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Increased Proliferation in Keloid Fibroblasts Wounded *in Vitro*

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A keloid is a pathological overgrowth of scar expanding beyond the boundaries of the initiating skin wound. Ultimately, this expansive scar is a result of excess collagen synthesized by fibroblasts within the wound. The processes that lead to this collagen excess remain unknown. An *in vitro* wound model was developed to test the hypothesis that fibroblasts isolated from keloid tissue and wounded *in vitro* might proliferate more rapidly than similarly wounded normal dermal fibroblasts. Keloid fibroblasts (KF) and normal human dermal fibroblasts (NDF) were grown to confluence and quiescence in flexible-bottomed culture plates. Wounds were created in a standardized fashion using a specially designed jig. The jig utilized a 25 gauge needle to reproducibly ablate 16–20% of cells from confluent cell sheets. Wounded and nonwounded cells were labeled with ^{3}H-thymidine at 24, 48, 72, and 96 hr postwounding to measure DNA synthesis. Wounded KF and NDF demonstrated increased ^{3}H-thymidine incorporation compared to nonwounded control cultures, and wounded KF demonstrated significantly higher levels of ^{3}H-thymidine incorporation than wounded NDF both 24 and 48 hr after wounding. A similar trend was seen in cell counts. The wounded KF also showed a statistically greater labeling index quantitated by autoradiography than did wounded NDF. The increased commitment to DNA synthesis in response to wounding *in vitro* in keloid fibroblasts correlates with pathology seen *in vivo*. Keloid fibroblasts may have a lower inherent threshold for S phase entry than do normal fibroblasts contributing to the increased proliferation of keloid fibroblasts in response to wounding *in vitro*. © 1996 Academic Press, Inc.

INTRODUCTION

Keloids are scars with excessive fibrous tissue that result as a response to skin injury [1, 2]. Keloid scars are distinguished from normal scars by excessive growth and invasion beyond the site of original injury [1]. The mechanism for keloid scar formation is still unknown [2–5].

A number of observations have been made concerning differences in cellular activity between fibroblasts in keloids and those in normal dermis or a mature scar. Mancini and Quaife observed a prolonged, hyperplastic fibroblasts response in wounds destined to become keloids [6]. Matsuko *et al.* demonstrated a greater density of fibroblasts in keloids than in normal granulation tissue suggesting that fibroblast proliferation *in vivo* is increased in keloids [7]. Kischer has alluded to the hypercellular nature of keloids early in their development in several studies [8–10]. This difference disappears in later phases of keloid development where cell numbers are the same or less than in normal scar or dermis [6, 11]. These observations suggest that keloid fibroblasts *in vivo* proliferate more rapidly than normal dermal fibroblasts exclusively in the early postwounding period.

Although this histologic evidence and the aggressive clinical behavior of keloids suggest that keloid fibroblasts undergo hyperplasia, previous studies have demonstrated similar growth kinetics for keloid-derived and normal dermal fibroblasts under standard culture conditions *in vitro* [11–15]. Diegelmann *et al.* found no differences between keloid and normal fibroblasts in log phase or stationary phase growth in the four cell isolates evaluated [13]. Similarly, McCoy *et al.* found no difference in the life span of keloid-derived fibroblasts compared to normal dermal fibroblasts under standard culture conditions [14].

We hypothesized that the inconsistencies between these two sets of observations might relate to the stable culture conditions utilized in the *in vitro* studies. We further hypothesized that keloid fibroblasts might proliferate more rapidly if wounded *in vitro*. The present study was designed to examine the mitogenic response of keloid-derived fibroblasts to a "wound" *in vitro,* and to examine whether keloid fibroblasts respond to wounding with greater vigor than normal dermal fibroblasts.

