Comparative study of keloid formation in humans and laboratory animals

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Summary

Background:
Keloids are common lesions arising from sites of a previous trauma, and are distinguishable from scars by the presence of continuous growth over the borders of the original injury. The objective of this article is to improve methods for studying keloids using experimental animal models, which may help to promote wound-healing research and to attain suitable management of keloids.

Material/Methods:
This study consisted of two parts: animal and tissue culture experiments. Experimentally induced wounds in animal models were used to investigate keloid formation. Tissue cultures of cells in a conditioned medium were used to compare the growth rates of fibroblasts obtained from normal skin and wounds of experimental animals or from normal human skin and keloids.

Results:
Since keloids are common in humans, hypertrophic scars rather than keloids were observed in animal skin wounds. Data from the tissue culture study demonstrated an increase in fibroblasts cells in human keloid cultures, but not in animal wound cultures.

Conclusions:
Studying keloids in experimental animals may be more efficient, cheaper, and more practical than to study them in humans. Furthermore, the use of tissue culture is a suitable medium in which to study keloid cell behavior in order to understand the mechanisms leading to the formation of keloids and to attain appropriate, effective management.

key words: keloid • hypertropic scars • fibroblasts • tissue culture


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BACKGROUND

Wound healing is a complicated process encompassing an organized sequence of catabolic and anabolic events that depend on cellular and biochemical actions. Inflammation is carried out mainly by macrophages, which secrete cytokines and growth factors that mediate various cellular and biochemical events responsible for healing. The migration and proliferation of several cell types primarily occur during the proliferative phase of healing, leading directly to the repair of tissue integrity. As the fibroblasts and vascular endothelial cells migrate into the provisional matrix, they begin to proliferate and the cellularity of the wound increases. The remodeling is the most important phase in plastic surgery because it determines the final appearance of scars. It depends mainly on two factors: the reorientation of collagen fibers and granulation tissue contraction. The growth factors, such as fibroblast growth factor (FGF) and transforming growth factor beta (TGF-β), interfere with wound contraction. FGF inhibits the production of collagen by fibroblasts, whereas TGF-β increases the expression of different kinds of collagen and may thus play a role in the formation of pathological scars, such as keloids [1,2].

Keloidal scars are abnormal healing responses to injury that present a therapeutic problem. The etiological factors that determine how a scar becomes a keloid remain unknown. Familial predisposition and immunological causes have been implicated [3,4]. Keloid formation is associated with an over-deposition of collagen, fibronectin, and extracellular matrix by proliferating fibroblasts. The ensuing cicatrix is composed of randomly aligned collagen bundles that grow beyond the boundaries of the original wound [5]. Cytokines were said to be implicated in keloid formation. Kamamoto (2003) and Liu (2002) found that TGF-β might be involved in keloid formation [1,6,7].

Tenascin C, undulin, collagen XIV, and fibronectin are extracellular matrix glycoproteins. During embryogenesis, tenascin C is abundant in mesenchymal tissues, but its distribution in human adult tissue is severely restricted. The levels of tenascin C expression are enhanced with skin inflammation, wound healing, and hyperproliferative skin diseases. These levels return to normal in normal scar tissue after wound contraction is completed [8,9]. Recently, Lim (2002) demonstrated a significant increase in normal fibroblast proliferation when co-cultured with keloid keratinocytes (KKs) compared with single-cell culture or co-culture with normal keratinocytes [10]. Thus, keloid fibroproliferation appears to be influenced by epithelial-mesenchymal interactions between KKS and keloid fibroblasts (KFs). Transmission electron microscopy of fibroblasts co-cultured with KKS showed an extracellular matrix (ECM) appearance similar to in vivo keloid tissue, an appearance not seen when normal fibroblasts were co-cultured with normal keratinocytes [10–13].

