Control of Hypertrophic Scar Growth Using Antibody-Targeted Photolysis

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Hypertrophic scar is marked by excess collagen accumulation secondary to an increased vascularization response in the scar and an increase in fibroblast cell density. It is currently the most debilitating long-term complication of the surviving burn patient, and at present, there is no routinely effective form of therapy. In this study, we investigated the potential use of antibody-targeted photolysis (ATPL) in treating hypertrophic scars. An immunoconjugate consisting of a photosensitizer (Sn-chlorin e6) linked to a monoclonal antibody that binds to human myofibroblasts (PR2D3) was prepared, which in response to photoactivation produces singlet oxygen in close proximity to the target cell surface. The model used for these studies consisted of 1-mm³ human hypertrophic scar tissue implants in athymic mice. These implants increase approximately 20-fold in volume over a period of 15 days. Four days after implantation immunoconjugate was injected directly into scar implants and allowed to diffuse throughout for 24 hr before implants were illuminated with laser light at 630 nm (120 J/cm²). ATPL treatment caused a significant reduction in total growth compared to the untreated controls (P < 0.05). No effect was observed when an irrelevant conjugate (anti-Pseudomonas aeruginosa) was used. Histological examination of the ATPL-treated implants 24 hr post-ATPL revealed the presence of a large number of lipid droplets indicative of massive cell damage and infiltration by mononuclear cells and neutrophils.

INTRODUCTION

As a result of advances in the care of the acutely injured burn patient, more patients now routinely survive to face the long-term consequences of moderate to severe burn injury [1]. Hypertrophic scars (HS) are one of the most debilitating long-term complications of these injuries. HS differ from normal skin and normal scars by exhibiting increased vascularization, increased fibroblast cell density, and a thickened epidermal cell layer [2]. During formation of HS, collagen accumulates to levels far in excess of that needed to replace the original volume of lost dermis. Once formed, the fate of HS is unpredictable; there may be partial or complete resolution, or the HS may remain permanently [3]. This complication is especially severe in children and is a major cause of functional, cosmetic, and psychological morbidity [4, 5]. There is currently no clearly unifying theory to explain the pathogenesis of HS. Investigators have, however, identified the collagen nodule as the structural unit of HS [6]. The nodule contains a high density of fibroblasts and unidirectional collagen fibrils. Microvessels encircle the main body of the nodule as a net with only a few vessels appearing within it. No such nodules are found in mature scar.

Our ability to control HS formation is limited, and no routinely effective method is available. Large lesions may be treated by surgical excision and skin grafting. However, because of the 55% recurrence rate [3], combination therapy is usually required. Elastic compression therapy is widely used with HS. Although the precise mechanism of the compressive action is not known, it appears to be related to a combination of factors that result in fibroblast degeneration and/or an alteration of the collagen anabolism/catabolism ratio favoring catabolism [7]. A major drawback of compression therapy is that pressure must be maintained day and night for a minimum of 4 to 6 months [8]. The therapeutic effect of the application of various gel sheets in treating HS has recently drawn considerable attention [9]. It has been postulated that the skin surface coverage of these gels increases the skin temperature which increases the rate of collagenase activity and thus the rate of collagen breakdown. Investigators have also injected HS and other fibrotic lesions with gamma-interferon with up to a 50% reduction in size in the lesions [10, 11]. Gamma interferon inhibits types I and III collagen synthesis by dermal fibroblasts by decreasing the levels of cellular mRNA for these proteins [12]. Other HS...
therapies include steroid injection, topical silicone, and radiation [3, 7, 10]. These can attenuate HS, but are severely limited in their ability to restore normal function and appearance. It is evident that better clinical methods for preventing and treating HS are needed.

A possible alternative approach for treating HS involves targeting and destroying fibroblasts, the collagen-producing cells in HS. Using specific antibodies coupled with cytotoxic agents, one can selectively target these cells and hopefully reduce or prevent the formation of these scars while minimizing side effects and damage to surrounding tissue. Antibody-targeted photolysis (ATPL) is a technique which uses an immunoconjugate consisting of a photosensitizer covalently linked to a specific antibody. The cytotoxic effect is based upon the photosensitizer's ability to selectively absorb light at a specific wavelength and to initiate a chain of photochemical reactions that culminate in the production of phototoxins such as singlet oxygen. Antibody-mediated localization of the photosensitizer limits the production of cytoxins to the immediate vicinity of the targeted cell. By confining the illumination to the scar region, the cell killing can be limited to the target fibroblast population, while sparing other cell types within the scar and nontarget fibroblasts residing in other tissues.

