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EFFECTS OF BIOSYNTHETIC HUMAN EPIDERMAL GROWTH FACTOR ON WOUND HEALING

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We are investigating the ability of biosynthetic epidermal growth factor (EGF) to accelerating healing of cutaneous injuries including middermal wounds and incisions. In preclinical animal studies, EGF significantly increased the rate of epidermal regeneration 2-fold in pigs following second degree thermal injuries or middermal incisions. EGF also induced hyperplasia of the epidermal cell layer which remodeled to normal skin architecture two weeks after complete healing. Two peptide growth factors structurally related to EGF, vaccinia growth factor (VGF) and transforming growth factor-α (TGF-α), also accelerated epidermal regeneration in middermal burns. A single application of EGF increased the tensile strength of cutaneous incisions in rats during the early phases of healing but only when formulated in a slow release vehicle such as liposomes. The rapid loss of EGF from the area of incisions when formulated in saline or viscous materials such as hyaluronic acid probably prevents its actions. Clinical trials with EGF in donor site middermal incisions are underway to evaluate its clinical usefulness.

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STATEMENT OF PROBLEM UNDER STUDY

At the present time, accelerating healing of major injuries such as middermal burns (second degree) or incisions is beyond the scope of clinical medicine. However, there is no doubt that much of the morbidity, mortality, and cost associated with such major injuries is closely related to the length of time required for them to heal. The major goals of this contract are to develop and evaluate new methods to accelerate healing of burns and incisions using biosynthetic peptide growth factors to stimulate normal healing processes.

BACKGROUND AND REVIEW OF APPROPRIATE LITERATURE

Epidermal regeneration. Patients with extensive second and third degree burns are at increased risk of developing life-threatening infections and other complications until their epidermal layer has regenerated or been replaced with autograft skin. The limiting factor in covering major third degree burns is the availability of autogenous skin grafts. Although cadaver and porcine skin or placental membranes are reasonable temporary substitutes, they are limited by the inevitable generation of immune responses to the foreign tissue. Similarly, synthetic skin, which has been difficult to produce in large amounts, eventually requires grafting with the patient's own skin. Since the only permant solution currently available is autografting, an agent that would accelerate healing of skin graft donor sites would decrease the morbidity and mortality of severely burned patients.

Epidermal growth factor (EGF), a small peptide (5458 mw) found in urine and blood of man and other animals, has potent mitogenic effects on epidermal cells in vitro (1). Multiple studies have tested the effect of EGF on epithelial cells in vivo. Brightwell et al. (2) reported that repeated topical dosing of EGF accelerated epithelial regeneration of primate corneas, and in unpublished results, EGF treatment produced total healing of patients with chronic corneal epithelial defects (R. Eiferman, personal communication). Results of studies investigating action of EGF on healing cutaneous wounds have been inconclusive and conflicting. Greaves (3) reported that brief (5 min) exposure of suction-induced epidermal wounds to EGF in saline solution once daily failed to accelerate wound closure. Arturson (4) also failed to find enhanced healing of middermal thermal burns in rats treated once daily for 20 seconds with EGF in saline. Thorton et al. (5) applied EGF once daily as a mist to middermal burns on rats and did not observe an increase in rate of healing. Franklin and Lynch (6), however reported a qualitative improvement in closuer of full-thickness wounds in rabbit ears when treated with EGF in an ointment. The studies which failed to show an acceleration of healing may have not provided adequate continuous exposure of the residual epidermal cells to the EGF. Studies in vitro have clearly shown that the mitogenic effect of EGF requires continuous exposure of cells to EGF for a minimum of 6 to 12 hours (7).

