VASCULAR PHOTODYNAMIC THERAPY

A MULTIFACTORIAL APPROACH TO

INHIBIT INTIMAL HYPERPLASIA

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PHOTODYNAMISCHE THERAPIE VAN DE VAATWAND
EEN MULTIFACTORIËLE BENADERING OM
INTIMA HYPERPLASIE TE VOORKOMEN

PROEFSCHRIFT

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Cover: front, rat carotid artery 4 weeks after balloon injury; back, injured carotid artery after PDT.

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Aan Susanne
Aan mijn ouders

PREFACE

The Wellman Laboratories of Photomedicine at the Massachusetts General Hospital in Boston is an internationally known institute for the development and application of laser in medicine. Besides fundamental research which investigates the interaction of light with cells and molecules, a great deal of studies are performed towards clinical application to treat human diseases. For this reason, all known medical specialties are involved in research programmes examining the use of laser for their purposes: from ophtalmologists, using photodynamic therapy to treat choroidal neovascularization, oncologists to photochemically target and remove breast cancer cells from bone marrow, urologist to detect bladder cancer with fluorescence, gastroenterologists to treat Barret's oesophagus to dermatologists removing tattoes and treating multiple skin diseases with the laser. The application of laser to treat vascular diseases was started decades ago, but more recently the concept of photodynamic therapy was introduced by the research group of Dr. LaMuraglia to prevent restenosis. As a research fellow in surgery I joined this group in 1994 and our research goal was to better understand how photodynamic therapy affects the vascular wall. The results from these investigations are presented in this thesis. The first part gives an outline of the problem of restenosis, the concept of photodynamic therapy and aims of the study. The following chapters describe how photodynamic therapy interacts with biological factors that regulate the vascular healing process. Finally, considerations for possible clinical use are discussed.

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CHAPTER 1

GENERAL INTRODUCTION

RESTENOSIS AND INTIMAL HYPERPLASIA

End organ and limb damage as a consequence of ischemic vascular disease leads to significant morbidity and is the major cause of death in the western society. Currently, the only way to treat established atherosclerotic stenotic lesions is by vascular interventions, which include balloon angioplasty, stenting, atherectomy, endarterectomy and bypass grafts. All of these procedures involve some mechanical alteration of the vessel wall or obstructing lesion that result in an injury to the tissues and subsquent vascular repair. Unfortunately, the long-term patency of these procedures is limited by the development of restenosis. Although the restenosis rate is procedure and site specific, after coronary balloon angioplasty 30-60% of patients will re-develop ischemic symptoms after an initial satisfactory procedure (McBride et al., 1988; Bauters et al., 1996). Approximately 30% of peripheral bypass grafts develop significant stenotic areas and the vast majority of these lesions develop within the first year (Grigg et al., 1988; Idu et al., 1992). Restenosis is also a major problem after balloon angioplasty and stent treatment of renal artery disease (Tullis et al., 1997). It can easily be inferred from these numbers that restenosis is a significant health problem and poses an enormous financial burden on health resources.

The pathogenesis of restenosis is complex and not well understood (Schwartz et al., 1995). After balloon angioplasty, acute thrombus deposition and vascular recoil, are early events that can lead to acute narrowing of the vessel lumen. Drugs directed towards interference with the coagulation cascade or platelet functions, such as heparin and aspirin, have been succesfully implemented to reduce acute failures following these procedures. However, the subacute and long term development of restenosis is mediated by multiple mechanisms and anticoagulation therapy has not been effective to inhibit its occurence (Bauters et al., 1996). Histopathological studies in both experimental animal models and human tissue suggest that intimal hyperplasia (IH) plays a dominant role in the development of restenosis (Ross, 1993; Schwartz et al., 1995). The cellular events following injury of the vascular wall have been studied in great detail in the rat carotid injury model (Reidy et al., 1992). Intimal hyperplasia develops as part of the healing response to the procedurally-related vessel wall injury. Shortly after an intervention to the vessel wall which results in denudation of the endothelial layer and medial wall damage, smooth muscle cells (SMC) in the vessel media shift from a contractile to a synthetic phenotype, start proliferating in the media and migrate to the intima. This acute phase of the healing process is followed by continued SMC proliferation in the intima and sustained production and deposition of large amounts of

extracellular matrix (ECM) by intimal SMC. It was recently demonstrated that the extent of the hyperplastic response correlates with the degree of injury to the arterial wall (Indolfi et al., 1995).

The vascular repair process shares many similarities with normal wound healing and is regulated by a complex interplay between platelets, inflammatory cells, SMC, fibroblasts and endothelial cells (Kovacs and DiPietro 1994). These cells are in close relation to the ECM and their function is coordinated by a wide array of growth factors, cytokines and vasoactive proteins. Injury to the vessel wall results in cell death with release, activation and increased production of several bioactive proteins that are involoved in the stimulation of SMC migration, proliferation and production of extracellular matrix (Ross, 1993). Although this healing process can occur and remain self contained, an exaggerated fibroproliferative response leads to severe intimal thickening which encroaches on the vessel lumen.

Besides the established role of IH in the pathogenesis of restenosis, there has recently been much attention to the process of arterial remodeling (Gibbons et al., 1994). This concept followed the observation that arteries undergo adaptive enlargement in response to progressive plaque expansion to maintain a stable lumen size. This compensatory process may limit the effect of plaque or intimal thickening on lumen narrowing. It is thought that failure of this adaptive response contributes to the development of restenosis. The mechanisms of remodeling and the vascular constriction process after angioplasty are not well understood. Furthermore, the role of this remodeling process in intimal hyperplasia associated with bypass grafts and arteriovenous fistels is not known.

A major challange in the cardiovascular field is the discovery of a therapeutic strategy to prevent restenosis. To date most clinical trials have fallen short to achieve this goal (Bauters et al., 1996). A reason to explain this is that animal models designed to illuminate the nature of IH have not been able to simulate the complexity of the clinical situation. Thus, although various pharmacological compounds were effective in inhibiting IH in animal models they failed to prevent restenosis in humans. Furthermore, most pharmacological agents attempted so far, such as ACE inhibitors and platelet antagonists, target only one or limited pathophysiological pathways that may have a role in the development of restenosis (Pratt and Dzau 1996). Considering the multifactorial character of IH and restenosis with involvement of different cell types and multiple cellular mediators, it may be essential to simultaneously modulate several pathways involved in the healing process to effectively deal with this problem. A number of novel strategies, including antisense oligonucleotides for inhibition of cell-cycle regulatory mRNA translation or other forms of gene-therapy, antibodies to growth factors, and gamma irradiation are being investigated with varied results (Bauters et al., 1996). The introduction of stents in the vascular wall is another approach to deal with restenosis by creating a more satisfactory immediate result and preventing the effect of vascular constriction after a conventional angioplasty (Serruys et al., 1993). Among

the great effort of research in this field, vascular photodynamic therapy has gained interest as a means to inhibit injury-induced IH.

PHOTODYNAMIC THERAPY

Photodynamic therapy (PDT) utilizes visible wavelength of light that is absorbed by an otherwise relatively inert light-sensitive dye called a photosensitizer. When a photosensitizer absorbs light of the appropriate wavelength it is converted from a stable electronic chemical structure (ground state) to an excited state (Figure 1.1). The photosensitizer has the unique property of undergoing a high percentage conversion to a triplet electronically excited state which has a lifetime of ns to ms. From the triplet state either of two reactions follow: in the type I reaction, the photosensitizer can react directly with other molecules (such as the amino acids, tryptophan, cysteine, or quenchers such as beta carotene or NaN_3) forming free radicals or chemical conversions; in the type II reaction, the photosensitizer reacts directly with O_2 to produce singlet oxygen ($^1O_2$), a chemical species that is highly reactive in biological systems (Henderson and Dougherty, 1992).

The reactive species generated by these photochemical processes are highly cytotoxic by causing damage to cellular and organelle membranes (Henderson and Dougherty, 1992). Cell damage mediated by free radicals will occur close to its site of generation. Due to the wide and varied sensitizer distribution within cells, the free radical reaction can affect virtually all cellular components. Photochemical-induced peroxidation of membrane cholesterol and other unsaturated phospholipids leads to changes in membrane permeability, loss of fluidity, cross-linking of aminolipids and polypeptides, and inactivation of membrane associated enzyme systems and receptors. Depending on the chemical structure of the photosensitizer and its distribution in the cell, different cellular targets can initiate cell death. Whatever the primary insult(s) may be, the consequence is a rapid loss of cell integrity if a lethal PDT-dose is administered. Dependent on the PDT-dose, cells can also be damaged by the photochemical reaction but not result in cell death due to cellular repair processes that restore integrity and function. With lower levels of PDT, apoptotic cell death has also been described in certain cell types (Noodt et al., 1999).

PDT maintains spatial selectivity because the reactive molecules are generated only in the irradiated field and have an extremely short half-life and diffusion distance, resulting in a localized effect. Furthermore, the proclivity of the photosensitizer to preferentially accumulate in proliferating cells and the ability to irradiate with laser light only over an area of interest has provided the concept to develop and utilize PDT as a means to cause targeted, localized tissue destruction. Therefore, PDT has gained substantial interest as an alternative approach to either treat or serve as a palliative technique for the management of several malignancies and is curently in clinical trials for cutaneous, head and neck, endobronchial, esophageal, gastrointestinal, genitourinary, and gynaecological cancers (Pass, 1993). Recently, the application of PDT has been

extended and tested experimentally as a method to deal with other non-malignant cellular proliferative disorders such as psoriasis (Calzavara et al., 1996), arthritis (Trauner and Hasan, 1996), keloid (Wolfort et al., 1996) and intimal hyperplasia.

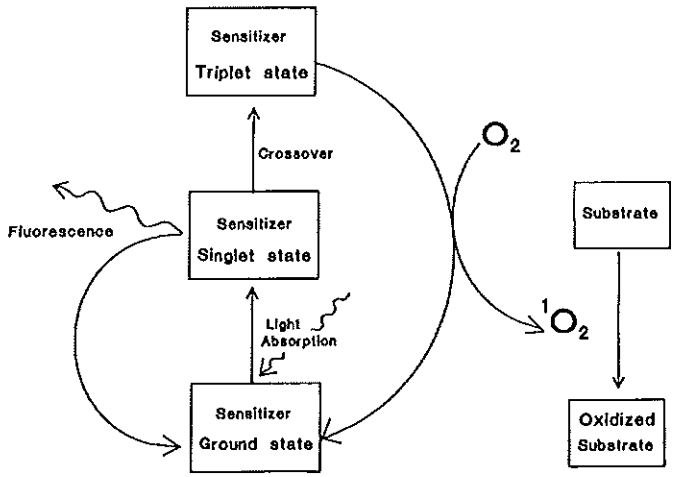


Figure 1.1.
Schematic representation of the type II photochemical reaction, which is thought to be the most important mechanism of PDT cytotoxicity. O_2, oxygen $^1O_2$, singlet oxygen.

CHAPTER 2

VASCULAR PHOTODYNAMIC THERAPY AND STUDY AIMS

Vascular Photodynamic Therapy

The rationale to utilize PDT as a means to inhibit IH has emerged from the standpoint that local eradication of SMC at the site of vascular injury would eliminate the effector cells responsible for IH. In 1983, Spears et al demonstrated for the first time that a photosensitive hematoporphyrin derivative selectively accumulated in experimental atherosclerotic plaques (Spears et al., 1983). This observation, confirmed by others, demonstrated the increased uptake of the photosensitizer porphyrin in both human and experimental atheresclerotic plaques, which was attributed to the high mitotic activity of smooth muscle cells in atheroma (Litvack et al., 1985; Neave et al., 1988). These findings initiated the investigation of high energy laser light as a means to ablate photosensitizer impregnated atherosclerotic plaques. However, these studies were not encouraging, based on the inability of the laser and photosensitizer to ablate the remaining calcified noncellular matrix material (Pollock et al., 1987; Tang et al., 1993). The finding of Dartsch et al, that SMC in culture derived from atherosclerotic plaques, were selectively inhibited by PDT using the photosenstizer Photofrin II, suggested the suitability of PDT for the treatment of hyperproliferative restenotic lesions (Dartsch et al., 1990).

In 1992, the first work of PDT as a method to treat experimental IH for the clinical application of restenosis was published (Ortu et al., 1992). That study concentrated on the acute and subacute effects of PDT on the vascular wall and established that injury-induced IH could be inhibited by PDT. Using the rat carotid balloon injury model to induce IH, PDT of the arteries with the photosensitizer chloroaluminum sulfonated phtalocyanine (CASPc) was performed at day 2 and 7 after the inflicted balloon injury. The injured vessel was irradiated externally with laser light with a total energy of 100 J/cm^2. PDT resulted in a significant decrease of IH assessed after 14 days with the absence of medial SMC or inflammatory cells in the treated area. Electron microscopy analysis showed early evidence of massive PDT-mediated cytotoxicity at 4-hours, no sign of collagen or elastic tissue structural alterations and only few platelets present at the intimal surface. This study concluded that PDT-induced SMC eradication was an effective means to locally eliminate the effector cells responsible for IH and can be employed without causing thrombosis, inflammation, or loss of structural vessel wall integrity. Subsequent experiments characterized the preferential distribution of the photosensitizer CASPc in IH tissue using laser-induced fluorescence and spectofluorimetric analysis (LaMuraglia et al., 1993). There was approximately 60% lower uptake and retention of the photosensitizer CASPc by normal arterial tissue as com-

pared to injured arteries. This data indicated that IH, has an increased accumulation and retention of CASPc compared to normal artery, and therefore therapeutic targeting of this cellular population is theoretically possible with PDT.

These promising data were followed by a study that addressed the long-term effects of PDT on the injured vascular wall (LaMuraglia et al., 1994). Consistent with the previous study, PDT of the rat balloon injured carotid resulted in complete local depletion of medial SMC. Sequential scanning electron microscopy of PDT-treated arteries, demonstrated that there was complete endothelial cell covering of the intima at 4 weeks (Figure 2.1) and adventitial repopulation with (myo)fibroblasts from week 1-16. However, even at 16 weeks, there was minimal SMC repopulation of the media or intima in the PDT-treated arteries and effective inhibition of IH (Figure 2.2). Similar to control balloon-injured arteries, PDT-treated arteries did not show any change in vessel diameter over the time period studied, which suggested preservation of structural integrity and no aneurysm formation.

Several other investigators, using different PDT-parameters, have confirmed the finding that injury-associated IH can be successfully inhibited by PDT. Hsiang et al employed a rabbit iliac artery balloon-injury model to study the effects of the photosensitizer Photofrin II on IH development (Hsiang et al., 1995). Nyamekye et al used 5-amino-levulinic acid, a precursor of the photosensitizer protoporphyrin IX, to perform PDT with external light irradiation in the rat carotid model (Nyamekye et al., 1995). More recently, Gonschior er all published data on PDT of injured porcine arteries using endovascular irradiation (Gonschior et al., 1996). The common findings from these experimental studies were that PDT of the vascular wall can result in eradication of medial SMC without causing thrombosis or an inflammatory reaction. The depletion of medial SMC after PDT persisted for periods of up to six months and is associated with effective inhibi-

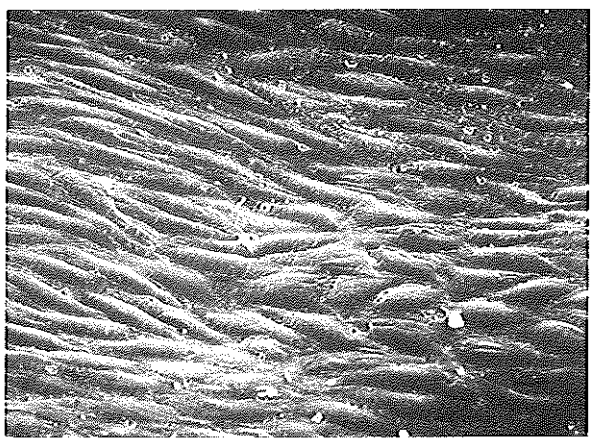


Figure 2.1.
Scanning electron micrograph of balloon-injured artery surface 4 weeks after photodynamic therapy. Surface is completely recovered by endothelial cells. Bar is 10 μm.

tion of injury-induced IH. There have been also studies which found less favorable results after PDT to inhibit IH (Sobeh et al., 1995 ; Hsiang et al., 1995). The reason of these failures was not clear and not well documented.

The effective depletion of cells from the vessel wall by PDT with the preservation of the structural integrity has prompted study to evaluate other vascular applications of PDT. One study investigated the effect of PDT on IH of bypass grafts using a rat vein graft model (LaMuraglia et al., 1995a). Similar to the previous studies in the balloon-injury model, the results demonstrated that PDT-treatment of vein grafts before implantation resulted in significant inhibition of IH in the body of the vein graft without thrombosis. However, at the anastomosis there was no difference in the degree of IH between the PDT and control groups. This indicated that at the anastomosis arterialization with SMC from the untreated artery supervenes.