In this study, we used a model of human HS tissue implanted subcutaneously in athymic mice [13–16]. To test the potential of ATPL for treating HS, antifibroblast immunoconjugates were injected percutaneously into the implants and, following irradiation, scar growth was monitored for 21 days. The results clearly indicate that ATPL treatment was able to effectively limit HS growth when compared to control treatments.

MATERIALS AND METHODS

Materials. PR2D3 is a monoclonal antibody (MAb) that was originally raised against a crude homogenate of human colorectal mucosa and found to bind human smooth muscle cells in culture and stain myofibroblasts in tissue sections of gut mucosa [17]. This antibody was produced from a mouse hybridoma cell line kindly supplied by Walter Bodmer (Director's Laboratory, Imperial Cancer Research Fund, Lincoln's Inn Fields, London). HS tissue was obtained from athymic mice [13–16]. Briefly, under sterile conditions, bilateral, linear, supracapsular incisions were made on the dorsum of the mice. The incisions were oriented transversely and were full thickness in depth. Curved microforceps were then introduced subdermally in the caudal direction to develop a pocket for the donor tissue. HS tissue pieces were blotted dry and placed in these pockets. The incisions were closed using two interrupted 5-0 chromic sutures. Each animal received two implants, one on each shoulder. A total of 40 animals were implanted with HS tissue using the tissue from three donors.

In vivo ATPL treatment. Four days after implantation, animals were lightly anesthetized with ether and the immunoconjugate was directly injected into each implant. Each implant received 200 μl of an immunoconjugate solution in PBS, pH 7.4. Conjugate concentration was varied from 200 to 1000 nM. Twenty hours after receiving conjugate, one implant on each animal was shielded with a black cloth (dark control) while the other was irradiated (see Fig. 1) with continuous exposure to 100 mW/cm² flux from a Kiton-red dye laser (Coherent, Palo Alto, CA). The animals were exposed to a surface fluence of 120 J/cm², corresponding to an exposure dose of 30 J/cm² across the skin thickness of the mouse [20]. Implant volumes were assessed at intervals of 3 days for up to 3 weeks using the method previously described by Waki et al. [16]. Briefly, calipers were used to measure the dimensions of the implant and implant volume estimated using the formula for an ellipsoid. Implants were clearly visible through the translucent skin of the animal. Since the implants appeared elliptical, volumes were calculated using the formula for an ellipsoid (D1 × D2 × D3) and thickness (D3) of the implant. These measurements were easily performed because the implants were clearly visible through the translucent skin of the animal. Since the implants appeared elliptical, volumes were calculated using the formula for an ellipsoid (D1 × D2 × D3). To measure D3, the skin containing the implant was gently retracted (easily performed in this loose-skinned animal) so as to permit the Ab to completely surround the implant. In none of our animals were the implants adherent to the suprascapular fascia. The skin thickness was measured from the thickness measurement to yield the D3 value. Implant volumes were measured independently by the first two authors, and the error in the calculated volumes between investigators was less than 5%. Data were analyzed using Analysis of the immunoconjugate revealed a single peak with an elution pattern similar to that of the unconjugated MAb. Immunoconjugates which were used in this study were shown to contain an average ratio of photosensitizer to MAb of 1.3 based on the absorbances at 410 nm for SnCe6 and 280 nm for the MAb. This conjugate has previously been shown to kill fibroblasts but not keratinocytes cultured in collagen lattices [19].