Incision healing. Treatment of sutured incisions is limited by the inability to easily reapply material in the tract of the incision once it has been closed. Thus, a single application of EGF in a saline vehicle might be ineffective if EGF was not retained in the incision tract for an adequate period. Buckley et al. (8) implanted polyvinyl sponge disks subcutaneously beneath the ventral panniculus carnosus in rats and measured collagen content 20 days later. Daily injections of EGF into the sponges increased collagen content 26% compared to saline injection. Since the EGF rapidly disappeared from the injection site (only 10% remained after 4 hours), EGF was formulated in slow release pellets which were placed in the implated in the sponges. At day 7, sponges receiving 10 ug of EGF released per day contained 41% increase in collagen content and pronounced increased in cellularity (DNA) and vascularity compared to saline control sponges. Similar results were reported by Laato et al. (9) following daily injections of EGF into cylindrical hollow sponges implanted subcutaneously on backs of rats,and by Grotendorst et al. (10) with collagen filled chambers containing EGF or platelet derived growth factor (PDGF).

RATIONAL USED IN CURRENT STUDY

Based on the in vitro and in vivo data discussed above, it seemed apparent that EGF could stimulate epidermal healing and cutaneous healing if applied in appropriate formulations. The model we chose for testing epidermal regeneration was middermal themal burns and split-thickness incisions (donor site injuries) in pigs. This animal model offered several distinct advantages over rats or guinea pigs. Pigs are large enough to sustain numerous test burns on the same animal without imparing the general health of the animal allows several test to be performed on the same animal. Thus, more direct comparisons can be made between experimental and control burns by minimizing variabilitiy in healing rates between different animals. Also, pig skin is very similar in structure and healing properties to human skin and pigs have been used extensively in burn research. The experimential protocol would be to test EGF in different formulations including saline, lanolin, and a commercially available antibiotic cream, Silvadene. Both split thickness incisions and middermal thermal burns would be utilized as models.

To study healing of cutaneous incisions, we chose to use interscapular incisions of rats. This animal model offered the advantages of low cost per animal and the ability to produce an incision long enough to obtain miltiple samples from each incision for tensile strength measurements. Also, rats have been used extensively for studies of cutaneous incision healing. The experimental protocol would be to test EGF in different vehicles including saline, single lamellar liposomes, multilamellar liposomes, and viscous polymer solutions. Tensile strength measurements and histology would be performed.

EXPERIMENTAL METHODS

Epidermal Regeneration. Experiments were conducted as described by Brown et al. (11). Eighty four (84) split-thickness cutaneous wounds (0.005 inches thick, 1x1 cm) were made on the dorsal thorax of four adult miniture pigs (Vita-Vet Laboratories, Marion, ID). Twice a day, 28 wounds were treated with EGF (10 ug/ml) in lanolin or Silvadene, 28 were treated with the cream alone, and 28 were untreated. Four (4) wounds from each treatment group on each pig were randomly selected each day and totally excised to a depth of 0.007 inches, including 5 mm of surrounding normal skin. Epidermal and dermal layers of the specimens were separated after incubation in trypsin solution overnight. Wounds were considered healed when no defect was present in the epidermal layer and not healed if any defect remained.

Middermal thermal burns were made by 10 seconds of contact with a brass template (3x5 cm, 430 g) heated to 70 C in a constant-temperature water bath to the depiliated dorsal skin of pigs (7-14 kg). After the resulting blister was removed, burns were treated twice a day with either EGF in Silvadene, Silvadene alone, or untreated. After 7 days, the fibrinous coagulum was removed and burns were photographed and biopsey specimens taken. The percentage of the original burn area that appeared to be reepithelized was calculated by computerized planimetry of enlarged photographs.