Keloid scars occur in animal species besides humans. Similar lesions are reported in horses, cows, and dogs [14]. Scars frequently cause tightness, pain, and itching for the patient. No efficient treatment exists for this pathology [1,14–16]. Many reviews and reports of different treatments have been published. However, the management of keloid scars and scientific analysis of the treatment options remain seriously hampered by suboptimal study designs of research on this phenomenon [14]. Simple surgical excision of keloid scars has a 50–80% risk of recurrence. A combination of surgery with either intrallesional corticosteroid injection or radiotherapy has been the mainstay of treatment [17]. Steroid therapy was used to inhibit fibroblast proliferation, promote apoptosis, and decrease collagen biosynthesis [18]. At the same time, it is important to distinguish between hypertrophic and keloid scars, since inappropriate management can lead to recurrence and larger scars [17]. The present study aimed to investigate the appearance of keloids in different species of laboratory animals such as mice, rats, and rabbits. The goal is to find another source of keloid specimens from experimental animals in order to develop a model for the management of keloid and scar formation.

MATERIAL AND METHODS

Minimal essential medium (MEM) is a rich, multipurpose medium that was used for the cultivation of mammalian cells (Table 1) [19]. Phosphate-buffered saline (PBS) is a phosphate-buffered physiological saline solution that is a calcium- and magnesium-free solution [19,20] (Table 2). The trypsin solution is show in Table 3.

Human samples

Human keloids

Human keloid samples obtained from female patients (age range: 12 and 14 years old) were used as raw materials for isolating human fibroblasts from keloids. The skin was obtained from scars excised during plastic surgery performed at King Abdulaziz University Hospital (KAUH).

Human skin fibroblast cells

The normal human specimens (human skin fibroblasts) were obtained at KAUH from female patients. The skin specimens excised from female patients were after breast reduction during plastic surgery. Written consent was obtained before operative excisions. The specimens were transported immediately after excision in previously prepared bottles of MEM (transport time between operating theater and the laboratory was approximately 5 min).

Animal samples

Three species of laboratory animals (mice, rats, and rabbits) were used in all experiments. Samples were obtained from adult BALB/c male mice weighing 25–30 g, adult Waster male rats (200–250 g), and adult Newland male rabbits (2,300–2,500 g). The animals were singly housed in standard cages at the animal house in King Faisal Medical Research Center (KFMRC) and allowed to acclimate to their surrounding for 7 days prior to the experiment. Deep cutaneous injuries were made by scalpel resulting in wound formations on the back regions of all animals after shaving their fur.

Three groups of animal wounds were examined in each species of animal: a) Group 1 was left to heal normally; b) Group 2 was closed surgically; c) Group 3 was daily irritated by scratching with a standard granular lotion (made in the present study’s lab) for mechanically induced friction to interfere with the healing process in order to investigate the appearance of kel-
oids in different species of laboratory animals. Post-injury observation was reported, complete with color slide photo-documentation of wounded animals at different times. The animals were sacrificed 1 to 7 weeks following surgery.

Cell cultures

Skin was removed under sterile conditions and washed three times with PBS and once with MEM (ICN) without serum. It was then cut into small fragments, minced, and gently agitated in trypsin solution (Table 3) (GIBCO) at a concentration of 0.25%, 0.10% glucose, and 0.02% ethylenediaminetetraacetic acid (EDTA) for 15 min. Trypsin action was quenched with MEM when intercellular separation was seen. The supernatant suspension containing the dissociated cells was removed and centrifuged at 1,500 rpm for 10 min, the cells were re-suspended in MEM containing 20% fetal calf serum (FCS; ICN), heat inactivated (56°C for 30 min), plus penicillin (10,000 U/ml) and streptomycin (10,000 U/ml) (GIBCO). Cells were adjusted to 1×10⁵ cells/ml and plated into 25-cm² tissue culture polystyrene flasks then incubated in a humidified incubator in an atmosphere of 5% CO₂ at 37°C [20]. The cells were grown in 25-cm² polystyrene flasks and passaged biweekly. The cells were suspended in PBS, centrifuged, and re-suspended in MEM [20]. Human keloid cells were plated and grown in MEM with 10% FCS and glucose without being supplemented. The keratinocyte-fibroblast co-culture obtained from human and rat-wounded skin was seeded at a density of 1×10⁵ cells/ml 90% confluent in monolayer [19,20].

The study groups of the human keloid and animal wound cultures were compared with normal ones at intervals of 1–12 weeks; cells in culture were imaged and saved in a PC.

