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Photodynamic Modulation of Wound Healing With BPD-MA and CASP

Selene G. Parekh, MD, Kenneth B. Trauner, MD, Bertram Zarins, MD, Timothy E. Foster, MD, and R. Rox Anderson, MD*

Wellman Laboratories of Photomedicine, Ellison Sports Medicine Laboratory, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114

Background and Objective: **Wound healing is an intricate process requiring the orchestration of cells, growth factors, cytokines, and the extracellular matrix. Cytokines, specifically TGF-β, are believed to be instrumental in sustaining the fibrotic process, which leads to scarring. Photodynamic therapy (PDT) uses potent photosensitizers, which induce a wide range of effects on cells and the extracellular matrix. The influences of PDT on wound healing are not well known.**

Study Design/Materials and Methods: **Seven full-thickness incisional wounds were placed on each of 24 hairless Sprague Dawly rats, three wounds on one flank serving as dark controls and four on the contralateral side treated with PDT. Wounds were created two days before, one hour before, or one hour after red light exposure with an argon ion pumped dye laser. Twelve rats were injected with 0.25 mg/kg or 0.5 mg/kg of the PDT drug, BPD-MA, and the other 12 with 5 mg/kg or 10 mg/kg of the PDT drug, CASP, 3 and 24 hours prior to irradiation of light, respectively. At low doses of both photosensitizers, animals were irradiated with 1, 5, 10, and 20 J/cm^2. At higher doses of BPD-MA and CASP animals were treated with 10, 20, 50, and 100 J/cm^2 of light. Wounds were examined each day for 14 days and noted for edema, erythema, inflammation, necrosis, and quality of scarring. Wounds were also photographed at day 0, 2, 5, 8, and 14 post-irradiation. All animals were sacrificed 14 days after irradiation and the wounds were evaluated by light microscopy.**

Results: **Grossly, animals treated with 0.25 mg/kg BPD-MA showed no effect with PDT. Animals treated with 0.5 mg/kg BPD, and 5 and 10 mg/kg CASP showed responses that varied with both light and drug dose. Erythema, edema, inflammation, and necrosis attributed to PDT were all observed, but there was no apparent influence of PDT on either the rate or final appearance of wound healing. Histologically, there were no apparent differences between treated and untreated sites, regardless of the drug, dose of light, or time of irradiation.**

Conclusion: **A single PDT treatment given before or after skin wounds does not apparently alter wound healing even when PDT caused brisk inflammatory reactions. PDT may have effects that were not detected. We conclude that PDT does not**

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*Correspondence to: R. Rox Anderson, 50 Blossom St., Massachusetts General Hospital, Boston, MA 02114.
E-mail: AndersonR@helix.mgh.harvard.edu

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greatly influence incisional skin wound healing in the rat model. Lasers Surg. Med. 24:375–381, 1999.

Key words: cytokines; rat incisional wound model; photosensitizer; scarring

INTRODUCTION

Photodynamic therapy (PDT) has been used in the treatment of a variety of conditions including tumors, arthritis, and skin disorders. The technique is based upon the injection, ingestion, or topical application of a photosensitizer followed by activation of the compound with light. The photosensitizer is excited by absorption of a photon, to an excited singlet state. Subsequently, one of two events occurs. The photosensitizer can decay back to its ground state via fluorescence or nonradiative decay, or it can enter an excited triplet state by intrasystem crossing (change in the excited electron spin), where it maintains a slightly lower energy level than the singlet state and is longer-lived. Chemical reactions tend to occur from the triplet state. In type I reactions, an electron or hydrogen atom is transferred to surrounding substrates. Type II reactions involve the transfer of energy, usually to oxygen. This generates singlet oxygen, which can then proceed to oxidize other molecules. The singlet oxygen is short-lived and therefore oxidative damage is very localized. Most PDT drugs operate predominantly by type II reactions.

