Activities of Human Acidic Fibroblast Growth Factor in an In Vitro Dermal Equivalent Model

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Acidic fibroblast growth factor is a potent mitogen for human dermal fibroblasts in an in vitro three-dimensional collagen matrix, the “dermal equivalent.” Both cell numbers and DNA synthesis are optimally stimulated by daily doses of 1 ng/ml of the pure human mitogen in the presence of heparin, which binds to, and stabilizes, the protein. Under daily treatment by 1 ng/ml aFGF, the fibroblast mitogenic response is marked but transient, and decreases steadily when fibroblasts mature in the collagen matrix. aFGF mitogenic stimulation also results in a decrease in cellular volume and inhibition of fibroblast-mediated contraction of the collagen gel. Various dosing regimes indicate that, although the greatest mitotic response was generated by daily dosing, nearly optimal responses can also be achieved with either a short duration of early daily dosing or longer-term intermittent treatment. J Invest Dermatol 97:793–798, 1991

Fibroblast growth factors (FGF) are a family of homologous proteins that were initially recognized in tissue homogenates over 50 years ago as a fibroblast mitogenic activity uniquely abundant in brain [1,2] and later also shown to be present in pituitary [3]. Brain [4] and pituitary [5,6] were reported to contain at least two mitogenic activities distinguished by apparent acidic and basic isoelectric points. Both acidic FGF (aFGF), the mitogen recovered from brain in greater abundance and basic FGF (bFGF), the principal FGF of pituitary, were purified to homogeneity [7–9], sequenced, and recognized to be homologous to one another [10,11]. Basic FGF has subsequently been found in a variety of tissues (reviewed in [12]), perhaps, in part, reflecting its presence in vascular endothelial cells [13,14]. Acidic FGF appears to be less widely distributed, although, in addition to brain and retina, it has been identified in kidney [15], bone [16], and heart [17]. Both FGF, which compete at one or more common receptors [18,19], stimulate division of many types of cells of mesodermal and ectodermal origin [12,20].

Over the past several years, five additional members of the FGF family, Int-2 [21], HST/K-FGF [22,23], FGF-5 [24], FGF-6 [25], and KGF [26], have been identified, cloned, and sequenced. These mitogens all appear to bind to heparin-affinity resins, presumably reflecting their physiologically significant partitioning to cellular and basement membrane heparin proteoglycans in vivo [27,29]. Heparin binding appears to stabilize both aFGF and bFGF from certain types of denaturation [30–34]. This stabilization results in enhanced activity in vitro and in vivo of the relatively labile aFGF compared to the more stable bFGF, which is fully active without heparin. Tight heparin binding is the basis for the alternative FGF nomenclature of heparin-binding growth factors.

Fibroblasts, the principal resident cell of the dermis, are induced to divide in serum-free medium in culture by aFGF [35]. In vivo, fibroblasts live and grow in a matrix of collagen that they secrete. In response to injury, they are able to contract the dermal collagen that is in its maximum manifestation (keloids) results in disfigurement. This fibroblast-mediated contraction of collagen can be mimicked in vitro in the three-dimensional skin equivalent model culture system first described by Bell and colleagues [36]. In this “dermal equivalent” model, fibroblasts can reach a high degree of differentiation, exhibiting a bipolar morphology, controlled cell division [37], regulated synthesis of macromolecules [38], and both membrane permeability and prostaglandin-H synthetase activity levels [39] similar to those found in vivo. The effect of recombinant human aFGF stimulation on human dermal fibroblasts was evaluated in this three-dimensional collagen matrix culture system.