Cutaneous incision healing. Adult male Spraque Dawley rats were anesthetized with Ketamine and Rompum and a single incision 5 cm long was made in the dorsal midline at the caudal portion of the back of each rat. Test solutions were placed in the tract of the incision, and incisions were closed with five (5) evenly placed interrupted horizontal mattress sutures of 4-0 nylon. Rats were housed in individual cages with unrestricted feed and water. At intervals after surgery, rats were sacrificed by carbon dioxide inhalation, and incisions together with surrounding normal skin were dissected to the panniculus carnosum, then placed on ice-cooled dishes until tested for tensile strength. For each incision, three strips of tissue were cut perpendicular to the original incision, and measured for tensile strength using a commercial instrument (UNITE-O-MATIC tensiometer). The values of the three strips were averaged to determine the tensile strength for each incision. For each experimental condition, the tensile strength was calculated by averaging the tensile strength for all incisions in that test group. Typically, ten (10) rats were used for each experimental condition at each time point. Specimens for histology were placed in 10% neutral buffered formalin, processed through paraffin, sectioned, and stained with hematoxyline and eosine.

Multilamellar liposomes containing EGF were prepared by adding lecithin (50 mg) to a 12x75 glass test tube and the hexane vehicle was evaporated under a stream of nitrogen. One ml of phosphate buffered saline (PBS) containimg 1mg of EGF was added and immediately vortexed and sonicated, then the liposomes were

separated from the solution by centrifugation at 20,000 x g for 20 min or by gel filtration through a 25 cm column of Agarose 4B. The amount of EGF entraped in liposomes was calculated by measuring the amount of a trace ^{125}I-EGF present in the purified liposomes. Single lamellar liposomes also were prepared as described previously (12). Briefly, a mixture of lipids containing lecithin, phosphatidylglycerol, and cholesterol were added to a round bottom flask, 5ml of ether added and dried under a nitrogen stream. Five ml of ether was added followed by 1ml of EGF (1mg/ml) in PBS then immediately sonicated. The organic phase was removed by rotary evaporation and the single lamellar liposomes were formed, then purified by gel filtration or centrifugation as before.

To measure the retention of EGF in incisions, multilamellar and single lamellar liposomes, as well as a solution of EGF in PBS, were prepared as above with a trace amount of 125I-EGF included to enable measurement of EGF by scintilation counting. Incisions were made as before and a known amount of EGF was placed in the incision tract then the incision was closed with sutures. At intervals, the incisions and surrounding skin were excised and counted.

RESULTS

Epidermal regeneration. As shown in Figure 1, 50% of the split-thickness incisions treated with EGF in lanolin or in Silvadene were healed after two (2) days of treatment, whereas vehicle-treated or untreated groups required greater than four (4) days for 50% of the wounds to heal ($p<0.05$, chi-squared analysis). Histological evaluation of healed split-thickness wounds were similar in appearance for all three groups wounds with a 10 to 12 cell layers of stratified epithelium characteristic of normal pig skin and no evidence of metaplastic transformation.

EGF-treatment of middermal thermal burns significantly ($p<0.01$, Tukey's analysis of variance) increased the area of regenerated epidermis at 7 days after injury (Table 1). Biopsy specimens taken 7 days after burn injury confirmed complete epidermal regeneration of EGF-treated burns with stratification of cell layers. In contrast, very little epidermal regeneration was observed in vehicle-treated or untreated burns. Specimens taken 35 days after burr injury appeared totally normal histologically.

Response of middermal burns to varying doses of EGF is shown in Figure 2. All burns treated with EGF (0.1, 1, and 10 ug/ml) were significantly more healed than untreated burns, but only burns treated with EGF at 10 ug/ml were significantly more healed than vehicle-treated burns ($p<0.05$, analysis of variance).

Treatment of middermal burns with TGF-α or VGF produced results similar to those observed with EGF-treatment. As shown in Table 2, treatment with TGF-α or VGF resulted in substantially more area re-epithelized than untreated or vehicle-treated burns. Also, low levels (0.1 ug/ml) of TGF-α appear to be more effective

than low levels of EGF in stimulating epidermal regeneration (Figure 3).