Another study utilized PDT for the development of allogeneic vasular bioprosthesis (LaMuraglia et al., 1995b). PDT was postulated as an innovative method to blunt or obviate the immunological response by eliminating vascular cells and possibly altering immunogenic antigens. Using inbred rats of two histocompatibly-disparate strains, PDT was used to treat the aorta

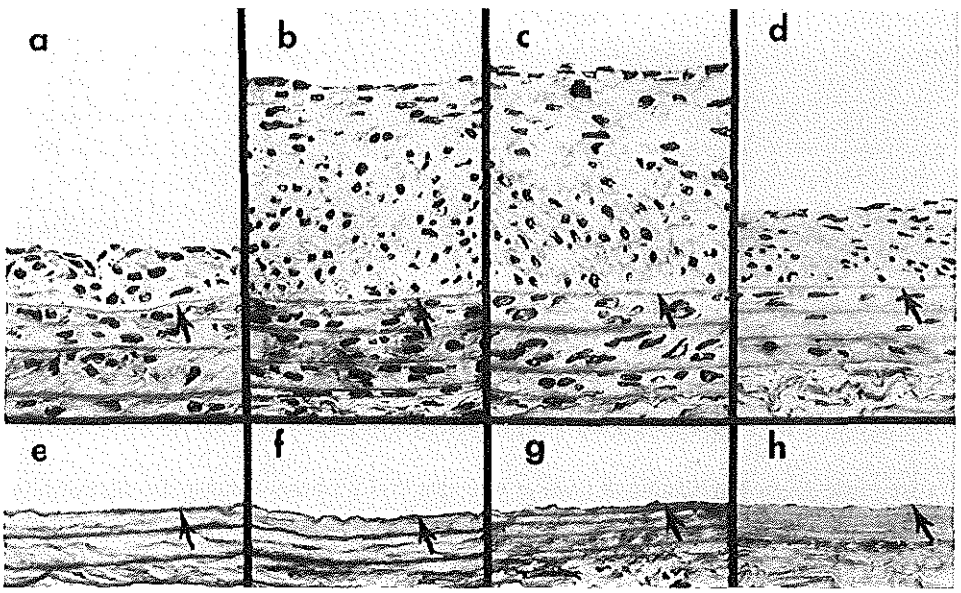


Figure 2.2.
Composite of light micrographs from balloon-induced IH of carotid arteries treated with laser light controls (a to d) or with PDT (e to h). Internal elastic lamina (arrow) is noted. a,e: 1 week after laser treatment; b,f: 2 weeks after laser treatment; c,g: 4 weeks after laser treatment; d,h: 16 weeks after laser treatment. In controls, IH is seen at 1 week (a), peaks at 2 to 4 weeks (b,c) before receding at 16 weeks (d). Cellularity of media is unchanged. In PDT-treated series, no IH was seen at any time point (e to h) in most animals. Media was cell free in most instances. (Original magnification x 800).

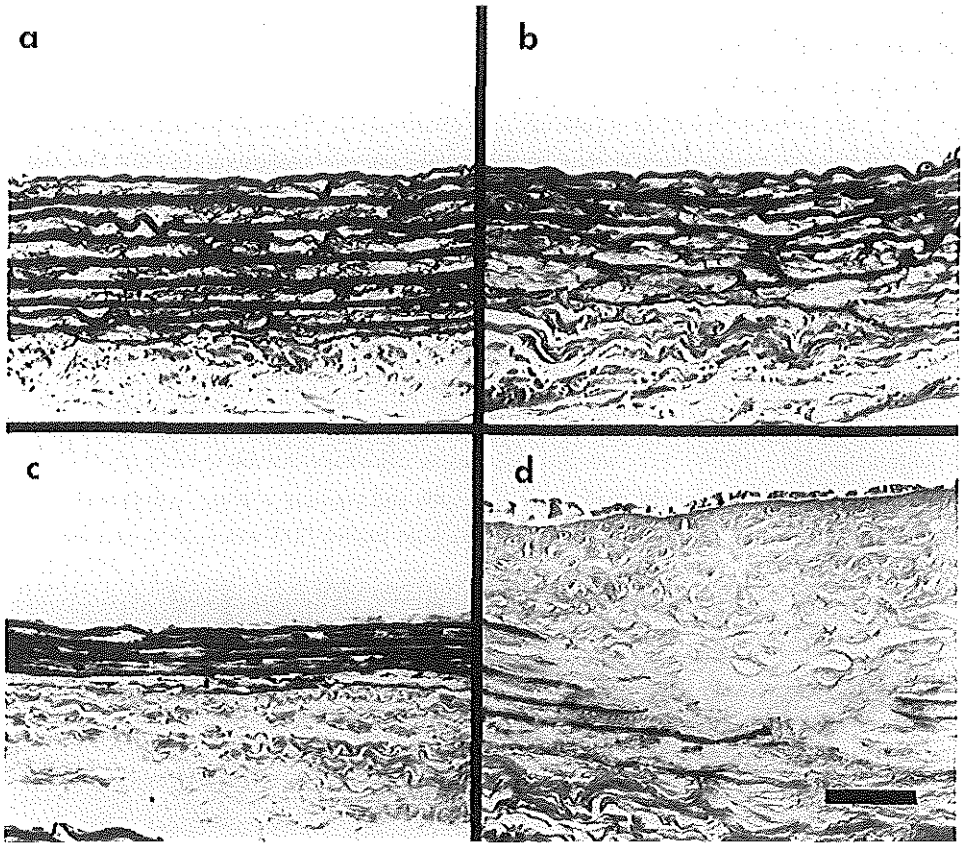


Figure 2.3.
Composite of photomicrographs depicting histological cross sections of rat aorta. a, Normal thoracic Lewis rat aorta; b, isograft 8 weeks after implantation; c, photodynamic therapy-treated allograft 8 weeks after implantation; and, control allograft 8 weeks after implantation. Note the framentation of elastic laminae and significant intimal hyperplasia. Stained with Verhoeff's elastin. Magnification x 310. Bar represents 50 μm.

before implantation. The significant findings were that PDT treatment of allografts supressed histology markers of arterial wall immune injury: adventitial inflammation, aneurysmal dilatation and development of IH (figure 2.3). In addition PDT treated grafts resulted in rapid and complete reendothelialization compared to controls. These data indicate that PDT seems to be a safe method of producing a biocompatible and nonthrombogenic arterial scaffold for use as a bypass graft.

AIMS OF THE STUDIES

Although to date many experimental studies have tested PDT as a method to inhibit IH in vivo, the mechanisms underlying its effects on the vascular wall are not understood. Because inhibition of injury-induced IH by PDT has been correlated with depletion of medial SMC, it is generally assumed that local eradication of these effector cells could explain how PDT works. However, this idea is simplistic and it seems paradoxical to apply a cytotoxic method to inhibit IH with the knowledge that this fibroproliferative disorder is caused by vascular cell injury. As described above, denudation of the endothelial layer and medial SMC damage are the key elements that trigger the healing process. In this regard, PDT could represent an additional traumatic insult to the vessel wall potentially promoting the entire cascade of events that leads to IH from the initial injury. Thus, mere PDT-induced cytotoxicity does not seem to be a plausible mechanism to explain the potential favorable healing response after vascular PDT. With proper implementation of PDT, this form of cytotoxic insult to the vessel wall is characterized by absence of an inflammatory response, rapid endothelial and adventitial repopulation but minimal repopulation of the medial wall. This pattern of vascular repair suggets that, besides cytotoxicity, PDT-induced photochemical effects can alter the biological process that normally causes a fibroproliferative reaction after vascular injury.

The search to better understand the effects of PDT on the vascular wall and the mechanisms involved in the inhibition of IH by this technique was the subject of this thesis. A profound insight in PDT-parameters to favorably affect vascular tissue healing will allow refinement of vascular PDT for clinical use. The hypothesis and aims of the different studies were as follows:

1] Importance of the PDT treatment field. To address the premise that with adequate parameters, PDT-induced cytotoxicity itself does not result in IH, it is necessary to study the effects of PDT on the normal vessel wall. In addition, since mechanical injury to the vessel wall does result in IH, it is necessary to define how important the extent of the PDT treatment field is to effectively control IH. Therefore, the purpose of this study was to 1) examine the healing characteristics of a normal vessel subjected to PDT and 2) examine the repair process of a PDT-treated balloon-injured artery in which the PDT-treatment field did not target the entire injured area (Chapter 3).

2] PDT of extracellular matrix. Because the extracellular environment is known to modulate specific cell functions, this in-vitro study tested the hypothesis that PDT can alter the extracellular matrix and affect the physiology of vascular endothelial- and smooth muscle cells (Chapter 4).

3] PDT inhibits matrix-associated TGF–β. PDT of the rat carotid artery and vein-graft is followed by expedient reendothelialization (Chapter 2) and PDT of extracellular matrix in-vitro stimulates EC growth (Chapter 4). This study explored one possible mechanism underlying these findings by investigating the effects of PDT on matrix-associated Transforming Growth Factor–β (TGF–β), a potent inhibitor of EC growth (Chapter 5).

4] Effects of PDT on TGF–β activity associated with vascular SMC-injury. The multifunctional cytokine, TGF–β, has been demonstrated to play an important role in the pathogenesis of IH. This study compared the effects of mechanical and PDT-induced SMC injury on TGF–β activity (Chapter 6).

5] PDT inactivates smooth muscle cell-associated bFGF. Injury of the vessel wall results in cellular release of basic Fibroblast Growth Factor, a potent mitogen of vascular SMC. PDT results in massive SMC eradication and yet this is not followed by a proliferative response. This study investigated the effects of PDT on cellular bFGF (Chapter 7).

6] Effects of PDT on the vascular fibrotic response. Excessive deposition of extracellular matrix proteins play a key role in vascular injury-induced IH. Cytokines, such as Platelet Derived Growth Factor, released after SMC injury and deposited in the ECM are known to stimulate SMC production of matrix proteins. This study examined whether PDT can inhibit the fibrotic response associated with vascular SMC injury (Chapter 8).

CHAPTER 3

IMPORTANCE OF THE PDT TREATMENT FIELD

Adapted from: Statius van Eps RG, ChandraSekar NR, Hasan T, LaMuraglia GM. Importance of the Treatment Field for the Application of Vascular Photodynamic Therapy to Inhibit Intimal Hyperplasia. *Photochem Photobiol* 1998; 67: 337-342.

INTRODUCTION

Vascular PDT represents a novel experimental approach to inhibit injury-induced IH. This technique is based on the activation of relatively inert photosensitive dyes by wavelength specific light to produce cytotoxic free-radicals. To perform PDT of the vascular wall, a photosensitizer is administered and the area of treatment is illuminated with a light source. Absorption of light by the photosensitizer that has accumulated in the vessel wall results in free-radical production and eradication of the sensitized cells. The ability to irradiate with light over a limited specific area and the short diffusion distance of the generated free radicals provide the concept of a local treatment modality (Chapter 2).

Recent studies from this laboratory described the acute and chronic effects of vascular PDT in the rat carotid balloon injury model (Ortu et al., 1992, LaMuraglia et al., 1994). It was demonstrated that with an effective PDT-dose, there is acute depletion of medial SMC in the targeted segment, without causing thrombus formation or inflammation. The healing response of the vessel wall following PDT demonstrates reendothelialization and adventitial repopulation, but surprisingly minimal SMC repopulation of the medial wall at the site of treatment by 16 weeks. These studies and several other experimental investigations have reported effective inhibition of IH with PDT-mediated SMC depletion at the site of balloon arterial injury.

These encouraging experimental findings have raised several questions regarding the mechanisms by which vascular PDT inhibits IH, and which elements are essential to achieve these results. The ultimate clinical goal is to apply PDT as an adjunctive treatment to prevent restenosis after invasive vascular interventions, such as endarterectomy, balloon angioplasty, and bypass-grafting. The degree and extent of vessel wall injury inflicted by these invasive procedures are dependent on several factors, including the type of procedure and the size of the occlusive lesion (Davies., 1994). For this reason, it is necessary to define how important the extent of the PDT treatment field is to effectively control IH.

In the initial experimental studies performed by this laboratory, PDT was performed of a segmental balloon-injured rat carotid artery with an external laser light irradiation that illuminated beyond the site of injury. Thus, in those studies both the injured artery and an healthy uninjured margin were included in the treatment field. In order to gain more understanding of the repair process of vessels subjected to PDT and to assess the importance of the treatment field, this study systematically investigated the healing characteristics of normal PDT-treated

arteries and balloon-injured arteries in which the PDT-treatment field did not target the entire injured area.

MATERIAL AND METHODS

Balloon injury induction of intimal hyperplasia

Male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) weighing 400 ± 40 gm were anesthetized with intramuscular ketamine (35 mg/kg), atropine (40 mg/kg), and xylazine (5 mg/kg). Exposure of the left carotid artery was obtained using microsurgical techniques. The entire common carotid artery (CCA), including the intrathoracic and cervical carotid segment to the bifurcation, was balloon injured in order to induce IH. A 2F Fogarty arterial embolectomy catheter (Baxter Health Care Corp., Edwards Div., Irvin, Ca) was introduced via an arteriotomy in the external carotid artery and passed into the thoracic aorta. The balloon was inflated with 0.4 ml of air and withdrawn to the carotid bifurcation three times before ligation of the external carotid artery. After closure of the neck incision, animals were recovered and had free access to standard rat chow (Purina rat chow 5001; Ralston Purina, St. Louis, MO) and water while maintained in a standard 12-hour light/dark cycle. The animal procedures were approved by the Institutional Animal Care Committee and complied with "Principles of Laboratory Animal Care" and the "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 80-23, Revised 1985).

Photodynamic therapy

The photosensitizer chloroaluminum sulfonated phthalocyanine (CASPc, Novartis, Switzerland) was diluted to a concentration of 5 mg/ml in phosphate-buffered saline (PBS) and administered intravenously via the femoral vein at a dose of 5 mg/kg at the time of balloon injury. Control balloon-injured animals received an equivalent volume of saline solution. The uninjured, PDT-control animals received CASPc 24-hours prior to light irradiation.

Twenty-four hours after the CASPc administration, a segment of the cervical CCA was irradiated to produce PDT as previously described (LaMuraglia et al., 1994). In brief, an Argon-pumped dye laser (Coherent INNOVA I 100 and Coherent CR 599, Coherent, Palo Alto, CA) was tuned to emit light at 675 nm, at an irradiance of 100 mW/cm^2 to illuminate the surgically exposed vessel segment with a total fluence of 100 J/cm^2. The area of light irradiation, confined to a segment of the cervical CCA, with a spot size of 2 cm^2, did not include the carotid bifurcation or the injured thoracic CCA. After treatment, the area of light exposure was superficially marked (proximal and distal border) with india ink, so the areas of PDT could be easily identified. The control for PDT-treated arteries included balloon injured arteries subjected to light irradiation only, without prior photosensitizer administration. Prior studies from this laboratory have documented that

CASPc administration alone did not affect the healing characteristics of balloon-injured carotid arteries, and therefore this control was not included in this study (Ortu et al., 1992). As a separate group, PDT and light-irradiation only using the described parameters were also performed of normal CCA's, to examine the vascular healing response of uninjured PDT-treated arteries as compared to balloon-injured PDT-treated arteries.

Harvest

Animals were sacrificed sequentially at 1, 2, 4, and 16 weeks after intervention by an overdose of intravenous pentobarbital. The thoracic aorta was flushed with 10 ml of saline solution and perfusion fixed in situ at 90 ± 10 mm Hg for 15 minutes with 10% buffered formalin for paraffin embedding light microscopy or with 1.5% glutaraldehyde in cacodylate buffer for electron microscopy. The entire left CCA and matching control from the right CCA were excised and placed in fresh 10% formalin or 4% glutaraldehyde.

Light microscopy

Formalin-fixed specimens of the entire CCA were transversely cut into three segments of the thoracic CCA and three segments of the cervical segments and sectioned at 4 mm thick cross sections. Morphometric analysis was performed of all three segments (proximal-mid-distal) of the PDT-treated and light-control areas, as previously described with a digitizing camera lucida system (LaMuraglia et al., 1994). The injured thoracic segments, which did not receive PDT or light-control treatment, were also examined for the presence of IH. Occasional CCA specimens were sectioned longitudinally to microscopically examine the progression of the hyperplastic lesion.

Immunocytochemistry

Four-micrometer thick histologic sections were deparaffinized to perform immunocytochemistry for the detection of smooth muscle actin with the antibody HHF-35 (Biogenics, San Ramon, CA) and visualization with the Vectastain Elite Kit (Vector, Burlingame, CA) as previously described (LaMuraglia et al., 1994).

Electron microscopy

To examine the effects of PDT treatment on ultrastructural characteristics of the arteries, transmission electron microscopy (TEM) was performed on arterial samples from 6 arteries. Specimens were fixed overnight, postfixed in 2% OsO_4 and embedded in epon. Thin sections were stained with uranyl acetate and Sato's lead stain, and examined with an electron microscope (model CM10, Philips, Eindhoven, The Netherlands).

Statistics

Data are expressed as means ± SEM. Statistical analysis was performed with a two-tailed Student t test for comparison of morphometric differences between control and PDT-treated arteries and a p value less than 0.05 was considered to be significant.

RESULTS

Histologic and morphometric analysis

Two rats developed thrombosis 24-hours following balloon injury and were deleted from further analysis. All rats treated with PDT appeared healthy, without signs of complications, skin photosensitivity and none developed thrombosis.

PDT of balloon-injured arteries. In control balloon-injured arteries, there was evidence of IH at 1 week, with a progressive increase in intimal area occurring at two weeks. At two weeks the hyperplastic lesion stabilized in size and remained equivalent at 4 and 16 weeks (Fig. 3.1 and 3.2). The medial vessel wall layer appeared normal with presence of SMC, and there was no sign of internal elastic lamina disruption (Fig. 3.2). The cervical carotid segments, which were injured and light-irradiated only and the thoracic and proximal carotid segments which were injured only demonstrated equivalent patterns of IH development (data not shown). Thus, laser- irradiation alone (cervical segment) did not affect the healing response of the injured artery.