MATERIALS AND METHODS
Preparation of the Dermal Equivalent Cultures. The dermal-equivalent cultures were prepared by a method modified from Bell et al [36]. Ten different fibroblast strains, all obtained from healthy donors, were grown as previously described and used between the fifth and eighth passages [40]. The cells were released from their substratum by treatment with 0.025% trypsin, 0.2% EDTA, and rapidly mixed with a solution of pepsin-extracted human placental collagen (gift from Institut Merieux, Marcy l’Étoile, France). Fibroblasts were suspended at a concentration of 4 × 10⁶ cells/ml of Eagle’s minimum essential medium with Earle’s salts (EMEM, Boehringer) containing 10% fetal calf serum (FCS) and antibiotics as previously described [40]. One volume of this cell suspension was gently stirred with a solution containing 5 volumes of 3 mg/ml human collagen, 2 volumes of 5X EMEM, and 1 volume each of FCS and distilled water. Petri dishes (10 cm diameter) containing 10 ml each of this mixture were placed in an incubator equilibrated with 5% CO₂ at 37°C and 100% relative humidity for 2–3 h until the gel that formed could be detached from the wall of the Petri dish by mild shaking. All cultures were done in triplicate.

Characterization of Dermal Equivalents. Fibroblast-induced collagen contraction was assessed by daily measurement of the diameter of the dermal equivalent disk. Fibroblast mitogenesis was monitored by incorporation of (3H) thymidine (0.5 μCi/ml) into DNA for 24 h. Experiments were terminated by rinsing the dermal equivalent with phosphate-buffered saline (PBS) and lysing the collagen gel with 2 mg/ml of collagenase (Sigma type 1A 69891) in PBS at 30°C with gentle shaking until the collagen was dissolved. Collagenolytic activity was stopped by addition of FCS to 10% final

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volume. Fibroblasts were suspended in 1 ml PBS and their number and size distribution was determined using a Model 2M Coulter Counter. Finally, aliquots were hydrolyzed with an equal volume of 2N NH₄OH, 4% triton (9/1, v/v) at 100°C for 1 h, and 400 μl was precipitated with 1 ml of 10% trichloroacetic acid on a filter for β scintillation counting (LKB 1212 RackBeta) of the radiolabeled DNA.

For flux cytometry, dermal equivalents were treated daily until day 14 with 10⁻⁹ gr/ml aFGF or control. Fibroblast aliquots were obtained at days 0, 5, 7, 11, 22, and fixed in pure ethanol (Merck) at −20°C. Prior to analysis, rehydration was undertaken in 25% (v/v) ethanol for 1 h, followed by PBS and overnight incubation in 100 μg/ml Mitomycin (Sigma) for DNA staining. Fluorescence was measured with an ATC 3000 ODAM Cytosensor equipped with an argon laser beam, at 457 μm, providing cell counts in Phase GO-G3S and G2.

Preparation of Recombinant Human aFGF The 140 amino acid microheterogeneous form of recombinant human aFGF was expressed in Escherichia coli from a synthetic gene carried in a pKK2.7 plasmid. The cells were lysed and the recombinant product purified as described [41], with the exception that the protein was eluted from the final C4 reversed-phase HPLC column with a gradient of ethanol in 10 mM phosphoric acid instead of the potentially toxic acetonitrile in 10 mM trifluoroacetic acid. The protein was eluted with approximately 35% (v/v) ethanol and stored at −70°C as a 0.45 mg/ml stock solution. Identical solution devoid of the aFGF served as control. Immediately before use, 30 μl of the aFGF stock solution, or of the control solution, were thawed, diluted tenfold in 35% ethanol, 10 mM phosphoric acid, and mixed with 500 μl of EMEM containing 10% FCS and 50 μg/ml heparin. These solutions were sterilized through a filter (Millex GV, SLGV 0.25 S, Millipore) previously rinsed with 500 μl of the same EMEM/FCS/heparin solution, and any residual aFGF was flushed through the filter with 500 μl of the same medium. The combined 1.3 ml solution containing 10 μg/ml of aFGF (or the control solution) was used for subsequent serial dilutions performed with the EMEM/FCS/heparin medium. The aFGF filtration and all subsequent serial dilutions were performed using Nunc polyethylene tubes to minimize adsorption. A possible biologic effect of the ethanol and phosphoric acid components in the stock solution was eliminated by the demonstration that, at their maximum concentrations present in the highest aFGF dose used (100 ng/ml), they did not elicit any change in the dermal equivalents. For each treatment of dermal equivalent, 100 μl of EMEM/FCS/heparin medium was used with the quantity of aFGF, or of control solution, necessary to obtain the chosen final concentration.

