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Research Article

Formulation Design of Acidic Fibroblast Growth Factor

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The design of an aqueous formulation for acidic fibroblast growth factor (aFGF) requires an understanding of the type of compounds that can either directly or indirectly stabilize the protein. To this end, spectrophotometric turbidity measurements were initially employed to screen the ability of polyanionic ligands, less specific compounds, and variations in solution conditions (temperature and pH) to stabilize aFGF against heat-induced aggregation. It was found that in addition to the well-known protection of aFGF by heparin, a surprisingly wide variety of polyanions (including small sulfated and phosphorylated compounds) also stabilizes aFGF. These polyanionic ligands are capable of raising the temperature at which the protein unfolds by 15–30°C. Many commonly used excipients were also observed to stabilize aFGF in both the presence and the absence of heparin. High concentrations of some of these less specific agents are also able to increase the temperature of aFGF thermal unfolding by as much as 6–12°C as shown by circular dichroism and differential scanning calorimetry. Other compounds were found which protect the chemically labile cysteine residues of aFGF from oxidation. Aqueous formulations of aFGF were thus designed to contain both a polyanionic ligand that enhances structural integrity by binding to the protein and chelating agents (e.g., EDTA) to prevent metal ion-catalyzed oxidation of cysteine residues. While room-temperature storage (30°C) leads to rapid inactivation of aFGF in physiological buffer alone, several of these aFGF formulations are stable *in vitro* for at least 3 months at 30°C. Three aFGF topical formulations were examined in an impaired diabetic mouse model and were found to be equally capable of accelerating wound healing.

KEY WORDS: acidic fibroblast growth factor; protein stability; polyanions; protein formulation.

INTRODUCTION

Acidic fibroblast growth factor (aFGF) is a member of a family of structurally related proteins capable of stimulating mitogenic, angiogenic, and chemotactic responses in a variety of cell types (1,2). Fibroblast growth factors are especially distinguished by their ability to bind heparin and have thus been designated heparin-binding growth factors. Due to their wide range of receptor-mediated biological activities, many potential clinical applications have been suggested for these proteins. In particular, the effect of growth factors on the process of wound healing has generated considerable interest (3). To develop efficacious formulations of a polypeptide growth factor such as aFGF for topical application to wound sites, it is necessary to understand the physical properties of aFGF and their relationship to both *in vivo* and *in vitro* stabilization.

The two prototypic heparin binding growth factors, acidic and basic fibroblast growth factor, possess nearly identical three-dimensional structures of 12 antiparallel β-strands arranged with approximate threefold internal symmetry as determined by X-ray crystallographic analysis (4–7). These two growth factors can be differentiated, however, by their molecular weights, isoelectric points, and number of cysteine residues. Although heparin protects both proteins from a variety of inactivating conditions such as pH, proteolysis, and temperature (8–12), it uniquely enhances the mitogenic activity of aFGF (1,2). It has been proposed that FGFs are bound *in vivo* to heparan sulfates on the extracellular matrix (ECM), where their biological activity is regulated by release from the ECM by heparinases or other physiological stimuli (13). It has also been suggested that these growth factors can directly interact with polyanionic nucleic acids, consequently playing an ill-defined role in gene expression (14).

The successful stabilization, formulation, and storage of an environmentally sensitive protein such as aFGF require a design strategy somewhat more complex than usual. We have therefore developed the following four-part approach. First, a rapid screening procedure (based on turbidimetric measurements) was utilized to identify solution conditions, polyanions, and common excipients that stabilize aFGF against heat-induced aggregation. This approach is similar to that employed by others for T4 lysozyme and γ-interferon (15). Second, the most promising agents were combined with aFGF and the protein's thermal stability was more quanti-

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tatively characterized employing biophysical techniques such as circular dichroism and differential scanning calorimetry. Third, the most promising agents were then formulated with aFGF in an appropriate aqueous milieu and the *in vitro* stability of the protein was monitored at room temperature (30°C). Finally, the *in vivo* efficacy of aFGF topical formulations was tested by examining their ability to accelerate the healing of full-thickness wounds in a diabetic mouse model (16).

MATERIALS AND METHODS

Materials

Recombinant human aFGF (15.9 kDa) was expressed in transformed *Escherichia coli* cells as previously described (17,18). The protein was purified by a combination of ion-exchange, affinity, and preparative reverse-phase chromatography followed by a refolding step as described elsewhere (19,20). The resultant aFGF displayed a high purity (>99% by SDS-PAGE with silver staining) and specific mitogenic activity as well as well-defined spectroscopic properties (21). This 141-amino acid form of aFGF (amino terminal truncated) manifests very similar mitogenic activity, heparin dependence, and receptor binding compared to the full-length 154 amino acid form (22). Protein concentration was determined spectrophotometrically using an extinction coefficient of E (0.1%, 1 cm) = 1.2 at 280 nm.

Heparin (~16 kDa) was purchased from Hepar, and low molecular weight heparin (~5 kDa) from Calbiochem. Fucoidin samples were obtained from Kelco. Sulfated cyclodextrins were provided by American Maize. All other sulfated polysaccharides and polyanions as well as the disodium salt of ethylenediaminetetraacteic acid (EDTA) were purchased from Sigma. Other reagents were obtained commercially and were of the highest grade available.

Since the molecular weight of aFGF and heparin are similar, molar ratios of protein to heparin are approximately equal to weight ratios. All references in the text to ligand: protein ratios (i.e., 3× or ⅓×) are on a weight basis unless specifically stated otherwise.

Methods

Turbidity Studies. The kinetics of protein aggregation were monitored by the degree of light scattering at 350 nm using a Perkin Elmer Lambda 6 UV-visible spectrophotometer equipped with a six-cuvette holder at both the sample and the reference cell positions. Temperature was controlled by a circulating bath containing a water/ethylene glycol solution. Nine-tenths milliliter of a PBS solution (6 m*M* phosphate, 120 m*M* NaCl, pH 7.2) was placed into each cuvette and incubated at the appropriate temperature within the spectrophotometer. Once equilibrated, 100 μL of either a 1 mg/mL aFGF solution or a PBS buffer (both containing the appropriate amount of ligand) was added to the sample and reference cuvettes, respectively, and mixed manually by inversion. The change in optical density at 350 nm over time was monitored continuously. A dead time of about 30 sec occurred in each experiment due to the mixing of the six samples. During each set of experiments, one of the six samples tested contained unliganded aFGF in PBS buffer as a control to correct for any variations between experiments. Aggregate formation from these stressed samples was irreversible as determined by an inability to resolubilize fully the aggregate with denaturing and reducing agents.

