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Essential Fatty Acids Are Not Required for Wound Healing

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ABSTRACT. **Rats with essential fatty acid deficiency (EFAD) exhibit mild body growth retardation, diminished leukocyte influx in certain models of inflammation, and skin lesions characterized by ulceration, thinning and decreased pigmentation. In the present study we examined the role of EFAD in cutaneous wound healing, a process in which the inflammatory response and the macrophage play a central role. We reproduced the EFAD condition in Lewis rats (n = 35), and examined its effects in wound healing using the paired rat surgical incision model. Rats were compared with weight-matched controls, receiving standard chow diet. Skin samples harvested at days 5, 7, 14 and 21 post-wounding were evaluated for tensiometry and histology. EFAD rats exhibited all the characteristics of this condition, and the typical alteration of liver lipids. Skin samples harvested at different days post-wounding did not show difference in maximal breaking strength when compared to weight-matched controls. Histological evaluation of skin samples showed no difference in the cellular inflammatory infiltration in either EFAD rats or in weight-matched controls. Immunohistochemical studies revealed no difference in the influx of macrophages in the different groups of rats. Fatty acid supplementation of EFAD rats (n = 7), successfully reversed the EFAD state as assessed by the macroscopic skin and liver changes and liver fatty acid content, without modifying either tensile strength or cellular inflammatory infiltration. Our results suggest that EFAD does not alter the normal course of the cutaneous wound repair in rats, despite all the cutaneous alterations produced by this condition. We conclude that essential fatty acids (EFAs) are not essential for cutaneous wound repair.**

INTRODUCTION

The essential fatty acids (EFAs) are w-6 polyunsaturated fatty acids (PUFAs) that can not be synthesized de novo from other lipids, carbohydrates or amino acids by mammals. Linoleic 18:2 (n-6) acid is a fundamental EFA from which all others (n-6) PUFA are derived metabolically (1). The most important metabolite, arachidonate, is incorporated into membrane lipids, and serves as a precursor in the formation of prostaglandins and leukotrienes.

The essentiality of dietary fat was first demonstrated in 1929 by Burr and Burr who observed poor growth and skin lesions in rats raised on a fat-free diet (2). In experimental animals essential fatty acid deficiency (EFAD) diets induced diminished body growth, increased fragility and permeability of cellular membranes, and macroscopic changes in organs (3). Recent studies in rats have clearly demonstrated that EFAD can ameliorate the course of different pathophysiologic processes characterized by active inflammation such as autoimmune diabetes (4, 5) and immune mediated glomerulonephritis (6), associated with inhibition of monocyte infiltration (6, 7). Such studies have led to the proposal that EFAD results in decreased formation of an as yet unknown lipid chemoattractant for macrophages. Depletion of kidney resident macrophages by EFAD, diminished the allogenic potential of the kidney in transplanted rats (8). Prior studies on the role of macrophages in the normal process of wound healing indicate that these cells play an important role in wound repair (9, 10). The kinetics of the collagen synthesis during wound healing is highly related to the macrophage infiltration which peaks at about 5 days post-wounding (11). Abrogation of macrophage infiltration with total body irradiation or glucocorticoids resulted in significant decrease in wound repair (12–14).

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Cutaneous abnormalities associated with EFAD have been described such as increased transepidermal water loss, desquamation and thinning, diminished skin pigmentation, and increased tendency to hair loss (15). Studies confirmed that the cutaneous signs are also predominant in humans with EFAD induced by parenteral nutrition with poor fat content, and confirmed that α-linolenic and linoleic acids are essential nutrients to keep the cutaneous integrity (16). Those studies opened the possibility of abnormalities of wound repair associated with the EFAD state. However, the attempts to either prove or disprove such association are not convincing (17), and to date no conclusive data are available regarding the effects of EFAD on cutaneous wound repair.

The present studies were designed to elucidate the influence of essential fatty acids on cutaneous wound healing. We used two approaches: First, we studied the effect of EFAD on wound breaking strength as an expression of collagen deposition in EFA-deficient rats, control rats and rats with EFAD supplemented with linoleate. And second, we looked at the characteristics of the cellular inflammatory infiltration into the wound by using histology and immunohistochemistry.