Cutaneous incision healing. Treatment of incisions at the time of suturing with EGF in saline failed to produce an increase in tensile strength even at high levels of EGF (100 ug). In contrast, when repetitive doses of EGF were given three times a day for 5 days (75 ug per injection) via a porous indweling cathater underlying the incision, EGF-treated incisions were approximately 35% stronger than saline-treated incisions at 7 days ($p<0,01$, Figure 4). Furthermore, a single treatment with a total of 3 ug of EGF encapsulated in multilamellar liposomes (ML-EGF) caused approximately 300% ($p<0.05$, T-test) increase in tensile strength at 10 and 14 days post-incision compared to blank liposomes or untreated incisions (Figure 5). Histolgy of 7 day incisions treated with EGF in liposomes showed hypertrophy of the overlying epidermis and substantially more fibroblast-like cells in the area of the incision tract compared to control incisions. Electron microscopy of 14 day incisions treated with EGF showed many active fibroblasts present with extensive rough endoplastic reticulum and more new collagen present than control incisions. Specimens at 28 days treated with EGF showed large numbers of very active fibroblasts, extensive well organized collagen with sparse ground substance giving the overall appearance of a very strong, mature scar, while control specimens retained large amounts of edematous and spongy ground substance indicating earlier stages of wound repair.

These results suggested that a major factor in the effectiveness of EGF in vivo is the length of exposure of incisions to EGF. When EGF formulated in saline, hyaluronic acid, multilamellar or single lamellar liposomes was placed in tracts of incisions, and the percentage of EGF remaining in the region surrounding the incisions was measured daily was determined, the results shown in Figure 6 were obtained. After one day, only 20% of the EGF formulated in saline or hyaluronic acid remained in the region surrounding the incision. EGF encapsulated in single lamellar liposomes also was rapidly lost from the incision area, but EGF encapsulated in multilamellar liposomes was substantially retarded in the area of the incision with 40% still retained at three days.

DISCUSSION AND CONCLUSIONS

The effects of EGF in vitro on epidermal and stromal cells suggest that processes of wound healing might be accelerated in vivo by exposing tissues to EGF. Our results support this hypothesis and reinforce the need to appropriately formulate EGF to provide constant exposure of target cells with EGF. The failure of previous studies of epidermal regeneration following middermal burns or split-thickness incisions which used EGF in vehicles or regimens which limited exposure time of EGF may be explained on this basis. Similar principles must be considered in healing cutaneous incisions. No increase in tensile strength of incisions was found when EGF was formulated in saline or

hyaluronic acid which allowed rapid exit of EGF from incisions. In contrast, large increases in tensile strength were produced by EGF encapsulated in liposomes which delayed the release of EGF from incisions or when EGF was repeatedly infused into incisions.

The results reported here demonstrate the 'proof of principle' that biosynthetic peptide growth factors can accelerate wound healing in vivo. Additional research needs to be conducted including evaluation of EGF in combination with other well characterized growth factors such as PDGF and fibroblast growth factor (FGF). It is probable that synergistic actions would occur with combinations of growth factors producing even greater acceleration of healing. Another area for further investigation is development of better slow-release vehicles for EGF. Biodegradable microencapsulation materials which could be designed to release EGF at different rates would likely improve healing. Preliminary discussions with material scientists at Ethicon Inc. indicate that it is possible to incorporate EGF in microspheres of polyvicryl which can be constructed to have different rates of biodegradation. Additional valuable information will be obtained when the limited clinical trial using EGF in Silvadene to treat skin graft donor sites begins at Emory University in late 1986 under the direction of Dr. Gregory Brown.