Tissue preparation. The HS tissue was handled in the operating room after Operand iodine preparation solution (General Medical Corp., Prichard, WV) was applied to the skin. Tissue from three different donors were used in this study. The tissue was placed between two sterile gauze sponges soaked in normal saline and was kept at 4°C until it was transferred to a sterile dish containing Dulbecco's modified essential medium (Gibco BRL, Grand Island, NY) with 10% fetal calf serum (JRH Biosciences, Lenexa, KS) and 100 U/ml penicillin-streptomycin (JRH Biosciences, Lenexa, KS). Pieces were defatted and deepithelialized using a 15-0 scalpel and fine straight scissors. The remaining tissue (dermis) was minced into small (10 × 10 × 5-mm) blocks with a fresh 15-0 scalpel and washed twice with phosphate-buffered saline (PBS), pH 7.4. After washing, the samples were further minced into 1 × 1-mm cubes (measured using a metric-ruler scalpel handle) and prepared for implantation into athymic mice. Processing of tissue was performed in a laminar flow hood at room temperature and implantation occurred within 4 hr after harvesting.

Atrophic mouse hypertrophic scar model. HS tissue was implanted into athymic mice using techniques previously described by others [13–16]. Briefly, under sterile conditions, bilateral, linear, supracapsular incisions were made on the dorsum of the mice. The incisions were oriented transversely and were full thickness in depth. Curved microforceps were then introduced subdermally in the caudal direction to develop a pocket for the donor tissue. HS tissue pieces were blotted dry and placed in these pockets. The incisions were closed using two interrupted 5-0 chromic sutures. Each animal received two implants, one on each shoulder. A total of 40 animals were implanted with HS tissue using the tissue from three donors.

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Incident Light

Dark Cloth

FIG. 1. Schematic diagram of ATPL in athymic mouse HS implant model. Deepithelialized HS tissue was cut into 1-mm³ pieces and implanted subcutaneously in the shoulder of nude mice. Each animal received two implants. After allowing the implants to grow for 4 days, immunoconjugate was injected locally. This conjugate binds to cells within the implant (right inset), and after 24 hr, one implant site was exposed to 120 J/cm² of incident light at 630 nm while the other site was covered by a dark cloth to serve as a dark control.

Histology. Histological analysis of the implants was also performed. At 24 hr, 48 hr, and seven days post-laser treatment, the animals were anesthetized with intraperitoneal chloral hydrate (200 JLI of 4 mg/100 ml) and skin implants were excised and immediately fixed in 10% formalin. The samples were sectioned and processed with hematoxylin–eosin (H & E) stain for viewing under the light microscope.

RESULTS

In Vivo ATPL

HS implanted into athymic mice were assessed every 3 days for volumetric growth. Untreated implants exhibited continued growth for about 2–3 weeks, followed by a phase in which the implant volume subsided (Fig. 2). This growth pattern is similar to observations made by other investigators [14, 16].

A total of 12 animals, each receiving two implants, were used in an initial study to determine the effect of conjugate concentration on implant growth. Animals were injected with 200 µl of a 200 nM, 500 nM, or 1000 nM immunoconjugate solution in both implants. In each animal, one implant site received a light dose of 120 J/cm² while the other implant was shielded from light and served as a dark control. The effect of ATPL on implant growth is shown in Fig. 2. Implants receiving 200 nM PR2D3 conjugate plus light displayed growth similar to that of the untreated and dark control (i.e., given immunoconjugate, but no light) implants. Some inhibition was observed at 500 nM, but it was not statistically significant. However, a significant inhibition of growth occurred in implants receiving 1000 nM conjugate plus light irradiation. In the latter case, the volumes of the ATPL-treated implants were statis-
brane lipids from lysed cells. No vacuoles were observed in implants treated with unconjugated photosensitizer followed by laser irradiation (not shown), indicating that their formation was not due to thermal-ysis or nonspecific photolysis. ATPL-treated implants also exhibited an infiltrate of inflammatory cells consisting of predominantly monocytes and macrophages (~80%), with some neutrophils present as well (~20%).

**DISCUSSION**

We have demonstrated retardation of the growth of HS implants in the athymic mouse using ATPL. This model is the closest approximation to human HS and keloids in which to test therapeutic strategies for the control of aberrant scar growth [13–16]. The benefits of the system are that the scars grow in volume while maintaining their histological appearance, cellularity, and glycosaminoglycan distribution without being rejected by a cellular immune response. Implant growth can be attributed, at least in part, to an increase in collagen mass [14, 16], and pharmacological agents that inhibit collagen synthesis have been used to inhibit this growth. The main disadvantage of this model is that the scars naturally resorb approximately 3 weeks after implantation. Possible explanations for this resorption may involve rejection of the HS implants, production of collagenase by the mouse cells to degrade the HS tissue, or disappearance of the stimulus for excessive collagen synthesis by the HS fibroblast.

