Limitations of studying keloid scars using the nude athymic mouse model

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Keloid scars are benign fibroproliferative growths that respond poorly to treatment. This study sought to determine the efficacy of three different glucocorticoids (triamcinolone, methylprednisolone and dexamethasone) in altering human keloid scar tissue implanted in athymic mice.

Keloid tissue obtained from three patients (one man and two women) who sought cosmetic removal of their scars was implanted into athymic mice for a duration of 15 or 30 days. The keloid tissue was examined histopathologically and evaluated by a dermatopathologist who was blinded to sample identity and who was using predetermined qualitative scoring criteria. The appearance of central calcification, granulation tissue, foreign body granulomatous reaction and acute inflammatory reaction complicated the comparison of the keloid tissue samples. However, on the basis of observations reported in the present paper, it appears that triamcinolone should remain the treatment of choice for keloid scars. The athymic mouse model that is used for studying keloid scars is the best available approach to *in vivo* studies; however, limitations identified in this study confound the interpretation of experimental data. Ideally, promising and novel therapies should be investigated clinically.

Key Words: Athymic mice; Glucocorticoids; Keloid scars

Les limites du modèle de souris nues athymiques pour l'étude des cicatrices chéloïdiennes

RÉSUMÉ : Les cicatrices chéloïdiennes sont des tumeurs fibro-prolifératives bénignes, peu sensibles au traitement. La présente étude vise à déterminer l'efficacité de trois glucocorticoïdes (triamcinolone, méthylprednisone, dexaméthasone) à modifier du tissu cicatriciel chéloïdien humain implanté sur des souris athymiques.

Le tissu chéloïdien prélevé sur trois patients (un homme et deux femmes) désirant subir l'ablation chirurgicale de leurs cicatrices a été implanté sur des souris athymiques pour une période de 15 ou 30 jours. Le tissu chéloïdien a été soumis à un examen histopathologique, puis évalué par un dermatopathologiste à qui l'on avait caché l'identité et qui fondait son analyse sur des critères prédéterminés de notation qualitative. L'apparence de calcification centrale, le tissu de granulation, la réaction granulomateuse aux corps étran et la réaction inflammatoire aiguë ont compliqué les comparaisons entre les prélèvements de tissu chéloïdien. Toutefois, d'après les observations consignées dans le présent article, il semble que la triamcinolone soit encore le traitement à privilégier pour les cicatrices chéloïdiennes. Le modèle de souris athymiques utilisé pour l'étude des cicatrices chéloïdiennes est le meilleur qui soit actuellement pour les études *in vivo*; cependant, les limites relevées dans la présente étude rendent confuse l'interprétation des données expérimentales. Idéalement, les nouvelles thérapies prometteuses devraient être évaluées en clinique.

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Keloids (Figure 1) are fibrous scars that display an excessive proliferative growth pattern in predisposed individuals who have sustained a dermal injury (1). In those people, the physiological response is out of proportion to the extent of the injury (2). This results in the formation of keloids, characterized clinically by a raised area that extends beyond the boundaries of the original wound (Figure 2). The prolonged growth of tissue is the result of unchecked and uncontrolled collagen production, which causes the scar tissue to outgrow the original dermal connective tissue at the site of the wound. Exuberant deposition of collagen and extracellular matrix proteins (such as fibronectin, elastin and chondroitin-4-sulphate), combined with the decreased degradation of the extracellular matrix proteins, are thought to be responsible for this uncontrolled growth. These scars are permanent and do not regress over time (3).

Keloid scars are disfiguring cosmetically and present a major problem for clinicians because of their high rate of recurrence. Therapies include excision, direct glucocorticoid injections (usually triamcinolone), application of silicone gel sheets, interferon injections, lasers, radiation and combinations of these treatments. The most common therapy is the injection of triamcinolone, 20 to 40 mg/mL, either directly into the keloid tissue or intralesionally, after the keloid has been excised. These treatments are associated with recurrence rates that range from 3% to 87% (4,5).

Understanding the pathogenesis of keloid scars has been a challenge because of the lack of an ideal animal model that reproduces the histology and biochemical properties of keloid tissue. The nude athymic mouse represents the best animal model for the study of keloid scars at the present time. Human keloid tissue implanted into nude athymic mice has been subsequently demonstrated to remain viable, biochemically and histologically, after both short and long term implantation for up to 80 days (6,7). Waki et al (8) demonstrated the utility of this model in evaluating pharmacological therapies for keloid scars.