The potential application of PDT in modulating wound healing and scar formation has not been explored. Wound healing is an intricate process, requiring complex interactions between cells, growth factors, cytokines, and the extracellular matrix. Traditionally, the inflammatory process has been divided into three phases, emphasizing different components of the system. The inflammatory phase is initiated by the disruption of blood vessels. Platelets degranulate, latent cytokines found in the extracellular matrix such as platelet derived growth factor (PDGF), fibroblast growth factor (FGF), and transforming growth factor (TGF) are activated, and neutrophils, monocytes, and macrophages enter the local area. One to 10 days after the inflammatory phase is initiated, the proliferative/granulation tissue phase begins. Fibroblasts proliferate and migrate into the wound, loose connective tissue is deposited, macrophages release growth factors, angiogenesis and re-epithelialization occur, and myofibroblasts appear. Finally, during the matrix formation and remodeling phase, the extracellular matrix is degraded by enzymes, type I collagen is accumulated and organized, and myofibroblasts contract the wound.

The initiation and control of many of these concerted processes are governed by molecules that direct cell activity such as cytokines, growth factors, and adhesion molecules. In particular, the extracellular matrix growth factors TGF-β, PDGF, and bFGF appear to initiate and or sustain fibrosis. Specifically TGF-β appears to be a dominant cytokine governing the aggressiveness of scarring response. TGF-β has been implicated in hepatic and pulmonary fibrosis, scleroderma, and keloids. It stimulates collagen and fibronectin formation, suppresses collagenase, and induces production of collagenase inhibitors [1]. Elevated TGF-β levels, increased scarring, and rapid healing responses are associated with the disorientation and thinning of type I collagen fibers as well as the abnormal production of proteoglycans and gylcosaminoglycans in the extracellular matrix of the wound. Neutralization of TGF-β with inhibitory proteoglycans [2] or inhibition of TGF receptors with blocking antibodies [3] leads to scar resolution or decreased scar formation. Effects may be tissue specific as evidenced by the reduction in fibrosis of central nervous system astrocyte cell populations after exposure to TGF-β.

Alteration of wound healing by ultraviolet ionizing radiation, light, hypothermia, electrical stimulation, ultrasound, and oxidative stress has been documented. However, the effects of photodynamic photosensitizers on wound healing have not been examined, despite studies that suggest that photodynamic therapy alters or inactivates certain proteins and growth factors, especially TGF-β [4–7].

Photosensitizers such as benzoporphyrin derivative (BPD-MA) (Quadra Logic Technologies Inc., Vancouver, Canada) and chloroaluminum sulfophthalocyanine (CASP) (Ciba-Geigy, Basil, Switzerland) are currently under evaluation for the treatment of malignant lesions [8]. The excitation spectra for these molecules have a peak at 690 nm and 675 nm, respectively, wavelengths at which light has a high tissue penetration [9]. BPD and CASP are potent, efficient photosensitizers [10], which cause only brief skin photosensitiza-

tion [11] and are well tolerated in vivo. We therefore evaluated the effects of these two photosensitizers in a rat skin wound healing model.

MATERIALS AND METHODS

Animals

Twenty-four hairless Sprague Dawley rats were obtained from Charles River (Wilmington, MA). The rats were housed in groups of three and fed standard rat chow. Rats were divided into several groups based on the photosensitizer injected, delivered light fluence, and the time of skin incisions (wounding) relative to light exposure.

Preparation of Photosensitizers

Liposomal benzoporphyrin derivative monoacid ring A (BPD-MA) (Quadra Logic Technologies Inc., Vancouver, Canada) was obtained as a lyophilized powder and thawed for four hours at room temperature. Sterile water was added to obtain an isotonic solution of 0.25 mg/ml, which was then injected via an intracardiac puncture into the rats.

Chloroaluminum sulfophthalocyanine (CASP) (Ciba-Geigy, Basil, Switzerland) was obtained as a powder, dissolved in normal saline to yield a final concentration of 2.5 mg/ml, and injected via an intracardiac puncture.

Skin Wounding and PDT

Rats were weighed and then anesthetized by inhalation of metofane (Pitman-Moore, Mundelein, IL). Anesthesia was maintained with a metofane-soaked gauze pad in a 50 ml centrifuge tube which was placed over the snout.