Circular Dichroism. CD spectra were recorded with an AVIV 62 DS spectropolarimeter. Samples of aFGF (100 μg/mL) in PBS buffer containing various ligands were placed into 1-mm pathlength cells. To monitor thermal unfolding, the change in ellipticity at 228 nm as a function of temperature was measured by computer-controlled increases in the temperature of the water bath (2°C increments), followed by a 2-min equilibration period to allow the sample to reach thermal equilibrium (no further time-dependent change in ellipticity). Reproducibility of the unfolding temperature (T_m) in these experiments was of the order of ±2°C.

Differential Scanning Calorimetry. DSC studies employed a Hart 7708 calorimeter, protein concentrations of approximately 1 mg/mL, and a scan rate of 60°C/hr. Samples (0.65 mL) contained sections of glass tubes to minimize interference from exotherms due to protein aggregation (23). All thermograms were background corrected and transitions were shown to be irreversible as determined by rescanning.

Anticoagulant Activity. Clotting times were monitored by either a one-stage plasma prothrombin time assay (PT assay) or an activated partial thromboplastin time assay (aPTT) using an automated optical detection system (Coag-a-Mate-XC) manufactured by General Diagnostics. Clotting times were measured in the presence of varying amounts of heparin (0–50 μg heparin/mL plasma) to generate a standard curve and clotting times of other compounds (at equal weight amounts) were obtained relative to these standardized values.

Mitogenic Activity. Biological activity of the purified protein was monitored as the mitogenic response of Balbc/3T3 cell lines and followed by uptake of tritiated thymidine as described in detail elsewhere (17). The cell culture medium contained excess heparin (0.5 mg/mL) to maximize mitogenic response. The specific activity is defined as the midpoint of a plot of mitogenic response versus aFGF concentration which is normalized to an aFGF reference standard. Although biological assays lack the precision and reproducibility of biochemical and biophysical techniques (RSD ~20%), the maintenance of biological activity during stability studies is the single most important property of the growth factor from a pharmaceutical perspective.

SEC-HPLC. Protein mass was quantitated by size-exclusion HPLC using a Ranin HPLC system with Dynamax software and a Toso Haas G-3000 SWXL column. A 0.1 *M* phosphate buffer (pH 6.8) mobile phase containing 0.5 *M* cesium chloride was used with detection at 215 nm. Cesium chloride was included in the elution buffer to dissociate protein–ligand complexes and to reduce nonspecific interaction between protein and the column matrix. Test samples were filtered and diluted (1:10) into the mobile phase and chromatographic peak areas corresponding to monomeric protein were compared to an aFGF standard of known concentration.

In Vivo *Wound Healing in a Diabetic Mouse Model.* The effect of topical formulations of aFGF on the healing of

full-thickness wounds was assessed by employing a diabetic mouse model (female C57BL/KsJ db+/+db mice, 10 weeks old, from Jackson Labs, Bar Harbor, ME). The protocol for wounding and monitoring the rate and extent of healing has been described in detail (24). A dose of 3.0 μg/cm^2 aFGF in 1% hydroxyethyl cellulose in PBS containing 9.0 μg/cm^2 heparin or 3.0 μg/cm^2 aFGF in 1% hydroxyethyl cellulose in PBS containing 0.2 m*M* EDTA with either 15.0 μg/cm^2 inositol hexasulfate or 9.0 μg/cm^2 sulfated β-cyclodextrin was applied to middorsal, circular wounds (2 cm^2) on the day of the wounding (day 0) and days 3 and 7 postwounding. Placebo-treated mice received the same formulations without aFGF. There were 10 mice per treatment group. Wounds were protected by Bioclusive wound dressing (Johnson & Johnson, New Brunswick, NJ). Every 3–4 days the dressings were changed and wound perimeters traced in triplicate onto sterile microscope slides. All procedures were done under aseptic conditions. Progress in healing was expressed as a decrease in mean wound area (normalized to day 0) as a function of time postwounding. Statistical analysis was performed by a one-sided Student's *t* test for individual time points and by log-rank test or two-way ANOVA to quantitate the estimated median number of days necessary for 50% (HT50), 70% (HT70), or 90% (HT90) reduction in wound areas.

RESULTS AND DISCUSSION

Turbidimetric Monitoring of aFGF Stability

When purified aFGF is placed in a phosphate-buffered saline solution, the unliganded protein is partially unfolded under physiological conditions (21,25–27) and unstable (due to aggregate formation) after storage for several weeks at 4°C (unpublished results). The most straightforward approach to stabilize aFGF is to add the highly sulfated polysaccharide heparin, which is known to bind to fibroblast growth factors and protect them from a wide variety of environmental insults (8–12). This heparin-stabilized aFGF solution is stable (as measured by protein mass, mitogenic activity, and a variety of biophysical and biochemical techniques) for at least a year at 4°C but cannot be stored for extended periods of time at higher temperatures.

Two questions immediately arise based on the above information: Can better-defined polyanions be substituted for the heterogeneous heparin preparation and can additional excipients be used to increase further aFGF stability in the presence of heparin? In order to address these questions, a spectrophotometric turbidity method was initially employed. When an aFGF solution is heated at temperatures near or above its T_m (the midpoint of the temperature of the transition from folded to unfolded protein), the growth factor loses its native tertiary structure and unfolds. The thermally denatured form of aFGF is extremely insoluble and aggregates begin to form rapidly as unfolded protein appears. Thus, a simple way to characterize the structural stability of aFGF is by monitoring the kinetics of temperature-induced aggregation by measuring the degree of light scattering (turbidity) at 350 nm.

To compare the ability of various compounds to stabilize aFGF in both the presence and the absence of heparin, the thermal conditions required for the heat-induced aggregation of aFGF were optimized. First, aFGF with ⅓× heparin (an amount sufficient to induce near maximal stability) was incubated over a wide temperature range for varying lengths of time, and the relative degree of turbidity formation was monitored at 350 nm. Figure 1A shows that the rate of aFGF aggregation in the presence of heparin is strongly dependent on temperature, with rapid aggregation evident at or above 55°C. The inset of Fig. 1A is a replot of the data at