MATERIALS AND METHODS

Fibroblast cultures. Normal human dermal fibroblasts (NDF) were obtained from the Lineberger Cell Culture Facility (University of North Carolina, Chapel Hill, NC). Keloid fibroblasts (KF) were isolated from sterile biopsies of keloids. Cells from three separate isolates were used for these experiments. To isolate keloid fibroblasts from biopsies, epidermis and subdermal fat were removed from the specimens. Subsequently, the tissue was minced (2 mm), and incubated for 1 hr at 37°C in 0.5% collagenase (Sigma Chemical Company, St. Louis, MO) in minimum essential medium containing 20 m*M* HEPES pH 7.2, 100 U/ml penicillin, 100 μg/ml streptomycin and 2

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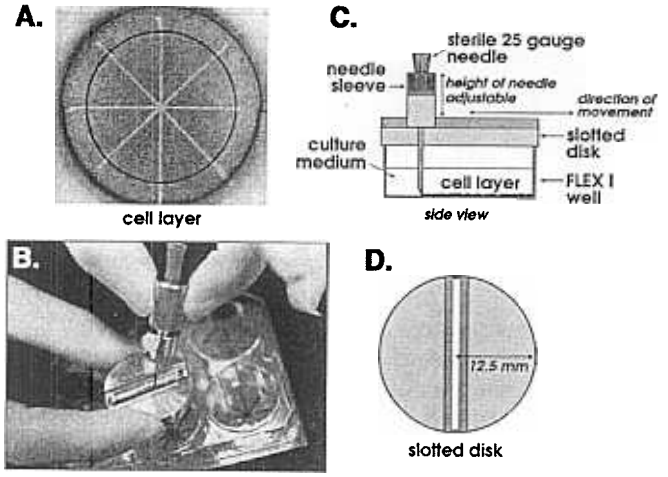

FIG. 1. (A) "Wounded" cells in culture dish, (B) wounding jig, (C) schematic top view of wounding jig, and (D) schematic side view of wounding jig.

μg/ml Fungizone. The tissue was then sedimented at 1000 × g for 10 min, and the supernatant fluid was discarded. The cell pellet was resuspended in Dulbecco's modified eagle's medium with high glucose (DMEM-H).

The cells were then cultured in DMEM-H supplemented with 10% fetal bovine serum, 1% glutamate, 1% sodium pyruvate, 20 m*M* HEPES pH 7.2, 0.5 m*M* ascorbate and antibiotics. They were incubated in an incubator with a 5% CO_2 humidified atmosphere at 37°C, and media were changed every 3 days (Napco 6300, CO_2 incubator). Normal dermal fibroblasts were grown under identical conditions. After 7 days, cells were plated at 75,000 cells/well (15,000 cells/cm^2) in sterile FLEX I, flexible-bottomed, collagen bonded tissue culture plates (Flexcell Corp., McKeesport, PA) [16]. The cells were grown to confluence, and then nutrient depleted for 5 days to establish quiescence.

Wounding procedure. *In vitro* wounds were created using a standardized jig designed in this lab (Fig. 1). This jig consisted of a slotted aluminum disc that could be rotated over a single well of a FLEX I plate. A standard, sterile, 1.5 in. long 25 gauge needle was placed in an aluminum sleeve that slid along the slot in the disc (Figs. 1B–D). This design allowed the needle to move across the rubber well surface in a standard pattern, leaving a defined center cut in the rubber that did not interfere with cell migration but that marked the wound track. When moved across the well diameter, the 25 gauge needle created a linear wound in the confluent cell sheet approximately 8 cells in diameter. Using a template beneath each well, wounds were created in north–south and east–west axes, as well as 45° between these locations (Fig. 1A). The wounding procedure removed 16–20% of the cell sheet as determined by cell counts before and after wounding. Half of the keloid fibroblast cultures (four plates, six wells/plate) and half of the normal dermal fibroblast cultures (four plates, six wells/plate) were wounded with this method. Nonwounded keloid and normal dermal fibroblast cultures served as controls.