Animals receiving BPD-MA were injected three hours prior to irradiation. Full-thickness, 2 cm incisions were created using a #10 scalpel blade and then apposed using a 3–0 nylon retention suture. Seven wounds were placed on each animal. Three wounds on the left flank served as dark controls, and four on the right serving as treated areas (Figure 1). The first two incisions on the right flank were allocated for one set of parameters, and the second for another. Six animals were treated with either 0.25 mg/kg or 0.5 mg/kg of BPD-MA. Light exposure fluences administered to 0.25 mg/kg animals were 1 and 5 J/cm^2 (three animals) or 10 and 20 J/cm^2 (three animals); fluences administered to the six 0.5 mg/kg animals were 10 and 20 J/cm^2 (three animals) or 50 and 100 J/cm^2 (three animals).

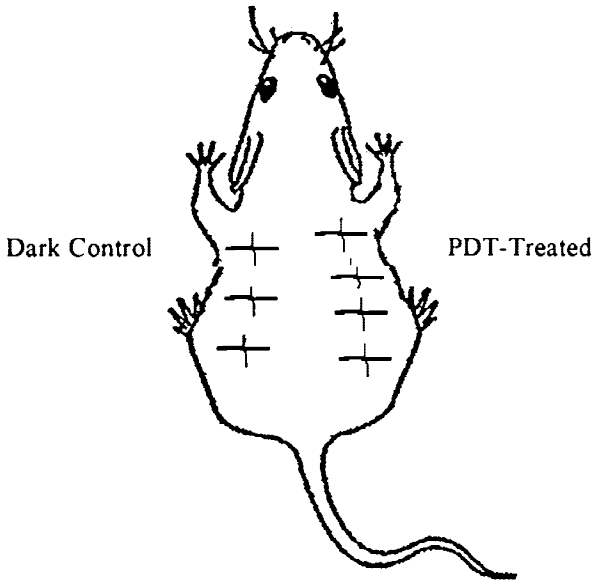

Fig. 1. Wound placements on the left and right flanks of the rat.

Animals receiving CASP were injected 24 hours prior to light exposure. Seven 2-cm incisions were created, with three on the left flank serving as the dark controls and four on the right serving as the treated samples. Doses of CASP evaluated were 5 mg/kg and 10 mg/kg. Six animals received CASP at 5 mg/kg. Three of these animals were treated with fluences of 1 and 5 J/cm^2, and three at 10 and 20 J/cm^2. An additional six animals received 10 mg/kg of CASP. Three animals were exposed to 10 and 20 J/cm^2, whereas another three animals were exposed to 50 and 100 J/cm^2 of light.

Light was produced with an argon ion pumped dye laser system (Innova 9000, Coherent, Palo Alto, CA). BPD animals were treated at a wavelength of 690 nm and CASP at 675 nm. The spot size was adjusted to an area of 4.9 cm^2 (radius of 1.25 cm), and a metal shield was used to avoid inadvertent exposure to surrounding tissue. The irradiance was 400 mw/cm^2 (690 nm), and 400 mw/cm^2 (675 nm); optical power was stable, measured periodically with a Coherent laser power meter (Palo Alto, CA). Groups of animals were irradiated two days after, one hour after, or one hour prior to wound production to examine the influence of timing between PDT and wound formation. Wounds were evaluated grossly every day for inflammation, edema, erythema, necrosis, and quality of scarring. Photographs of wounds

were taken day 0, 2, 5, 8, 10, and 14 post-irradiation.

Animals were sacrificed 14 days after light exposure by an overdose of metofane. Tissue samples were then obtained by excising a rectangular area surrounding the site of the healed wound. The samples were bisected with one-half immersed in formalin and the other used for frozen sections. Tissue samples fixed in formalin were then embedded in paraffin, sectioned at 5 or 10 μm intervals, stained with hematoxylin and eosin and examined by light microscopy. Sections were also stained with Mason's trichrome to evaluate collagen orientation in the tissue.

RESULTS

Gross Observations

Control. Control (no light exposure) samples for 0.25 mg/kg and 0.5 mg/kg BPD-MA injected animals and 5 mg/kg and 10 mg/kg CASP injected rats yielded similar results. No erythema or edema was present in these sites. The wounds had fully closed by day 5 and scars were hardly noticeable by day 8.

