The Antagonism of Glucocorticoid Inhibition of Wound Healing in Rats by Growth Hormone-Releasing Factor (43098)

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Abstract. **Daily therapeutic injections of cortisone to rats will cause weight loss and impaired wound healing. Weight loss is attributed to the catabolic effect of steroid, whereas impaired healing is associated with reductions in fibroplasia and connective tissue deposition. As the major structural protein component of connective tissue is collagen, its absence is responsible for the retarded gain in wound breaking strength. Cortisone also blocks wound closure by inhibiting wound contraction. An anabolic agent such as growth hormone may antagonize the effect of cortisone on the would healing process. Endogenous GH can be released from the pituitary by exogenous injections of growth hormone-releasing factor (GRF). Two synthetic GRF peptides, a natural 44-amino acid peptide of the human GRF sequence, GRF-44, and an N-terminally substituted analog 29 residues, GRF-29A, were studied. Each was given twice daily with a single daily injection of cortisone for a 7-day period. Concurrent administration of GRF-44 or GRF-29A and cortisone to rats had no effect on restored body weight loss or inhibited wound contraction. While GRF-44 restored collagen deposition and caused restored wound breaking strength, GRF-29A was ineffective in restoring either. GRF-44, a synthetic peptide that stimulates pituitary release of growth hormone, antagonized some of the inhibiting effect of steroid on wound repair by promoting fibroplasia and collagen deposition.** [P.S.E.B.M. 1990, Vol 194]

Wound healing is an essential biologic process that, in the animal kingdom, ranges from the complete regeneration seen in starfish to the scarring observed in humans. The healing process in mammals is composed of three major phases which appear in a chronological sequence with some overlap. The initial phase is the "lag phase" characterized by inflammation which protects the host from infection and inaugurates the next phase of healing. The "proliferative phase" is characterized by the migration and proliferation of fibroblasts, the deposition of a new connective tissue matrix, and the development of a new vasculature. The last phase of healing, the "remodeling phase," is characterized by the reorganization of the newly deposited tissue into scar tissue and the regeneration of an epidermal surface. The healing of a sutured wound involves newly deposited collagen fibers securing the edges of the wound together. The developing gain in wound-breaking strength is sufficient to prohibit the incision from coming apart following the removal of the sutures. The measure of gain in wound-breaking strength is the force required to pull the wound edges apart. The amount of new collagen deposited during the initial 14 days of healing is directly proportional to the gain in wound-breaking strength. After that period the degree of covalent chemical crosslinks developing between the collagen molecules augments further gain in wound-breaking strength. The closure of an open wound requires both the deposition of a new connective matrix as well as the process of wound contraction. The process of wound contraction is the pulling of uninjured surrounding skin over the defect. During this contractile process, the defect is filled with granulation tissue in the absence of a complete epidermal surface. Wound contraction gives rise to healed wounds covered with normal skin which reduces the amount of residual scar tissue. Recent studies have shown that forces accountable for fibroblast locomotion are responsible for wound contraction and not the contraction of a specialized cell the myofibroblast (1).

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To study the enhancement or stimulation of wound healing in laboratory animals such as rats is difficult because they heal so rapidly. One approach for investigating agents which may potentionally stimulate wound healing examines its antagonism of glucocorticoid-inhibited wound healing in rats. By measuring the gain in wound-breaking strength, new collagen deposition and wound contraction with the concurrent administration of cortisone, the capability of an agent to enhance wound healing is evaluated (2).

Rats receiving high therapeutic doses of glucocorticoids show diminished wound healing (3). This inhibition is linked to a reduction in fibroplasia and the deposition of a new connective tissue matrix. Where a scarcity of new deposits of connective tissue exists, the incremental progress of gain in wound-breaking strength of a sutured wound is deficient. Initial gains in wound-breaking strength are not achieved as a result of deficits in collagen synthesis and deposition (4).