MATERIALS AND METHODS

Animals

Male Lewis rats were obtained as weanlings from Harlan Sprague Dawley (Indianapolis, IN). A fat-free diet was purchased from Purina Test Diets (Richmond, IN). The composition and fatty acids content of this diet have been previously published (18). EFAD animals were made EFA-deficient by feeding them a fat-free diet for at least 8 weeks. Controls were weight-matched, and received standard chow diet.

Linear incision model

Full thickness paired incisions of 6 cm in length were performed as described (19, 20). Incisions were coapted with three surgical clips, and harvested at days 5, 7, 12 and 21 post-wounding. Maximum wound breaking strength was measured using three 8 mm strips from each wound with a tensometer (Tensometer 10, Monsanto Co, St Louis, MO).

Histological and immunohistochemical analysis

Samples were obtained for histological evaluation by standard hematoxylin and eosin staining. Additional samples were harvested and prepared in standard frozen fashion for immunoperoxidase labeling (21). After fixation, endogenous peroxidase activity was suppressed. The samples were sequentially incubated with rabbit serum blocking solution (Vector Laboratories, Burlingame, CA), followed by anti-rat macrophage primary antibody which recognizes specific antigens present in the membrane of macrophages (22), (Sera Laboratories, Sussex, UK). Negative controls consisted in parallel sections incubated with comparable dilutions of irrelevant primary antisera. After rinsing, biotinylated mouse anti-rat IgG (Vector Laboratories, Burlingame, CA) was added and the slides incubated with streptavidin peroxidase. Sections were exposed to diaminobenzidine tetrahydrochloride (DAB) (Sigma Chemical Co, St Louis, MO) and counter-stained with hematoxylin.

Fatty acid repletion experiments

EFAD rats were selectively repleted with (18:2 [n-6]) *cis*-linoleic acid methyl ester (Sigma Chemical Co, St Louis, MO) as described (7). Animals were injected with 1 g/kg/day BW i.p. of the fatty acid methyl ester for 1 week and received standard rat chow diet during 15 days prior to the studies.

Experimental groups

Nine experimental groups of rats were studied. The influence of EFAD on wound healing at days 5, 7, 14 and 21 was evaluated on groups 1 (n = 7), 3 (n = 6), 5 (n = 7) and 7 (n = 7). Rats from groups 2 (n = 7), 4 (n = 6) and 6 (n = 7) were used as weight-matched controls for the EFAD rats, and they received standard rat chow and were sacrificed at days 5, 7, 14 and 21 post-wounding. Rats from group 9 consisted of seven rats with EFAD that were repleted with linoleate as described above, to reverse the EFAD condition. These rats were wounded in the same manner and harvested at day 7 post-wounding.

Validation of the EFAD

To validate the deficiency state biochemically, fatty acid analysis was performed as previously described (7) on livers from normal, EFAD, and linoleate-repleted animals. Liver lipids were extracted using a Bligh-Dyer extraction and were then transmethylated (along with heneicosanoic acid as an internal standard), and the resultant fatty acid methyl esters were isolated by thin layer chromatography and characterized by gas chromatography (18).

Calculations and Statistics

Wound-breaking strength

Six samples were obtained from each rat and the

results for the wound-maximal breaking strength measurements were averaged in the EFAD and control rats. Results are expressed as mean ± standard error of the mean, and comparisons between groups were made by means of ANOVA.

Histological analysis

The cellularity of the EFAD and control wounds was estimated by two independent observers using a scale of 1–4; 1 representing baseline cellularity in unwounded dermis and 4 representing maximum cellularity at the time point tested (19, 20). Results were averaged for statistical analysis.

Immunoperoxidase

Wound sections were examined microscopically, and positive staining was evaluated for the estimation of the cells stained as macrophages using a scale of 1–4 depending on the approximate number of macrophages observed; 1 representing the minimum number of macrophages and 4 the maximum number of macrophages.

RESULTS

Validation of EFA-deficiency

Animals placed on the EFA-free diet were clinically and biochemically fatty acid deficient. They exhibited the characteristic mild growth retardation, macroscopic changes in the liver, and cutaneous changes such as diminished skin pigmentation and thinning, ulcers on the dermis and hair loss. To validate the deficiency state biochemically, fatty acid analysis was performed on liver. Liver lipids showed a 90% depletion of arachidonate and a 20:3 (n-9) to arachidonate ratio of 3.1 with EFA-deficiency (Table 1). A 20:3 (n-9) to arachidonate ratio of >0.4 is the biochemical criterion for EFAD (23).