Figure 1) Excised keloid tissue stained with routine hematoxylin and eosin (original magnification $\times 40$). Broad bands of randomly oriented collagen, containing some hyaline bands, are apparent

The goal of the present study was to compare systematically and prospectively the effectiveness of three glucocorticoids (triamcinolone, dexamethasone and methylprednisolone) for the amelioration of keloid scars by using the nude athymic mouse model.

MATERIALS AND METHODS

Experimental surgery and patient selection

Male nude mice (BALB/cAnNCrI-nuBR nu/nu, Charles River, USA) were housed in presterilized cages (one per cage) in a sterile environment. Their diet consisted of sterilized mouse chow ad libitum and sterilized water.

Human keloid tissue samples were obtained from excess surgical tissue from the earlobes of a 19-year-old North American Indian man and a 25-year old black woman, and the neck of a 29-year-old black woman.

The keloid tissue samples were implanted into an athymic mouse host within 4 h of being excised from the patients. Before implantation, keloids were kept on saline-moistened sterile bandages. Representative samples were taken for morphological analysis before being implanted into the mice.

Implantation of human keloid scar tissue followed the methodology that was outlined by Shetlar et al (7).

Two sections of de-epithelialized human keloid, approximately $2 \times 2 \times 2$ mm, were implanted into each mouse at the following sites:

- Subjacent to the subcutaneous muscle located at the midline, caudal to the scapulae
- In the midline lumbar area between the hind legs

The keloid tissue samples that were obtained from the three volunteers were subdivided into 48 pieces that were subsequently implanted into 24 mice. The implants were removed from the mice at 15 and 30 days in a random fashion.

Figure 2) Nodular keloid scar located on the ear of a female patient

TABLE 1

The number of implanted keloid samples affected by central calcification, foreign body granulomatous reaction, granulation tissue and acute inflammation

Histological characteristic	Control		Triamcinolone		Methylprednisolone		Dexamethasone	
	15 days	30 days	15 days	30 days	15 days	30 days	15 days	30 days
Central calcification	3 of 6	4 of 6	4 of 5	3 of 4	4 of 6	4 of 6	2 of 5	3 of 6
Granulation tissue	5 of 6	5 of 6	1 of 5	2 of 4	6 of 6	6 of 6	5 of 5	3 of 6
Foreign body granulomatous reaction	6 of 6	5 of 6	1 of 5	2 of 4	4 of 6	5 of 6	5 of 5	4 of 6
Acute inflammation	1 of 6	2 of 6	2 of 5	1 of 4	1 of 6	1 of 6	2 of 5	1 of 6

Treatments

The mice were randomly assigned to the following treatment groups:

- **Triamcinolone group:** Six athymic mice (12 human keloid implants) received 40 mg/mL triamcinolone acetone (Kenalog, Bristol-Myers Squibb, Canada)
- **Methylprednisolone group:** Six athymic mice (12 human keloid implants) received 40 mg/mL methylprednisolone succinate (Solu-Medrol, Pharmacia Canada, Canada)
- **Dexamethasone group:** Six athymic mice (12 human keloid implants) received 8 mg/mL dexamethasone phosphate (Hexadrol, Organon Teknika, Canada)
- **Control group:** Six athymic mice (12 human keloid implants) received no treatment and served as the control group

Each site of keloid implantation received 0.025 mL of glucocorticoid solution, which equates to 1 mg of triamcinolone, 1 mg of methylprednisolone and 0.2 mg of dexamethasone per injection. When it was not possible to directly inject the implanted keloid tissue, the steroid solution was injected into the wound cavity to bathe the tissue sample. The mice in the control group did not receive any injections.

Fifty per cent of the implanted keloid samples were removed from the athymic mice at 15 days and the remaining 50% were removed at 30 days. Each sample was processed for routine histology. Sections were obtained from paraffin embedded tissue and were subsequently stained with hematoxylin and eosin.

Sample analysis

The criteria for judging the efficacy of each glucocorticoid were qualitative. Untreated keloid scar tissue is characterized by eosinophilic, irregular, broad hyalinized bands of

collagen; collagen oriented in random patterns; and the appearance of nodules or 'whorls' in the scar tissue. After treatment, it was anticipated that these features would become less prominent as the orientation of collagen bundles became parallel to the epidermis (9). The efficacy of glucocorticoid treatment was evaluated at 15 and 30 days by histological comparison using the criteria listed above. A dermatopathologist who was blinded to the identity of the samples made each comparison.

Statistical analysis

Statistical analysis of the data in Table 1 was accomplished by making paired comparisons of each treatment at 15 and 30 days using Fisher's Exact test. The computations were carried out using StatXact-3 (version 3.0.2, Cytel Software Corporation, USA). $P \leq 0.05$ was considered to be significant.