^{3}H-Thymidine incorporation. ^{3}H-Thymidine incorporation was evaluated in wounded and nonwounded NDF and KF 24, 48, 72, and 96 hr postwounding. ^{3}H-Methyl thymidine (2 μCi/well) (ICN Biochemicals, Irvine, CA) was added to cultures 24 hr prior to each collection point, and incubated at 37°C under the standard conditions described above. At the selected collection times, media were aspirated, and each plate was gently washed three times with 3 ml of buffered saline. The plates were then fixed and washed exhaustively with ice-cold 5% trichloroacetic acid. The culture plates from the 24, 48, and 72 hr collection times were frozen at −20°C until the final collection. After the 96 hr collection, samples were thawed, and the flexible-bottomed wells of all plates were removed. Each well bottom was placed in 15 ml of Scintiverse Bio-HP and counted in a liquid scintillation counter (Beckman LS 600TA) for 5 min. Mean dpm's/1000 cells were calculated for each group (n = 6/group), and differences between means were evaluated using ANOVA with a Fisher PLSD test. Experiments were replicated twice, and the results of one data set are shown.

Cell counts. Cell counts were determined in all groups evaluated for ^{3}H-thymidine incorporation. To measure cell counts, media were aspirated and cells were rinsed with warmed buffered saline. They were then trypsinized (0.01% trypsin, Lineberger Cancer Research Center) for 10 min at 37°C, resuspended in DMEM-H and placed in cell counting vials with filtered Isoton. Cell numbers were determined using an electronic cell counter (Elzone 180, Elmhurst, NY). Mean cell counts were calculated for each group (n = 3/group), and differences between means were evaluated using ANOVA with a Fisher PLSD test.

Autoradiography. Cultures from the 24 hr time point were evaluated by autoradiography. Media were aspirated from the wells, and the cells were rinsed twice with warmed buffered saline. The cells were then placed on ice and fixed with a 1:3 solution of acetic acid and methanol for 10 min. They were subsequently rinsed with ice-cold 10% trichloroacetic acid solution three times (allowing 10 min between rinses), treated with methanol, and dried. The well bottoms were removed from the FLEX I plates, coated with autoradiography emulsion (Kodak NTB2), and placed in a sealed desiccated box. The emulsions were then developed, and labeling indices were determined by counting labeled nuclei within standardized areas along the wound tracks utilizing a video-microscopy system. [Image I analyzer, Universal Imaging Corp.] The mean number of labeled nuclei per unit area were calculated for each group (n = 6/group). Differences between means were evaluated using ANOVA with a Fisher PLSD test.

RESULTS

^{3}H-Thymidine

Wounded KF and NDF incorporated significantly more ^{3}H-thymidine/1000 cells than did nonwounded controls at the 24 and 48 hr time points after wounding ($P < 0.01$) (Fig. 2). At the 48 hr time point, the mitogenic response of wounded KF was 3.3-fold greater than that of non-wounded KF. The response of wounded NDF was 2.3-fold greater than that of the nonwounded NDF at 48 hr. The response observed in wounded KF was significantly greater than NDF at 24 and 48 hr ($P < 0.05$), and at the 48 hr time point, it was 1.3-fold greater than that demonstrated by wounded NDF. By 72 to 96 hr postwounding, ^{3}H-thymidine incorporation in both keloid and normal dermal fibroblasts decreased. There were no statistical differences observed at these later time points.

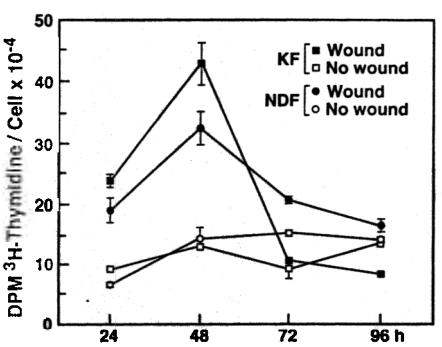

FIG. 2. DNA Synthesis in wounded and nonwounded keloid-derived fibroblasts and normal dermal fibroblasts.