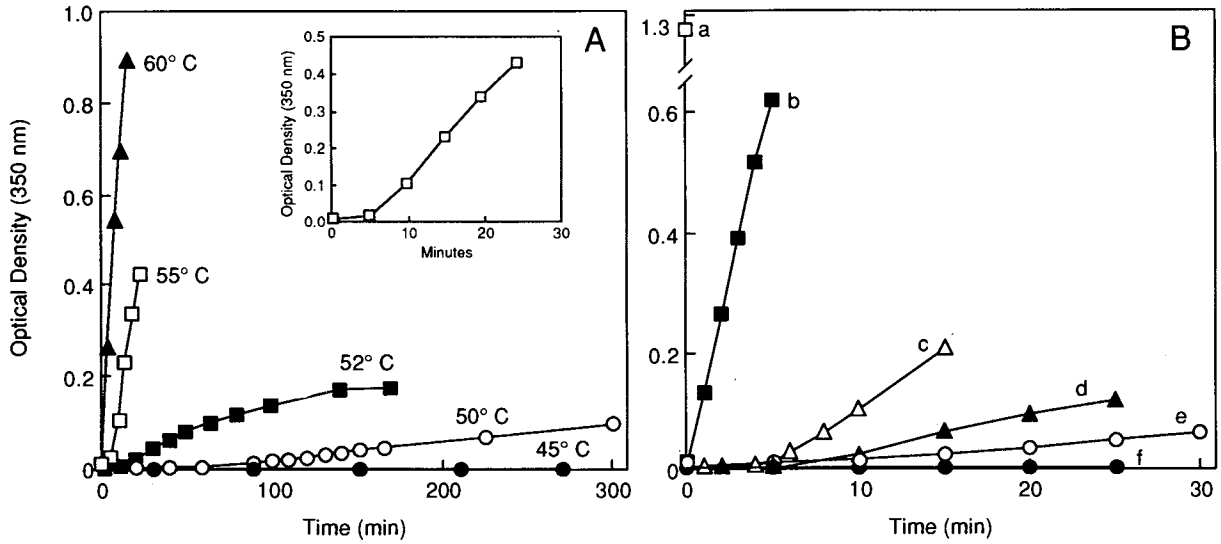

Fig. 1. Turbidity measurements of the heat-induced aggregation of aFGF in the presence of heparin. (A) Temperature dependence of turbidity formation in the presence of ⅓× heparin (by weight). The inset is a more detailed view of the time course of turbidity formation at 55°C. Samples contained 100 μg/mL protein in a PBS buffer, pH 7.2. (B) Time course of the heat-induced aggregation of aFGF at 55°C in the presence of varying amounts of heparin. Samples contained 100 μg/mL protein in a PBS buffer, pH 7.2, with (a) no heparin, (b) 10 μg/mL or 0.1×, (c) 20 μg/mL, (d) 33 μg/mL, (e) 100 μg/mL, and (f) 300 μg/mL heparin. The curve is drawn through the data for clarity only.

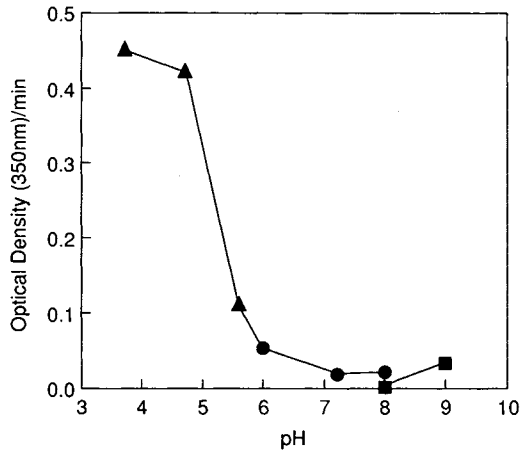

Fig. 2. pH dependence of the heat-induced aggregation of aFGF in the presence of ⅓× heparin (by weight) at 55°C. Experiments were performed in either citrate (▲), phosphate (●), or borate (■) buffer (10–50 m*M*) containing 120 m*M* NaCl. The *Y* axis represents the maximum rate of turbidity formation ($\Delta OD_{350\ nm}$/min). Samples contained 100 μg/mL protein.

55°C and demonstrates a linear change in turbidity with time between 5 and 25 min, suggesting that the slope in this region can be used as a convenient measure of protein stability. Second, the effect of heparin concentration on the rate and extent of heat-induced aggregation at 55°C is shown in Fig. 1B. As expected, increasing the heparin concentration significantly protects aFGF from heat-induced aggregation. For example, in the presence of 3× heparin, no aggregation is observed after 30 min at 55°C. Similar turbidity experiments were carried out with unliganded aFGF in a PBS buffer at 40°C. The rate of the turbidity formation was slower ($OD_{350\ nm}$ ~0.45 after 40 min) compared to 55°C ($OD_{350\ nm}$ ~1.3 at time 0; see Figure 1). Thus, turbidity measurements at 40°C were employed to test the ability of various polyanionic substances to stabilize aFGF in the absence of heparin, while 55°C turbidity measurements were used to examine the effect of various compounds on aFGF stability in the presence of heparin.

The selection of an appropriate pH is an initial important step in identifying the best solution conditions for *in vitro* storage since pH affects both the stability and the solubility of proteins. When an aFGF solution with ⅓× heparin was incubated at 55°C at various pH values and the change in turbidity was measured, low pH was observed to destabilize aFGF dramatically. As shown in Fig. 2, aFGF is most stable in the neutral to near alkaline pH range at elevated temperatures. Spectroscopic analysis of aFGF at low pH shows a nonnative structure presumably leading to the observed destabilization (not illustrated). All experiments were therefore carried out at pH 7.2 both to maximize aFGF solution stability and to maintain potential topical formulations of the growth factor near physiological conditions for application to wound sites.