RESULTS

Figures 3 and 4 show keloid tissue samples that were removed at 15 and 30 days, respectively. The implanted keloid samples removed at 15 days (Figure 3) and 30 days (Figure 4) did not display any evidence of improvement in any of the treatment groups compared with control samples or the keloid tissue excised from the patients (Figure 1).

In Figure 3, notice the mild granulation tissue on the left border of the control keloid tissue. The keloid tissues that were treated with triamcinolone and dexamethasone appeared largely unchanged from the time of implantation into the athymic mice; however, granulation and granulomatous reaction began in the dexamethasone-treated tissue. In the methylprednisolone-treated tissue, granulation and granulomas surrounded the keloid section. Calcium deposits were seen throughout all groups.

The control keloid tissue and the dexamethasone- and methylprednisolone-treated keloid tissues did not change appreciably over the 30 days (Figure 4). Extensive calcification was observed throughout the triamcinolone-treated keloid tissue.

There was little difference in keloid morphology between the sample that was taken at the time of clinical

Figure 3) Keloid implants removed from athymic mice after 15 days

Figure 4) Keloid implants removed from athymic mice after 30 days

excision (Figure 1) and the samples that were removed from the athymic mice at 15 (Figure 3) and 30 (Figure 4) days. The distinctive collagen 'whorls' and random deposition of collagen were still observed at 30 days in all treatment groups, with the exception of the appearance of extensive central calcification, granulation tissue, foreign body granulomatous reaction and acute inflammation of the keloid tissue (Table 1). The keloid histology of most tissue samples was obscured by the appearance of these unexpected histological changes. Evaluation of outcomes was problematic because many of the keloids were no longer visible histologically.

Table 1 outlines the frequency of the unexpected histological findings that occurred during the course of the study. There were a total of 44 keloid samples analyzed. Two mice in the triamcinolone group died, one before the 15-day sample and one before the 30-day sample. The keloid samples implanted in the former mouse were not analyzed, and those in the latter mouse were analyzed. A 30-day keloid implant from a mouse in the dexamethasone-treated group was damaged during preservation of the tissue, producing an unsatisfactory sample that could not be analyzed.

Fisher's exact test was used to compare treatment outcomes at 15 and 30 days. At 15 days, samples treated with triamcinolone showed less granulation tissue than did those samples treated with methylprednisolone ($P=0.015$) and dexamethasone ($P=0.024$). Also, at 15 days, triamcinolone-treated samples displayed less granulomatous reaction than did samples treated with dexamethasone ($P=0.024$) and samples from the control group ($P=0.015$). No other statistically significant differences were identified.

Figure 5) Two large areas of central calcification are evident in the keloid implant in the control group after 15 days (original magnification $\times 40$). The keloid tissue is visible only surrounding the dense calcium deposits on the right and upper perimeter

Central calcification was observed in many of the implanted samples. At least some degree of central calcification was observed in all experimental groups at 15 and 30 days. More than 50% of the samples in the treatment and control groups showed central calcification. Table 1 displays the number of samples within each group that scored positive for central calcification. Figure 5 shows a keloid

implant from the control group that was removed from an athymic mouse at 15 days. The interior of the implant was replaced by two large calcium deposits.

Another significant observation was the appearance of a granulomatous reaction of the foreign body type, characterized by the presence of multinucleated giant-cell histiocytes (or macrophages). The majority of samples in the treatment groups showed granulomatous reaction, apart from the samples that were treated with triamcinolone, as shown in Table 1.

The majority of the keloid samples in the experimental groups demonstrated an augmented granulation tissue reaction during the implantation period, with the exception of those samples that were treated with triamcinolone (Table 1). In many of the samples, the keloid tissue was no longer visible because of the formation of granulation tissue.

In addition, approximately 25% of the samples showed a nonspecific acute inflammatory reaction associated with necrosis of the grafted tissue. The frequency of acute inflammation was generally lower in each of the treatment groups than that in the control group (Table 1).

DISCUSSION

Until the development of the athymic mouse model for the study of keloid scars by Shetlar et al (6), there was no animal model that reliably reproduced all the characteristics of the keloid scar. The ideal model would require an animal that is capable of forming a keloid scar on its own; however, animals do not form keloid scars (10). Implanting human keloid scar tissue into athymic mice addresses this limitation. This indirect model proved to be a major advance in the study of keloid scars *in vivo*.