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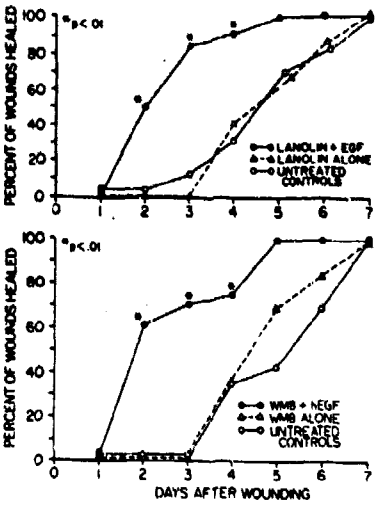

FIGURE 1. Effects of hEGF and vehicles on healing of split-thickness wounds. Each point represents the data from 16 wounds treated with hEGF (10 μg/ml) in the indicated vehicles, with vehicles alone, or with no treatment. Values were compared for statistical significance by χ^2 analysis. WMB, water-miscible base (Silvadene).

TABLE I

Effect of hEGF and Vehicle on Healing of Partial-thickness Burns

Treatmen.	Percentage of burn area healed
hEGF	93 ± 6
Control	36 ± 18
Untreated	17 ± 11

$p < 0.01$ using one-way analysis of variance and Tukey's Honest Statistical Difference (HSD) test. Quantitative planimetry measurements were performed on photographs of partial-thickness burns that were treated for 7 d with hEGF in Silvadene (10 μg/ml), Silvadene alone (control) or untreated. Values are mean ± SD for eight wounds.

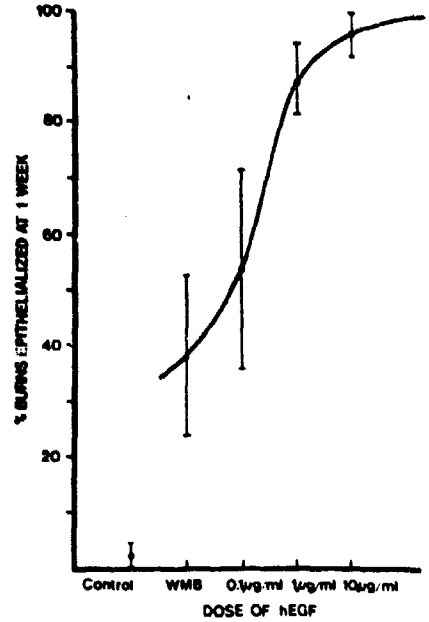

FIGURE 2. Response of partial-thickness burns to varying doses of hEGF. Partial-thickness burns (3 × 3 cm) were treated for 7 d with Silvadene alone, Silvadene containing hEGF (10 μg/ml, 1 μg/ml, 0.1 μg/ml), or were not treated. The percentage of original burn area that had healed was measured by quantitative planimetry of photographs. Each data point is the mean ± SD for four burns. WMB, water miscible base (Silvadene).

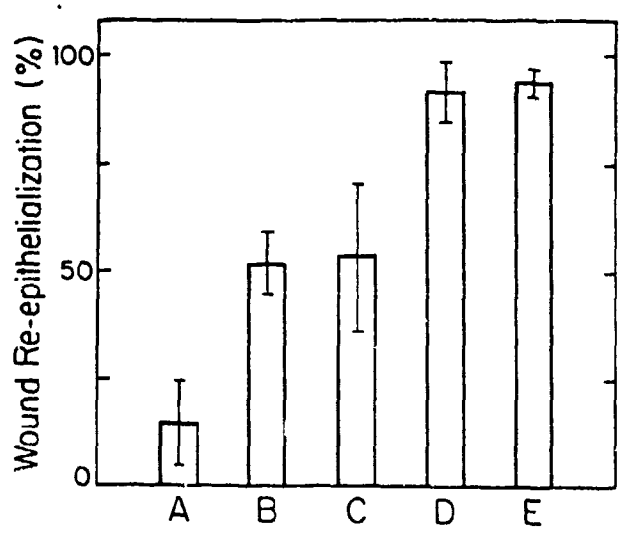

Figure 3. Relative abilities of various treatment regimens to facilitate epithelial wound healing. The growth factors were all tested at 0.1 μg/ml. This concentration has previously been shown to be suboptimal for EGF (2). Conditions are: A. Untreated; B. Silvadene® alone; C. Silvadene® and EGF; D. Silvadene® and TGF; E. Silvadene® and VGF. Results were scored at 9-10 days and are the average of two or more experiments using different test animals. Error bars are mean ± standard deviation.