RESULTS

Morphological observations

- In Group 1 it was noticed that untreated deep incision wounds in rabbits and rats started to heal during the first week and reached complete healing with the observation of a line scar after two weeks. Figures 1 and 2 show the deep injuries in the skin of a rabbit.
- In Group 2 the closed surgical wounds in rats and rabbits were completely healed after two weeks, and fur grew around the scars; no apparent skin contraction was observed in this group.
- In Group 3 it was noticed that the scratched wounds of the rabbits’ skin took longer to heal than the other groups, with the formation of hypertrophic scars (Figures 3, 4). However, the scars contracted by the end of 5 weeks.
- In mice of Groups 1 and 2 the treated and untreated deep wounds showed complete healing within 10 days with the appearance of scar lines; Figure 5 shows the injury in the skin of mice after 10 days and Figure 6 the line scars in mice after two weeks.

Microscopic observations

Tissue culture of human samples

After one week of fibroblast-keratinocyte co-culture, conditioned media were subjected to continuous examination of fibroblasts proliferation in all the studied groups. The results of these examinations are explained as follows:
- After 2 weeks of culture, examination of human dermis revealed an increase in the number of both KFs and associated keratinocytes (Figure 7) compared with normal dermal culture (Figure 8).
- The KF number continued to increase to the third week, when the fibroblasts showed a high density of well-spread- ing cells with the appearance of some keloid melanocytes and KKS (Figures 9, 10).
- After 5 weeks of culture, normal human dermis showed a dramatic decrease in the number of normal fibroblasts in comparison with a high density of KFs (Figures 11, 12).

Table 1. The components of 1 liter of minimal essential medium (MEM).

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Component</th>
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<tbody>
<tr>
<td>1 bottle</td>
<td>Prepared MEM medium as powder (9.39 g) (ICN)</td>
</tr>
<tr>
<td>2.2 g</td>
<td>NaHCO₃ (Powder)</td>
</tr>
<tr>
<td>0.3 g L</td>
<td>Glutamine (Powder)</td>
</tr>
<tr>
<td>10 ml</td>
<td>Non essential amino acid (100×)</td>
</tr>
<tr>
<td>10 ml</td>
<td>Hepes (100×) solution</td>
</tr>
<tr>
<td>10 ml</td>
<td>Antibiotic mix (100×) solution</td>
</tr>
<tr>
<td></td>
<td>Up to 1 Liter Deionised – distilled water</td>
</tr>
</tbody>
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Table 2. The components for one liter of prepared phosphate buffer saline solution (PBS).

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Component</th>
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<tbody>
<tr>
<td>9.00 g</td>
<td>NaCl</td>
</tr>
<tr>
<td>0.20 g</td>
<td>KCl</td>
</tr>
<tr>
<td>1.44 g</td>
<td>Na₂HPO₄</td>
</tr>
<tr>
<td>0.20 g</td>
<td>KH₂PO₄</td>
</tr>
<tr>
<td>1.00 g</td>
<td>NaHCO₃</td>
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Table 3. The components of 100 ml of trypsin solution.

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Component</th>
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<tbody>
<tr>
<td>70 ml</td>
<td>PBS</td>
</tr>
<tr>
<td>0.25 g</td>
<td>Trypsin</td>
</tr>
<tr>
<td>0.02 g</td>
<td>EDTA</td>
</tr>
<tr>
<td>0.10 g</td>
<td>NaHCO₃</td>
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<table>
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<tr>
<th>0.10 ml</th>
<th>Phenol Red (0.5 %)</th>
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Completed to 100 ml by adding PBS. Stirrer 1 hr at room temperature, pH adjusted to (6.8–7.4). Sterile-filtered through a 0.22 µm filter and stored at +4°C. EDTA is ethylenediaminetetraacetic acid disodium salt [16,17].
After 8 weeks, some KFs appeared to degenerate. After 12 weeks of culture, normal human dermis showed a loss of cells that revealed degeneration and detachment of the normal fibroblast in the culture (Figure 13); whereas the KFs were regenerated in the derived keloid culture, with an increase in the number of cells (Figure 14).

These results indicate that fibroblast proliferation increased in keloid sites, which revealing its important role in keloid formation.
Comparisons of keratinocyte-fibroblast co-culture obtained from rat-wound with those of control animals revealed the following:

- Melanocyte cells appeared early in the experimental culture of animal wounds, after one week, before its appearance in the controlled skin. It was also noticed that nor-
animal fibroblast proliferation is higher than that of animal wounds (Figures 15, 16).