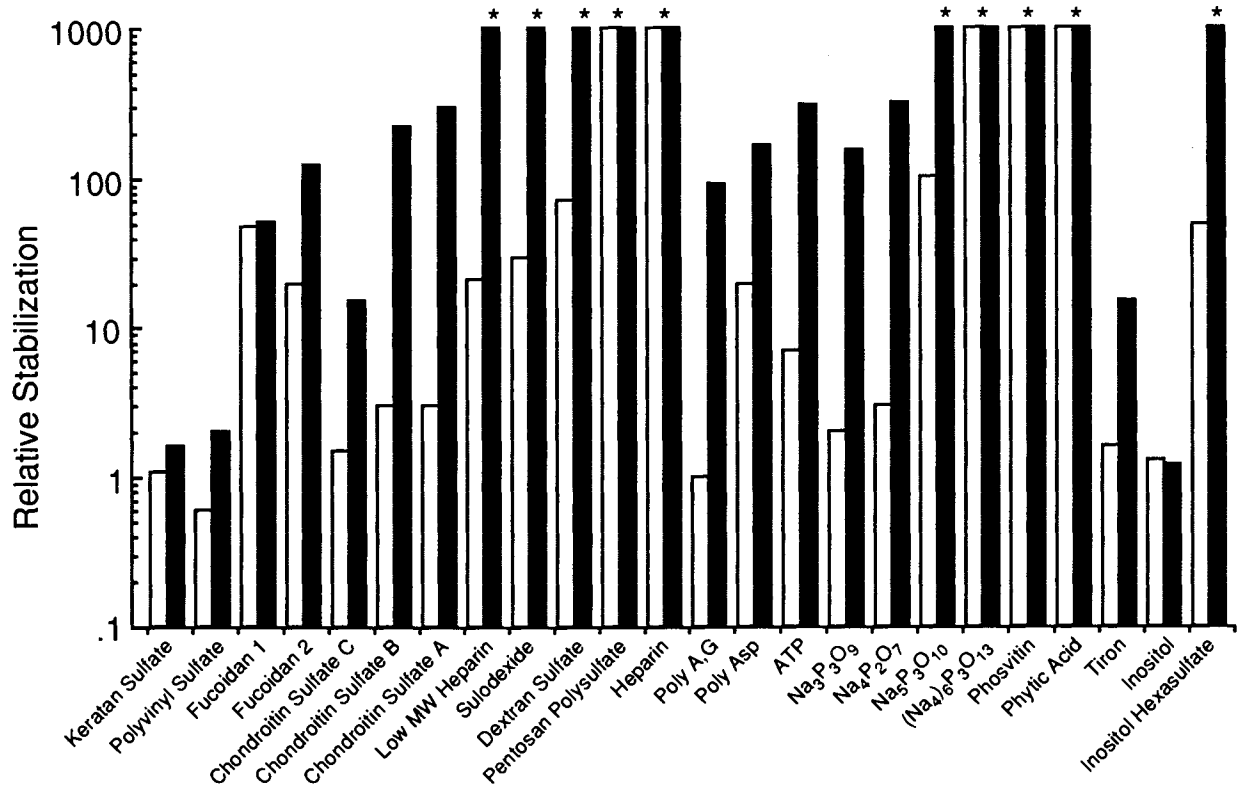

Fig. 3. Stabilization by polyanions of aFGF against heat-induced aggregation at 40°C. The relative stabilization is the extent of aggregation ($\Delta OD_{350\ nm}$ at 15 min) of aFGF in the presence of 0.5× (□) and 10× (■) ligand (by weight) normalized to aFGF in buffer alone. Samples contained 100 μg/mL protein in a PBS buffer, pH 7.2. (*) No aggregation was observed during the time course of the experiment.

Effect of Other Polyanions on aFGF Stability

To assess the ability of polyanions other than heparin to stabilize aFGF, the turbidimetric method (40°C) was used to screen a wide variety of heparin-like sulfated polysaccharides, small sulfated and phosphorylated molecules, and many other highly charged compounds. These polyanions were incubated with aFGF at 0.5× and 10× weight excess and the rate and extent of aggregate formation were followed as described above. A summary of these experiments in terms of the extent of aggregation is illustrated in Fig. 3. The heparin-like molecules, i.e., sulfated polymers, varied significantly in their ability to stabilize aFGF. For example, at high concentrations of ligands (10× by weight), heparin, pentosan polysulfate, and dextran sulfate were more effective (no aggregate formation after 30 min) than chondroitin sulfates (10–100× stabilization), which were in turn more potent than polyvinyl or keratan sulfate (in the range of destabilization to 1–10× stabilization). Many small, highly charged sulfated and phosphorylated molecules such as ATP, inorganic phosphates, and various inositol compounds also significantly decreased the extent (and rate; not illustrated) of turbidity formation (Fig. 3). A combination of fluorescence spectroscopy and circular dichroism was utilized to show that many of these polyanionic ligands, along with other well-defined polyanions such as sulfated β-cyclodextrin and sucrose octasulfate, stabilize aFGF by significantly increasing the temperature of thermally induced unfolding by 15–30°C (26,27). The nature of the aFGF polyanion binding site was then characterized by varying the size and charge of various stabilizing ligands (27).

It was of interest to determine the relative anticoagulant properties of some of the nonheparin polyanionic ligands which are capable of stabilizing aFGF. The ability of these molecules (on a weight basis) to lengthen clotting times was roughly proportional to their molecular weight, with values found from about one-half (dextran sulfate, low molecular weight heparin, sulfated β-cyclodextrin) to less than one-tenth (inositol hexasulfate, phytic acid and tetrapolyphosphate) that of heparin (results not illustrated). With the exception of heparin and dextran sulfate, however, these polyanionic compounds are not currently approved for clinical use and may have as of yet undetected biological activities.

Effect of "Nonspecific" Agents on aFGF Stability

Several categories of excipients known to stabilize proteins nonspecifically including sugars, polyols, and amino acids were screened for their ability to stabilize further aFGF in the presence of ⅓× heparin at pH 7.2. As an initial experiment the ability of the disaccharide sucrose to inhibit the heat-induced aggregation of aFGF at modest and higher concentrations of sugar (50 m*M* and 2 *M*) was demonstrated (Fig. 4). The effect of a variety of other compounds on aFGF stability (in the presence of ⅓× heparin) was then tested and the results are summarized in Table I. It can be seen that several categories of compounds stabilize aFGF to an extent beyond that induced by heparin alone. Only the most effective agents from each class are listed in Table I. Compounds such as mannose, ethylene glycol, and several amino acids (lysine, arginine, aspartic and glutamic acid) were much less effective then their illustrated counterparts. The wide variety of compounds that stabilize aFGF (Table I) suggests that multiple mechanisms may be involved in this effect. Stabilization with compounds such as sugars, amino acids, and polyols are well documented with other proteins (28,29). These solutes may act by increasing solution surface tension (28). Table I also shows that inert polymers (PEG 8000), detergents (i.e., the zwitterionic Chaps), and proteins (albumin and phosvitin) inhibit the heat-induced aggregation of aFGF. Several potential modes of action may explain these apparent stabilizing effects including solubilization of the unfolded form of aFGF, a steric effect which effectively screens unfolded proteins from each other, or prevention of surface adsorption (which may nucleate aggregation). In the case of proteins, serum albumin also has reducing activity (due to its free thiol group), while phosvitin (see Fig. 3) contains multiple phosphate groups capable of either direct interaction with the growth factor or chelation of trace metal-ions (see below).

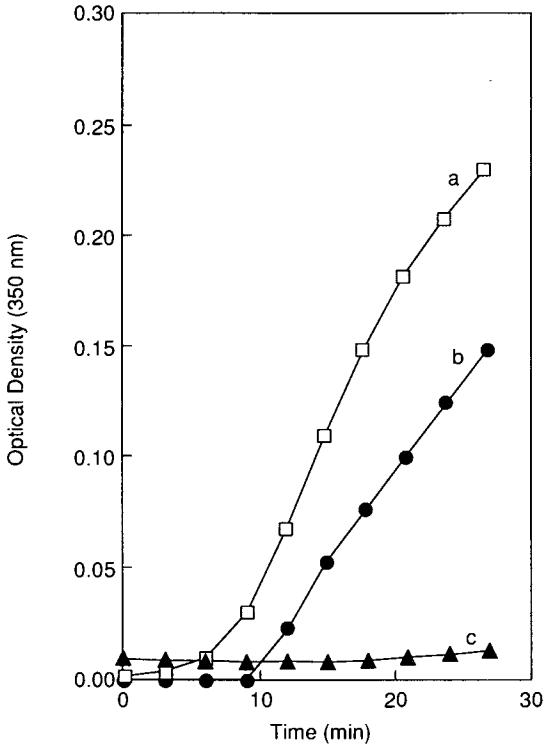

Fig. 4. Time course of heat-induced aggregation of aFGF in the presence of ⅓× heparin (by weight) at 55°C in (a) buffer alone and in the presence of (b) 50 m*M* and (c) 2 *M* sucrose. Solutions contained 100 μg/mL protein in PBS buffer, pH 7.2.