This study, however, identified limitations that could interfere with interpreting treatment results generated from the athymic mouse model. The original goal was to compare the efficacy of triamcinolone, methylprednisolone, dexamethasone and no treatment in ameliorating human keloid tissue that had been implanted in athymic mice for 15 and 30 days.

Although triamcinolone seemed to be more effective than the other treatments, it was not possible to determine with certainty whether any of the glucocorticoid therapies were more effective than the others because of the appearance of unexpected histological findings. Many of the implanted keloid samples developed extensive central calcification, foreign body granulomatous reaction, granulation tissue and acute inflammation at 15 and 30 days after implantation into the athymic mice. These problems were likely due to the experimental model and not to the glucocorticoid treatments because all the histological findings were observed in each treatment group, including the control group. Furthermore, there did not appear to be a temporal effect, because each of the histological markers was seen at both 15 and 30 days.

Previous studies have shown that keloid scar tissue implanted into athymic mice maintained its morphological

characteristics for up to 246 days (11). However, a convincing keloid model must also demonstrate biochemical similarity. Chondroitin-4-sulphate is a component of the extracellular matrix that appears in markedly increased amounts in keloid tissue compared with normal skin (12). A study examining the components of the extracellular matrix of keloid scars implanted in athymic mice showed that the implanted tissue remained similar to active keloid scars for approximately 80 days (7). Thus, the athymic mouse model provided approximately 80 days in which to study keloid scars in the athymic mouse before the attenuated murine immune system began to reject the implanted tissue.

The appearance of central calcification, granulation tissue, granulomatous reaction and acute inflammation in this study, as early as 15 days and at 30 days in all experimental groups, indicates that this model does not always represent a reliable reproduction of a human keloid scar over time. It also indicates the absence of a treatment-specific or time-dependent effect. The histology of the experimental samples suggests that some of the implants did not remain viable for even 15 days.

No reports of calcification in keloid scar tissue implanted in athymic mice appear in the literature, and this reaction is not known to occur in human keloid scars. The appearance of granular calcium deposits in the keloid tissue samples is likely the result of pathological calcification secondary to necrosis of the graft keloid, therefore representing dystrophic calcification (13).

In a previous study of hypertrophic scar and keloid viability and morphology in the athymic mouse model (11), one incident of necrosis was reported. The authors concluded that the necrosis was due to the large size of the implant. In their study, a standardized tissue sample of approximately 5×8×5 mm was used. However, all the tissue segments used in the present study were small (approximately 2×2×2 mm). It may be concluded that size was unlikely to have been the cause of the necrosis.

Granulation tissue contains many proangiogenic and mitogenic cytokines that are released from platelets and macrophages during the wound healing process. These growth factors are responsible for the first steps of re-establishing dermal integrity after a wound (14). In the present study, the formation of granulation tissue around the keloid implants likely indicates that the murine wound repair process had begun.

Athymic mice are unable to produce T lymphocytes (15); however, the lack of cell-mediated immunity has no effect on the mouse's ability to produce histiocytes. The presence of histiocytes is characteristic of a foreign body granulomatous reaction. Macrophages are responsible not only for destruction of foreign material, but also for the constant production of cytokines or growth factors during the wound repair process (14). This partial immune response may explain the occurrence of granulation tissue and granulomatous reaction in many of the samples. In many samples, original keloid tissue was no longer observable because of the quick (within 15 days) murine immune response.

Triamcinolone was effective in preventing the formation of granulation tissue at 15 days compared with treatment with methylprednisolone and dexamethasone. Triamcinolone also prevented granulomatous reaction more effectively than dexamethasone. The sample sizes used in this study may have been too small to define real differences among the glucocorticoid treatment groups. Glucocorticoids would be expected to prevent the formation of granulation tissue, granulomatous reaction and acute inflammation because of their well known inhibitory effects on the components of the immune system (16). On the basis of observations reported here, and in spite of limitations in the model employed, it appears that triamcinolone should remain the treatment of choice for keloid scars.

CONCLUSIONS

The discovery of central calcification, granulation tissue, granulomatous foreign body reaction and acute inflammation

indicates that the nude athymic mouse model for the study of keloid scars has serious limitations and requires further refinement. The use of nude athymic rats to study implanted keloid tissue has also been reported (17). This use of athymic rats may not result in the same complications that were encountered in the present study. A macrophage-depleted athymic mouse would likely decrease the granulomatous reaction that occurs in response to the grafted keloid tissue. It would also ensure that the host remained neutral to the implant for longer periods of time, which would permit systematic study of chronic palliative or curative therapy.

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