- Two weeks later, most cells in wounded skin culture were seen to be converted to adipocytes (Figure 17).
- After five weeks, the cells started to degenerate in the experimental and control cultures (Figure 18).

These results of tissue culture samples revealed that the reaction of fibroblasts obtained from animal wounded skin are distinguished from the reaction of human keloid fibroblasts. Furthermore, the human tissue culture samples were improved by the regeneration and re-growth of KFs whilst the cells were re-cultured for a lengthy period of time; whereas the animal fibroblasts degenerated in just a short period of time.

**DISCUSSION**

There was a difference between the growth rates of human KFs and the fibroblasts derived from animal wounds. Since there was no significant increase in the proliferation of fibroblasts derived from animal wounded skin in this study, the fibroblasts of human keloids appeared to be more proliferated than normal skin. Earlier results from other studies concerning the growth rate of fibroblasts are contradictory; indicating an increase in normal fibroblast proliferation compared with that of KFs [20], the similarity of fibroblast proliferation [21,22], or that normal fibroblast prolifera-
tion occurred more slowly than that of KFs [23–25]. This diversity may reflect differences in culture conditions at the time of study.

The findings in the present study may give an explanation of what was reported by other researchers. Grinnel, Desmouliere, Chipev, and Simon demonstrated that normal wound healing requires fibroblast proliferation and migration into the wound bed followed by tightly regulated matrix deposition and contraction [26–28]. Aberrations in these processes can lead to excessive collagen accumulation, as found in keloids. These scars extend beyond the original wound margins. When grown, in vitro KF cultures contain a high percentage of α-smooth muscle actin-expressing cells, myofibroblasts, which are a major constituent of the granulation tissue. The presence of myofibroblasts can be regulated by both TGFβ and ECM. However, reduction of tension in the matrix is the critical determinant. This predicts that the tension in the wound bed determines the type of scar at different human body sites. This study may, in addition, determine the types of scars in different species of animals and human beings.

Chipev and Simon [28] found that the glabrous skin of keloid-prone individuals tends to cause a large disordered accumulation of collagen that extends beyond the original margins of the wound, which may account for the observed location of keloids in addition to other abnormalities in KFs. This also may account for the absence of keloids in animals in the present study.

Fibroblasts of human keloid cultures that were incubated for a long period of time showed regeneration again after the appearance of some degenerative changes due to the effect of KKS, which may secrete growth factors that cause the regrowth of fibroblast. These results are consistent with reported data [10,13].

Based on the present observations, the lack of extensive scar formation in laboratory animals could be related to different interactions. The question here is do keratinocytes located in the skin of furred animals functionally react to injury differently from how human skin does? Or do they lack the proposed interaction with dermal fibroblasts that are reported to exist in humans [10]? The explanations of these questions are discussed as follows:

- In keloids, increased tensin C expression was observed especially in the reticular dermis associated with collagen fibrils sharply demarcating the limit of the lesion. Corresponding to the in vivo findings, tensin C expression was increased in KFs compared with normal fibroblasts in vitro, whereas undulin/collagen XIV and fibronectin expressions in keloids were similar to those in normal tissue. Therefore, tensin C is a marker associated with keloids [9].
- Chiquet-Ehrismann et al., Alvarez-Dolado et al., and Watt studied the role of tensin C during embryogenesis, finding that mice lack a functional tensin gene. This may explain the poor development of keloids in injured experimental animals in the present study [29–31].
- In a recent study, Bloor et al. [32] mentioned in their findings that the normal expression of keratin K2e in the upper spinous and granular layers of interfollicular epidermis was increased in keloid scars. It was also found that the fur of animals affected the keratin proliferation, which needs further investigations in the future.

CONCLUSION

The aim of this study was to improve methods for studying keloids using experimental animal models, which may help to promote wound-healing research and to attain suitable management of keloids. Comparisons of normal and keloid human confluent cultures revealed an increase in fibroblast, keratinocyte, melanocyte, and macrophage cells in human keloid cultures of epidermis and dermis. The present results indicated that inducing keloid development in animal models may be species dependent or may need more complicated wound procedures, such as inducing deep inflammation in a cut wound or burning with certain reagents, which need further investigation.

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