TABLE 1

Cell Counts in Wounded and Nonwounded Keloid-Derived Fibroblasts and Normal Dermal Fibroblasts at 24 and 48 hr

		24 hr	48 hr
Keloid	Wound	28.0% ± 12.1[a,b]	44.3% ± 8.1[a,b]
	No wound	10.0% ± 2.6	16.3% ± 7.6
NDF	Wound	17.7% ± 7.6	23.7% ± 2.3
	No wound	9.3% ± 5.1	19.3% ± 3.8

[a] Wound > no wound, $P < 0.05$.
[b] Wounded keloid > wounded NDF, $P < 0.05$.

Cell Counts

At 24 and 48 hr postwounding, the percent increase in cell counts in wounded KF and NDF was greater than the percent increase in cell counts in nonwounded controls. (Table 1). The percent increase in cell counts in wounded KF was 2.8-fold and 2.7-fold greater than those for nonwounded KF at 24 and 48 hr, respectively, while the percent increase in counts in wounded NDF was elevated 1.9- and 1.2-fold at the two time points. Only the differences between wounded and nonwounded KF reached statistical significance ($P < 0.05$). The percent increase in cell counts in wounded KF was significantly greater than the increase in wounded NDF at both the 24 and 48 hr time points ($P < 0.05$). The percent increase in counts for wounded KF was 1.6- and 1.9-fold greater than those for wounded NDF at 24 and 48 hr. At the 72 and 96 hr time points, there were no significant differences between wounded and nonwounded KF and NDF, and these data are not included in Table 1.

Autoradiography

Greater numbers of labeled nuclei were detected by autoradiography in the wounded cultures of both cell types as compared to nonwounded controls (Fig. 3). In both cell types, the labeled nuclei were predominantly located adjacent to the wound site. Although both cell lines responded to *in vitro* wounding with increased nuclear labeling in the wound region, the labeling index in wounded KF cultures was significantly greater than that in wounded NDF cultures ($P < 0.01$). The number of labeled nuclei/area in KF cultures was twice that in NDF cultures. (Fig. 3A) There was also an increase in the distance from the wound in which labeled nuclei could be found in KF as compared to NDF (not statistically evaluated).

DISCUSSION

The results of this study support the hypothesis that keloid-derived fibroblasts respond to *in vitro* wounding by increasing their rate of cellular proliferation to a greater degree than normal dermal fibroblasts. These results suggest that keloid fibroblasts have an intrinsic ability to respond to wounding more vigorously than their normal dermal counterparts.

Our data demonstrate increased cell proliferation by keloid fibroblasts in response to wounding in the first 48 hr. By 72 and 96 hr, the growth kinetics of keloid and normal fibroblasts showed no statistical differences whether wounded or not. This finding may be due to the fact that the cell sheet had filled in the ablated region by 72 hr. The *in vitro* wound had essentially "healed" by that time. It is likely that keloid fibroblasts may continue to proliferate much beyond the 4 days examined in this study *in vivo,* with larger wounds and longer healing periods. It will be necessary to increase the size of the *in vitro* wounds and the time period evaluated in order to assess whether this hypothesis is plausible.

Previous work suggested that growth characteristics of keloid-derived fibroblasts and dermal fibroblasts are similar under standard conditions in culture [11–15]. This finding did not explain why increased numbers of fibroblasts are seen in biopsies of younger keloids [6, 7]. Data from the current study suggest keloid-derived fibroblasts have a lower threshold for entering S phase than normal dermal fibroblasts when wounded. This provides an explanation for why cellularity is increased soon after wounding in keloids. The lack of an increased mitogenic response at later time points may explain why cell numbers in keloids diminish over time as they mature as well [6, 11].

In wounded keloid cultures examined by autoradiography, not only was the density of labeled nuclei greater, but increased labeling density was seen in cells over a larger area. The mechanism by which keloid cells relatively removed from the original site of injury