The effect of the concentration of stabilizing agent on the inhibition of aFGF aggregation was examined in greater detail. A representative experiment is shown in Fig. 5, which illustrates that sorbitol stabilizes aFGF in the presence and absence of ⅓× heparin. An IC_{50} value can be defined as the concentration of excipient required to reduce the rate or extent of turbidity formation by 50%. A significant stabilization of aFGF is observed, with an IC_{50} value of 160 in the presence of ⅓× heparin (55°C) and 310 m*M* in the absence of heparin (40°C). Values of IC_{50} for many of the aforementioned compounds are reported in Table II. In the presence of ⅓× heparin at 55°C, the IC_{50} values for sugars, salts, or

Table I. Stabilization of aFGF (with ⅓× Heparin by Weight) Against Heat-Induced Aggregation at 55°C by Various Nonspecific Excipients[a]

Class of compound	Example	Concentration	Stabilization ratio
Salts	Ammonium sulfate	1 *M*	5
		2 *M*	—
Amino acids	Histidine	0.1 *M*	4
	Glycine	2 *M*	20
Detergents	Chaps	0.01%	3
Polymers	PEG 8000	0.5%	2
	Albumin	1%	11
Polyols	Sorbitol	0.05 *M*	3
		2 *M*	—
	Mannitol	0.05 *M*	1.2
		2 *M*	—
	Xylitol	0.05 *M*	1
		2 *M*	—
	Glycerol	0.05 *M*	2
		2 *M*	20
Sugars	Sucrose	0.05 *M*	1.5
		2 *M*	46
	Dextrose	0.05 *M*	1.4
		2 *M*	61
	Trehalose	0.05 *M*	1
		1.5 *M*	81
Reducing agents	β-Mercaptoethanol	2 m*M*	5
		10 m*M*	—
	Dithiothreitol	2 m*M*	—
		10 m*M*	—

[a] The stabilization ratio is the extent of aFGF aggregation ($\Delta OD_{350\ nm}$ at 30 min) in a buffer containing an additive normalized to aFGF in buffer alone. (—) No aggregation within the time course of the experiment. Samples contained 100 μg/mL aFGF in a PBS buffer at pH 7.2 and the indicated amount of compound.

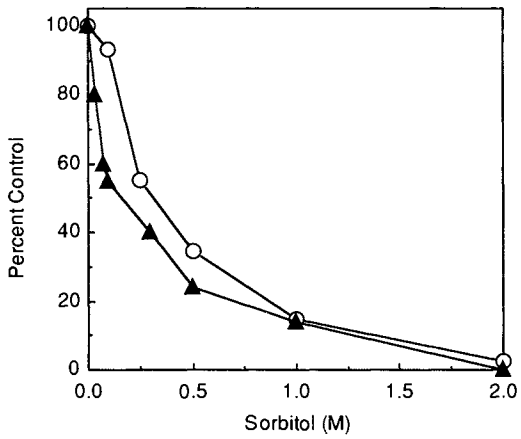

Fig. 5. The effect of sorbitol on the heat-induced aggregation of aFGF in the presence of ⅓× heparin (by weight) at 55°C (▲) and in buffer alone at 40°C (○). Samples contained 100 μg/mL protein in PBS buffer, pH 7.2. The *Y* axis represents the maximum rate of turbidity formation ($\Delta OD_{350\ nm}$/min) normalized to a sample without sorbitol.

Table II. Inhibition of Heat-Induced Aggregation of aFGF in the Presence of Varying Amounts of Excipients (A) in the Presence of ⅓× Heparin by Weight and (B) in PBS Buffer Alone[a]

Compound	IC_{50} (m*M*)
(A) aFGF in the presence of ⅓× heparin (by weight) at 55°C	
Sodium sulfate	690
Histidine	45
Glycine	70
Sorbitol	160
Dextrose	350
Trehalose	440
EDTA	0.3
(B) aFGF in the absence of heparin at 40°C	
Sodium sulfate	0.55
Ammonium sulfate	0.43
Ammonium chloride	4.2
Histidine	1.6
Glycine	180
Sorbitol	310
Dextrose	250
Trehalose	155
EDTA	0.5

[a] IC_{50} is the concentration of excipient at which the rate of heat-induced aggregation (ΔOD_{350}/min) of aFGF is 50% of the sample in the absence of excipient. All solutions contained 100 μg/mL aFGF in PBS buffer at pH 7.2 and the indicated amount of compound.

amino acids required to stabilize aFGF further are in the range of 50 to 700 m*M* (except EDTA; see below). In the absence of heparin at 40°C, these compounds also significantly stabilize aFGF but the concentration required is more varied (IC_{50} values of approximately 0.5 to 300 m*M*).

It is important to distinguish between compounds that stabilize aFGF through nonspecific effects (e.g., preferential hydration) and those that bind to the polyanion binding site. As summarized in Table II, sodium sulfate is required in large amounts to stabilize aFGF further (e.g., like sugars) in the presence of heparin, but in relatively small amounts (unlike sugars) in the absence of heparin. High concentrations of sodium sulfate are probably required to stabilize aFGF-heparin through an indirect "salting-out" type of effect (28,30). In the absence of heparin, sulfate molecules can presumably also bind to basic residues in the polyanion binding site of aFGF and this is reflected in the much lower concentration of sodium sulfate necessary to stabilize aFGF under these conditions. In fact, tightly bound sulfate ions are clearly seen in the crystal structures of FGFs (4–6). Thus, in the absence of heparin, sodium sulfate (or ammonium sulfate; Table II) is primarily a specific ligand that binds and stabilizes aFGF like many of the polyanions described above, although its lower affinity requires a high molar concentration (for example, the IC_{50} value for heparin under the same conditions is 0.13 μ*M*).