RESULTS

Fibroblast Proliferation and Cell-Matrix Interactions Daily addition of aFGF to fibroblast cultures in the dermal equivalent collagen matrices results in increased cell number (Fig 1A) and DNA synthesis (Fig 1B) on day 9. The cell number approximately doubles from 4 × 10⁴ to 8 × 10⁴ in the serum-containing medium without aFGF. The optimal 1 ng/ml dose of aFGF results in slightly greater than one additional doubling, to over 17 × 10⁴ cells. DNA synthesis, monitored by the 24-h incorporation of [³H] thymidine, is enhanced approximately fourfold over the solvent control-treated cells by day 9. Both the cell proliferation and DNA synthesis dose-response curves are bell-shaped, revealing a high concentration inhibition by aFGF. Fibroblast-mediated collagen contraction was inhibited by aFGF in a dose-dependent manner (Fig 1C). Inhibition was observed at 100 pg/ml and reached a plateau of 1–100 ng/ml. No high-concentration inhibition of contraction was noted, indicating that the cells were still viable and responsive to these elevated concentrations of aFGF. Contraction as a function of time is shown in Fig 1C (inset), both with and without daily doses of 100 ng/ml aFGF. Significant inhibition of contraction by aFGF was observed as early as day 5.

The fibroblast DNA synthetic and proliferative responses to daily 1 ng/ml doses of aFGF over a 21-d time course are shown in Fig 2. Cell numbers increase over the serum-stimulated controls beginning on day 4 and steadily grow through day 14, corresponding to 8.4 times more cells or approximately three population doublings (Fig 2A). In contrast, the control cultures grown in the presence of 10% FCS with no aFGF increase only 2.7 times or 1.4 population doublings. The 9-d cell proliferation dose-response data presented in Fig 1A can be seen to occur near the middle of the response curve as a function of time. As expected, DNA synthesis (Fig 2B) precedes cell division. The cells respond to aFGF stimulation by day 2, reaching a maximal response plateau in the rate of DNA synthesis from days 4 through 7, which is approximately 3 times that of the control.

Figure 1. Dose-response of aFGF on the dermal equivalent. Human collagen dermal equivalents initially containing 4.5 × 10⁵ human dermal fibroblasts supplemented with 10% fetal calf serum were seeded daily with human aFGF. The response at each concentration of aFGF is the average of triplicate determinations and the error bars represent SEM. (A) Cell number was determined after 9 d of treatment. (B) [³H]Thymidine was added 24 h before measuring its incorporation into DNA on day 9. (C) Collagen gel disk diameters were measured on day 9. (Inset) Diameter measured as a function of time at 0 (³) and 10⁻⁹ (•) gr/ml aFGF per day.
Figure 2. Response kinetics of fibroblasts to daily stimulation by aFGF. Human fibroblasts in the collagen gel dermal equivalents were dosed daily for 21 d with either 0 (○) or 1 (●) ng/ml aFGF. The mitogenic responses are monitored directly by cell number (A), [3H]thymidine incorporation into DNA (B), and mean cell volumes (C). Error bars, SEM of triplicate determinations.

cells maintained in 10% FCS. Although the DNA synthetic rate decreases sharply after day 7, it remains greater than in the control cells at all times. Cell number continues to increase linearly for some days, but the slope then flattens and appears to reach a plateau at day 22.