Anabolic agents, such as vitamin A, and anabolic steroids have been shown to antagonize the inhibition of collagen deposition by systemic cortisone therapy, leading to increases in wound-breaking strength (5, 6). Hypophysectomized rats have been shown to heal normally (7). In another study additions of thyroxine, testosterone, and ACTH to hypophysectomized rats healed better than intact rat controls (8). In addition, these hypophysectomized rats showed enhanced wound healing when supplemented with only bovine growth hormone as compared with intact controls. Growth hormone (GH) therapy, which produced a somatic growth response in a normal rat, did not produce enhanced healing (9). A pilot study showed that the combination of human growth hormone and daily systemic glucocorticoid treatment resulted in nearly normal healing in rats.

This report investigates the ability of growth hormone-releasing factor (GRF) to reverse the inhibition of wound repair by daily systemic injections of glucocorticoids. The wound-healing model utilized in these studies measured the rate of open wound closure, gains in wound-breaking strength, the amount of new collagen deposited, and histologic variances. Another objective of this study was to compare the antagonistic effects of a synthetic preparation of human GRF, GRF-44, and a synthetic N-terminally substituted analogue containing 29 residues, GRF-29A on restoring wound healing in rats treated with glucocorticoids. The ability of GRF-44 and GRF-29A to promote wound healing in untreated rats was also examined.

GRF-44, a peptide hormone secreted by the hypothalamus, stimulates the release of growth hormone from the pituitary gland (10). Hormone peptides with growth hormone-releasing activity were first characterized in a pancreatic tumor (11). GRF-44 was later purified and identified. A number of synthetic GRF peptides with increased biologic activity have been produced (12). GRF (1–44)-NH_2 has a primary structure identical to human hypothalamic GRF (13). GRF (1–29)-NH_2 was shown to have full intrinsic activity and nearly the same potency as GRF (1–44)-NH_2 (14), demonstrating the importance of the amino terminus in biologic activity. GRF (1–27)-NH_2 had a 12% decrease in intrinsic activity, while GRF (1–21)-NH_2 had full intrinsic activity but low potency. The 21-amino acid peptide was the shortest active fragment. GRF analogues with unprotected —COOH at the carboxyl terminus had half the potency of the corresponding amidated peptides (14). Deletion fragments such as GRF (1–15) (20–44)-NH_2 and GRF (1–15) (18–44)-NH_2 were shown to be inactive (15).

In spite of the structural differences of GRF among species, it has been shown that the human sequence not only stimulates the release of GH in humans, but also in a number of other species (16). Rat GRF is more potent than human GRF in its ability to stimulate GH release from the rat pituitary (17). Synthetic peptides without this species-specific nature have been developed to release growth hormone from the species tested. Homology has been demonstrated between different species of hypothalamic GH releasing peptides (18).

Materials and Methods

Male Sprague-Dawley rats (Taconic Farms, Germantown, NY) weighing between 300 and 350 g were maintained on a diet of commercial rat chow with water *ad libitum*. After being weighed, animals were anesthetized with ether, then given an intraperitoneal injection of sodium pentobarbital (50 mg/kg body wt). The back and the abdomen of each rat were shaved and washed with 70% ethanol. Wound-breaking strength, collagen deposition, and histologic evaluation utilized the implantation of circular polyvinyl alcohol sponge (Unipoint Labs; High Point, NC) discs, 1.2 cm in diameter by 0.2-cm thick. For histology a polyvinyl sponge disc was sandwiched between two outer discs (1.2 × 0.2 cm) cut from a silicone rubber stopper and stapled together before being subcutaneously implanted under the dorsum. This modified implant for histology studies limits cell migration and deposition of new granulation tissue to the exposed peripheral edge of the disc. Implants were harvested at 7 days, and the polyvinyl center discs were separated from the outer silicone discs before fixing in formalin. The fixed tissues were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and viewed.

Other polyvinyl discs were modified by cutting them in half and then stapling the two halves back together prior to implantation. Two such modified implants were subcutaneously placed under the abdominal surface. The discs were harvested, at Day 7 the

staples were carefully removed, and the implant's breaking strength, developed at the sutured edges because of collagen deposition, was measured. Two pairs of hemostats were used to test wound-breaking strength. One pair was clamped to one half of the staple-free sponge implant and to a stationary object. The other clamp was clamped to the other half of the implant and secured by a string to a graduated cylinder hanging freely. Water was added at a constant rate to the graduated cylinder until the sponge halves tore apart. The container of water was weighed to the nearest gram and the result recorded as the breaking strength (2).