Acidic FGF was examined in the presence of some of the more effective "nonspecific" compounds listed in Tables I and II by circular dichroism (CD) and differential scanning calorimetry (DSC). The far UV circular dichroism spectrum of aFGF is characterized by positive ellipticity at 228

nm and a negative band at 205 nm (21) as generally manifested by β-structure-rich proteins of the β-II type (31,32). The effect of temperature on the secondary structure of aFGF in both the presence and the absence of heparin can be detected by changes in the CD ellipticity maximum at 228 nm. As shown in Fig. 6A, the midpoint of the thermally induced unfolding (T_m) of aFGF in the presence of ⅓× heparin is 64°C. Upon addition of 2 *M* sorbitol, the midpoint shifts to 72°C. Similarly, in the absence of heparin, the T_m value for aFGF alone is 47°C, while in the presence of 2 *M* sorbitol the T_m increases to 54°C. Thus, high concentrations of sorbitol increase the T_m values of aFGF in both the presence and the absence of heparin. Similar results were observed for aFGF in the presence of the disaccharide trehalose (Fig. 6B) and were also detectable by fluorescence spectroscopy (not illustrates).

Differential scanning calorimetry (DSC) was utilized as an independent, complementary technique to measure the thermal unfolding of aFGF by monitoring the heat absorbed during the temperature-induced unfolding of the protein. As shown in Fig. 7, the transition temperature of aFGF (with ⅓× heparin) rises from 63 to 67 to 74°C as the concentration

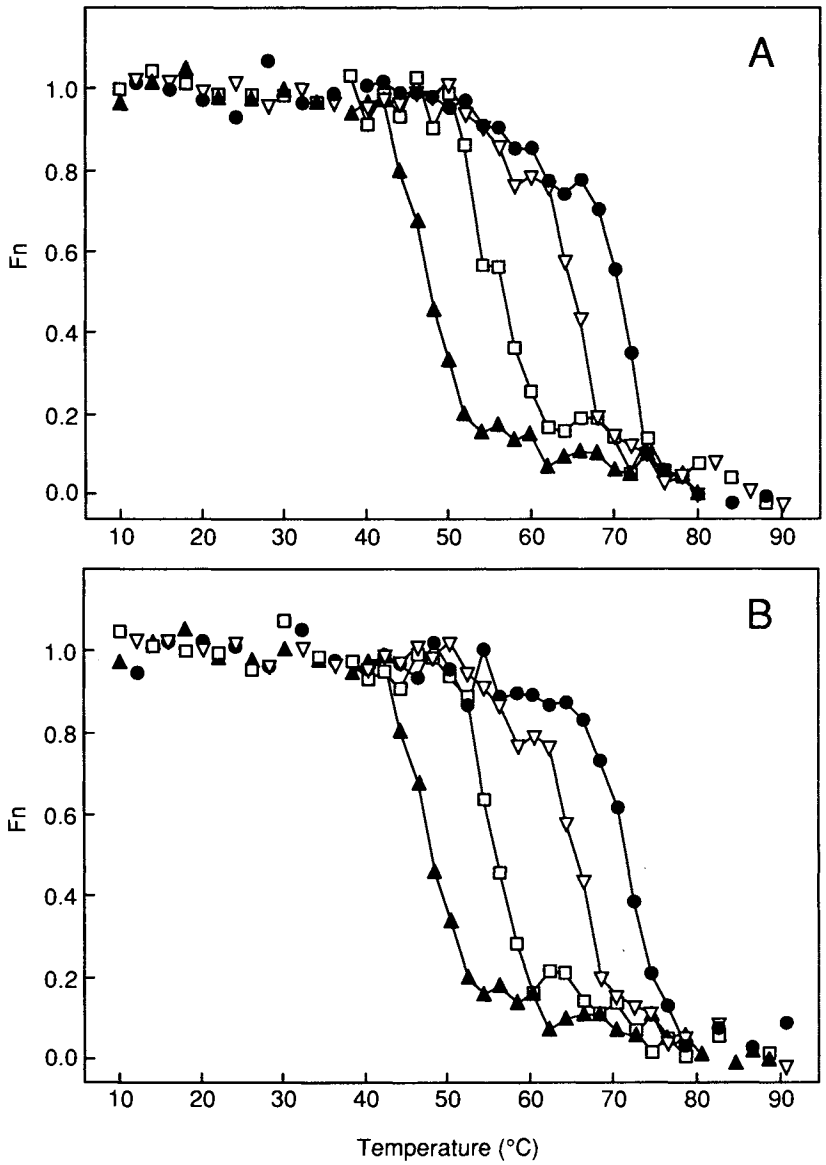

Fig. 6. Effect of sorbitol (A) and trehalose (B) on the thermal unfolding temperature (T_m) of aFGF in both the presence and the absence of heparin as measured by far-UV circular dichroism. Samples contained 100 μg/mL protein in PBS buffer, pH 7.2, in (▲) buffer alone, (□) buffer containing either 2 *M* sorbitol or 1.5 *M* trehalose, (▽) buffer containing ⅓× heparin (by weight), and (●) buffer containing both ⅓× heparin (by weight) and either 2 *M* sorbitol or 1.5 *M* trehalose. The Y axis represents the fraction of unfolded protein as measured by the change in the optical ellipticity of aFGF at 228 nm.

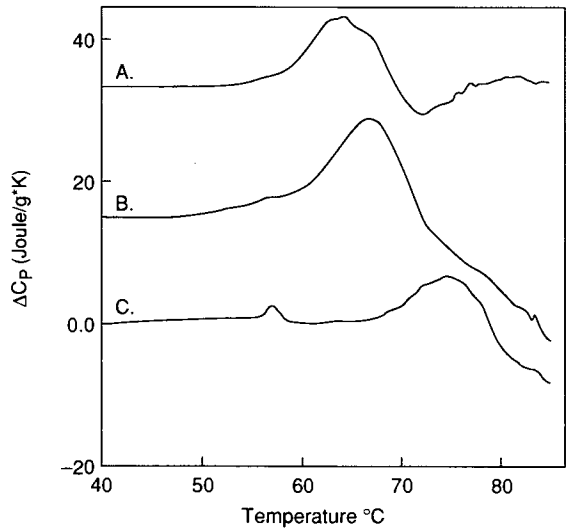

Fig. 7. The effect of trehalose on the DSC endotherm of aFGF in the presence of ⅓× heparin (by weight). All samples contained 1 mg/mL protein in a PBS buffer, pH 7.2: (a) buffer alone; (b) 0.5 *M* trehalose; (c) 1.5 *M* trehalose.

of trehalose is increased from 0 to 0.5 to 1.5 *M*, respectively (T_m of unliganded aFGF is 45°C). Table III shows that low to modest concentrations of various sugars, polyols, and amino acids have no effect on the T_m of aFGF in the presence of heparin. In contrast, higher concentrations (e.g., 1.5–2 *M*) of these compounds result in a 6–12°C increase in the T_m of heparinized aFGF as measured by both CD and DSC.