Interestingly, fibroblasts treated with aFGF exhibit an initial 1.7-times increase in volume on day 3, followed by a shrinkage starting on day 4 that continues until, on day 22, the cells are slightly smaller than on day 0 (Fig 2C). The control cells show virtually identical increases and decreases on days 3 and 4, but appear to rebound to maintain a doubled volume through day 14. Thereafter, the mean volumes of both cell populations decrease in a similar fashion. The
decrease could be elicited by a blockage in the G2 phase but this hypothesis was ruled out by flow-cytometry analysis (Fig 3A,B). Interestingly, cell counts in the S and G2 phases reach a maximum within a few days but decrease steadily thereafter despite continued aFGF until day 14, supporting the hypothesis that aFGF mitogenic effect is marked but transient.

The reversibility of the aFGF-induced inhibition of matrix contraction was investigated by terminating the 1 ng/ml aFGF daily additions after 4 d while continuing to monitor fibroblast numbers and collagen gel contraction up to day 22 (Fig 4). Despite the cessation of treatment on day 4, aFGF-mediated inhibition of contraction was clearly visible on day 8 and persisted through day 22 (Fig 4A). By day 4, aFGF induced slightly more than one additional doubling in cell number compared to those receiving no aFGF. This difference was also maintained at day 22 (Fig 4B). Once again, the mean cellular diameter of the aFGF-treated cells remained smaller (12.9 μm) on day 22 than the control cells (16.5 μm).

Effect of aFGF Dosing Regimes The effect of daily aFGF dosing (1 ng/ml) for 14 d was compared with the efficacy of a) four intermittent doses every third day, b) a single application during the preparation of the dermal equivalent, and c) a 3-h exposure of trypsinized cells to the mitogen followed by a change of medium prior to their incorporation into the dermal equivalent. As before, daily dosing of aFGF induced an additional cell doubling (Fig 5A), an approximately 4.5-times increase in the rate of DNA synthesis (Fig 5B), and a clear inhibition of matrix contraction (Fig 5C) measured on day 15. The four intermittent doses of aFGF supported almost as
numbers were determined and compared to untreated control cultures of the same age (Fig 6). The rather weak mitotic stimulation by aFGF in the freshly prepared dermis might be attributable to the high death rate known to occur in the initial phase of culture. The mitotic response over control values was greatest from weeks 1–3, with quick decreases in weeks 4 and 5 as the dermal equivalents aged.

**DISCUSSION**

Cells such as dermal fibroblasts live in a three-dimensional collagenous matrix in vivo. Their degree of differentiation and response to stimuli are modulated by such cell–matrix interactions in ways not mimicked in typical monolayer cell culture [39]. Fibroblast cultures in three-dimensional collagen matrices in vitro have been developed that better simulate this potentially important aspect of normal dermal physiology. This in vitro dermal equivalent model was used to evaluate the effect of aFGF on fibroblasts and their interaction with the collagenous matrix in which they were embedded.

Dermal fibroblasts are significantly stimulated by aFGF in the presence of heparin, a glycosaminoglycan that binds and stabilizes the mitogen. Interestingly the speed of this proliferative response varies slightly among the fibroblast strains tested (Figs 1A and 2A). The dose-response curve for both cell number and DNA synthesis is bell-shaped, with a peak at approximately 1 ng/ml, as was previously seen with fibroblast cell lines in monolayer culture [43]. Therefore, this three-dimensional matrix system did not significantly attenuate either the mitotic stimulation or its inhibition by high concentrations of aFGF. Although the mechanism of this inhibition is unknown, it correlates with the induction of process formation and cell migration in a subconfluent BaB/c 3T3 fibroblast monolayer culture system as monitored by time-lapse cinematography and Boyden chamber checkerboard analysis, rather than toxicity. The mitotic response did plateau, however, after an approximately eightfold increase in cell number corresponding to three doublings. The limitation to additional cell divisions may occur very fast as demonstrated by flux cytometry analysis, implying that this phenomenon is less likely to be associated with nutrient depletion or waste accumulation than with decreased receptor expression as a result of increased cell density or matrix-induced cell maturation. The magnitude of the mitotic response to this daily dose of 1 ng/ml of aFGF appears to be a function of the time the cells have been in the collagen matrix, perhaps reflecting an influence of their state of maturation and duration of quiescence. Thus, aFGF would have a stimulating effect in the early phase of wound healing, subsiding quickly, however. It would be interesting to investigate whether aFGF can induce a mitotic response in resting dermal fibroblasts in vivo or not, i.e., in cells distant from any lesion.