PDT of the balloon injured cervical carotid segment resulted in complete acute depletion of cells over the area of treatment. The media remained acellular, but occasional cells were noted at 16 weeks (Fig. 3.2). The depletion of medial SMC after PDT was associated with a lack of IH at 1 and 2 weeks at the site of treatment (Fig. 3.1). However, despite the absence of medial SMC, there was significant IH development at 4 and 16 weeks (Fig. 3.1 and 3.2) in the PDT-treat-

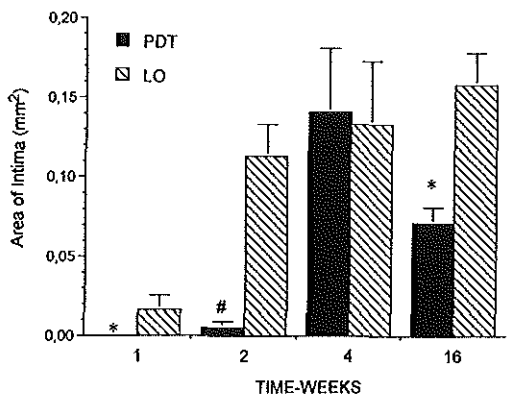


Figure 3.1.
*The size of intimal hyperplasia in PDT-treated and control injured carotid arteries as determined by morphometric analysis at 1, 2, 4 and 16 weeks. The area of intima was measured at the proximal, mid, and distal site of the injured cervical carotid segment and averaged for each artery (means ± SEM). The area of measurement included only the cervical carotid portion that was injured and light irradiated. * denotes $p < 0.05$, # denotes $p < 0.005$, values are three arteries except 16 weeks are four arteries.*

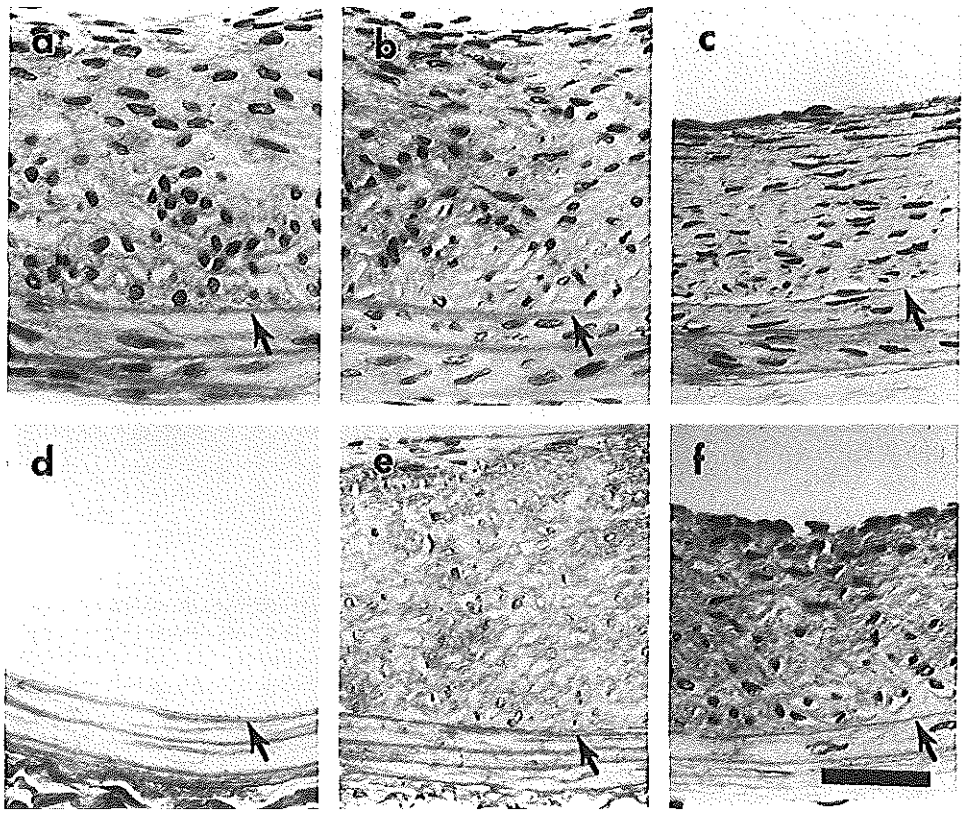


Figure 3.2.
Composite of light micrographs from representative cross sections of the cervical segment of balloon injured carotid arteries treated with laser light controls (a to c) or with PDT (d to f). Internal elastic lamina (arrow) is noted. a,d: 2 weeks after treatment; b,e: 4 weeks after treatment; c,f: 16 weeks after treatment. In PDT-treated arteries there was a delay in IH development, with a peak at 4 weeks in contrast to the peak at 2 weeks seen in control arteries. Note further the depletion of vascular cells after PDT at 2 weeks and the presence of IH at 4 and 16 weeks in PDT treated arteries despite minimal repopulation of the medial layer with vascular SMC. Hematoxylin and eosin stain, bar is 50 μm.

ed cervical segments. The thoracic segments of these arteries exhibited the same hyperplastic response as in the control injured arteries with significant IH development at 1 and 2 weeks.

Analysis of longitudinal sections of the PDT-treated segments at 4 weeks, revealed a wave of IH traveling over the acellular medial wall (Fig. 3.3). This wave of IH progressed from the injured proximal and distal carotid areas that did not receive PDT-treatment towards the PDT-treated acellular segment. Immunocytochemistry staining for smooth muscle cell specific-actin demonstrated positive staining for cells in the intimal hyperplastic lesions.

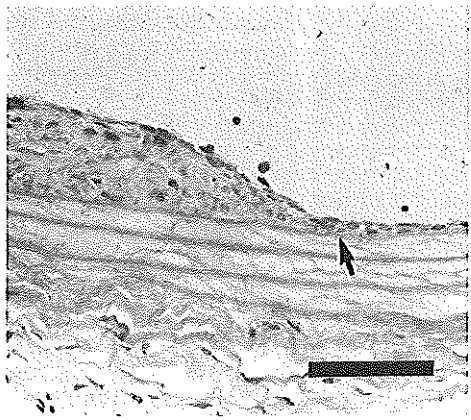


Figure 3.3.
Light micrograph of longitudinal section from balloon-injured artery 4 weeks after PDT shows propagation of the intimal hyperplastic lesion from distal (left) to proximal (right) site of the cervical carotid segment. Note the absence of medial SMC. Arrow indicates internal elastic lamina. Hematoxylin and eosin stain, bar is 100 μm.

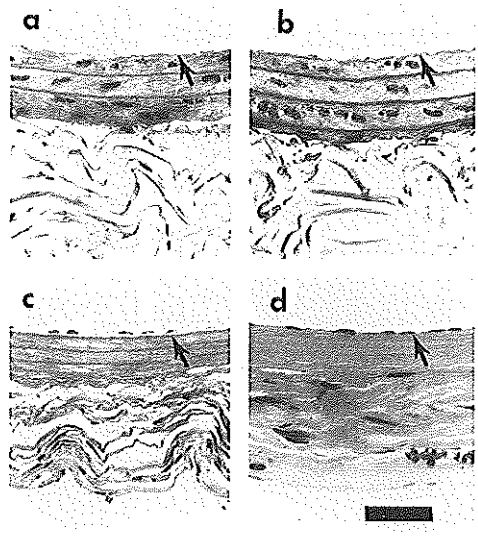


Figure 3.4.
Composite of light micrographs (hematoxylin and eosin stain) from uninjured carotid arteries treated with laser light controls (a,b) or with PDT (c,d). Internal elastic lamina is indicated with the arrow. a,c: 2 weeks after treatment; b,d: 16 weeks after treatment. Although the PDT-treated arteries demonstrated endothelial and adventitial cell repopulation, the medial wall remained depleted of SMC up to 16 weeks. Note that neither laser light control or PDT resulted in IH. Bar = 50 μm.

In both PDT and control arteries, there was an increase in the medial area from 1 to 4 weeks. This increase persisted at 16 weeks in the control group and slightly regressed in the PDT group (Table 3.1). The diameter of the arteries was equivalent between the control and PDT-treat-

Table 3.1 Comparison of IH areas, media areas and artery diameters between PDT and control balloon-injured arteries*

	1 week	2 weeks	4 weeks	16 weeks
Area of IH (mm^2 ± SEM)				
PDT	0.0 ± 0.0†	0.005 ± 0.004‡	0.141 ± 0.040	0.071 ± 0.010†
Control	0.016 ± 0.009	0.113 ± 0.020	0.133 ± 0.040	0.158 ± 0.020
Area of media (mm^2 ± SEM)				
PDT	0.040 ± 0.040	0.040 ± 0.003§	0.090 ± 0.030	0.050 ± 0.008§
Control	0.050 ± 0.005	0.060 ± 0.006	0.090 ± 0.020	0.090 ± 0.004
Diameter (mm ± SEM)				
PDT	0.97 ± 0.03	0.93 ± 0.04	0.92 ± 0.04	1.02 ± 0.10
Control	1.09 ± 0.04	0.96 ± 0.02	0.89 ± 0.04	0.82 ± 0.04

*Values are three arteries except PDT 16 weeks are four arteries.
†$P < 0.05$ PDT *vs* control.
‡$P < 0.001$ PDT *vs* control.
§$P < 0.01$ PDT *vs* control.

ed arteries throughout the time period studied.

PDT of normal uninjured arteries. Similar to PDT of a balloon-injured artery, PDT of a normal artery resulted in complete depletion of vascular cells. Despite swift reendothelialization and adventitial repopulation of the PDT-treated area, the media remained essentially accelular up to 16 weeks. After PDT of normal uninjured arteries, there was no IH development (Fig. 3.4).

Electron microscopy

Transmission electron microscopy analysis of PDT-treated balloon injured and uninjured arteries at 16 weeks, demonstrated depletion of medial smooth muscle cells without evidence of inflammation or structural matrix deterioration. In the medial wall, cellular debris was

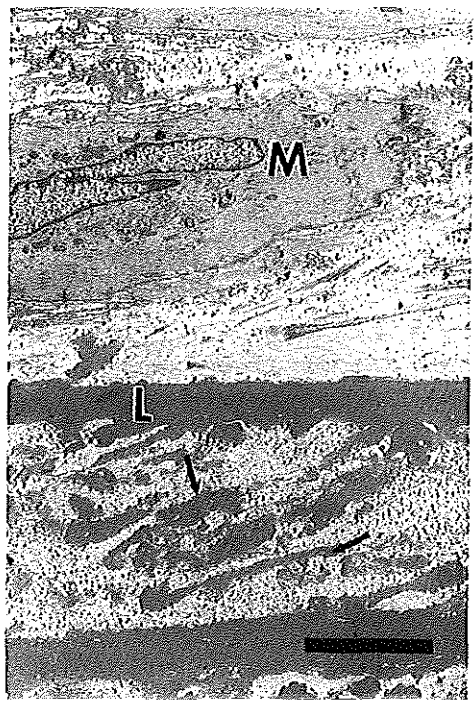


Figure 3.5.
Transmission electron micrograph of a balloon-injured carotid artery 16 weeks after PDT. Note the presence of intimal SMC (M) above the internal elastic lamina (L). The media remains depleted of SMC and shows evidence of elastin tissue (arrow). Bar is 5 μm.

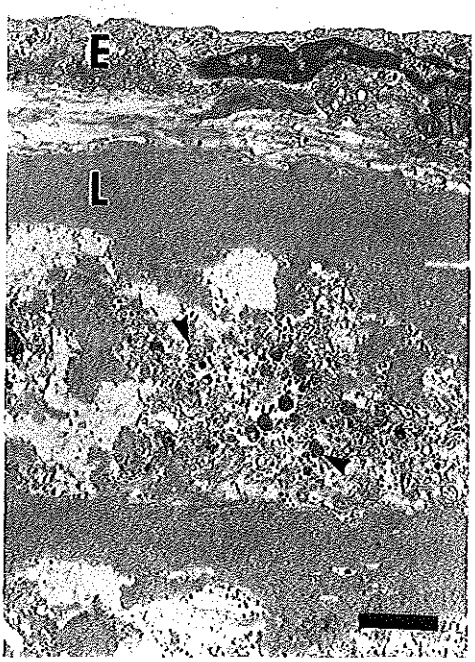


Figure 3.6.
Transmission electron micrograph of a normal uninjured carotid artery 16 weeks after PDT. The luminal surface is covered with endothelial cells (E). The internal elastic lamina (L) is intact and the medial layer remains devoid of SMC with some evidence of cellular debris (arrowhead). Bar is 1 μm.

seen between normal appearing parallel bands of elastin and interspersed with collagen and elastic tissue (Fig 3.5 and 3.6). In the PDT-treated balloon injured arteries, intimal SMC were observed above the internal elastic lamina (Fig 3.5), and in the uninjured PDT-treated arteries, endothelial cells covered the internal elastic lamina (Fig 3.6).

DISCUSSION

The development of restenosis after vascular interventions is a disturbing iatrogenic complication mediated by injury to the vessel wall. The local nature of this problem, in which the area of injury is known, makes the application of a local approach to prevent this complication attractive. The potential utility of PDT to prevent IH relates in part to the ability to target the area of interest. For this purpose it is important to know which area of the injured vessel wall should be included in the PDT-treatment field in order to control IH. The principal finding of this study is the failure of PDT to inhibit balloon injury-induced IH, when the injury extends beyond the area of PDT-treatment. This finding complements the results of a previous study from this laboratory, which demonstrated long term inhibition of balloon injury-induced IH when the PDT-treatment field included the entire injured area (LaMuraglia et al., 1994). Together with the additional finding from the present study, which showed that PDT-mediated depletion of vascular cells itself did not induce IH, it can be reasoned that inclusion of the complete injured area or an uninjured margin in the PDT-treatment field is essential to inhibit balloon injury-induced IH.

In the previous study, there was significant IH development in only two of the eight PDT-treated injured arteries at four weeks. It is tempting to speculate that the PDT-treatment field may have been inadequate in those arteries which resulted in IH development from the injured vessel margin that did not received PDT-treatment. Another recent experimental study, described effective inhibition of balloon injury-induced IH at four weeks using the protoporphyrin precursor 5-amino-levulinic acid (Nyamekye et al., 1995a). In that study, balloon injury was performed of the entire carotid artery including the thoracic segment, which, similar to the present study, precluded PDT-treatment of an uninjured margin at the distal end. In a subsequent report, that group presented their data with analysis of PDT-effects on the injured vessel wall extending to longer periods of 12 and 26 weeks. At these time points, all arteries presented with significant IH despite persistent depletion of medial SMC (Nyamekye et al., 1995b).

The present study also showed a delay in IH development over the area of PDT-treatment in injured arteries. Since there is consistent histological evidence that the medial vessel wall remained depleted of SMC at the site of PDT-treatment, it can be inferred that the hyperplastic lesion developed from the distal injured segment that did not receive PDT. This notion is supported by the longitudinal examination of PDT-treated segments, which showed a wave of intimal SMC traveling over the cell depleted medial wall (Fig. 3.3). The mechanism underlying this occurrence is not understood but may

involve haemodynamic factors. At the site of injury without PDT-treatment there is IH development within a week, which develops from the proliferation and migration of the underlying injured medial SMC's. Over the PDT-treated segment, depletion of the medial SMC population precludes this rapid onset of IH. This results in disproportionate IH development over a short area, which may result in changes of shear stress. These changes will trigger an adaptive response of the vessel wall to achieve normal shear stress, which may occur by spreading of the intimal hyperplastic lesion (Gibbons et al., 1994). This study also illustrates that an intimal hyperplastic lesion does not necessarily evolve from the underlying medial layer, but may progress from adjacent proliferating and migrating intimal SMC.

The consistent histologic finding presented in this report and described by others (Nyamekye et al., 1995a, Grant et al., 1994) that PDT itself, in contrast to mechanical injury and other forms of vessel wall injury, does not induce IH development is intriguing. It has been well documented in the balloon injury model of the rat carotid that medial SMC injury and widespread SMC death play a dominant role in the fibroproliferative response that leads to IH (Reidy et al., 1992). After adequate doses of PDT, there is complete eradication of medial SMC and yet this is not followed by a proliferative response (Fig. 3.4). In the central area of the PDT-treatment field, severe depletion of medial SMC may contribute to the lack of a SMC proliferative response. However, at both sides of the PDT-treated segment there is an interface in which SMC depletion occurs adjacent to normal medial SMC not included in the PDT-treatment field. Since SMC injury and death are important triggers for the activation of surviving SMC, the absence of a significant proliferative response at this PDT-normal artery interface is surprising (Chapter 1). This is highlighted by the observation that in the presence of mechanical vessel wall injury, significant IH develops from this interface as was demonstrated in this study.

The lack of IH development after PDT-mediated eradication of vascular cells is associated with a characteristic vascular healing response. Although there is rapid endothelial cell regrowth and adventitial repopulation (Fig. 3.4), the medial layer remains essentially devoid of SMC for periods up to 16 weeks. The mechanism underlying this favorable healing response after PDT is not understood and requires further investigation.

The present data showed that the favorable vascular healing response after PDT is overridden if there is mechanically injured tissue with cells near the PDT-treatment field, resulting in IH. On the other hand, it was previously demonstrated that even after mechanical injury, this favorable healing response can be obtained, if the PDT treatment field includes all the injured artery or extends beyond the site of injury and includes an uninjured vessel margin (LaMuraglia et al., 1994). If this is achieved, there is, similar to PDT of a normal uninjured artery, rapid endothelial cell regrowth and a lack of IH. These findings provide important insights into basic principles required to perform effective PDT in vivo and are of great value for the application of PDT to prevent restenosis in future

human studies. Besides other PDT-parameters such as light dosimetry, photosensitizer concentration and type of photosensitizer, which will determine the degree of SMC eradication, the area of PDT-treatment is of great importance. The field of treatment can be adjusted by modifying the laser-light illumination area and the photosensitizer distribution in the vessel wall. Therefore, careful planning of the treatment field should be considered in the evaluation of vascular PDT to prevent restenosis.