Effect of Chelators and Reducing Agents on aFGF Stability

Acidic FGF contains three cysteine residues whose chemical lability leads to loss of biological activity. Site-directed mutants of aFGF (in which the cysteine residues have been replaced by serines) display increased stability and less heparin dependence than wild-type protein (17,18). Similar results have been found with bFGF (33). The copper-catalyzed oxidative formation of a disulfide bond in both wild-type (via intermolecular oxidation) and mutant (via intramolecular oxidation) aFGF has been shown to inactivate the protein as measured by an *in vitro* mitogenic assay (17,18,34).

Since thiol groups can be oxidized spontaneously by atmospheric oxygen in the presence of trace-metal ions (35), it was of interest to examine the ability of reducing and chelating agents to stabilize aFGF. Reducing agents such as dithiothreitol and β-mercaptoethanol stabilize aFGF solutions containing ⅓× heparin as measured by turbidity formation at 55°C (Table I). Furthermore, small amounts of a chelating agent such as EDTA stabilize aFGF against heat-induced aggregation in both the presence and the absence of heparin (Table II). Thermal unfolding experiments employing CD and DSC analysis find that low concentrations of reducing agents or EDTA (1 m*M*) do not affect the T_m of aFGF (not illustrated). Therefore, these agents do not influence the structural integrity of the protein but probably prevent trace-metal ion-catalyzed oxidation of aFGF thiol groups. The stabilizing effect of the previously described polyanionic ligands may be due partially to their ability to bind trace-metal ions in addition to their direct binding to aFGF. These polyanions, however, unlike reducing and chelating agents, do significantly raise the growth factor's thermal unfolding temperature.

Table III. Effect of Excipient Concentration on the Thermal Unfolding Temperature (T_m) of aFGF (in the Presence of ⅓X Heparin) as Measured by Differential Scanning Calorimetry (DSC) and Circular Dichroism (CD)[a]

Compound	T_m (°C) DSC	T_m (°C) CD
Ammonium sulfate		
0.5 *M*	62	
1.5 *M*	70	73
Glycine		
0.1 *M*	62	
2 *M*	69	72
Sorbitol		
0.5 *M*	65	
2 *M*	75	70
Dextrose		
0.5 *M*	66	
2 *M*	75	78
Trehalose		
0.5 *M*	67	
1.5 *M*	74	72
Buffer alone	63	64

[a] DSC experiments were conducted at 1 mg/mL aFGF and CD samples at 100 μg/mL aFGF, both in PBS buffer, pH 7.2, in the presence of the indicated amount of solute.

Real-Time Probe Stability Studies of aFGF in Topical Formulations

Three major categories of aFGF stabilizing agents have been identified as described above: specific ligands that directly bind to the aFGF polyanion binding site, nonspecific agents that either bind weakly to aFGF or exert their effect through perturbation of solvent, and reducing and chelating agents that prevent oxidation of the growth factor's three thiol groups. The next step in the formulation design strategy was to determine the real-time stability of aFGF in the presence of these stabilizing compounds. Acidic FGF was formulated in a buffered solution containing hydroxyethyl cellulose (HEC). This polymer provides sufficient viscosity to permit aFGF to be applied to wound sites without loss due to runoff. This inert polymer was found to have no direct effect on the stability of aFGF as determined by circular dichroism, fluorescence, differential scanning calorimetry, and mitogenic activity (not illustrated). In addition, aFGF has been shown to be completely released from a 1% HEC solution (24). The protein concentration was adjusted to 100 μg/mL, which is sufficient to minimize any potential adsorptive losses to the container surface (36).

As shown in Fig. 8, the combination of a polyanion (either heparin, sulfated β-cyclodextrin, or tetrapolyphosphate) and 1 m*M* EDTA stabilizes aFGF in a 1% HEC solution for 3 months at 30°C as measured by size exclusion chromatography and mitogenic activity. It can also be seen in Fig. 8 that some of the low molecular weight sulfated

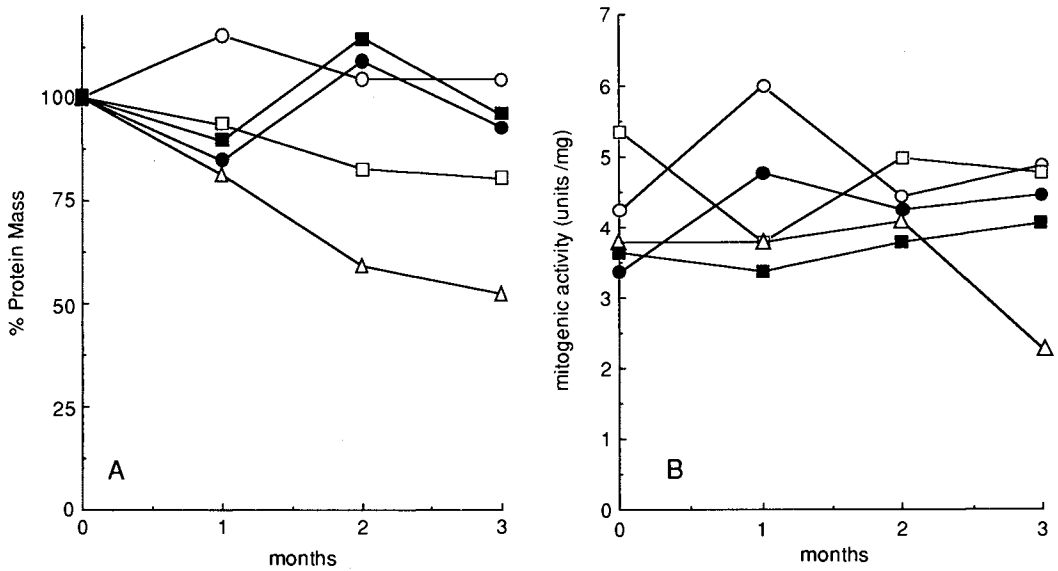

Fig. 8. Room temperature stability (30°C) of aFGF formulations. Samples contained 100 μg/mL aFGF in a PBS buffer at pH 7 containing 1 m*M* EDTA, 1% hydroxyethyl cellulose, and the following stabilizing polyanions: (○) 3× heparin by weight, (□) 3× inositol hexasulfate by weight, (●) 3× sulfated β-cyclodextrin by weight, (■) 10 m*M* tetrapolyphosphate, and (△) 120 m*M* sodium sulfate (this sample lacks the 120 m*M* NaCl component of PBS buffer). Protein mass was measured by (A) size exclusion HPLC and (B) mitogenic activity by the stimulated uptake of ^{3}H-thymidine by mouse fibroblast 3T3 cells.

compounds such as inositol hexasulfate and sodium sulfate were not as effective as other sulfated polyanions. In contrast, aFGF viscous formulations containing either EDTA or polyanionic ligands separately were much less stable, with significant loss of protein mass and mitogenic activity after 1 to 3 months at 30°C (data not shown). When aFGF formulations containing both heparin and EDTA were prepared with 0.3 *M* sorbitol instead of 0.15 *M* NaCl (equivalent osmolarity), no differences in the stability of aFGF were observed. It should be pointed out that the enhancement of aFGF thermal stability by either sugars, polyols, or amino acids (in the presence of heparin) required sufficiently high concentrations (2 *M*) that they are not practical in topical formulations as a consequence of the high osmolarity of such solutions.