After testing the breaking strength, the sponge halves were pooled and further used to measure collagen deposition by extracting intact native collagen and purifying it by salt precipitation. Each pooled group of polyvinyl sponges was homogenized in 0.5 *M* ice-cold acetic acid containing 0.1 mg of pepsin/ml. The homogenate mixtures were stirred for 24 hr at 4°C and centrifuged; the supernatants were saved. Noncollagen proteins are either precipitated by the acid conditions or the pepsin cleaves the soluble proteins except collagen. The native solubilized collagen was precipitated by adding sodium chloride to 10% w/v. The pepsin and other peptides remain soluble in the salt solution. The precipitated collagen was collected by centrifugation (10,000*g* for 10 min), taken up in 145 m*M* potassium phosphate buffer (pH 7.6), exhaustively dialyzed against 10 m*M* HCl, and lyophilized, after which its dry weight was weighed on a balance and recorded. This technique requires pooling polyvinyl sponge implants in order to have enough material to weigh. Collagen purity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. It was found that no protein-stained bands other than those attributed to collagen and blue dye front could be identified.

Two full excision wounds (1.5 × 1.5 cm^2) were made by sharp excision on the back of each rat. After surgery, the wound edges were tattooed and measured. The area within those marks was measured again 1 week later. The weight of each rat was recorded on Day 0 and again at Day 7, the termination point of the experiment.

The synthetic GRF peptides were provided by Hoffmann-La Roche Inc., Nutley, NJ. [Desaminotyr^1, ala^{15}]-GRF(1–29)-NH_2, a shortened N-terminally substituted analog of GRF-44, which possesses high potency *in vitro* (12), was referred to as GRF-29A. The other peptide, which was identical to the natural sequence of human GRF containing 44-amino acids, was referred to as GRF-44. In order to maximize serum GH plasma concentrations without exhausting the reserves of pituitary growth hormone peptide, GRF-29A at 15 μg/dose and GRF-44 at 83 μg/dose were injected intramuscularly every 12 hr for 6 days. This schedule was selected because GRF-29A is 5 to 10 times more potent than GRF-44 (12) *in vitro* and it was anticipated that this dose would provide the maximum attainable pituitary GH response without depleting the gland of all of its GH reserves.

Four treatment groups were made. Rats whose initial body weight was between 310 and 330 g were housed in pairs for these studies.

Group 1. *Control.* Five rats were given intramuscular injections of 0.3 ml of saline twice daily at 12-hr intervals. Intramuscular injections of 0.2 ml of saline were given once daily. Doses were given on Day 0, the day of surgery, to Day 6.

Group 2. *Cortisone only.* Eight rats were given intramuscular injections of 0.2 ml (10 mg) of cortisone acetate once daily, as well as intramuscular injections of 0.3 ml of saline at 12-hr intervals. Two rats expired before 7 days, leaving six rats surviving for analysis at 7 days.

Group 3. *GRF only.* Six rats were given intramuscular injections (0.3 ml) of 83 μg of GRF-44 twice daily at 12-hr intervals. Five rats received twice daily intramuscular injections (0.3 ml) of 15 μg of GRF-29A at 12-hr intervals. All 11 rats were given daily 0.2 ml intramuscular injections of saline at a distant site.

Group 4. *Combination.* Seven rats were injected with GRF-29A and seven with GRF-44 (as described for Group 3) twice daily at 12-hr intervals, as well as concurrent injections of 0.2 ml (10 mg) of cortisone acetate once daily.