Effects of EFA-deficiency on body weight

It has been demonstrated by several investigators that EFAD affects weight and body growth (24, 25). In a pilot study, we observed that rats of the same age under EFA-free diet had mild growth retardation in comparison with rats receiving standard chow diet 287 ± 12 vs 363 ± 14 g. Total body weight is an important factor influencing wound healing, when weight loss exceeds one-third of the normal body weight the healing is severely affected (26). Since total body weight is an important factor modulating wound repair and the EFAD state alters the body weight, we used weight-matched rats that had been on a standard chow diet as controls. The difference in age was approximately 15 days to 1 month. Age is a factor that also influences wound repair, natural delays in the healing of the elderly have been demonstrated (27), but in general during the adulthood in healthy individuals the healing is not modified by differences in age.

Effects of EFA-deficiency on wound tensile strength

The tensile strength measurements for EFAD and controls rats at days 5, 7, 14 and 21 post-wounding are depicted in Figure 1. EFAD rats and matched weight controls showed similar values for tensile strength at different days-post wounding, suggesting that the lack of essential fatty acids in the diet does not affect wound healing in rats, as determined by tensile strength measurements.

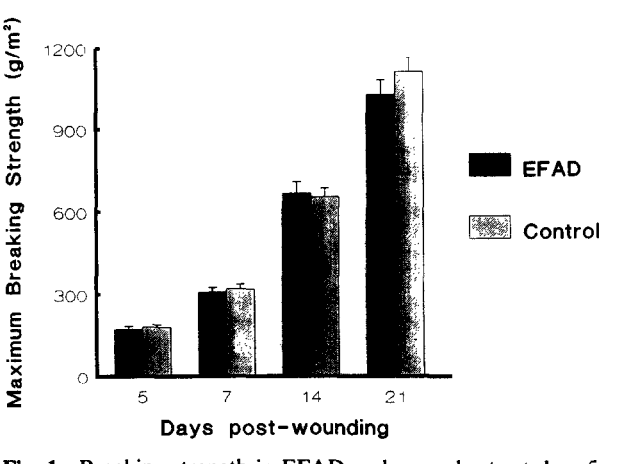

Fig. 1 Breaking strength in EFAD and normal rats at days 5, 7, 14 and 21 post wounding. For each rat six samples were obtained (three from each wound) and tested on the tensometer for maximum wound breaking strength. Results are expressed as mean ± SEM. Comparisons between EFAD and control rats were not statistically different at any time point examined.

Table 1 Liver lipid fatty acid analysis (*mol%*)

	(n)	16:0	16:1	18:0	18:1	18:2	20:3	20:4	20:3/20:4	22:6
EFAD	(3)	31 ± 0.3	17 ± 2.3	6 ± 1.1	4.1 ± 0.4	ND	4.0 ± 0.9	1.2 ± 0.1	3.1 ± 0.4	ND
Control	(3)	26 ± 4	1.0 ± 1	31 ± 7	13 ± 2	12 ± 3	ND	14 ± 2	0.00	3 ± 1
Repleted	(3)	35 ± 1	ND	1.3 ± 1	14 ± 1	18 ± 1.2	ND	12 ± 1	0.00	3 ± 0.4

Liver lipids from control, EFAD, and EFAD/linoleate supplemented animals were extracted using a Bligh-Dyer extraction. The constituent fatty acids were then transmethylated, isolated by thin-layer chromatography and characterized and quantified using gas chromatography.
ND = not detected, EFAD = essential fatty acid deficient.

Effects of EFA-deficiency on wound inflammatory infiltration

Hematoxylin and eosin sections from control and EFAD rats were analyzed. No significant difference in wound inflammatory cellular influx between the two groups was seen by two independent observers at post-wounding days 5, 7, 14 or 21 (Table 2), suggesting that EFAs are not essential to trigger the acute inflammatory response in cutaneous wound healing.