CHAPTER 4

PDT OF EXTRACELLULAR MATRIX

Adapted from: Adili F, Statius van Eps RG, Karp SJ, Watkins MT, LaMuraglia GM. Differential Modulation of Vascular Endothelial and Smooth Muscle Cell Function by Photodynamic Therapy of Extracellular Matrix: Novel Insights into Radical-Mediated Prevention of Intimal Hyperplasia. *J Vasc Surg* 1996; 23: 698-705.

INTRODUCTION

Photodynamic therapy of arteries and vein grafts has been demonstrated to entirely eradicate cells from the vascular wall and to inhibit the development of IH. This effect, however, is not accompanied by thrombosis, structural deterioration of the artery wall, or myointimal proliferation. Despite total cell eradication immediately after the treatment, histological examination of arteries subjected to PDT reveals a good healing response with complete reendothelialization and almost no medial repopulation (Chapter 2 and 3). The favorable vascular healing response after PDT is overridden if there is mechanically injured tissue with cells near the PDT-treatment field, resulting in IH. Therefore, it is of great importance to include an uninjured margin in the treatment field (Chapter 3). These findings cannot be explained by mere free-radical induced cytotoxicity but suggest that PDT may affect other biological components involved in the healing process. Complex interactions between cells and extracellular matrix (ECM) proteins are thought to be in part responsible for the modeling of the arterial wall and other tissues (Madri et al., 1991; Raghow, 1994). Because the ECM is known to modulate specific cell functions, this in-vitro study was devised to ascertain whether PDT of isolated ECM affects the physiology of vascular endothelial cells (EC) and SMCs.

MATERIAL AND METHODS

Primary bovine aortic SMC and EC cultures were established from aortas of freshly slaughtered calves. ECs were obtained by scraping the intimal aortic surface and dispersion in 0.1% CLS II collagenase (Worthington; Freehold, N.J.) The EC identity was confirmed by the polygonal, monolayer shape seen on phase-contrast microscopy and by uptake of the fluorescent probe Di-I-Ac-LDL (Biomedical Research Technologies, Inc.; Stoughton, M.A.). SMC cultures were established with the explant technique from strips of aortic media (Grünwald et al., 1984). Their identity was verified by inderect immunoflourescence with an anti-α actin antibody (Biomedical Research Technologies). Both cell types were kept in 37 °C incubator in the presence of 5% CO_2 and were fed every 48-hours with complete Dulbecco's Modified Eagles Media supplemented with 10% calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.6 mol/L L-glutamine (Gibco; Grand Island, N.Y.). Cells were passed at a ratio of 1:5 using 0.05% trypsin / 0.125% ethylenediamine tetraacetic acid upon reaching confluence and used during passages 2 through 6.

Preparation of ECM

ECs were subcultured in six-well plates (Falcon, Becton Dickinson; Lincoln Park, N.J.) and left confluent for 8 to 10 days. For isolation of the ECM, the cell-monolayer was removed after 30 minutes of incubation in phosphate-buffered saline solution (PBS) containing 0.5% Triton X-100 (Sigma Chemical; St. Louis) and 20 mmol/L NH_4OH, and after three rinses with PBS (Gadjusek et al., 1989). These steps were carefully monitired by phase-contrast microscopy. The resultant ECM that coated the cell-culture plates was covered with 1.5 ml PBS and stored at 40°C for use within 48-hours. The presence of ECM was verified by scanning electron microscopy. One 13-mm-round platic coverslip (Nunc, Inc.; Naperville, Ill) was placed in each well, and ECM was prepared as described above. After removal of the cells, the cover-slips were transferred into 4% glutaraldehyde in 0.1 mol/L cacodylate buffer and incubated for one hour. After dehydration in graded series of alcohol and rinsing in hexamethyldisilazane, the specimens were allowed to air-dry, were coated with gold-palladium in a cold sputter coater, and were examined with an scanning electron microscope (Amray 1400).

Photodynamic Therapy

ECMs were covered with 1.5 ml of the photosensitizer drug CASPc in PBS (5 mg/ml) just before irradiation (Figure 4.1) with thermoneutral light (fluence 100 J/cm^2; irradiance, 100 mW/cm^2; λ = 675 nm) delivered by an Argon-pumped dye laser (Coherent). After PDT, the wells

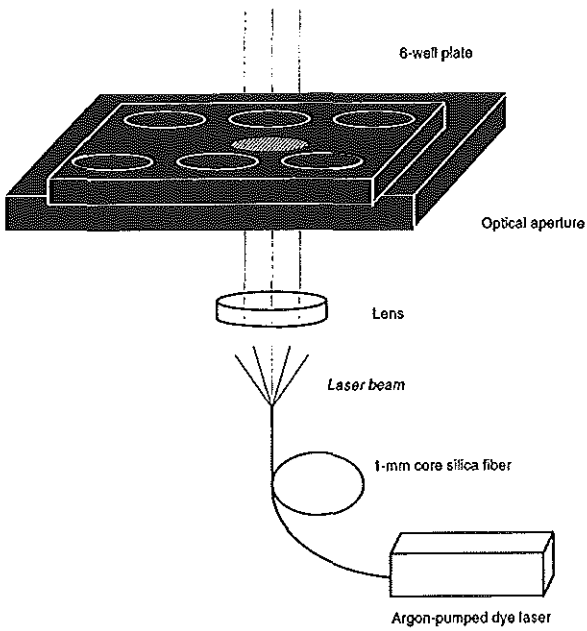


Fig. 4.1.
Experimental setup to irradiate isolated ECM in six-well plates with laser light. The six-well plate is moved on a light-impervious block with an aperture that provides illumination of one well at a time, keeping other wells dark.

were rinsed three times with PBS, covered with aluminium foil to avoid additional exposure to ambient light, and stored at 40°C. Controls included untreated ECM, ECM-free plates, and ECM exposed to light irradiation only. To access possible dark-effects of the photosensitizer, matrices were incubated for 2 hours with CASPc in the absence of light.

Attachment assay

ECs or SMCs were plated at a density of 2 x 10^5 cells per well. The plates were gently agitated to ensure homogenous dispersion of the cells and were incubated for 2-hours at 37°C. The unattached cells were gently washed off and transferred into isoton (Isoton II, Coulter Corp.; Miami). The cell numbers were counted with an electronic cell counter (Multisizer, Coulter Electronics Ltd.; Luton, United Kingdom), and the percent attachment was derived from the number of unattached cells.

Proliferation assay

SMCs or ECs were plated at a density of 1 x 10^5 cells per well. After 24-hours of incubation in serum-poor (0.5%) media, 1 mCi ^{3}H-thymidine (New England Nuclear; Boston, M.A.) was added to the cells for 5-hours. Serum-poor media was used to ensure that ECM was not subjected to serum constituents that might bind to the ECM and elicit potential effects on cellular proliferation. After three washes with PBS, 1.5 ml 0.5% trypsin was added for at least thirty minutes. The cells were than vigorously washed, the cell count assessed with a hemocytometer, and the radioactivity in each well was quantitated with a scintillation counter (Beckman Instruments, Inc.; Fullerton, Calif.). The resulting data, expressed as counts/cell/minute, were normalized for untreated ECM and reported as the percentage of thymidine incorporation.

Migration assay

For the migration assay, a circular metal fence that prevented the cells from leaking out after inoculation was placed into the central portion of each well. On the first day, 7.5 x 10^4 cells suspended in serum-poor media were seeded into the central fence of the dish, where they were constrained on an area of 0.62 cm^2. After 4 hours of incubation at 37°C, the unattached cells were gently removed, and fresh media was added. Cell migration was initiated after release of contact by removal of the fence. Confluent cell cultures with sharply defined margins were obtained. The distances between the baseline mark and the confluent cell front in four centrifugal directions were measured daily with a calibrated microscope eyepiece reticle and averaged. The total migration distance after 7 days was reported as percent of cell distance migration compared with untreated ECM.

Immunostaining of ECM

To verify the structural integrity of ECM in the cell-culture dishes, normal and PDT-treated ECM were incubated for 1 hour at room temperature with a mouse anti-human fibronectin antibody (Gibco). The second antibody was a fluorescein-conjugated goat-affinity purified mouse immunoglobulin G (Cappel; Westchester, Pa.). Excitation wavelenghts between 450 and 490 nm and an emission band-pass between 515-565 nm were used for fluorescence imaging. Fibronectin coated six-well plates served as positive control and empty plates as negative control specimens.

Statistical analysis

All data are expressed as mean ± SEM and were analyzed with a one-way analysis of variance and Tukey's Honest Significant Difference post hoc test for multiple comparisons (Statistica, Statsoft; Tulsa Okla.). Resuls were considered statistically significant if $p < 0.05$.

RESULTS

At a magnification of x5000, bovine aortic EC-ECM appeared as a ubiquitous, fibrillar, and nonhomogeneous network (Fig. 4.2). The presence of fibronectin, a major constituent of EC-ECM, was evaluated with an antibody against the cell-binding domain of fibronectin. As demonstrated in figure 4.3, untreated ECM yielded a strong fluorescence signal after the cells were removed.

To determine how the presence of ECM would generally affect vascular SMC and EC physiology in our model, SMCs and ECs were plated on untreated ECM or directly on tissue-culture plastic. The attachment of either cell-type to ECM did not differ from SMC and EC attachment to plastic (Fig. 4.4). SMC proliferation on ECM, however, increased by 70% and migration increased approximately 85% when compared with SMCs on tissue-culture plastic ($p < 0.001$; $n = 15$). Conversely, EC proliferation and migration were slightly, but significantly, diminished on ECM ($p < 0.01$; $n = 15$) when compared with plastic. (Fig 4.4, Table 4.1).

Table 4.1 SMC and EC functions on different treated matrixes

	Attachment		*Proliferation*		*Migration*	
	SMC (n = 15)	EC (n = 12)	SMC (n = 15)	EC (n = 15)	SMC (n = 6)	EC (n = 9)
Control	95 ± 0.4	86 ± 0.6	100 ± 1.4	100 ± 6.2	100 ± 0.9	100 ± 0.8
Drug only	90 ± 1.0	88 ± 0.6	94 ± 4.7	113 ± 9.0	103 ± 1.3	104 ± 1.1
Light only	95 ± 1.4	88 ± 0.6	99 ± 4.9	102 ± 1.6	96 ± 1.4	105 ± 1.2
PDT	86 ± 0.4*	88 ± 0.6	46 ± 0.5	129 ± 6.2*	40 ± 1.0*	118 ± 1.2*

Data expressed as mean percentage ± SEM.
*$p < 0.05$ when compared with control group.

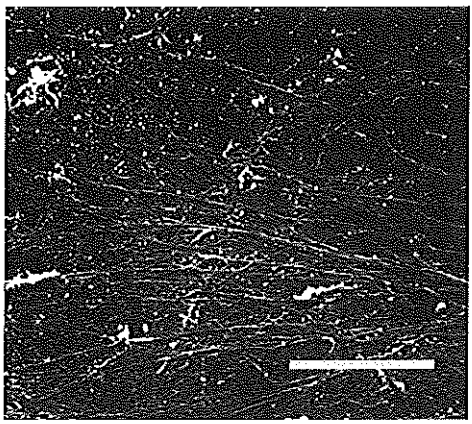


Fig. 4.2.
Scanning electron micrograph of isolated EC-ECM in plastic tissue-culture dish (original magnification x5000). A fibrillar, nonhomogenous network of ECM covers the plastic tissue-culture dish. Bar equals 5 μm.

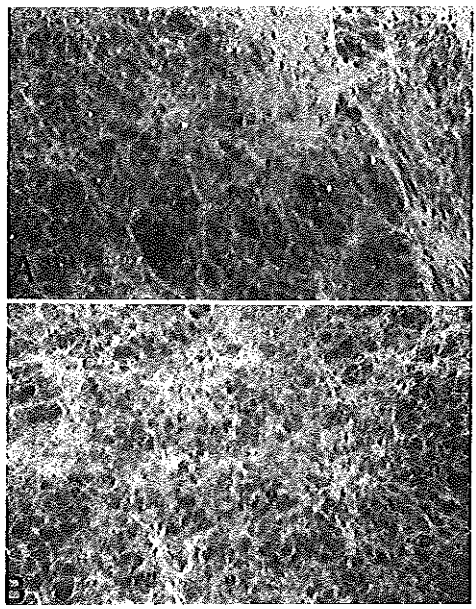


Fig. 4.3.
Photomicrograph of bovine aortic EC-ECM (original magnification x32). Immunostaining with anti-fibronectin IgG (1:100) and fluorescein-labeled secondary antibody before (A) and after (B) PDT. Note that staining patterns and intensities before and after PDT do not differ.

PDT of ECM

Immunostaining of ECM with antibodies against fibronectin before and after PDT demonstrated a clearly identifiable three-dimensional network with an unchanged fluorescence intensity and similar morphological pattern, which validated the presence of ECM (Fig. 4.3). Nevertheless, despite apparently unchanged fluorescence labeling of the cell-binding domain in the fibronectin molecule, SMC attachment to PDT-treated ECM was diminished by approximately 10% ($n = 15$; $p < 0.005$). SMC proliferation and migration also decreased markedly after PDT (table 4.1).

In contrast to SMCs, EC proliferation and migration both were significantly potentiated after PDT of ECM ($p < 0.001$ and $p < 0.01$, respectively; $n = 15$; Table 4.1). EC attachment, however, was unaffected. To elucidate which, if either, of the two components required for PDT (drug and light) was particularly relevant for the biologic effects seen after PDT of ECM, normal ECM

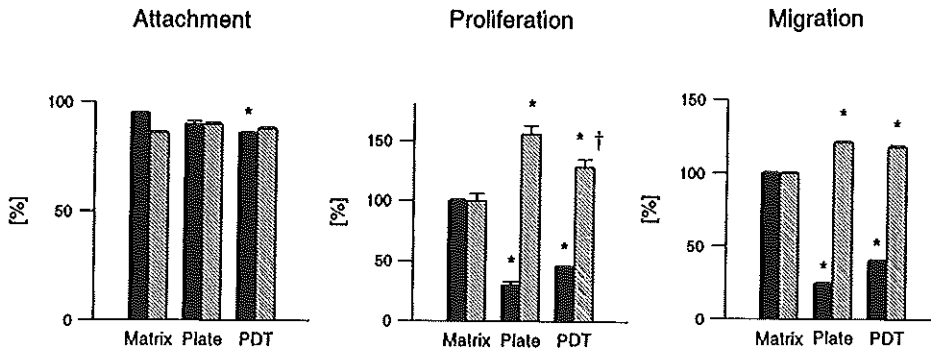


Fig. 4.4.
*Attachment, proliferation, and migration of bovine aortic SMCs (solid fill) and bovine aortic Ecs (hatched fill) on normal ECM (Matrix), plain tissue-culture plastic (Plate), and PDT-treated ECM (PDT). Data are expereessed as mean percentage ± SEM and normilized for values obtained on normal ECM. *Statistically significant differences in which $p < 0.05$ when compared with "Matrix" for the respective cell type. † $p < 0.05$ when compared with "Plate" for ECs.*

was subjected to either incubation with CASPc only, or sole irradiation with 675-nm light (Table 4.1). In the absence of light, exposure of normal ECM to CASPc resulted in no alteration of SMC and EC attachment, proliferation, or migration. Likewise, attachment, proliferation, and migration of SMCs and ECs on matrixes irradiated with thermoneutral light at a fluence of 100 J/cm^2 was similar to untreated ECM.

DISCUSSION

ECs and the underlying SMCs are the primary cell types that comprise the vascular wall. If SMCs are completely eliminated by PDT early at the onset of IH, obstructing lesions most likely will not develop (Chapter 2). PDT of fully developed vascular stenoses, on the other hand, has not been found to significantly diminish luminal narrowing despite considerable cell depletion in and beyond the obstructing lesion (Hsiang et al., 1995). These data appear to indicate that the effects of PDT are primarily based on cytotoxicity rather than on structural alteration of ECM. Although the ECM does not physically change after PDT, four consistent histological findings have been reported that imply functional changes in the extracellular environment. First, after balloon injury and PDT no cells are present in the arterial media after 6 months, whereas the intimal surface is completely lined with normal appearing endothelium by 2-weeks. Second, despite the presence of cellular debris in the vascular wall that is attributable to PDT-induced cytotoxicity, no inflammatory reaction with infiltration of phagocytizing macrophages has been observed. Third, at the interface between normal and PDT-treated vessel segments, where live cells, such as ECs, SMCs, and fibroblasts remain present, there is no indication of an increased proliferative or migratory activity of SMCs. Fourth, no thrombus formation has been noted

after balloon injury of arteries and subsequent PDT except for one recently published article (Eton et al., 1995). In summary, these in-vivo observations suggest that vascular PDT leaves behind an environment that favors expedient reendothelialization but inhibits SMC growth and subsequent development of IH. It was therefore hypothesized that in addition to cell eradication, PDT may induce changes in the extracellular portion of the vascular wall. These changes may modulate the function of repopulating ECs and SMCs.

Cultured bovine vascular ECs produce an ECM that is similar in organization and macromolecular composition to the naturally occuring subendothelium (Korner et al., 1993). This ECM does not function only as an inert structural support, but rather regulates attachment, proliferation, migration, and differentiation of cells (Rogelj et al., 1989). The effects on cell behaviour are to a large extent ascribed to the native composition and three-dimensional structure of the ECM that induce a permissive change in the shape of cells and allow them to respond more readily to physiologically occurring hormones and growth factors in serum and plasma (Vlodavsky et al., 1987). In most cases, isolated single-matrix components failed, even when applied in various combinations, to elicit the biologic response induced by a naturally produced ECM (Fridman et al., 1985). Because the in-vitro reconstitution of ECM from its isolated constituents into the correct, highly ordered structure that it represent would be a formidable task, isolated ECM produced by bovine ECs was used in our study.