Results

The results are summarized in the tables. Changes in body weight appear in Table I. The minimal weight gain observed in control rats and in those treated with GRF was attributed to the trauma of surgery and the frequent handling necessitated by the injection schedule. The rats appeared to be healthy and continued to eat and drink water, but weight gain was modest during

Table I. Changes in Rat Body Weight at 7 Days

	Group change	Initial weight (g)	Final weight (g)	%
1	Control alone (n = 5)	302 ± 15	314 ± 10	+3.9
2	Cortisone alone (n = 6)	292 ± 11	248 ± 17	−14.8[a]
3	GRF-44 alone (n = 6)	314 ± 23	344 ± 39	+9.2
	GRF-29A alone (n = 5)	286 ± 33	336 ± 34	+8.7
4	GRF-44 + cortisone (n = 7)	310 ± 27	270 ± 31	−13.0[a]
	GRF-29A + cortisone (n = 7)	308 ± 26	270 ± 26	−11.9[a]

[a] Significant difference using Dunnett's test ($P \leq 0.05$).

the week before testing. Rats in groups 2 and 4, which received cortisone alone or in combination with releasing factors, lost between 18 and 22% of their body weight. This expected finding results from the catabolic effects of glucocorticoid therapy in rats. The concurrent administration of GRF did not reverse the glucocorticoid-induced weight loss.

GRF-44 was seen to antagonize the effects of glucocorticoids on the deposition of collagen (Fig. 1). That is, animals in Group 2, which received cortisone alone, had the least amount of collagen deposited, 480 μg/implant. Rats in Group 4, which received concurrent cortisone and GRF-44 injections, had 590 μg of collagen deposition/implant. The concentration of collagen deposited in the combination group receiving GRF-29A was similar to that of Group 2, cortisone alone. Of interest is the finding that the concentrations of collagen in implants in the combination group that received GRF-44 are similar to those of controls. Both preparations of GRF alone, 790 μg of GRF-44 and 890 μg of GRF-29A, enhanced collagen deposition. Glucocorticoid-alone therapy, in Group 2, showed a reduction in collagen deposition to 33% of that of the control Group 1.

Pepsin digestion extracts only native collagen. Other proteins and denatured collagen will be cleaved by pepsin digestion. Collagen newly deposited in the

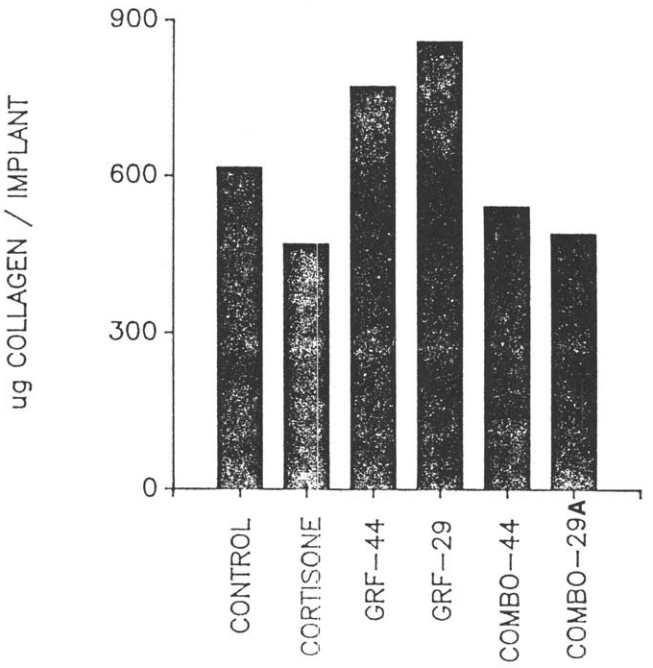

Figure 1. Collagen content of sponge implants. Implanted polyvinyl sponges were harvested at 7 days. After testing for breaking strength, the ruptured sponge halves were pooled to make six treatment groups. Collagen was extracted by limited pepsin digestion, salt fractionated, dialyzed against 1 m*M* HCl, frozen, and lyophilized. The salt-free lyophilized collagen was weighed and the total weight was divided by the number of sponges in that treatment group. No statistical analysis was possible because the collagen content measured was a total of all of the sponges in each group.