Table 2 Wound cellularity of EFAD and control wounds

Days post-wounding	EFAD	Control	$EFAD_s$	t-test
5 (n = 14)	1.58 ± 0.1	1.66 ± 0.1		NS
7 (n = 19)	3.00 ± 0.1	2.91 ± 0.1	3.01 ± 0.1	NS
14 (n = 14)	2.07 ± 0.2	2.00 ± 0.2		NS
21 (n = 14)	2.14 ± 0.1	2.21 ± 0.3		NS

Cellularity of hematoxylin and eosin histologic sections were scored by two blinded observers. A scale of 1–4 was used; 1 representing baseline cellularity in unwounded dermis, 4 representing maximum cellularity at the time point tested. The blinded observations were averaged and unpaired Student's t-test analysis revealed (NS) no significant difference in cellularity. ($EFAD_s$) EFAD supplemented rats, these rats were repleted with linoleate as described in methods. EFAD rats n = 26, Controls n = 26, $EFAD_s$ n = 7.

Table 3 Wound macrophages of EFAD and control wounds

Days post wounding	EFAD	Control	$EFAD_s$	t-test
5 (n = 14)	3.24 ± 0.2	3.32 ± 0.1		NS
7 (n = 19)	2.28 ± 0.1	2.14 ± 0.1	2.18 ± 0.1	NS
14 (n = 14)	ND	ND		NS

Samples were stained with immunoperoxidase using anti-macrophage antibody, and scored by two blinded observers. A scale of 1–4 was used; 1 representing the lower minimum number of macrophages observed in the samples, 4 representing maximum number. The blinded observations were averaged and unpaired Student's t-test. Analysis revealed no significant difference in the number of macrophages.

Abbreviations: NS, not significant; ND, not detected; EFAD, essential fatty acid deficient; EFA_s, EFAD supplemented rats, these rats were repleted with linoleate as described in Methods.

Effects of EFA-deficiency on wound macrophages

Further evaluation of the presence of macrophages with immunohistochemistry studies showed no difference between the EFAD and control groups (Table 3), suggesting that the lack of EFAs in the diet does not modify the infiltration of macrophages necessary for an adequate cutaneous wound repair.

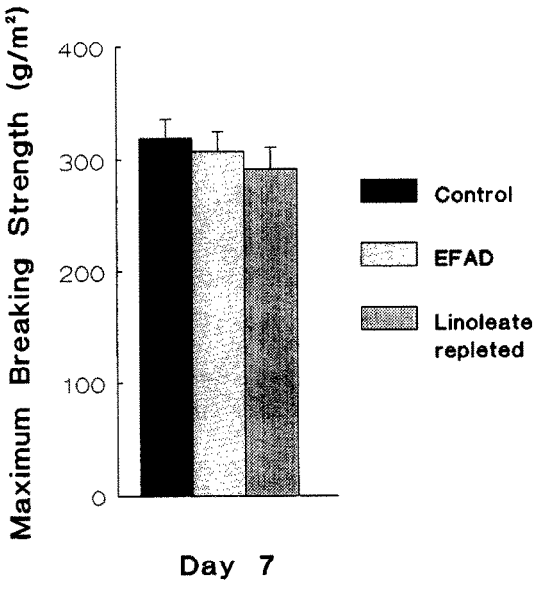

Fig. 2 Breaking strength in EFAD, control, and EFAD supplemented rats at day 7 post-wounding. For each rat six samples were obtained (three from each wound) and tested on the tensometer for maximum breaking strength. Results are expressed as mean ± SEM. EFAD supplemented rats were repleted with linoleate as described in Methods. Comparisons between EFAD, control and EFAD supplemented rats were not statistically different at day 7 post-wounding.

Fatty acid repletion

Repletion with linoleate reversed all the cutaneous changes produced by the EFAD, as well as the macroscopic changes in the liver. Liver lipids returned to normal values indicating that the EFAD was reversed. Wound breaking strength values at day 7 post-wounding in rats with EFAD given linoleate were not significantly different from values in either control or EFAD rats. Supplementation with linoleate did not affect the tensile strength values, when compared supplemented rats, with EFAD rats non supplemented and with normal controls (Fig. 2). Along with those results, histological evaluation did not show differences in the inflammatory infiltration among the three groups of rats studied (Table 2).