Our results amply corroborate the importance of cell-derived ECM for SMC and EC function. In addition to structural constituents, EC-derived ECM is known to contain growth factors such as platelet-derived growth factor (Kelly et al., 1993) and basic fibroblast growth factor (Casscells et al., 1992a) that represent important mitogens for SMCs. It was therefore not surprising to find SMC proliferation and migration increased on ECM as compared to tissue-culture plastic. Recently, other investigators have described radical changes in the functional character of vascular SMCs in vitro when isolated from normal ECM and cultured in a ECM-free environment, that is, as a monolayer on plastic (Casscells et al., 1992a).

In contrast to this report, these experiments also authenticated diminished EC proliferation and migration on normal ECM. The foundation of this behavior of ECs, however, is not clear. Conflicting findings that demonstrated increased SMC and EC growth on ECM when compared with plastic could possibly originate from different culture conditions (e.g., numbers of cells seeded and cell passage) and experimental design (Gospodarowicz et al., 1980). In this study the absence of exogenous growth factors and the use of serum-poor cell-culture media were thought to facilitate the identification of ECM-mediated effects. Particular culture conditions are known to have profound consequences for the cell growth and composition of the deposited ECM (Gospodarowicz et al., 1980). Even the temporary absence or presence of ECM-associated EC growth stimulators, such as basic fibroblast growth factor, or EC inhibitors, such as type V collagen, glycoproteins, glycosaminoglycans (Davies, 1993), TGF–β (Taipale et al., 1995), and nitric oxide (Sarkar et al., 1995), can result in decreased EC-growth.

The existence of a broad variety of growth stimulators and inhibitors in conjuction with experimental data suggests redundancy of the different systems and also additive effects (Thyberg et al., 1990). Because redundancy may limit the efficacy of antibodies to a single growth factor to block cell attachment, proliferation and migration, the use of novel treatment methods such as PDT, which potentially targets multiple growth factors, growth inhibitors, hormones and matrix constituents at the same time, has theoretical appeal.

PDT has primarily been used for its cytotoxic biologic effects and has therefore had its greatest development in the treatment of cancer. As PDT of neoplastic and other proliferative disorders such as arthritis and IH progressed to clinical trials, new photosensitizers with fewer side effects have been developed and advocated. Compared with the traditionally used hematoporphyrin derivative (HpD), which is currently under clinical evaluation to treat different cancers, second-generation photosensitizers such as CASPc have distinct advantages. They appear to have less dark toxicity than HpD in vitro and to generate greater cytotoxicity, but less systemic toxicity, in vivo than HpD (Koshida et al., 1993). Finally, these drugs are activated with light wavelengths-for example, 675 nm light for CASPc-that have a deeper tissue penetration depth than the 630 nm light used to activate HpD.

When added in aqueos solutions, CASPc binds avidly to protein molecules (Rosenthal, 1991). On illumination, free radical moieties are generated that alter proteins. It is therefore conceivable that PDT may also depleted other relevant, biologically active components in the ECM and subsequent caused altered EC and SMC function. These in vitro findings of differential cell modulation on isolated ECM support this hypothesis.

When compared with untreated ECM, PDT-treated ECM significantly compromised SMC attachment, proliferation and migration, and therefore corresponds with the in vivo prevalence of a persistently acellular vessel media after PDT. Because SMC penetration through the media into the subendothelium is instrumental for the development of intimal thickening, it can be reasoned that in addition to cytotoxicity, alteration of ECM may also represent a cornerstone of PDT-mediated prevention of IH.

Interestingly, EC proliferation and migration were significantly potentiated after PDT of isolated ECM. To rule out the possibility that PDT physically removed the entire ECM in the culture dish, which might have explained the similarities of the changes seen with SMCs and ECs on PDT-treated matrixes versus ECM-free plates, immunostaining of ECM with a monoclonal antibody directed against fibronectin was performed both immediately before and after PDT (Fig. 4.3). The flourescence signal before and after PDT did not produce marked differences, which implies that strucurally comparable matrixes were present before and after treatment. Despite the problems with transferring in vitro data to the in vivo situation, our study firmly suggests that rapid reendothelialization of PDT-treated vessel segments is largely

independent from the absence of SMCs. Conversely, inhibition of SMC ingrowth into the subendothelium may not be solely mediated by the presence of an EC monolayer.

This report establishes altered vascular cell function caused by PDT-induced changes in the ECM, and opens a new line of investigation that may not only provide further insights into the mechanisms of vasular PDT, but may also help to gain a better understanding of the various interactions between the cells and their immediate environment in vascular remodeling.

CHAPTER 5

PDT INHIBITS MATRIX-ASSOCIATED TGF–β

Adapted from: R.G. Statius van Eps, F. Adili, M.T. Watkins, R.R. Anderson, G.M. LaMuraglia. Photodynamic Therapy of Extracellular Matrix Stimulates Endothelial Cell Growth by Inactivation of Matrix-Associated Transforming Growth Factor–β. *Lab Invest* 1997; 76: 257-266.

INTRODUCTION

Treatment of atherosclerotic arterial occlusive disease with balloon angioplasty, endarterectomy or bypass grafting is accompanied by endothelial denudation and medial vessel wall injury. The rapidity and extent of reendothelialization will, in part, modulate the degree of medial smooth muscle cell (SMC) migration, proliferation and matrix synthesis, the dominant events in intimal hyperplasia (IH) development (Casscells, 1992). Lack of endothelial cell (EC) regrowth is believed to contribute to the longterm failure of invasive vascular procedures (Davies et al., 1993).

Vascular photodynamic therapy (PDT) is a novel experimental technique to prevent IH development (Chapter 2). Following vascular cell eradication with this method, the vascular healing process is characterized by rapid and complete endothelial regrowth but a lack of medial SMC repopulation at the site of treatment (Chapters 2 and 3). Considering the importance of an intact endothelium for the attenuation of neointimal thickening (Asahara et al., 1995), it is thought that, besides eradication of medial SMC, expedient reendothelialization after experimental vascular PDT is essential for the successful inhibition of IH.

The control of EC growth after denudation of the basement membrane is complex and not well understood. Factors such as the extent of endothelial loss and the magnitude of medial wall injury seem to affect the degree of EC regrowth (Lindner et al., 1989). Furthermore, the underlying and surrounding extracellular matrix and various growth factors are known to affect endothelial cell behavior (Madri et al., 1991). Of special interest is the finding that transforming growth factor β-1 (TGF–β), a multifunctional cytokine produced by platelets and local vascular cells, is a potent inhibitor of EC proliferation and migration (Heimark et al., 1986). Release of this cytokine by adhering platelets and intimal SMC at the site of vascular injury could influence EC regeneration (Heimark et al., 1996; Madri et al., 1989; RayChaudhury et al., 1991).

The mechanisms by which PDT of the vascular wall could affect EC repopulation are not known. However, a recent in vitro study from this laboratory has shown that PDT of isolated endothelial cell extracellular matrix (ECM) resulted in inhibition of ongrowing SMC but stimulation of EC proliferation and migration (Chapter 4). This data suggested that PDT-induced photochemical reactions could target and affect specific structural ECM components or ECM-associated biologically active proteins causing differential effects on vascular cell function.

Since TGF–β is known to profoundly affect EC function, it was the aim of this in vitro

study to investigate the effect of PDT on matrix-bound TGF–β and how this interaction could affect EC proliferation. The presence of TGF–β in isolated ECM was verified and the effect of PDT on this cytokine quantitatively assessed. In addition, the functional significance of matrix-bound TGF–β on EC proliferation and how PDT could alter this effect were further examined.

MATERIAL AND METHODS

Culture of Endothelial Cells

Primary bovine aortic EC cultures were established from aortas of freshly slaughtered calves and characterized as described in Chapter 4. Cells were kept in a 37°C, 5% CO2 incubator, refed every 42-72 hours with DMEM and the necessary supplements. Cells were passed at a ratio of 1:5 using 0.05% Trypsin / 0.125% EDTA (Gibco) upon reaching confluence and used during passages 2-6.

Preparation of Extracellular Matrix

To prepare ECM, endothelial cells were seeded on tissue culture plates (9.6 cm^2) at a density of 5 x 10^5 cells, grown to confluence and left for 8-10 days. Isolation of the underlying ECM, with 0.5% Triton X-100 and 20 mmol/L NH4OH was performed as described in Chapter 4.

Fibronectin (2 $\mu g/cm^2$), (Collaborative Biomedical Products, Bedford, M.A.) coated plates (4.5 cm^2) were prepared after a 2 hour incubation time at room temperature and stored at 4°C in PBS.

TGF–β Binding to Fibronectin

Binding of human TGF–β (R&D Systems, Minneapolis, MN) to immobilized coated fibronectin (Fn) was performed as described by Mooradian. In brief, human TGF–β (1 ng/ml) in a 0.1% protease-free Bovine Serum Albumin (BSA, Sigma Chemicals) PBS solution was added to Fn coated plates and incubated for four hours. Unbound TGF–β was removed by two washes with PBS. This method yielded a 59.2 ± 3.8% (mean ± SD) binding of the administered TGF–β to Fn coated plate based on extraction of bound TGF–β and quantitated by ELISA (data not shown).

Photodynamic Therapy

Photodynamic therapy of matrices (ECM, Fn and Fn-TGF–β) was performed as described in Chapter 4. The matrix-coated plates were covered with the photosensitizer and illuminated with laser light. Controls included untreated matrices and matrices exposed to the photosensitizer or light only. The free radical scavenger, sodium azide (100mM, Sigma Chemicals), was used to determine whether PDT-effects on matrix-bound TGF–β were mediated through the generation of free radicals (Freeman et al., 1982).

To perform PDT of TGF–β in solution, a specific amount of TGF–β was diluted in a CASPc solution in serum free medium. These samples were placed in tissue culture wells and subjected to PDT as described above. To directly examine PDT-effects on TGF–β under these conditions, these samples were analysed by gel electrophoresis and the functional activity determined by an EC proliferation assay.

Determinations of TGF–β

To extract TGF–β from ECM, the matrix preparations were covered with 1ml of 0.1% BSA in PBS and exposed to acid (30 µl of 1N HCL) treatment (pH 1.5-2.5) for one hour prior to neutralization with 30 µl of 1N NaOH (Mooradian et al., 1989). TGF–β concentrations were calculated per 10 cm^2 of ECM.

To determine TGF–β concentrations in conditioned media of EC growing on ECM or matrix-free plates, 2.5 x 10^5 EC were seeded on these substrates in serum-free medium and grown for 24-hours. After this time the conditioned media was collected, clarified by centrifugation, and divided into two fractions. One fraction was exposed to acid (see above) in order to activate latent TGF–β to obtain total TGF–β concentrations and the other fraction remained untreated to measure only the active TGF–β.

Measurement of TGF–β concentration was performed with an ELISA kit (Promega, Madison, WI). In this immunoassay, TGF–β in the test sample is sandwiched between an anti-TGF–β monoclonal antibody coated on the microtiter plate and a second polyclonal anti-TGF–β antibody. A species-specific antibody conjugated to horseradish peroxidase is used as a tertiary reactant for color formation with a chromogenic substrate. The color intensity of the samples was measured at 450 nm and compared with a standard curve to obtain TGF–β concentrations .

Proliferation Assay

Endothelial cell proliferation was indirectly assessed using a mitogenesis assay based on cellular ^{3}H-thymidine incorporation (Battegay et al., 1990). ECs were seeded on the matrix preparations at a density of 1 x 10^5 or 5 x 10^4 cells/well in 0.5% calf serum medium and incubated 24-hours at 37°C. In separate experiments, a neutralizing polyclonal antibody against active TGF–β (rabbit IgG, R & D Systems) or a non-immune control antibody (10 µg/ml of normal rabbit IgG, R&D Systems) was added to the medium of EC on ECM during the incubation time. After the incubation time, 2.5 µCi ^{3}H-thymidine (New England Nuclear) was added to the medium and incubated with the cells for five hours. Unbound ^{3}H-thymidine was subsequently removed by three washes with PBS. Cells were then dissolved in 0.1N NaOH and placed in Ready Gel scintillation fluid (Beckman Instruments, Inc.) and cell incorporated radioactivity was determined by a scintillation counter (Beckman Instruments,Inc). The resulting data expressed as, counts/minute,

were normalized to the control untreated group and reported as percentage thymidine incorporation. Cell plating efficiency was verified to be equal on the different treated matrices as previously noted (Chapter 4) and therefore ^{3}H-thymidine counts were not corrected for the number of cells.

TGF–β Protein Gel Electrophoresis

To characterize whether PDT of TGF–β results in protein cross-links and changes in its molecular weight, sodium dodecyl sulfate polyacrylamide gel (15%) electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (Laemmli., 1970) to analyze TGF–β. PDT of carrier-free human recombinant TGF–β (500 ng, R & D Systems) was performed with the photosensitizer CASPc (0.012g) in the absence and presence of sodium azide (100 mM). Molecular-mass standards [Lysozyme (14.4 kDa), Carbonic anhydrase (31 kDa), Ovalbumin (45 kDa), Serum albumin (66.2 kDa), (Bio Rad, Hercules, CA)] were run with the samples as markers. The seperated proteins were visualized by silver staining as recommended by the manufacturer (Bio Rad).

Statistical Analysis

All data is expressed as mean ± standard deviation (SD). For data comparison between two groups, a two-tailed Student's t-test for independent variables was performed. For comparison of means between multiple groups, an one-way analysis of variance and Tukey's HSD post hoc test for multiple comparisons was applied (Statistica). p-values of less than 0.05 were considered significant.

RESULTS

Effect of PDT on Matrix-Associated TGF–β

To determine whether the photochemical reaction induced by PDT could affect matrix-associated TGF–β, the concentration of total TGF–β (active + latent) present in the EC-derived ECM preparations was analyzed by ELISA. Untreated ECM contained 85.4 ± 10.2 pg/10 cm^2 of TGF–β. In contrast, after PDT of ECM barely detectable levels of TGF–β (0.2 ± 0.5 pg/10 cm^2) could be measured (figure 5.1). To explore whether this effect was mediated by only light or photosensitizer exposure, ECM was subjected to either incubation with CASPc only, or only irradiation with 675 nm laser light. Whereas light irradiation only did not affect the matrix-associated TGF–β content, exposure of the matrix to the photosensitizer CASPc only resulted in reduced measurable levels of TGF–β (figure 5.1).

Functional Significance of PDT Effects on Matrix-Bound TGF–β

To assess whether the reduced immunoreactivity of matrix-bound TGF–β after PDT and CASPc exposure had any functional effect, a mitogenesis assay was utilized to examine EC proliferation. TGF–β was bound to the extracellular matrix molecule fibronectin (Fn) to specifically

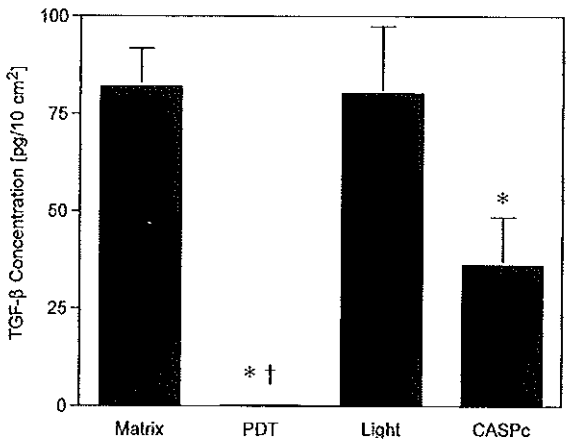


Figure 5.1.
Effect of photodynamic therapy (PDT) on the level of matrix-associated TGF–β. The concentration of TGF–β in pg/10cm² extracted from the matrix deposited by endothelial cells is depicted for PDT treated (PDT) and control matrices: untreated (matrix), laser-light exposure only (light), drug exposure only (CASPc). Values are mean ± SD, n=8 in the untreated group and n=5 in PDT, light and CASPc. "" denotes p< 0.0005 versus matrix and light and "†" denotes p< 0.001 versus CASPc.*

study the effect of matrix-bound TGF–β on EC proliferation.

Endothelial cell proliferation on Fn-TGF–β coated wells was significantly inhibited (44.1 ± 11.7%, p< 0.0005) as compared to EC proliferation on Fn (100 ± 5.9 %). This inhibition of EC proliferation was completely reversed by PDT of the Fn-TGF–β coated well (Figure 5.2). The PDT effect was mediated by inactivation of the Fn-bound TGF–β, since PDT of Fn alone did not affect EC proliferation (103.7 ± 2.1%, n=3). In addition, replenishment of TGF–β by administration of the initial concentration of TGF–β to PDT treated Fn-TGF–β, restored the inhibitory effect on EC proliferation (Figure 5.2).

