenous cell type that they can be used as an in vitro model of the disease. For this reason, we have examined the

production of basic FGF in DC-derived cell types to correlate these findings with those obtained with fresh tissue.

Northern blot analysis shows that the mRNA for basic FGF is present in these cells. Accordingly, the cells synthesize basic FGF that can be detected by Western blot analysis of heparin-Sepharose–purified cell extracts. Basic FGF activity is modulated through a high-affinity receptor,[13, 25] and we here report the presence of these receptors at relatively high levels on the surface of the Dupuytren's cells and the proliferative response of the cells to exogenously added growth factor. Cross-linking studies and analysis of stimulation of tyrosine phosphorylation show the receptor to be similar to that reported on other cell types.[23, 25, 34, 35] Thus, all the conditions are present for an autocrine role for basic FGF in the proliferation of DC cells. Such an autocrine stimulation has been suggested for the transformation of melanocytes to melanomas[36] and could explain, in part, the observation by Gabbiani and coworkers that cultured fibroblasts from Dupuytren's tissue have characteristics intermediate between normal human fibroblasts and sarcomatous or virus-transformed cells.[37] If this were the case, then specific therapies, mediated through the basic FGF receptor, might provide a mechanism to inhibit cell proliferation.

We have recently reported immunohistochemical staining and in situ hybridization studies of lesions from patients who had undergone surgery for DC.[38] Basic FGF was distributed throughout the lesions and also in normal tissue of the palmar aponeurosis. In contrast, synthesis of the mRNA for basic FGF and for the basic FGF receptor was detected in the lesions but not in the normal hand tissue.[38] This work suggests that there is an ongoing synthesis of both the receptor for basic FGF and the growth factor in the disease and that these events could drive the observed cell proliferation. This provides further rationale for treatment of the disease utilizing the basic FGF receptor.

We have previously described several basic FGF antagonists whose activities are mediated through the basic FGF receptor.[11, 12, 39] One antagonist we have developed is a chemical conjugate between basic FGF and saporin, a potent ribosome-inactivating protein from *Saponaria officinalis*.[40] Saporin is targeted to the basic FGF receptor by the FGF moiety, is internalized by the receptor, and inhibits protein synthesis in the cell, which results in cell death. The observation that DC cells express the basic FGF receptor and are sensitive to the mitotoxin indicates that the proliferating myofibroblasts in the palmar aponeurosis may also be sensitive to FGF antagonist therapy. This novel approach offers a potential treatment of DC, for which there is now only surgical treatment, and may also help in preventing the approximately 50% recurrence rate that is seen in the disease after surgery.[9]

Ribosome-inactivating proteins have been used clinically, both nonconjugated for treatment of AIDS[42, 43] and as immunotoxins for treatment of tumors[44-46] or graft-versus-host disease.[47] Problems that have arisen include lack of efficacy,[44] collateral toxicities,[45] and development of neutralizing antibodies to the foreign protein.[46] The suggestion of the use of basic FGF-saporin must be given with the necessary caveat of these problems. DC in the hand may be a disease in which many of these problems could be circumvented. Local treatment might avoid some collateral toxicities and the formation or access of neutralizing antibodies. Also, we propose this molecule as a model for treatment of DC. Alternative pharmaceutical agents, which would rely on the basic FGF receptor for targeting, may be constructed from approved cytotoxic or cytostatic materials.

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