The athymic mouse implant model has previously been used to test various pharmacological agents such as triamcinolone, acetylcysteine, colchicine, and penicillamine [16]. ATPL resulted in an approximately 81% inhibition of implant growth compared to 72% for triamcinolone, 43% for acetylcysteine, 54% for colchicine, and 20% for penicillamine. In addition, ATPL has the advantage of minimizing systemic toxic reactions by exposing only the implants to the laser irradiation. The high specificity of ATPL for the target cell population was clearly shown by control experiments which were performed using PR2D3, photosensitizer, and nonspecific conjugate, with and without light exposure. Since no differences were observed in the growth curves in these controls compared to untreated implants, we can conclude that both aspects of ATPL (cell targeting via antibody/antigen binding and light-induced generation of phototoxins) were necessary to inhibit implant growth. This is the first demonstration of using ATPL to control HS growth in an animal model.

The host response to the ATPL-treated scar implants was characterized by the presence of inflammatory cells within the implants. These inflammatory cells were predominantly (roughly 80%) monocytes and macrophages, with the remaining being neutrophils. In addition, an increasing number of vacuoles was observed as a function of time after treatment, which is consistent with a necrotic reaction within the implant. Consequently, the necrotic reaction caused by ATPL may
Fig. 4. H & E stain of HS implants. Implants were injected with 200 µl of a 1000 nM immunoconjugate solution on Postoperative Day 4. ATPL-treated implants were subjected to laser therapy on Postoperative Day 5. (A) Dark control implants showing fibroblasts (f) within the implant. (B) ATPL-treated, 24 hr after laser therapy. Vacuoles are readily apparent. (C) ATPL-treated, 7 days after laser therapy, demonstrating an increased number of vacuoles. (D) ATPL-treated, 24 hr after laser therapy, exhibiting an infiltrate of monocytes (m). Bar, 200 µm.

...play an important role in the observed implant growth inhibition.

Although the PR2D3 MAb was initially reported to bind myofibroblasts in tissue sections of human gut mucosa [17], we have recently found that the PR2D3–PS conjugate, but not the anti-Pseudomonas–PS conjugate, efficiently kills fibroblasts cultured in fibroblast-populated collagen lattices [19]. In addition, we found that the PR2D3 conjugate does not kill cultured keratinocytes, which supports the hypothesis that a prime target for ATPL was the fibroblasts in the scar implant. Examinations of histological sections reveal that 100% killing of fibroblasts was not achieved at the light dose and immunoconjugate concentrations used in this study. These doses have been previously shown to kill approximately 10⁸ fibroblasts in a fibroblast-populated collagen lattice model [19]. This is of the same order of magnitude as the number of fibroblasts in the 1.0-mm-thick implants at the time of ATPL treatment (estimated by counting the number of fibroblasts in 5-µm-thick histological sections of several implants, and assuming that the fibroblasts are uniformly distributed in the implant). Undoubtedly, there is some loss of the injected immunoconjugate over the 24-hr period prior to laser treatment. In addition, scavenging of singlet oxygen by endogenous proteins in the scar mass could potentially decrease the net cytotoxic yield. Future work to investigate immunoconjugate transport is warranted in order to determine the optimum conjugate concentration, light dose, and irradiation time after conjugate injection for inhibiting scar growth.

Although the specific mechanism of action of ATPL on implant growth needs further clarification, the results presented in this paper demonstrate the potential of this approach in the treatment and/or prevention of HS. Since a suitable animal model of HS formation does not exist, the true test for the technique of ATPL would be in treating HS in a clinical trial in burn patients. This would most likely involve the local injection of a surgically debulked HS with immunoconjugate, followed by laser irradiation of the lesion after 24 hr with the intention of eradicating those fibroblasts at the borders of the lesion that could be responsible for the overproduction of extracellular matrix.

The lack of a basic understanding of the mechanism of HS formation has prevented the development...
of effective therapies. ATPL is a potentially valuable tool for basic science research in this area because of its ability to selectively and efficiently eliminate targets. By using immunonjugates against different cells, ATPL could be used to provide new knowledge of the relative importance of certain cell types on HS formation.

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