first 2 weeks of healing is responsible for increases in wound-breaking strength. During the later stages of healing, after 2 weeks, collagen covalent crosslinks are responsible for the continuing gain in wound-breaking strength. The inhibiting effect of glucocorticoids on collagen deposition can be seen in the reduced gains in wound-breaking strength (Table II). Rats in Group 2 (cortisone alone) showed impaired progression in wound-breaking strength, 354 g of compared with 587 g for controls. Concurrent administration of GRF-44 to cortisone-treated rats restored gains in wound-breaking strength to normal levels (Table II). The breaking strength of sponge halves from rats receiving GRF-44 concurrently with cortisone was 19% greater than those implants from rats receiving cortisone alone; this difference was statistically significant by Dunnett's test. Under the same conditions, GRF-29A ineffectively antagonized cortisone's inhibition of gain in wound-breaking strength. This group of rats was statistically identical to the cortisone-alone rats (Group 2). Rats receiving GRF alone (Group 3) showed that GRF-29A produced no enhancement but, in fact, caused an insignificant reduction in the measured gain in wound-breaking strength. In comparison, GRF-44 modestly enhanced gain in wound-breaking strength compared with controls, an increase that was not statistically significant.

At 7 days, an open wound in an untreated rat contracted by 80% to 20% of its initial size (Table III). Rats treated with cortisone alone (Group 2) showed only a 56% decrease in wound size. Concurrent therapy with either GRF-44 or GRF-29A (Group 4) did not restore wound contraction to normal levels. Wound contraction in Group 4 (the combination groups) was identical to that of Group 2 (the cortisone-alone treated rats, Table III). Systemic injections of GRF alone (Group 3) did not promote wound contraction. Wound

Table II. Implant Breaking Strength at 7 Days

	Group	Breaking strength (g)
1	Control (n = 9)	587 ± 203
2	Cortisone alone (n = 6)	354 ± 92[a]
3	GRF-44 alone (n = 8)	678 ± 74
	GRF-29A alone (n = 8)	488 ± 194
4	GRF-44 + cortisone (n = 10)	422 ± 178
	GRF-29A + cortisone (n = 12)	362 ± 139[a]

[a] Significant difference between these groups and controls based on Dunnett's test ($P \leq 0.05$).

Table III. Changes in Area of an Open Wound at 7 Days

Group change	Initial size (mm^2)	Final size (mm^2)	%
1			
Control ($n = 5$)	1990 ± 200	410 ± 150	−79.8
2			
Cortisone alone ($n = 6$)	1930 ± 410	850 ± 170	−55.7[a]
3			
GRF-44 alone ($n = 12$)	2410 ± 820	790 ± 490	−67.2
GRF-29A alone ($n = 10$)	2130 ± 790	620 ± 260	−71.0
4			
GRF-44 + cortisone ($n = 10$)	2110 ± 350	1340 ± 550	−37.5[a]
GRF-29A + cortisone ($n = 9$)	2000 ± 360	980 ± 220	−51.2[a]

[a] Significant difference between these groups and controls based on Dunnett's test ($P \leq 0.5$).

contraction in the GRF-alone rats (Group 3) was the same as the controls (Group 1).

Examples of histologic sections of modified polyvinyl sponge implants are shown in Figure 2. Figure 2A shows a control with an ingrowth of granulation tissue. In Figure 2B the limited amount of granulation tissue is remarkable, but expected; cortisone therapy inhibits inflammation and the proliferative phase of healing is, therefore, compromised. In Figure 2C granulation tissue appears to be similar to that of controls in the group treated with GRF-29A alone. It was not possible to appreciate an enhancement of tissue deposition in implants from rats receiving GRF-44 injections compared with untreated controls (Fig. 2D). When Figure 2E is compared with 2B the differences are striking. Concurrent therapy with GRF-44 restored the proliferative phase of healing to cortisone-treated rat wounds. In rat wounds treated with GRF-29A and cortisone, some promotion of the proliferative phase of healing was observed (Fig. 2F), but this was of minor note when compared with GRF-44-treated rats.