The aFGF induction of mitosis was accompanied by an apparent persistent decrease in mean cellular diameter that is of uncertain physiologic significance. Cell volume would clearly halve immediately following mitosis so, in part, the aFGF-induced decrease might simply reflect a more rapid rate of cell division compared to the untreated cells. In the presence of a potent and persistent aFGF mitogenic signal, the cells might not pause to fully recover their original size prior to committing to a subsequent round of division. This explanation is consistent with a nearly 50% decrease in apparent mean cell volume following daily dosing compared to the relatively quiescent cells not stimulated by aFGF. In addition, aFGF-treated fibroblasts conserve their markedly lower cell volumes even days after cessation of mitotic exposure (data not shown); it would appear that unknown factors involved in cell volume control may be modulated by aFGF.

Despite previous observations that contraction of the matrix is positively correlated with the number of mature cells present [36],

* B. Coulomb (personal communication).
† K. Thomas (unpublished results).

**Figure 4.** Long-term responses of the dermal equivalents to four early daily doses of aFGF. Dermal equivalents were treated daily for 4 d with either 0 (open bars) or 1 ng/ml (solid bars) of aFGF. (A) Contraction of the dermal equivalent was inhibited on days 8 and 22 by prior aFGF treatment. (B) Cell number was increased over controls after four daily doses of aFGF but little additional response of aFGF-treated versus controls occurred by day 22. Error bars, SEM of triplicate determinations.
aFGF induces an increase in cell number accompanied by a decrease in matrix contraction. The aFGF-induced rise in cell number and DNA synthesis and the decrease in matrix contraction are consistent with micro-cinematography experiments indicating that fibroblasts do not contract the matrix during mitosis. The inhibition of contraction is not strictly dependent, however, on mitosis, because high concentrations of aFGF, which do not support cell division, still inhibited contraction. Induction of rapid (2–3 h) fibroblast-mediated collagen matrix contraction appears to be principally dependent on serum platelet-derived growth factor (PDGF) AB and BB isoforms [44]. Similarly, platelet-derived transforming growth factor-β (TGF-β) can induce contraction [44,45] after a 1–2-d delay, perhaps by induction of PDGF synthesis [46]. Basic FGF has been reported to promote contraction of preformed collagen gels within 3 d [47], a period within which we see no effect of aFGF on the gel disk diameter. The basis for the apparently opposite results seen with aFGF and bFGF is not known. Regardless of the mechanism, aFGF-mediated inhibition of fibroblast contraction could contribute to its potential therapeutic efficacy by inhibiting excessive dermal contraction, which can represent a major complication in the healing of wounds and burns.

If a growth factor such as aFGF is to be used clinically to promote the repair of dermal injuries, an indication of not only dose response but also dose frequency would be useful. Typically, therapeutic agents are given in the minimum amounts that achieve full efficacy to avoid undesirable side effects. Although long-term daily aFGF dosing elicits the greatest mitotic response, either four early consecutive daily doses or four intermittent doses, each 3 d apart, are almost as effective. A single dose at the time of gel formation, however, causes little, if any, response. Therefore, from these results, either an early short duration or a longer intermittent dosing regime might be clinically desirable.

The human dermal equivalent model has enabled us to demonstrate several new activities of aFGF, to speculate on the mechanisms of these effects, and to suggest possible therapeutic dosing regimes to promote dermal healing. The validity of this strategy, early treatment for a limited period or intermittent dosing over a longer duration, the in vivo efficacy of the inhibition of fibroblasts contraction, and finally, the validation of the predictivity of the dermal equivalent as a new pharmacologic model, can only be fully assessed through in vivo studies.

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REFERENCES