The combinations of polyanions and antioxidants are effective stabilizers of aFGF in viscous, topical formulations. Despite the effectiveness of smaller molecular weight polyanions such as inositol hexasulfate and sodium sulfate as aFGF stabilizers during accelerated stability studies, aFGF formulations containing these sulfated polyanions were less stable during real-time stability studies (Fig. 8) compared to larger sulfated polyanions (e.g., heparin). When heparin is added to aFGF at a 3× ratio of heparin to protein, one globular aFGF molecule (15.9 kDa) binds on the average to a single elongated heparin chain (~16 kDa) as measured by static and dynamic light scattering (37,38). Thus, these conditions may prevent aFGF molecules from directly interacting with one another in solution. Conversely, aFGF molecules bound to lower molecular weight polyanions may collide in solution more frequently in inactivating orientations, leading to an increased probability of aggregation and/or intermolecular oxidation.

Although conformational instability is the most likely initial cause of aFGF inactivation, labile cysteine residues can still oxidize at temperatures well below the T_m of heparinized aFGF, with concomitant or subsequent conformational changes in the protein molecule. Both intra- and intermolecularly cross-linked species are evident in unreduced SDS-PAGE of copper-oxidized aFGF samples (results not illustrated). By eliminating or minimizing one cause of protein inactivation, another, slower degradative pathway may then become the rate-limiting step in determining the protein's shelf life and storage temperature. For example, preliminary experiments suggest that stabilized solutions of aFGF containing polyanions and EDTA may deamidate during prolonged storage at room temperature. These inactivation pathways may also vary as a function of the exact storage conditions including the nature of the vial, protein concentration, solution pH, etc.

In Vivo Efficacy of Topical aFGF Formulations

The *in vivo* efficacy of topical formulations of aFGF stabilized by either heparin, inositol hexasulfate, or sulfated β-cyclodextrin (containing 1% hydroxyethyl cellulose in PBS with 0.2 m*M* EDTA in the latter two formulations) was examined in a diabetic mouse model (16,24). The decrease in the mean wound area for full-thickness wounds treated with each active formulation is shown in Fig. 9. It can be seen that differences among topical aFGF formulations stabilized by any of the three polyanionic compounds are not statistically significant. Thus, lower molecular weight polyanions such as sulfated β-cyclodextrin and inositol hexasulfate may be potential substitutes for heparin in topical aFGF formulations. Comparisons among these three aFGF topical formulations and their corresponding placebos (combined data) revealed statistically significant differences for the number of days

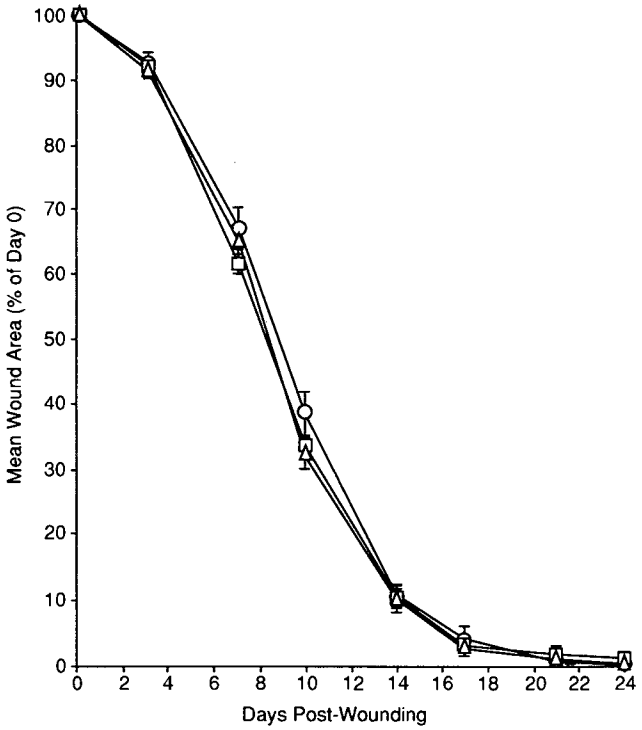

Fig. 9. The time course of wound healing in diabetic (db+/+db) mice treated with three applications of 3.0 μg/cm² aFGF with either (○) 9.0 μg/cm² heparin, (□) 15.0 μg/cm² inositol hexasulfate, or (△) 9.0 μg/cm² sulfated β-cyclodextrin. Formulations contained 1% HEC in a PBS buffer with the latter two formulations containing 0.2 m*M* EDTA. Values are presented as percentage of original wound area (mean ± SD).

required for 70% healing (HT70) and 90% healing (HT90) ($P < 0.001$ and $P = 0.002$, respectively, as determined by two-way ANOVA; not illustrated). It should be pointed out that although HT90 differences between active and placebo were always statistically significant, the window between active and placebo varied between studies. For example, differences in the response to identical treatments of a heparin-containing aFGF formulation ranged from 2.4 to 7.8 days to reach HT90 (24).

CONCLUSIONS

Due to the dramatic dependence of the stability of purified aFGF on its solution conditions (21,25–27), topical formulations of the growth factor require excipients to stabilize the protein during storage. Not only is formulation design for aFGF a challenge in terms of *in vitro* stability, but also there exists the possibility of potentiating the biological activity and efficacy of the growth factor through excipient selection. Despite the effectiveness of heparin as a stabilizer of aFGF, it is an inherently heterogeneous material derived from animal sources with well-known anticoagulant activity (39). In contrast, the ideal aFGF stabilizer might be a synthetic, homogeneous molecule with reduced anticoagulant activity, proven safety, and tolerability. It should have the ability to stabilize, and perhaps potentiate, the biological activity of aFGF. This work demonstrates the potential for utilizing better-defined polyanionic compounds in aFGF formulations. The final choice of a formulation polyanion for aFGF, however, should not only be based on the protein's stability, but must also consider the safety and tolerability of all the components as well as any effects on the biological and clinical efficacy of aFGF. For this purpose, topical formulations of aFGF containing various polyanionic stabilizers and EDTA have been evaluated in a diabetic mouse wound healing model. Using this model, topical formulations of aFGF containing either inositol hexasulfate or sulfated β-cyclodextrin were as effective as aFGF-heparin in promoting wound healing, suggesting that these polyanions may be suitable replacements for heparin in topical aFGF formulations.

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