Discussion

The release of GRF stimulated by the hypothalamus results in the pituitary release of growth hormone. Circulating growth hormone stimulates the secretion of the hormone-mediating factor somatomedins (19). Insulin-like growth factor or somatomedin C suppresses the synthesis of pituitary growth hormone (20, 21). Somatomedins stimulate cell proliferation in a variety of cell types. Human fibroblasts make somatomedin-like peptides in culture. Growth hormone and other growth factors may promote fibroblast proliferation by this mechanism (22).

GRF maximizes the release of growth hormone in a species-specific manner. Natural homologues from other species may elicit a GH release response, but it is of lesser magnitude in the nonhost species. Synthetically derived homologues have greatly decreased species specificity and appear to have equal potency in maximizing the release of growth hormone.

GRF-44 alone promoted a small increase in wound-breaking strength compared with controls. GRF-29A alone produced a smaller decrease in wound-breaking strength compared with controls. These differences were found not to be significant. GRF-29A treatment increased collagen deposition compared with GRF-44 treatment. Because measurements were made of pooled samples, no statistical analytical manipulations could be employed, but GRF therapy appeared to enhance collagen deposition. This enhancement has a possible negative effect or, at best, a modest promotion on gains in wound-breaking strength.

GRF-44 and GRF-29A alone produced enhanced collagen deposition. However, in regard to gain in wound-breaking strength they were the same as controls. The reduction exhibited with GRF-29A therapy was attributed to very low values from two implants, which is reflected in the high standard error reported. This difference appears to be related to a technical problem with these two implanted sponges, rather than an inhibiting action of the GRF-29A. GRF alone had no promoting activity on enhancing wound contraction. These findings are expected since anabolic agents, like vitamin A alone, have shown only modest effects in promoting rat wound healing (3). In another study with the same rat model used here, a promoter of macrophage infiltration at the wound site stimulated wound contraction in the presence of cortisone therapy (2). Cortisone's inhibition of wound contraction was reversed by the concurrent administration of tetrachlorodecaoxygen. This was an unexpected finding. Here, the concurrent administration of an anabolic agent did not reverse the cortisone-induced inhibition of wound contraction.

The synthetic natural GRF-44 and the shortened peptide analogue, GRF-29A, both would be expected to elicit a growth hormone release from the pituitary. GRF-29A reportedly releases 5 to 10 more GH than GRF-44 but it was not as effective in this wound-healing study. One possibility is that GRF-29A is ineffective at releasing GH in the presence of glucocorticoids. Another is that GRF-44 is more effective than GRF-29A because it can sustain a release of GH over a long period of time. This sustained increased release of GH may effectively enhance the proliferative phase of healing, in the presence of systemic glucocorticoids. It may be that GRF-29A exhausts GH reserves in an acute manner and only a spiked release of hormone occurs. This short interval of release of GH does not maintain