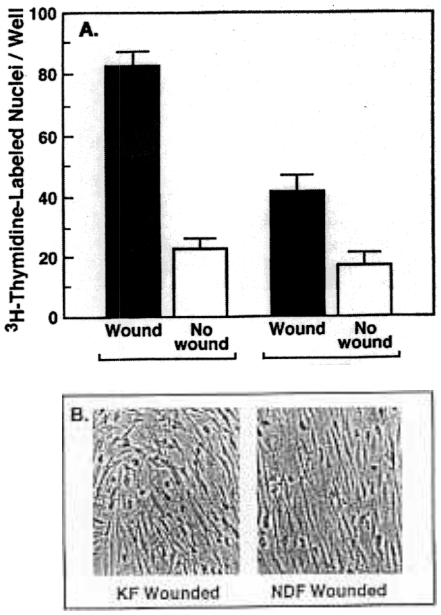

FIG. 3. (A) ^{3}H-Thymidine labeled nuclei in wounded and nonwounded keloid-derived fibroblasts and normal dermal fibroblasts. (B) Histology demonstrating labeled nuclei in wounded keloid-derived fibroblasts and normal dermal fibroblasts.

respond to wounding is unknown. It may be due to increased receptor sensitivity to cytokines or to increased postreceptor signal transduction. It may also be that the wounding stimulus induces dividing fibroblasts adjacent to the wound to migrate farther into the surrounding area in keloids than in normal wounds.

This study only examined the mitogenic response to *in vitro* wounding. Keloid fibroblasts are known to synthesize more collagen and other matrix components *in vitro* than do normal dermal fibroblasts under standard culture conditions [17–24]. Cohen *et al.* were the first to demonstrate increased rates of collagen synthesis in keloid cultures compared to normal skin or scar [17], and this finding has been corroborated by several studies [18–22]. Kischer *et al.* [23] and Babu *et al.* [24] have shown that keloid fibroblasts also produce more fibronectin than normal fibroblasts. These studies suggested that the increased quantities of matrix in keloids resulted solely from increased synthesis per cell. The current study suggests that increased quantities of matrix may result from not only increased matrix production by the individual cells, but also greater cell numbers due to this greater mitogenic response to wounding.

The mechanism for this pathologic response is still unknown. One possibility is that keloid cells are particularly sensitive to cytokines. Russell *et al.* demonstrated that keloid-derived fibroblasts were more responsive to stimulation with low concentrations of serum than dermal fibroblasts, and in addition showed that the two cell types responded differently to a combination of epidermal growth factor (EGF) and transforming growth factor-β (TGF-β) [25]. Harper showed that keloid-derived fibroblasts were more responsive to EGF than dermal fibroblasts [26], and this finding was confirmed by Kikuchi *et al.* [27]. Kikuchi *et al.* also considered other cytokines including platelet-derived growth factor (PDGF) and TGF-β and could not demonstrate differential effects with these factors. Haisa *et al.*, in contrast, demonstrated increased mitotic rates in keloid-derived fibroblasts when stimulated by any of the PDGF isoforms [28] though not with EGF. These studies suggest that keloid-derived fibroblasts do respond differently to cytokine stimulation than dermal fibroblasts do. Russell *et al.* suggested that keloids may result from the untimely expression of a developmentally regulated growth-control mechanism [25].

The cytokines to which the fibroblasts respond are, at least in part, provided by serum. It is also possible that the keloid fibroblasts themselves produce an unknown autocrine factor, which causes them to divide more rapidly and synthesize greater quantities of matrix in response to wounding. Identification of the exact cytokine to which wounded cells respond awaits further study.

The greater mitogenic response to wounding *in vitro* seen in keloid-derived fibroblasts as compared to normal dermal fibroblasts may partially explain why keloids develop *in vivo*. More fibroblasts may be generated in the early healing phase of wounds destined to become keloids. This increased rate of proliferation in response to wounding, combined with the known ability of keloid cells to synthesize increased amounts of collagen and other matrix proteins, may contribute to the proliferation of excessive fibrous tissue in keloids.

In summary, this study shows that keloid fibroblasts exhibit greater cell growth in response to wounding *in vitro* than do normal dermal fibroblasts. This increased mitogenic response supports previous observations of increased fibroblast activity *in vivo* in early keloid scars, and suggests that increased keloid-derived fibroblast proliferation in response to wounding contributes to the pathogenesis of keloid scar formation.

ACKNOWLEDGMENTS

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