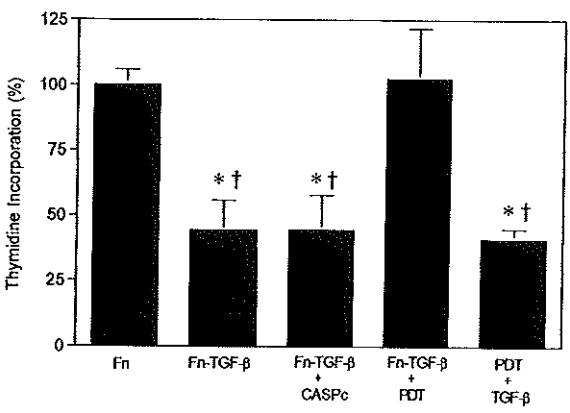


Figure 5.2.
Photodynamic therapy (PDT)-mediated inactivation of matrix-bound TGF–β stimulates endothelial cell (EC) proliferation. EC proliferation, determined by ³H-thymidine incorporation, on non-treated coated fibronectin (Fn) is compared with EC proliferation on coated Fn-TGF–β complex (Fn-TGF–β), photosensitizer exposure of Fn-TGF–β only (+ CASPc) and PDT of Fn-TGF–β (+ PDT). PDT + TGF–β represents restoration of the Fn-TGF–β complex after PDT of Fn-TGF–β by replenishment of TGF–β.Values are mean ± SD, n=9 in the Fn and Fn-TGF–β groups and n=6 in CASPc, PDT and PDT + TGF–β. "" denotes p< 0.0005 versus Fn and "†" denotes p< 0.0005 versus PDT.*

Because exposure of ECM to CASPc resulted in decreased levels of measurable TGF–β, it was necessary to verify whether CASPc itself could functionally affect matrix-associated TGF–β. This was not the case, since EC proliferation on Fn-TGF–β coated plates was not affected by CASPc only exposure (Figure 5.2). This suggests that the interaction of CASPc with the TGF–β molecule caused interference with the ELISA for TGF–β measurements, without affecting its functional activity.

To confirm that PDT-induced inactivation of TGF–β was free radical mediated, a specific free radical scavenger, sodium azide, was added to the Fn-TGF–β coated well during laser irradiation. Whereas PDT completely removed the inhibitory effects of matrix-bound TGF–β on EC proliferation (102.3 ± 19.3%), there was still significant inhibition of EC proliferation (71.3 ± 8.5%, $p < 0.01$) if PDT was performed in the presence of the scavenger. These results demonstrate that the free radical scavenger substantially protected matrix-bound TGF–β from PDT-inactivation.

Importance of Matrix-Associated TGF–β for EC Proliferation

To determine whether EC growing on EC-derived ECM could release matrix-associated TGF–β, total and active TGF–β in the conditioned media of EC on ECM were measured and compared to TGF–β concentrations in the conditioned media of EC growing on matrix-free plates. Significantly more active and total TGF–β could be detected in the conditioned media of EC growing on ECM as compared to EC on matrix-free plates (Table 5.1). The percentage of active TGF–β was also significantly increased in the conditioned media of EC on ECM (Table 5.1).

Table 5.1 Active and Total TGF-β Concentrations in Conditioned Media of Endothelial Cells Growing on Extracellular Matrix and Plate

	Active (pg/ml)	Total (pg/ml)	Active (%)
Matrix	166.5 ± 17.2*	1033.6 ± 171.3*	16.6 ± 3.4*
Plate	39.4 ± 4.9	662.0 ± 126.2	6.2 ± 1.3

Data expressed as mean ± so, $n = 8$.
* denotes $p < 0.001$ versus plate.

To further delineate the role of TGF–β on EC proliferation growing on ECM, a neutralizing antibody against active TGF–β was used to block its activity. Adding the antibody to the medium during the incubation time significantly increased EC proliferation on ECM as compared to EC proliferation on ECM in the absence of the antibody (Figure 5.3). This increase in EC pro-

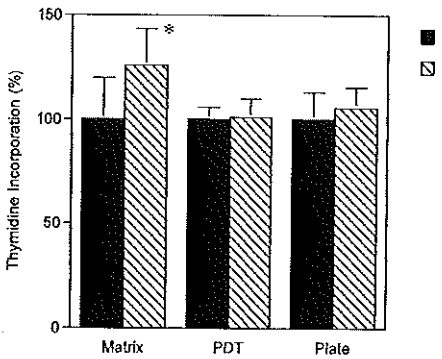


Figure 5.3.
Effect of anti-TGF–β neutralizing antibody on endothelial cell (EC) proliferation. EC proliferation was determined by ^{3}H-thymidine incorporation for EC seeded on untreated matrix (matrix), PDT-treated matrix (PDT) and matrix-free plate (plate) in the absence (- antibody) or presence (+ antibody) of an antibody against TGF–β (10 μg/ml). For each group, thymidine incorporation in the non-antibody experiments was normalized to 100% for comparison to the experiments with antibody. Values are mean ± SD, n= 8 in the matrix group and n= 6 in the PDT and plate groups. "" denotes p < 0.05 versus matrix without antibody (t-test).*

liferation was not due to an antibody effect on TGF–β present in 0.5% calf serum medium (9.1 ± 4.5 pg/ml, n=5), because the antibody failed to promote proliferation in cells grown on matrix-free plates (Figure 5.3). Similarly, the antibody mediated effect on EC proliferation was not due to a non-specific effect since the addition of a non-immune antibody did not alter EC proliferation (104 ± 13.2%, n=3).

Consistent with previous results (Chapter 4), EC proliferation on PDT treated ECM was significantly increased (176 ± 14.4 %, p< 0.0001, n=6) as compared to EC proliferation on untreated ECM (100.2 ± 14.8 %, n=6). Because matrix-associated TGF–β was inactivated by

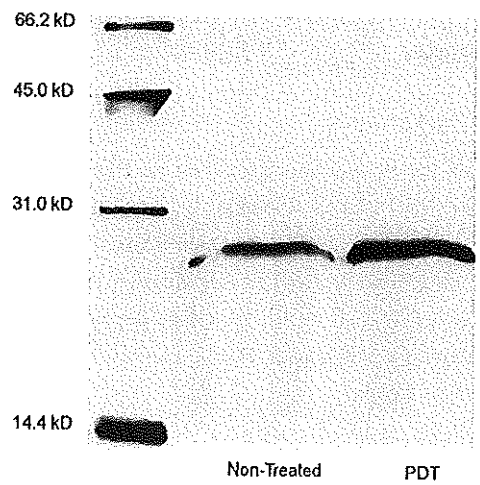


Figure 5.4.
Gel electrophoresis analysis of PDT-treated TGF–β. To conduct PDT of TGF–β in solution, a solution containing TGF–β and the photosensitizer CASPc was illuminated by 675 nm laser-light with a fluence of 100 J/cm^2. Non-treated and PDT-treated TGF–β-CASPc solutions were analyzed by SDS-PAGE under non-reducing conditions and silver stained. Molecular-mass standards (see methods) were run with the samples as markers. Note that there is no change in the 25 kD TGF–β band after PDT.

PDT, it was postulated that adding the TGF–β antibody to EC growing on PDT treated ECM, would not affect EC proliferation. As expected, addition of the antibody to EC on PDT treated matrix did not alter EC proliferation (Figure 5.3).

Effects of PDT on TGF–β in Solution

To gain more understanding how PDT modifies TGF–β on a molecular level, PDT was performed of TGF–β in solution and analysed by SDS-PAGE under non-reducing conditions. Figure 5.4 demonstrates that there was no change in the molecular weight of TGF–β after PDT. This experiment was performed twice with consistent results. Addition of the scavenger sodium azide during irradiation did not affect these results (data not shown).

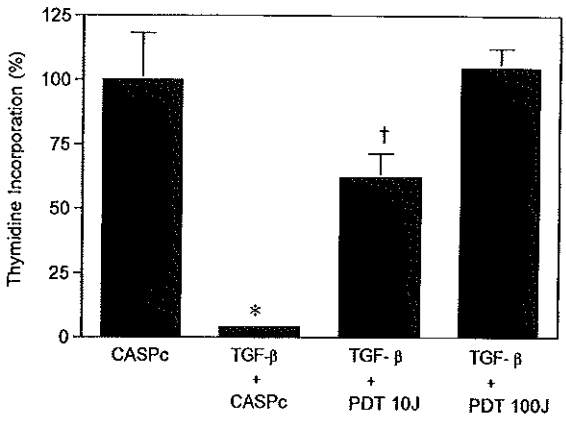


Figure 5.5.
PDT-mediated functional inactivation of TGF–β in solution. The functional activity of TGF–β in solution was determined with an EC proliferation assay (^{3}H-thymidine incorporation). PDT of TGF–β in solution (1 ng/ml) was performed with fluences of 10 and 100 J/cm^2. Negative control included a 5 mg/ml CASPc only solution (CASPc), which was compared to EC proliferation exposed to an untreated TGF–β-CASPc solution (TGF–β + CASPc), as the positive control, and PDT-treated TGF–β-CASPc solutions (TGF–β + PDT 10 and 100J). Values are mean ± SD, n= 3. "" denotes p < 0.001 versus CASPc, and TGF–β + PDT 10 and 100J, and "†" denotes p < 0.02 versus CASPc and TGF–β + PDT 100J.*

To assess whether PDT could inactivate the EC-inhibitory function of TGF–β under similar conditions, PDT-treated TGF–β in solution was used to determine EC proliferation. As compared to medium with only the photosensitizer CASPc, there was a significant ($p< 0.001$) inhibition of EC proliferation with medium containing CASPc and TGF–β (Figure 5.5). PDT of TGF–β, caused a reversal of this inhibitory effect of TGF–β on EC in a dose dependent way (Figure 5.5). These results clarify that the EC-inhibitory function of TGF–β can be completely inactivated by PDT, without an effect on its molecular weight.

Discussion

In contrast to the lack of total EC regrowth after widespread denudation injury of the rat carotid artery (Reidy et al., 1983; Lindner et al., 1990), complete reendothelialization has been consistently observed after PDT of balloon injured rat carotid arteries and normal rat femoral arteries (LaMuraglia et al, 1994; Grant et al, 1994). Since an intact endothelium is known to provide a non-thrombogenic lining and modulate the underlying medial SMC behavior, rapid and complete EC regeneration after vascular PDT may be a mechanism by which IH is successfully inhibited. Furthermore, since in the clinical situation adjunctive PDT to inhibit IH after invasive vascular procedures will likely cause additional EC denudation, better understanding of the reendothelialization process after vascular PDT is imperative. The mechanisms by which PDT of the vascular wall could influence EC function are not known, but a recent study from this laboratory demonstrated that PDT-induced alterations in the extracellular matrix deposited by EC in vitro, favorably affected EC proliferation and migration (Chapter 4). The present study was undertaken to elucidate the mechanisms underlying these findings.

The knowledge that free radicals generated by PDT can directly alter proteins and inactivate enzymes (Grossweiner, 1976; Freeman et al, 1992), prompted us to investigate whether PDT of matrix resulted in depletion of a biologically active inhibitor of EC function. Cell deposited matrix is a complex reservoir of bioactive substances including growth factors and inhibitors, adhesion molecules and modulators of coagulation and fibrinolysis (Fridman et al, 1985; Korner et al, 1993; Rogelj et al, 1989). This study concentrated on the effects of PDT on matrix-associated TGF-β. This multifunctional polypeptide is produced by a variety of cells in culture including EC and is known to be one of the most potent inhibitors of EC proliferation and migration (Madri et al, 1991; Heimark et al, 1986; Raychaudhury et al, 1991). TGF-β has been shown to be present in the ECM deposited by EC in vitro (Falcone et al, 1993; Benezra et al, 1993; Taipale et al, 1995) and to specifically bind to matrix molecules, such as fibronectin and laminin (Mooradian et al, 1989).

The first step in this study was to examine whether TGF-β could be detected in the EC matrix preparations. Since binding of TGF-β to the matrix molecule fibronectin has been shown to be strongly pH-dependent (Mooradian et al, 1989), an acidification procedure was used to dissociate TGF-β from matrix components. Utilizing this technique, TGF-β could be readily extracted from the EC matrix preparations. To assess whether PDT could affect TGF-β bound to ECM, PDT of the matrix was performed and TGF-β quantitatively measured with an ELISA. After PDT of ECM, levels of TGF-β were barely detectable. To determine whether loss of TGF-β immunoreactivity after PDT correleted with functional inactivation, the effect of fibronectin-bound TGF-β on EC proliferation was further examined. The finding that PDT could eliminate

inhibition of EC proliferation by TGF–β bound to fibronectin provides strong evidence to the hypothesis that matrix bound TGF–β is sensitive to reactive free radical moieties generated by PDT. The sensitivity of TGF–β to free radicals was confirmed by the finding that the free radical scavenger, sodium azide, significantly protected TGF–β from PDT-inactivation. The decrease in TGF–β immunoreactivity after CASPc exposure only was surprising but subsequent experiments revealed that the presence of this photosensitizer during TGF–β measurement interfered with the ELISA system by unknown mechanisms (data not shown). However, the interaction of CASPc with TGF–β without light did not alter the functional effect of inhibition of EC proliferation.

The precise mechanisms by which PDT inactivates TGF–β are not clearly understood. However, extensive research has been performed to characterize how photochemical reactions can alter and inactivate enzymes and other proteins (Grossweiner, 1976; Freeman et al, 1982). The photodynamic effect appears to be principally mediated by free-radical reaction with sensitive amino acids such as, histidine, methionine, tyrosine and tryptophan. This process leads to chemical changes of these amino acids directly involved in the active site or binding site of the protein or to conformational changes in the proteins, which could cause functional disturbance (Freeman et al, 1982). The results presented herein demonstrate that PDT of TGF–β in solution does not result in changes in its molecular weight as analysed by SDS-PAGE. This suggest that PDT of TGF–β in solution, with the applied PDT-dose and TGF–β concentration, does not induce intermolecular photochemical cross-linking and aggregation of the TGF–β protein as has been shown for PDT of spectrin (Verweij et al., 1981). However, this study further clarified that in a dose dependent way, PDT of TGF–β in solution inactivates the EC-inhibitory function of TGF–β. Therefore, it is likely that the functional inactivation and loss in immunoreactivity of TGF–β by PDT is mediated by free radical-induced conformational changes in the secondary or tertiary structure of the TGF–β protein (Freeman et al., 1982). Furthermore, the photochemical destruction of TGF–β is not specific for this polypeptide and other biological important proteins, such as basic fibroblast growth factor, have also been shown to be sensitive to this photochemical reaction (Chapter 7). The findings presented in this report elucidate the functional significance of free-radical mediated inactivation of an active cellular mediator in a biological system.

How and in what form TGF–β is bound to the matrix deposited by EC is not well documented. Since TGF–β is known to bind to several matrix molecules, such as b-glycan (Andres et al, 1989), decorin, (Yamaguchi et al, 1990), collagen, laminin and fibronectin (Mooradian et al, 1989) it is likely to bind to multiple sites in the matrix. Although unbound TGF–β is known to inhibit EC proliferation, the functional effect of TGF–β incorporated in the ECM on EC growth has not been previously studied. Most studies have focused on the growth-promoting effect of matrix-associated basic fibroblast growth factor on EC without considering the presence and function of TGF–β (Fridman et al, 1985; Rogelj et al, 1989). This study demonstrates the importance

of matrix-bound active TGF–β as it inhibits EC mitogenesis.

Most cells, including EC, secrete TGF–β in a biologically latent, high molecular weight form, that cannot bind to cellular TGF–β receptors and must be activated to elicit a biological response (Sporn et al, 1992). Moreover, a recent study demonstrated that the TGF–β present in the matrix deposited by human EC was predominantly in a latent form (Taipale et al, 1995). It was therefore necessary to delineate whether TGF–β incorporated in the ECM could affect EC proliferation. The activation of latent TGF–β is a critical step in the regulation of TGF–β action and can be achieved in a test tube by acidification, heat, proteolysis by plasmin and chaotropic agents (Sporn et al, 1992). Under physiological conditions, latent TGF–β can be activated by coculture of ECs with either pericytes or smooth muscle cells (Antonelli-Orlidge et al, 1989; Sato et al, 1989). The activation occurs on the cell surface or matrix, by localization of the latent TGF–β complex to specific binding sites and is mediated by tissue type II transglutaminase and plasmin derived from serum plasminogen via the action of cell surface plasminogen activators (Sato et al, 1989; Dennis et al, 1991; Flaumenhaft et al, 1993; Sato et al, 1993; Kojima et al, 1993). Since EC express cellular transglutaminase (Kojima et al, 1993) and plasminogen activators on their cell-surface (Moscatelli et al, 1988), it is feasible that EC growing on matrix can activate latent TGF–β bound to matrix molecules (Flaumenhaft et al, 1993).

The activation of latent TGF–β incorporated in the ECM by EC is supported by the finding that, as compared with EC growing on matrix-free plates, significantly more active TGF–β could be detected in the conditioned media of EC growing on matrix. This was accompanied by increased levels of total TGF–β, which may also implicate that TGF–β in the latent form was released from the matrix by EC. Although increased cellular production of TGF–β cannot be excluded, these results corroborate the findings of a recent study demonstrating that macrophages growing on EC matrix released increased amounts of total TGF–β in their conditioned media, which was not related to increased TGF–β mRNA expression (Falcone et al, 1993). The finding in the present study that the percentage of active TGF–β was increased in the conditioned media of EC grown on matrix compared to EC grown on matrix-free plates, suggests that under these conditions more activation of latent TGF–β occurs. Because of PDT inactivation of matrix-bound TGF–β, it was expected that, similar to EC on matrix-free plates, less TGF–β would be present in the conditioned media of EC growing on PDT treated matrix. This postulate could however not be directly verified, because of the interference of the photosensitizer CASPc with the ELISA. Instead, a neutralizing TGF–β antibody was used to indirectly determine functional TGF–β activity.

The addition of the TGF–β antibody to EC growing on either PDT treated matrix or matrix-free plates had no effect on EC proliferation, indicating that there was no functional TGF–β activity to be blocked. On the other hand, the addition of TGF–β antibody to EC grown on non-treated matrix increased EC proliferation. Thus, the activity of either active TGF–β in the matrix

or active TGF–β generated from EC-mediated activation of latent TGF–β was blocked by the antibody, decreasing its inhibitory effect on EC proliferation. Taken together, these data demonstrate that matrix-associated TGF–β, either directly or after activation, exert an acute inhibitory effect on EC proliferation. This proliferative inhibition can be reversed by an antibody against TGF–β or by PDT-induced inactivation.