DISCUSSION

Despite long-standing recognition of the state of EFA-deficiency and its known association with amelioration of inflammatory mediated processes, the influence of EFAs in cutaneous wound repair, a physiologic process in which the end result is determined by an organized and sequential acute inflammatory reaction, has not been properly defined. To the best of our knowledge the report by Hulsei et al more than a decade ago, has been the only

one that has partially addressed the effects of EFAD on experimental wound healing in rats (17). However, that report suffers from important deficiencies. First, the experimental rats were on an EFAD diet for only 3 weeks. We have repeatedly demonstrated that a minimum of 4 weeks on an EFAD diet is necessary to successfully reproduce the EFAD state. Second, the EFAD condition was not validated. It is accepted that a 20:3 (n-9) to arachidonate ratio of >0.4 is the biochemical criterium for EFAD (23). Third, that study lacked histological evaluation. In our study we have examined the effects of EFA-deprivation on cutaneous wound healing. The results of this study suggest that EFAs are not essential for cutaneous wound repair. This assertion is based on several lines of evidence derived from our mechanical, histological and immunohistochemical observations. First, maximum breaking strength measurements were not significantly different between normal rats and rats with EFA-deficiency (Fig. 1). This assumption is inferred from the lack of significant difference in wound tensile strength for the two groups, indicating that collagen synthesis by fibroblasts was similar in the EFAD rats and in the control rats. Normal wound healing is characterized temporally by immediate entry of inflammatory cells at the site of tissue injury; subsequent influx, activation and proliferation of wound fibroblasts, leading to new collagen synthesis; followed by a remodeling phase after 2–3 weeks (28–30). New collagen synthesis is directly proportional to the increase in wound breaking strength during the early phase of healing (31). Second, analysis of cellular migration into the wounds showed that the cellular inflammatory influx was similar at each time point examined, and not different from samples obtained from rats with EFA-deficiency (Table 2). The state of EFA-deficiency is characterized by a general decrease in tissue levels of arachidonate and decrease in the production of its metabolites (prostaglandins and leukotrienes) which are inflammatory mediators. Rats with induced EFA-deficiency healed in the same way as normal rats, suggesting that arachidonate metabolites are not necessary to activate the acute inflammatory response that characterizes normal wound healing with influx of neutrophils during the first days after wounding, followed by an influx of macrophages within days 2–3 and by an influx of fibroblasts by days 3–4 (11, 32). It has been demonstrated that the EFAD condition decreases the chronic inflammatory response in pathologic processes such as glomerulonephritis and diabetes (5–7). Experimental impairment in wound healing can be obtained with irradiation, which by producing bone marrow suppression results in 43–65% loss of wound tensile strength (12, 13). Glucocorticoid-induced wound healing deficit results in 30–74% decrease in wound tensile strength (14), probably as a consequence of severe alteration in the inflammatory response. In the present study the acute inflammatory response in cutaneous wound repair was not modified by the lack of EFAs, suggesting either an inflammatory response not mediated by arachidonic acid metabolites or a redundancy in the pathways and inflammatory mediators, in such a way, that suppression of the synthesis of metabolites of arachidonic acid does not affect the final outcome of the process.

Immunohistochemical studies with anti-macrophage antibody performed at different time points showed that macrophage influx into the wound was similar in samples obtained from rats with EFA-deficiency and from normal controls (Table 3). One of the most important cellular elements of the acute inflammatory response in wound healing is the macrophage. This cell participates in at least three aspects of tissue remodeling: first, as a source of growth factors that affect fibroblasts and other mesenchymal cells; second, as a source of angiogenic factors involved in neovascularization of the healing wound; and third, as a source of factors that modulate the production of proteins in the connective tissue matrix by other cells within the local environment (11). Thus, wound healing is a macrophage-dependent phenomenon (10). Convincing studies have reported that EFAD produces a marked depletion in the number of resident mesangial macrophages in the rat, without a decrease in circulating monocytes (7). However in the current study the influx of macrophages into the wound was not affected by the lack of EFAs, as evaluated by immunohistochemical studies, suggesting that macrophage migration into the wound is independent of chemotactic factors derived from arachidonic acid.

Supplementing EFAD rats with linoleate restored the lipid liver content and reversed the skin alterations, but it did not modify either the inflammatory infiltration nor the presence of macrophages. As a consequence the wound breaking strength values were not altered, indicating that the process of cutaneous wound repair is independent of the availability of EFAs and/or their metabolic products.

In summary, the current study establishes that the normal course of wound repair in the rat is not affected by lack of EFAs in the diet. We conclude that the process of wound healing or the cells involved in this response are not dependent on arachidonic acid metabolites.

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