Table 2. Epithelial regeneration in mid-dermal burns treated with TGF-α or VGF

	Percent of original burn area with regenerated epithelium				
animal 1					
				VGF (μg/ml)	
	Vehicle	Untreated	0.1	0.5	1.0
right side	68 ± 6	10 ± 1	96 ± 2	95 ± 1	85 ± 3
left side	76 ± 1	6 ± 4	92 ± 1	90 ± 2	68 ± 4
animal 2					
				rTGF-α (μg/ml)	
	Vehicle	Untreated	0.1	1.0	10
right side	45 ± 5	30 ± 6	98 ± 1	73 ± 1	90 ± 1
				hTGF-α (μg/ml)	
	Vehicle	Untreated	0.1	1.0	5.0
left side	58 ± 2	17 ± 3	86 ± 4	84 ± 4	66 ± 6

Mid-dermal thermal injuries were made on the dorsal thorax of anesthetized female Yorkshire pigs (30 lbs) whose backs had been shaved and dipilitated with commercial hair cream remover (2). A brass template (3 x 3 cm, 147 gm) was equilibrated in a 70°C water bath and placed in firm contact with the skin for exactly 10 sec and the resulting blister was removed. Five mid-dermal burns were placed on each side of the spine and were separated from each other by approximately one inch. Burns were treated twice a day with approximately 3 ml of vehicle cream (Silvadene®) alone or containing growth factor or were untreated. Synthetic TGF-α, both human and rat, was purified to homogeneity by reverse phase chromatography as previously described (5). VGF was purified

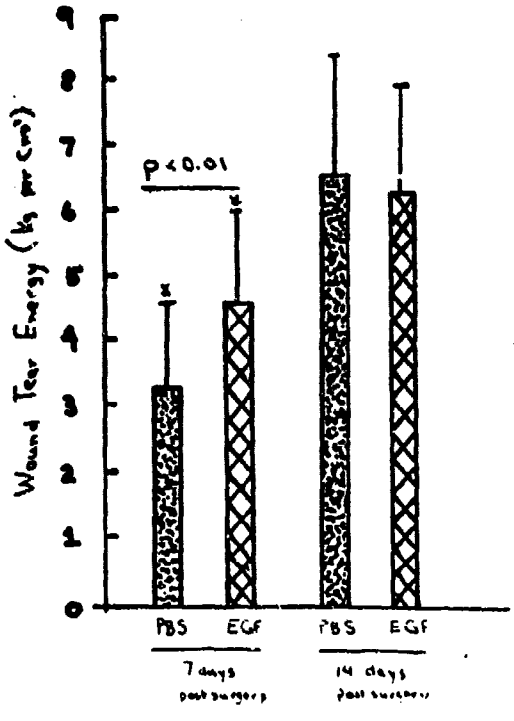

Figure 4. Tensile strength of incisions treated with EGF. Single incisions 5 cm long were made to the panniculus carnosum in the dorsal midline at the caudal portion of rats and closed with five interrupted sutures. EGF (75 µg) or saline was injected three times daily for five days via an indwelling perforated catheter underlying each incision, then incisions excised and tensile strength measured at 7 and 14 days. Values represented are mean and standard deviation of ten incisions. Paired-T test used for statistical comparison.