BPD-MA. No erythema or edema attributable to PDT was apparent in animals treated with 0.25 mg/kg, after exposure to all fluences and at all three time points for irradiation. By day 5 post-irradiation, wounds had completely closed and by day 8, scars were barely visible. No discernible difference in rates of healing or scar formation was noted between the treated and untreated wounds.

In animals receiving 0.5 mg/kg, there was no apparent difference between the light-exposed and control incisions at light exposures of 10 and 20 J/cm^2. At 50 and 100 J/cm^2, erythema and edema were present immediately after exposure in all animals receiving 0.5 mg/kg BPD-MA, and all of those incisions became indurated with little or no necrosis. Ten days after irradiation, treated wounds remained indurated and raised, however the gross appearance of the scar for treated and untreated samples was the same.

CASP. Animals treated with 5 mg/kg demonstrated dose-dependent light effects. At 1 and 5 J/cm^2, no erythema or edema was found after irradiation. Animals exposed to 10 and 20 J/cm^2 displayed erythema and edema immediately following irradiations. No necrosis was seen in any of these animals. Animals treated with 10 mg/kg also displayed dose-dependent light effects. At 10 and 20 J/cm^2, no erythema, edema, or differences were notable between treated an untreated sites. At 50 and 100 J/cm^2, for all time points, erythema and edema were apparent immediately after treatment with light. Two days after irradiation, the edema had subsided, however a reddish-yellow discoloration of the skin became apparent, with necrosis noticeable in the 100 J/cm^2 exposure sites. Five days after irradiation, the 50 J/cm^2 sites also became necrotic and the 100 J/cm^2 sites began to slough the necrotic tissue, however all wounds had closed. On day 8, discoloration of the wounds was less severe, necrotic tissue was till present, and the sites were indurated. By day 10, normal skin color had returned, necrotic tissue had almost completely sloughed off, and there was no apparent difference between treated and untreated sites except for induration (Figure 2). Induration was still present at day 14, when the animals were sacrificed by overdose of metofane.

The responses noted above were independent of the timing between PDT and incisional wounding for both drugs.

Histology

Control. Histologic evaluation of control samples for 0.25 mg/kg and 0.5 mg/kg BPD-MA injected animals and 5 mg/kg and 10 mg/kg CASP injected rats yielded similar results. Re-epithelialization and keratinization of the epidermis was complete by day 14. Dense bands of fibrotic tissue, oriented parallel to the surface of the skin, were present throughout the depth of the dermis. There was variation in the amount of fibrotic tissue based on the location of the wounds on the rat. Furthermore, the extent of fibrosis for some samples could not be appreciated due to confounding by the presence of hair follicles and sebaceous glands in the dermis.

Inflammatory cellular infiltrate was sparse throughout the epidermis and dermis. Fibroblasts were the predominant cells in the fibrotic tissue. At the junction of the dermal and sub-dermal tissue, and at the distal portion of the scar were an increased cellular infiltrate. A variety of cells were present in this area, including erythrocytes, inflammatory cells, and fibroblasts.

BPD-MA. There was no consistent difference between treated and control sites for both concentrations of BPD at all fluences and time points. Healing of the incisional wounds varied between rats and within rats. Histologic appearance of some of the wounds was confounded by sebaceous glands or hair follicles. One sample in-