The importance of TGF–β in affecting EC behavior has been suggested by an in vivo study utilizing the rat carotid balloon injury model, showing that EC regrowth after denudation injury is modulated by TGF–β and fibronectin (Madri et al, 1989). That study identified TGF–β staining in the intima of chronically de-endothelialized areas and proposed that TGF–β may be involved in eliciting and prolonging the chronic deendothelialization noted in this model. This effect could be mediated by acutely inhibiting EC proliferation and migration and chronically by modulating the matrix synthesized by local vascular cells. In regard to these findings, PDT-mediated inactivation of TGF–β associated with the intima may provide a mechanism to explain rapid EC regeneration after PDT of the vascular wall.

In summary, this study provides mechanistic insights into matrix-associated TGF–β inhibition of EC proliferation and how this response can be modulated PDT. For the first time, functional inactivation of TGF–β by PDT-generated free radicals is described. These results provide support to the concept that besides eradication of vascular cells, PDT-mediated changes in the ECM may play a pivotal role in determining the outcome of the vascular healing process after injury. Furthermore, the interaction of PDT with biologically active proteins may represent a tool to inhibit the excessive activty of these mediators when they are associated with disease states, such as intimal hyperplasia.

CHAPTER 6

EFFECTS OF PDT ON TGF–β ACTIVITY ASSOCIATED WITH CELL INJURY

Adapted from: RG Statius van Eps and GM LaMuraglia. Photodynamic Therapy Inhibits Transforming Growth Factor–β Activity Associated with Vascular Smooth Muscle Cell Injury. *J Vasc Surg* 1997; 25: 1044-1053.

INTRODUCTION

Cell migration, proliferation and enhanced production of extracellular matrix are important events in biologic repair processes to restore tissue integrity and physiological function after injury (Kovacs et al., 1994). However, failure to properly terminate this response may lead to progressive fibrosis and tissue damage (Border et al., 1994). The repair process is to a large extent mediated by the release of cytokines and growth factors in response to injury. Several lines of evidence point to transforming growth factor β1(TGF–β), as a key cytokine that regulates tissue repair and whose sustained production underlies the development of tissue fibrosis (Border et al., 1992, 1994).

One such fibrotic condition is intimal hyperplasia (IH) induced by vascular injury, a major cause of restenosis after invasive vascular interventions. TGF–β has been shown to be involved in IH after balloon injury in experimental models (Majesky et al., 1991; Wysocki et al., 1996), in human vascular restenosis lesions (Nikol et al., 1992), and in experimental vein graft IH (Hoch et al., 1995). The main effect of TGF–β in IH development is believed to be increased and sustained stimulation of matrix production and accumulation, which accounts for the bulk of the intimal lesion (Nabel et al., 1993; Rasmussen et al., 1995). Although all cells involved in IH, including smooth muscle cells (SMC), endothelial cells (EC), platelets and monocytes are known to produce TGF–β in vitro, it is thought that neointimal SMC are the major source of this cytokine during vascular repair (Rasmussen et al., 1995).

Strategies to suppress TGF–β activity may have an enormous clinical potential to inhibit IH and other fibrotic conditions associated with overproduction of TGF–β. In fact, antibodies against TGF–β have been shown to inhibit IH (Wolf et al., 1994) and several other experimental fibrotic conditions, such as glomerulosclerosis (Border et al., 1990) and skin scarring (Shah 1992). The complex regulation of TGF–β production and activity offers a number of targets for TGF–β suppression that may be more suitable than antibodies for use in humans. One important aspect of TGF–β regulation is its activation from its precursor latent form in order to elicit biological activity. TGF–β is produced and secreted as an inactive precursor protein, latent TGF–β, consisting of a latency-associated peptide (LAP) bound to the active protein (Sporn et al., 1987). Although it is not clear how TGF–β becomes activated in vivo, it is thought that protease cleavage by plasmin represents a physiological mechanism of TGF–β activation (Lyons et al., 1990). Interference with the TGF–β activation process or utilization of LAP-like proteins that specifically bind to TGF–β

are potential means to inhibit TGF–β activity (Border et al., 1992b). However, because of the essential systemic physiologic function of TGF–β (Wahl, 1994) only local inhibition of TGF–β at the site of overproduction and intended inactivation may be feasible.

An approach to locally interfere with the biological activity of important proteins, such as TGF–β, may be photodynamic therapy (PDT). Due to the short half life of PDT-induced reactive molecules, irradiation of laser light only over the area of interest provides a means to elicit a localized effect, and therefore spatial selectivity is maintained. It is known that free radicals can chemically react with lipids and proteins which may cause functional disturbance of biologic molecules (Freeman et al., 1982; Grossweiner, 1976). In fact, it has been recently demonstrated that the photochemical reaction induced by PDT profoundly alters the biologic characteristics of extracellular matrix deposited by EC in vitro (Chapter 4) and inactivates matrix-associated TGF–β (Chapter 5). Thus, besides its cytotoxic effects, PDT-generated free radicals may represent a method to locally interfere with the biologic activity of cellular mediators centrally involved in the healing response after tissue injury.

Since TGF–β is a key mediator of IH and other fibrotic states, this study concentrated on the effects of PDT on the biological activity of TGF–β. Utilizing a defined in vitro model with vascular SMC, this study examined the effects of SMC injury on the release and activation of TGF–β and whether this response could be modified by PDT.

MATERIAL AND METHODS

Cell Culture

Primary bovine aortic SMC and EC cultures were established from the aortas of freshly slaughtered calves and characterized as previously described (Chapter 4). Cells were kept in a 37°C, 5% CO_2 incubator, refed every 42-72 hours with Dulbecco's Modified Eagles Media (DMEM) supplemented with 10% calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.6 mol/L L-glutamine (Gibco, Grand Island, NY). Cells were passed at a ratio of 1:5 using 0.05% Trypsin / 0.125% EDTA (Gibco) upon reaching confluence and used for experiments between the 2th and 6th subpassages for EC and between the 2th and 4th subpassages for SMC.

Photodynamic therapy

To perform PDT of SMC in culture, the cells were seeded in full medium at a density of 2.5 x 10^4 /cm^2 on tissue culture plates (Falcon, Becton Dickinson, Lincoln Park, NJ) and allowed to attach for 24-hours. The photosensitizer chloroaluminum sulfonated phtalocyanine (CASPc), at a concentration of 5 µg/ml, was subsequently added to the cells in serum-free medium and incubated for 2-hours. After two rinses with phosphate buffered saline (PBS), the cells were irradiated with thermoneutral light delivered by an argon-pumped dye laser (Coherent Innova I and

Coherent CR 599, Coherent, Palo Alto, Ca) tuned at 675 nm for optimal absorption. The end-fiber irradiance was set at 100 mW/cm² to avoid any thermal effects and two different fluences (total light energies) were applied: subtherapeutic dose of 10 J/cm² and the in vivo therapeutic dose to inhibit IH of 100 J/cm². To confirm that PDT-cytotxicity was mediated by a photochemical reaction involving the activation of the photosensitizer by light, cells exposed to the photosensitizer only or light only served as controls.

Cell Viability Assay

Smooth muscle cell viability was determined 24-hour after PDT treatment and mechanical injury using a colorimetric assay based on the uptake of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide salt (MTT, Sigma Chemicals, St. Louis, MO) by viable cells (Mosmann, 1983). In brief, the MTT solution (0.5 mg/ml) was added to the cells and incubated at 37°C to allow cleavage of the tetrazolium ring by mitochondrial dehydrogenases and formation of blue formazan crystals. After 3 hours, the residual MTT was carefully removed and the crystals were dissolved by incubation with DMSO (Sigma Chemicals) for 30 minutes. The intensity of the developed color in each well was read by an ELISA reader at 570 nm. The optical density of untreated cells represented 100% viable cells and background color formation of MTT with DMSO added to an empty plate, 0% viable cells. The optical density from the treatment groups were fitted into a linear regression line obtained from the control groups to calculate percent viability.

Preparation of Conditioned Media

Conditioned media was collected from SMC that were PDT-treated or mechanically injured. Controls included media from untreated SMC and SMC that were exposed to the photosensitizer only. To induce mechanical injury, SMC were vigorously scraped from the well with a rubber policemen (McNeil et al., 1989). Cell scraping with a rubber policeman represents a form of barotrauma, which resembles in vivo mechanical injury, and causes cell membrane damage which could lead to either cell death or recuperation of cell integrity and survival. This form of cell injury was used to compare an in vivo relevant method of cell trauma with PDT-induced cytotoxicity and how these different forms of injury affect the release and activation of TGF–β. After PDT or mechanical injury, the cells were allowed to condition the medium for 24 hours at 37°C in serum free medium supplemented with 0.1% bovine serum albumin (Sigma Chemicals). The medium was then collected and clarified by centrifugation at 2,000 rpm for 15 minutes for TGF–β assay.

Determination of TGF–β protein levels

The concentration of TGF–β was measured in the conditioned media with a commercially available ELISA kit (Promega, Madison, WI), which employs the "sandwich" immunoassay technique. For measurement of TGF–β levels, the conditioned media was divided into two fractions. One fraction was exposed to acid (20 ml of 1N HCL, pH 1.5-2.5) treatment for 30 minutes prior to neutralization with 20 ml of 1N NaOH, in order to activate latent TGF–β and obtain total TGF–β concentrations. The other fraction remained untreated to measure only the active TGF–β portion in the conditioned media.

Mitogenesis assay

To assess functional TGF–β activity in the conditioned media of PDT-treated or mechanical injured SMC, an EC mitogenesis assay was used. EC mitogenesis is known to be strongly inhibited by TGF–β (Frater-Schroder et al., 1986). For this purpose, [^{3}H]-thymidine incorporation in EC was determined as an indicator of DNA replication (Klagsbrun et al., 1977). Endothelial cells were seeded in full medium at a density of 10 x 10^4 /cm^2 and allowed to attach for 24-hours. To overcome any depletion of essential nutrients, the serum free SMC-conditioned media was supplemented with calf serum to make a 10% calf serum conditioned media solution. This composite medium was subsequently added and incubated with the EC for 24-hours. In separate experiments, the conditioned media of mechanical injured SMC was pre-treated with a neutralizing antibody against TGF–β1 (R & D Systems, Minneapolis, MN) or a non-immune control antibody (normal rabbit IgG, R & D Systems) to determine if the functional effect of the conditioned media was mediated by TGF–β. For the last 5 hours of the incubation time, 2.5 mCi of [^{3}H]-thymidine (New England Nuclear, Boston, MA) was included in the medium. The cells were then washed 3 times with PBS, dissolved in 0.5 N NaOH and placed in ready gel scintillation fluid (Beckman Instrument, Inc., Fullerton, CA). Cell incorporated radioactivity was counted with a scintillation counter (Beckman Instruments, Inc.)

Statistical Analysis

All data is expressed as mean ± standard deviation (SD). For comparison of means between multiple groups, an one-way analysis of variance and Tukey's HSD post hoc test for multiple comparisons was applied (Statistica, Statsoft, Tulsa, OK). p-values of less than 0.05 were considered significant.

RESULTS

Smooth Muscle Cell Viability

To study the relationship between SMC-injury and the release and activation of TGF–β, SMC viability was assessed after PDT and mechanical injury. There was no SMC survival after

PDT with both 10 and 100 J/cm² (0.9 ± 1.2% and 0.0 ± 1.8%, respectively), while, exposure of SMC to either light (100J/cm²) or photosensitizer only did not affect cell viability (Fig. 6.1). Mechanical SMC disruption was also associated with a substantial decrease in SMC viability to 10.9 ± 5.6% (Fig 6.1).

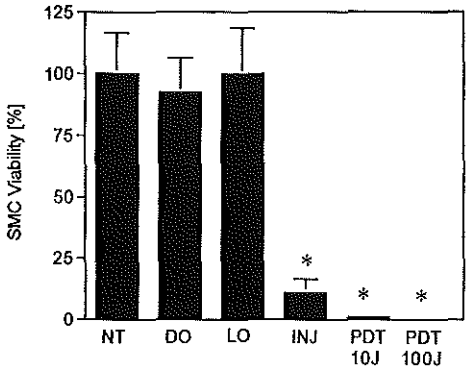


Figure 6.1.
*Effect of PDT and mechanical injury on smooth muscle cell viability. Viability, as determined by the tetrazolium salt conversion assay, of PDT-treated SMC with total fluences of 10J/cm² and 100J/cm², SMC exposed to either the photosensitizer (DO) or light (LO) only, and mechanical injured (INJ) SMC is compared to non-treated (NT) SMC viability, which represents 100% viable cells. Values are mean ± SD, * denotes p< 0.0005 versus NT, DO and LO (ANOVA, n = 6).*

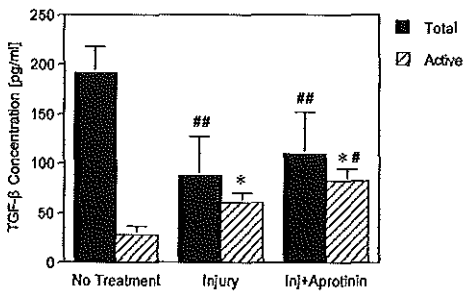


Figure 6.2.
*Effect of mechanical SMC injury on release and activation of TGF–β. The concentration of active and total TGF–β, as measured by ELISA, in the conditioned media of SMC is plotted for non-treated SMC, mechanically injured SMC (injury) and SMC that were mechanically injured in the presence of the plasmin inhibitor, aprotin (100 mg/ml). Values are mean ± SD, * denotes p< 0.0005 versus active no treatment, # denotes p< 0.0005 versus active injury, and ## denotes p< 0.01 versus total no treatment (ANOVA, n = 9 for NT and INJ, n = 6 for active INJ + APR, and n = 3 for total INJ + APR)*

SMC Injury-Associated Release and Activation of TGF–β

To determine whether SMC injury is associated with specific effects on the release or activation of TGF–β, the concentration of both active and total TGF–β was measured in the conditioned media of untreated and mechanical injured SMC (Fig. 6.2). The level of active TGF–β in the conditioned media of untreated SMC was low (27.7 ± 8.7pg/ 1 x 10^5 cells) as compared to the total amount (191.1 ± 26.7pg/ 1 x 10^5 cells). Although mechanical injury of SMC resulted in a decrease in the total amount of TGF–β released (86.9 ± 39.97pg/ 1 x 10^5 cells), this was associated with a significant (p< 0.001) increase in the level of active TGF–β (60.1 ± 10.1pg/ 1 x 10^5 cells).

To examine whether the increased levels of active TGF–β after SMC injury could be mediated by plasmin-mediated activation of latent TGF–β, the specific plasmin inhibitor aprotinin

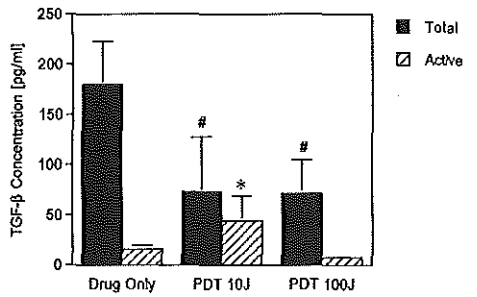


Figure 6.3.
*Effect of PDT on SMC release and activation of TGF–β. The concentration of active and total TGF–β, as measured by ELISA, in the conditioned media of SMC is plotted for SMC that were treated with the photosensitizer drug only, and PDT treated SMC with total fluences of 10J/cm² and 100J/cm². Values are mean ± SD, * denotes p< 0.02 versus active drug only and active PDT 100J, and # denotes p< 0.005 versus total drug only (ANOVA, n = 6).*

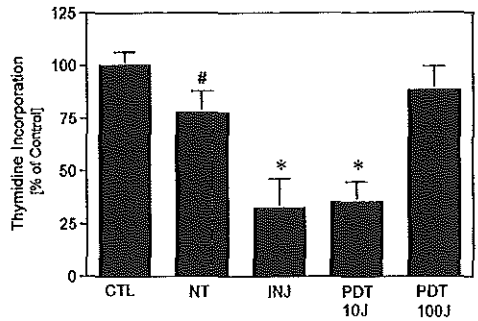


Figure 6.4.
*Inhibition of EC mitogenesis by SMC injury-associated increase in active TGF–β: Reversal by PDT with 100J/cm². Non-treated (NT) SMC, mechanical injured (INJ) SMC and SMC that were PDT-treated with 10J/cm² and 100J/cm², were allowed to condition their media for 24 hours. This conditioned media was used to assess TGF–β growth-inhibitory effect (³H-thymidine incorporation) on EC. Medium with 10% calf serum served as control (CTL). Values are mean ± SD, * denotes p< 0.0002 versus CTL, NT and PDT 100J, # denotes p< 0.005 versus CTL (ANOVA, n = 12 for CTL, n = 10 for INJ, and n = 6 for NT,*

was used to block plasmin activity. Surprisingly, the presence of aprotinin (100 mg/ml) in the medium at the time of injury resulted in a significant (p< 0.005) increase in the amount of active TGF–β , as compared to mechanical SMC injury without aprotinin. (Fig 6.2).

PDT Effects on SMC Release and Activation of TGF–β

To study the effects of PDT on SMC release and activation of TGF–β, the concentration of TGF–β was measured at different dosimetry in the conditioned media of PDT-treated SMC. Exposure of SMC with CASPc only, which did not affect SMC viability, served as control to correct for any interference of CASPc with TGF–β release or measurements. Similar to mechanical injury, PDT-mediated cytotoxicity with 10 J/cm² was associated with a significant increase (p< 0.02) in the level of active TGF–β (44.4 ± 22.4pg/ 1 x 10^5 cells), despite a decrease in the total amount (Fig. 6.3). In contrast, at higher doses of PDT (100 J/cm²), there was no increased level of active TGF–β (8.1 ± 3.5pg/ 1 x 10^5 cells) despite an equivalent level of total TGF–β (Fig. 6.3).