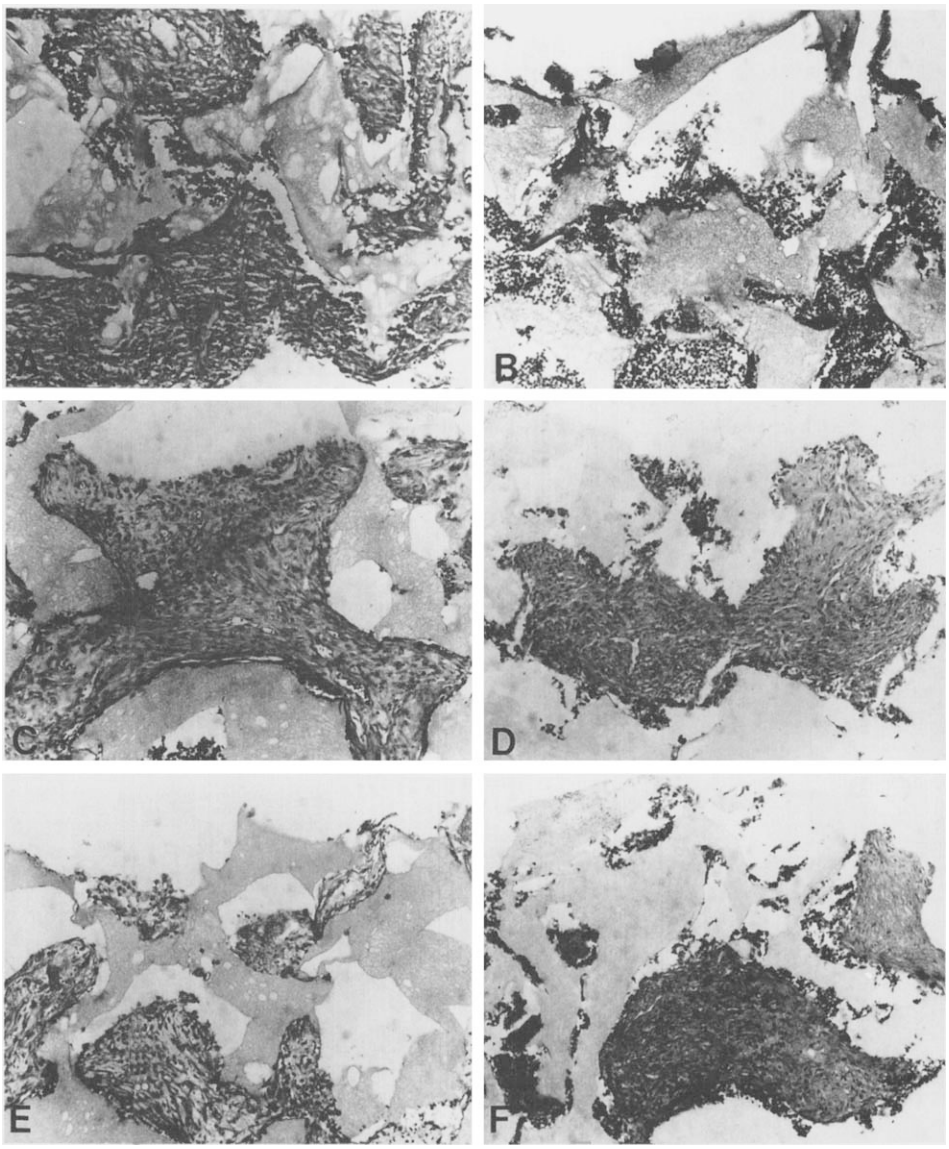

Figure 2. Histology of 7-day-old implanted polyvinyl sponges in rats. Implants were fixed, processed, and stained as described in Materials and Methods. The outer edge of the sponge appears at the top of the panel. The entire capsule covering the implant was removed prior to processing. (A). Control (Group 1) implant shows granulation tissue accumulating within the sponge interstices. (B) Cortisone-alone (Group 2) implant shows that very little granulation tissue has developed within the sponge. Interstices have some fibrin with few inflammatory cells. (C) GRF-29A-alone (Group 3) implant shows granulation tissue within the interstices of the sponge material. (D) GRF-44-alone (Group 3) implant shows granulation tissue deposits throughout the sponge. (E) Cortisone with GRF-29A (Group 4) implant shows few areas with granulation tissue; most areas are filled with fibrin with sparse cells within it. (F) Cortisone with GRF-44 (Group 4) implant shows numerous areas with granulation tissue deposits. The granulation tissue is less dense than controls but more dense than cortisone alone or cortisone with GRF-29A.

a sustained level of GH which is necessary for antagonizing cortisone's inhibition of wound healing.

Studies of GRF as an antagonist of cortisone inhibition of wound healing, measured by gains in wound-breaking strength and deposition of collagen, showed that GRF-44 was more effective than GRF-29A. The antagonism of cortisone by these agents is defined by gains in wound-breaking strength and new collagen deposition. The use of GRF is expected to increase systemic levels of growth hormone, which, in turn, would antagonize the cortisone inhibition of wound healing by promoting insulin-like growth factor, somatomedin, at the wound site. This may prove clinically beneficial, especially for the trauma patient whose elevated levels of glucocorticoids can be expected to contribute to a less than optimal wound-healing response. GRF may promote healing by intensifying the anabolic state of the patient. It is speculated that GRF works by promoting the proliferative phase of the healing process through increasing the levels of somatomedin at the healing site.

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