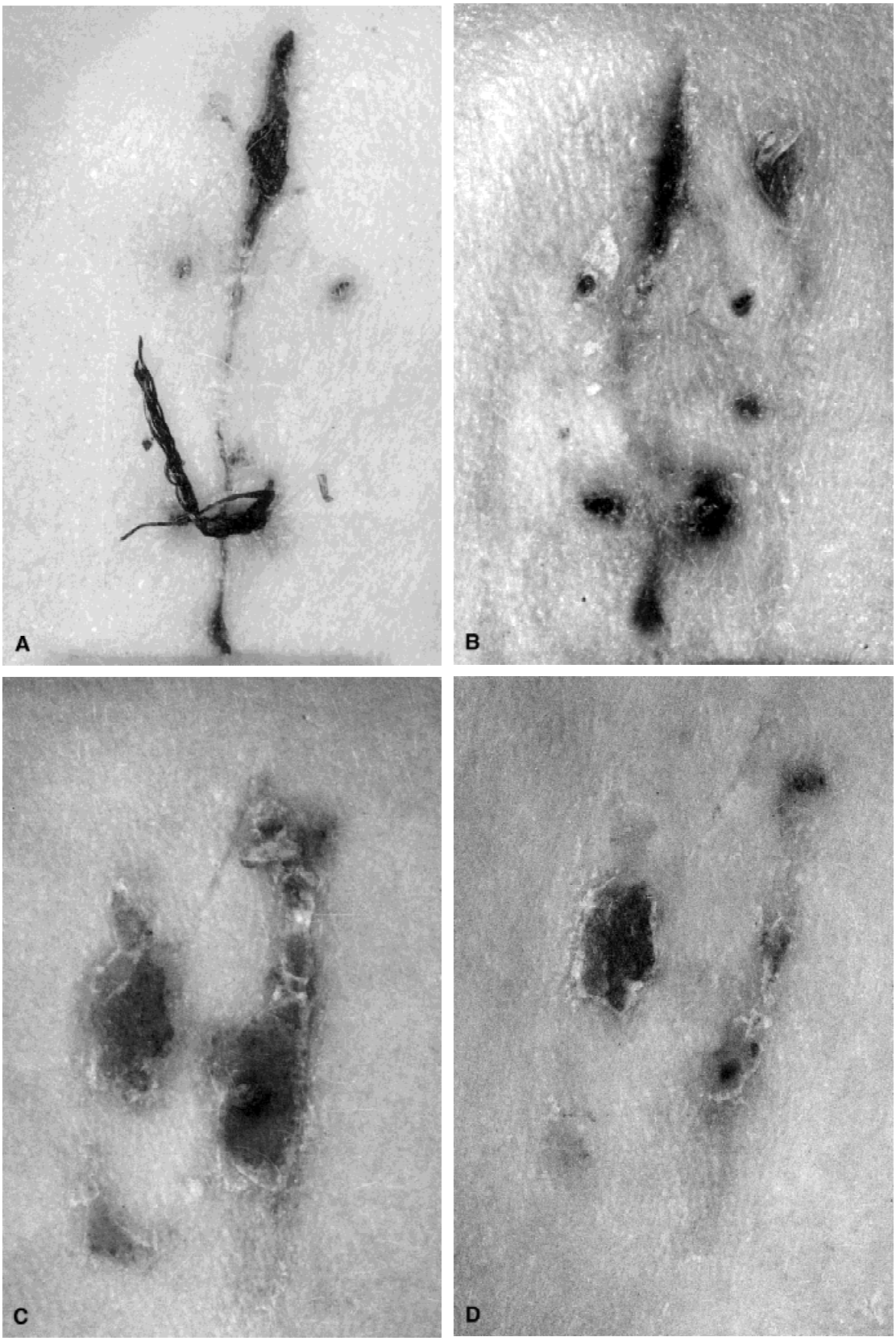

Fig. 2. Animal receiving 10 mg/kg of CASP and irradiated with 100 J/cm^2 at 675 nm 2 days after wound production. **A:** Wound two days prior to irradiation. **B:** Wound immediately after irradiation. **C:** Wound five days after irradiation. **D:** Wound 10 days after irradiation.

jected with 0.25 mg/kg of BPD and irradiated with 10 J/cm^2 1 hour after the incisions were made, demonstrated large cells at the junction of the dermis and the subdermal tissue. These eosinophilic cells contained a large nucleus and cloudy cytoplasm, and are likely to be myofibroblasts.

CASP. No consistent difference was noted between treated and untreated sites for both drug doses of CASP at all fluences and time points. There was large variability between wounds of different animals and within wounds on one animal.