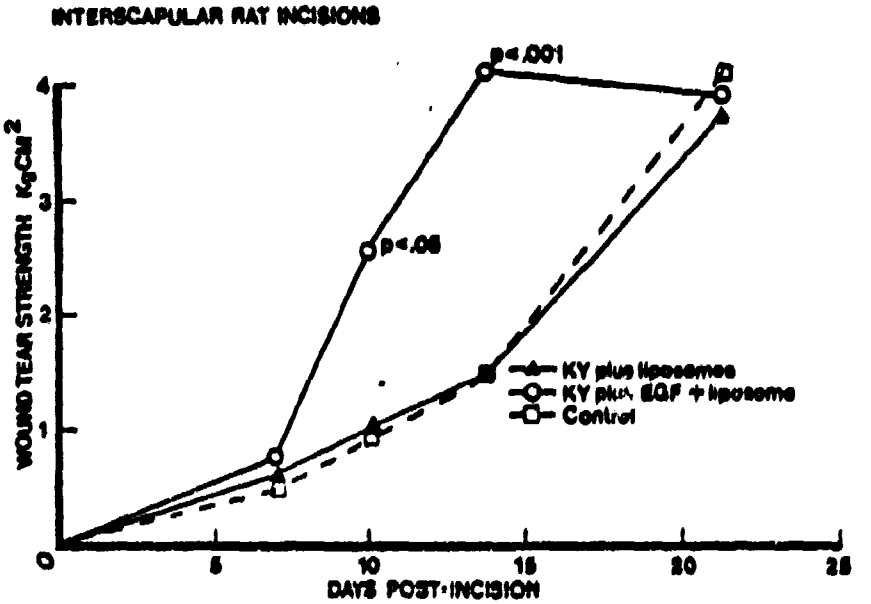

Figure 5. Tensile strength of incisions treated with EGF-liposomes. Single incisions 5 cm long were made to the panniculus carnosum in the dorsal midline at the caudal portion of rats and EGF (3 µg) encapsulated in multilamellar liposomes or blank liposomes placed in the tract of the incision which was then closed with five interrupted sutures. Incisions were excised at weekly intervals and tested for tensile strength. Values represented are the mean of seven incisions and T-test was used for statistical comparisons.

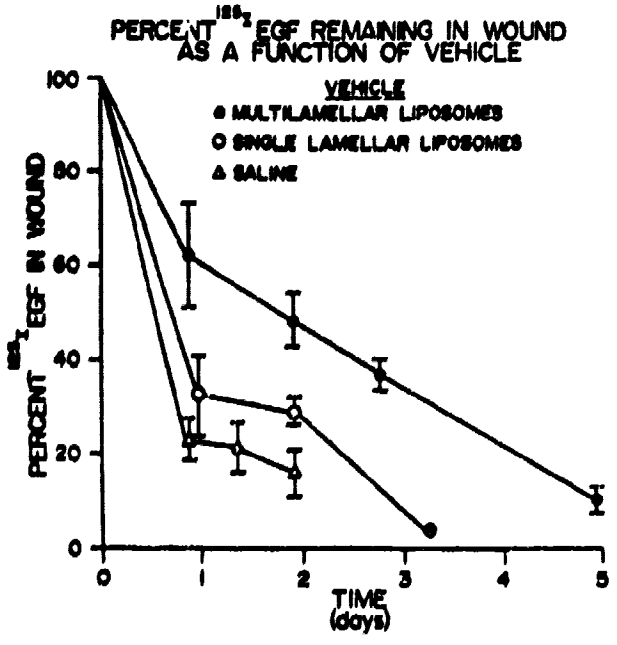

FIG. 6. MEASUREMENT OF EGF REMAINING IN INCISION SITE, IN VIVO. IODINATED EGF IN MULTILAMELLAR LIPOSOMES, UNILAMELLAR LIPOSOMES, OR SALINE WAS PLACED INTO THE TRACT OF A 4 cm INTERSCAPULAR INCISION WHICH WAS CLOSED USING 4.0 NYLON IN A RUNNING SUTURE. AT INDICATED TIMES, ANIMALS WERE SACRIFICED AND 0.5 cm OF TISSUE WAS EXCISED FROM EACH SIDE OF WOUND. TISSUE WAS WEIGHED AND IODINATED EGF WAS MEASURED USING A BECKMAN GAMMA COUNTER.