DISCUSSION

In this study, PDT caused a range of effects from little or no effect at low doses of light and photosensitizer to increased edema, inflammation, erythema, and induration with high doses of photosensitizer and light. PDT was given at three different periods in the wound healing process to evaluate potential effects in the rate and quality of healing. Irradiating animals one hour prior to incisional wounds may affect latent growth factors and cytokines in the extracellular matrix. Irradiating a site one hour after wounding may affect and/or alter the cellular response subsequent to wounding. Finally irradiating two days after production of the wound might alter newly produced cytokines and growth factors as well as late-phase inflammatory cells in the area.

PDT has been increasingly used in combination with surgery for the treatment of respiratory, gastrointestinal, urogenital, and cutaneous cancers. In these situations, PDT could potentially change the rate of healing and formation of scar tissue, leading to complications. Our study suggests that PDT in conjunction with surgery has no detrimental effect on either the rate of healing or the quality of the scar when compared to dark controls. This implies that the effects of a single PDT treatment on the wound healing process are either minor or transient. It is also possible that the rat incisional wound healing model is insensitive to the subtle effects that may occur when PDT with BPD-MA or CASP is used. Rats heal rapidly and with relatively little scar formation. Although the rat offers a good model for detecting inhibition of wound healing, any acceleration or improvement in wound healing would be difficult to detect.

Previous studies have suggested that laser light alone benefits wound healing at low powers [12–14] and delays the healing process at high powers [12]. Lasers are currently being adopted with other technologies in an attempt to improve the wound healing process, by either increasing the rate of repair, or diminishing the amount of scarring. Different laser systems have been studied for excision of keloids, however there has been no successful suppression of keloid regrowth [15]. By using 5 mg/kg of Photofrin and 630 nm light at 25, 50, and 75 J/cm^2, it has been shown that PDT of a rat skin flap results in delayed wound healing, causing effusions, discoloration of the flaps, necrosis, scab formation, and lower tensile strength [16]. Hypertrophic scars treated with antibody-targeted photolysis using a Sn-chlorin photosensitizer linked to a monoclonal antibody targeting human myofibroblasts led specifically to a reduction in growth of scar [17]. Recently, human albumin solder has been used as a vehicle to deliver TGF-β_1. This technique repeatedly accelerated wound healing following laser-welded wound closure, and increased the early tensile strength of laser welded wounds [18].

PDT effects on processes intimately involved with cytokines and growth factors have been studied over the past five years. Several years ago, our laboratory discovered that PDT inhibits arterial restenosis following endothelial injury in animals [7,19]. Using CASP activated by red light, dose-dependent killing of smooth muscle cells in the media was documented. It was also found that a range of moderate light exposure doses caused widespread cell necrosis in the artery and endothelium, but did not cause destructive inflammation (arteritis), thrombosis, or loss of structural integrity. Studies were then performed on the effect of PDT on extracellular matrix. The results demonstrated that PDT profoundly altered the biologic characteristics of the matrix and the growth of the smooth muscle cells was strongly inhibited, whereas endothelial cell growth was stimulated on the PDT-altered matrix [7]. The results suggest that free radicals produced by PDT may interfere with biologic proteins in the matrix, specifically TGF-β, which may lead to differential effects on different cell types.

In this study, two type II, injectable photosensitizers were evaluated. Grossly, animals treated with 0.25 mg/kg BPD-MA showed no effect with PDT. Animals treated with 0.5 mg/kg BPD, and 5 and 10 mg/kg CASP showed responses that varied with both light and drug dose. Erythema, edema, inflammation, and necrosis attributed to PDT were all observed, but there was no apparent influence of PDT on either the rate or

final appearance of wound healing. Histologically, there were no apparent differences between treated and untreated sites, regardless of the drug, dose of light, or time of irradiation. PDT using topical and other injectable photosensitizers, other than BPD and CASP, may alter wound healing. Furthermore, multiple doses of PDT treatment at varying time intervals may prove more efficacious in altering wound healing and scar formation than a single dose of PDT. Animal models of wound healing, which are more sensitive to alterations in the wound healing process should be explored. Finally, the possible role of antibody-mediated targeted photolysis of particular components in the wound healing process should be evaluated to specifically effect certain cells or proteins in the fibrotic process. This study implies that use of photosensitizers targeted against specific cells or growth factors will not suffer from nonspecific affects of PDT on normal healing.

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