Functional Effect of Cell Injury-Associated Increase in Active TGF–β

The biological activity of TGF–β in the conditioned media was determined using an EC

mitogenesis assay (Fig. 6.4). For this purpose, EC mitogenesis incubated with 10% calf serum medium served as a control. The conditioned media of untreated SMC resulted in a significant decrease (77.6 ± 10.3%, $p< 0.005$) in EC mitogenesis as compared to the control (100 ± 6.4%). However, there was significant ($p< 0.0002$) more inhibition of EC mitogenesis with the conditioned media of mechanical injured SMC (32.3 ± 13.8%) and PDT with 10 J/cm² (35.2 ± 9.6%), a fact that correlates with the increase in the level of active TGF–β after SMC injury. Furthermore, the conditioned media of mechanical SMC injury in the presence of aprotinin, which was associated with the highest level of active TGF–β, resulted in the greatest inhibition of EC mitogenesis (14.8 ± 5.6%, $p< 0.02$ versus injury without aprotinin). Aprotinin added to control medium did not affect EC mitogenesis (data not shown).

To confirm that the inhibition of EC mitogenesis was mediated by active TGF–β, a neutralizing antibody against active TGF–β was preincubated with the conditioned media of mechanical injured SMC. Addition of the TGF–β antibody (90 mg/ml) significantly ($p< 0.0005$) reversed the EC inhibitory effects of the injured SMC-conditioned media (Fig. 6.5), whereas presence of the antibody in control medium had little effect on EC mitogenesis (110.7 ± 13%, n=4).

Since PDT-mediated cytotoxicity with 100 J/cm² was not accompanied by increased levels of active TGF–β, it was postulated that the conditioned media from this group would not affect EC mitogenesis. As shown in figure 6.4, the SMC conditioned media of PDT with 100 J/cm² did not significantly affect EC mitogenesis (88.1 ± 11.4%), as compared to control media. This finding strongly indicate that with this dose, PDT-mediated cytotoxicity does not result in increased TGF–β activity.

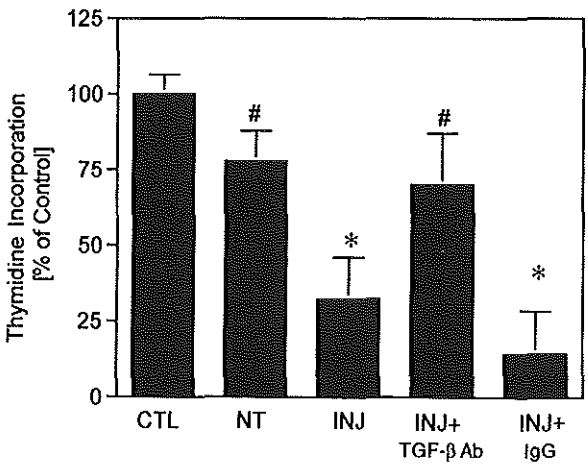


Figure 6.5.
*The effect of a neutralizing antibody against TGF–β on SMC injury-associated TGF–β activity. The conditioned media of mechanical injured SMC (INJ) was pre-treated with an anti-active TGF–β neutralizing antibody (90 mg/ml) or a normal rabbit IgG control (90 mg/ml), and used to assess TGF–β growth-inhibitory effect (³H-thymidine incorporation) on EC. CTL, NT and INJ as in figure 4. Values are mean ± SD, * denotes $p< 0.0005$ versus CTL, NT and INJ + TGF–β Ab, and # denotes $p< 0.005$ versus CTL (ANOVA, $n = 4$ for antibody goups).*

DISCUSSION

Several studies have now documented that PDT of the vascular wall in vivo is an effective method to inhibit injury-associated IH in experimental models (Chapter 2). The rationale of PDT as a means to prevent IH has been the local eradication of SMC in the vessel wall responsible for the fibroproliferative process. The free radicals produced by PDT are highly cytotoxic, thus upon wavelength-specific light illumination over an area of interest, vascular cells that have taken up the administered photosensitizer are lethally injured. However, it seems paradoxical to assume that mere eradication of SMC in the vessel wall could explain the effective inhibition of injury-associated IH by PDT. This assumption is unfitting because it is well documented that IH develops as an response of the vessel wall to many forms of injury. In fact, it has been demonstrated that the extent of IH is related to the degree of injury-induced SMC death in the medial layer of the vessel wall (Indolfi et al., 1995). With appropriate doses of PDT, there is eradication of SMC in the vessel wall, and yet this is not followed by an inflammatory or fibroproliferative response (Chapter 2). Instead, the vascular healing response after PDT is characterized by rapid and complete EC regrowth, but minimal repopulation of the medial layer with SMC. This consistent histologic finding after vascular PDT opened a new line of investigation to examine whether, besides cytotoxicity, free radicals produced by PDT could affect important biological mediators , such as TGF–β, and thereby profoundly modify the vascular healing response to injury.

The findings presented in this study demonstrate that lethal SMC injury under culture conditions results in increased biologic TGF–β activity. The increase in TGF–β activity associated with SMC injury can be prevented if the cells are treated with an adequate dose of PDT. Considering the important role of TGF–β in the vascular repair process after injury, these findings may help explain why PDT-mediated SMC eradication is not followed by an exaggerated fibroproliferative response. By interfering with biologic TGF–β activity, PDT may represent a method to inhibit fibrotic conditions associated with local overproduction of TGF–β. Because TGF–β strongly autoinduces its own synthesis (Kim et al., 1989), acute inhibition of TGF–β activity by PDT may disturb this positive feedback loop and therefore interfere with the overproduction of TGF–β. In addition, since TGF–β has been implicated to have an inhibitory effect on EC regrowth, PDT-inhibition of TGF–β activity may be a mechanism to explain the rapid EC recovery observed after experimental vascular PDT (Chapter 5). This conjecture is supported by the functional assay performed in this study demonstrating that, unlike mechanical SMC injury that promoted TGF–β activity and inhibited EC mitogenesis, with therapeutic PDT dosimetry, there was no inhibitory effect on EC mitogenesis. Of special interest in this regard is the finding of a recent in vivo study showing that the extent of EC recoverage after denudation injury is dependent on the degree of medial wall injury and SMC necrosis (Doornekamp et al., 1996). One could envision that under these circumstances there is increased local activation of TGF–β and inhibition of EC growth.

The precise mechanism by which PDT with 100J/cm² inhibited TGF-β activity associated with SMC injury in this in vitro model is not known. The data clearly indicate that the effect on TGF-β activity is not related to the degree of PDT-mediated cytotoxicity. PDT of SMC with the subtherapeutic dose of 10 J/cm², which reduced cell viability to the same extent as PDT with therapeutic 100 J/cm², was associated with a significant increase in active TGF-β. Likewise, vigorous mechanical SMC injury considerably affected cell viability which was also accompanied by an increase in TGF-β activity. The finding in the present study, and supported by others (Antonelli-Orlidge., 1989), that untreated bovine SMC predominantly produce and secrete TGF-β in a larger latent complex, strongly suggests that with SMC injury a significant portion of the latent TGF-β becomes activated. Although it has been demonstrated that TGF-β plays a role in the development of IH using experimental vascular injury models, it is not known how active TGF-β is generated from the latent complex under these conditions. For the first time it is demonstrated that SMC injury under a defined in vitro condition leads to increased TGF-β activity, which potentially represents a pathway of TGF-β activation after vascular injury. A possible mechanism to explain this may be that cell injury results in the release of proteolytic enzymes (van den Eijnden-Schrauwen., 1995) that could cleave the latent complex and liberate active TGF-β. Because plasmin is known to activate the latent TGF-β propeptide (Lyons et al., 1990), a plasmin inhibitor was used to block its activity. However, the addition of the plasmin inhibitor, aprotinin, did not affect the increased levels of TGF-β associated with SMC injury. The finding that there was in fact a slight increase in active TGF-β when the cells were injured in the presence of aprotinin is not understood. Possibly, this broad serine protease inhibitor may prevent enzymatic degradation of either active TGF-β or factors involved in TGF-β activation after cell injury. It remains to be determined which factors are involved in the activation of TGF-β after cell injury, but this may be a formidable task considering the abundance of proteolytic enzymes and other factors that could be released with cell injury. Since after PDT of SMC with 100 J/cm² there was, albeit decreased, measurable levels of latent TGF-β in the conditioned media, it could be speculated that this PDT dose inactivated the critical factors involved in the activation process. This is a likely assumption since it has been demonstrated that in a dose dependent way, PDT inactivates several enzymes, including plasmin, lysozyme and pepsin (Grossweiner, 1976).

The observed decrease in total TGF-β at 24-hours after both doses of PDT and mechanical injury is likely mediated by the substantial loss of cell viability resulting in decreased production of TGF-β. However, direct effects of PDT on cell-associated TGF-β cannot be excluded. The present study concentrated on the relationship between cell injury and its effects on TGF-β release and activation over a period of 24-hours, but did not assess whether PDT could affect intracellular TGF-β directly. Since after both doses of PDT, there was essentially no SMC survival but still measurable levels of total TGF-β, it can be reasoned that some TGF-β is stored in the cells which is released after cytotoxic injury. This notion is supported by a recent in vivo study that demonstrated histochem-

ically that there is some TGF–β present in untreated medial SMC of the rat carotid (Majesky et al., 1991). Because of the complex regulation of TGF–β activity and the difficulty of conventional immunohistochemical methods to monitor TGF–β activation in vivo, the present study did not assess the effects of PDT on TGF–β in the vessel wall. In chapter 5 it was shown that PDT can directly inactivate matrix-associated TGF–β. The significance of cell-associated TGF–β and whether it can be targeted directly by PDT remains to be investigated.

The reduction in TGF–β activity after PDT of vascular SMC in the model described is not a specific isolated effect. First, after PDT there is considerable reduction in SMC viability. Second, free radicals produced by PDT do not specifically affect enzymes that are involved in TGF–β activation or TGF–β itself. Free radicals react with sensitive amino acids, such as histidine, methionine, tyrosine and tryptophan and thus a myriad of proteins could be affected by the photodynamic affect (Freeman et al., 1982). This study examined the effects of PDT on TGF–β activity in a biological system with vascular SMC in an attempt to mimic the in vivo situation in which SMC are eradicated by PDT. Because free radicals travel a short distance of only nano to micometers, the chemical characteristics and cellular distribution of photosensitizers and the laser light parameters will intrinsically determine whether certain biological molecules will be affected by the PDT-effect (Henderson et al., 1992). The present study examined the effects of PDT with the photosensitizer CASPc, which is known to bind to proteins (Rosenthal, 1991), but whether other photosensitizers with different chemical characteristics could elicit the same effects is not known and requires further study.

The determination that PDT-mediated SMC cytotoxicity is accompanied by specific effects on biological active molecules, such as TGF–β, may be appealing for the clinical application of PDT to prevent restenosis after invasive vascular procedures. The pathogenesis of this clinical condition is multifactorial which may mandate a therapeutic approach that targets more than one pathobiologic factor. Vascular PDT represents a multifactorial approach in that, besides eliminating the effector cells responsible for IH, it may affect other critical biological mediators that regulate the excessive healing response associated with vascular injury. On the other hand, it was clearly demonstrated in the present study that, similar to mechanical injury, PDT mediated cytotoxicity with a subtherapeutic dose, results in increased TGF–β activity. In this way, inadequate PDT could be an additional injurious insult to the vessel wall with the whole sequel of events that lead to injury-associated IH. This finding has important implication concerning the dosimetry of PDT to inhibit IH. In fact, a recent experimental in vivo study indicated that with subtherapeutic doses of PDT, there is eradication of medial SMC, but with subsequent delayed IH development (Ortu et al., 1992). Taken together, it is conceivable that besides SMC eradication, PDT-mediated inactivation of key cellular mediators is pivotal for the successful application of PDT to prevent restenosis. Identification of PDT-parameters to achieve this effect will allow refinement of PDT for application in humans to prevent restenosis.

CHAPTER 7

PDT INACTIVATES CELL-ASSOCIATED bFGF

Adapted from: RG Statius van Eps, F Adili, GM LaMuraglia. Photodynamic Therapy Inactivates Cell-Associated Basic Fibroblast Growth Factor: A Silent Way of Vascular Smooth Muscle Cell Eradication. *Cardiovasc Res* 1997; 35: 334-340.

INTRODUCTION

The effectiveness of any vascular intervention for the treatment of occlusive arterial disease may be limited by restenosis due to intimal hyperplasia (IH), an exaggerated vascular healing response associated with injury to the vessel wall (Chapter 1). A key event in the initiation of IH formation is the injury-induced stimulation of medial smooth muscle cells (SMC) resulting in proliferation and migration to the intima. The extent of this response is known to correlate with the degree of injury to the arterial wall (Fingerle J et al., 1990; Indolfi et al., 1995). The proliferative response of SMC following mechanical injury is thought to be to a large extent mediated by endogenous mitogens, including basic fibroblast growth factor (bFGF), released from damaged cells in the vessel wall (Reidy et al., 1992).

With improved understanding of the IH pathobiology there has been development of novel experimental strategies to deal with this problem (Ross, 1993; Clowes and Reidy, 1991). It has been shown that antibodies to bFGF (Lindner et al., 1991; Nguyen et al., 1994), interference with signal-transduction pathways (Morishita et al., 1990), genetic modulation of the cell-cycle (Indolfi et al., 1995), and low-dose ionizing radiation (Sarac et al., 1995) can decrease the SMC proliferative response and inhibit experimental IH development. In addition, considerable interest has focused on photodynamic therapy (PDT) as a means to locally eliminate the SMC population responsible for the hyperplastic process (Chapter 2).

A remarkable finding after PDT-mediated cell removal to inhibit experimental IH is the lack of an excessive fibroproliferative process in response to injury. Despite extensive cytotoxicity, which represents a principal mechanism of endogenous mitogen release (Reidy et al., 1992), there is no increased proliferation or migration of viable SMC at the boundary between PDT and untreated vessel segment. PDT treated vessels are associated with minimal medial wall SMC repopulation and effective inhibition of experimental IH formation (Chapter 2).

The distinctive vascular healing response after PDT suggests that, besides causing cytotoxicity, PDT-generated free radicals may interfere with important biological mediators that initiate the repair process and thereby profoundly alter the vascular healing process (Chapters 2-5). This may be of particular importance in the vascular system since other means of injury to the vessel wall results in an exuberant healing response with formation of IH (Davies, 1994). The aim of this study was to investigate a mechanism that could explain why PDT-mediated eradication of vascular cells is not followed by a proliferative response. Since bFGF is considered to be a

"wound hormone" that is released from injured and dead cells to activate cell growth (Reidy et al., 1992), this study examined the acute effects of PDT on cell-associated bFGF and pure bFGF. Utilizing an in vitro model, PDT of vascular SMC was performed to test the hypothesis that, besides cytotoxicity, PDT-generated free radicals could target and inactivate cell-associated mitogens, such as bFGF, and thereby inhibit its activity following cell injury.

MATERIALS AND METHODS

Cell Culture

Primary cultures of bovine smooth muscle cells were obtained from the aortas of freshly slaughtered calves by using the explant technique (Chapter 4). Cells were kept in a 37°C, 5% CO_2 incubator, refed every 42-72 hours with DMEM supplemented with 10% calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.6 mol/L L-glutamine (Gibco). Cells were passed at a ratio of 1:5 using 0.05% Trypsin / 0.125% EDTA (Gibco) upon reaching confluence and used for experiments between the 2th and 5th subpassages.

Photodynamic therapy

To perform PDT of SMC in culture, the cells were seeded at a density of either 4 x 10^4 /cm² on 96-well tissue culture plates or 2.5 x 10^4/cm² on 6-well plates (Falcon) and allowed to grow in full medium for 24-hours . The photosensitizer CASPc, at a concentration of 5 µg/ml, was subsequently added to the cells in full medium and incubated for another 24-hours. After two rinses with phosphate buffered saline (PBS), the confluent cell layer was irradiated with thermoneutral light delivered by an argon-pumped dye laser (Coherent) tuned at 675 nm for optimal absorption. The end-fiber irradiance was set at 100 mW/cm² and three different fluences (total energies) were applied: 10, 50 and 100 J/cm². Controls included untreated cells and cells exposed to the photosensitizer or light only. In addition, PDT of SMC was performed in the presence of the free radical quencher, sodium azide (10 and 100mM, Sigma Chemicals, St. Louis, MO) as a separate control (Freeman et al., 1982).

To perform PDT of pure bFGF, a 1 ml serum-free medium solution containing 0.1% bovine serum albumine (Sigma Chemicals), 5 µg/ml CASPc, and 250 pg/ml bFGF (R&D Systems) was placed in the wells of 12 well tissue culture plates and irradiated as outlined above.

Cell Viability Assay

Smooth muscle cell viability was determined 24-hour after PDT treatment using a colorimetric assay based on the uptake of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide salt (MTT, Sigma Chemicals) by viable cells (Chapter 6).

Determination of bFGF Protein Levels

To determine whether PDT could have an acute effect on cellular levels of bFGF, the amount of bFGF protein in SMC lysates was measured using a bFGF immunoassay immediately after treatment. (R&D Systems). In this assay, bFGF in the test sample is sandwiched between a monoclonal antibody against human recombinant bFGF coated on the microtiter plate, and a second polyclonal antibody against bFGF conjugated to horseradish peroxidase. A set of known bFGF concentrations are analyzed in parallel to obtain a standard line with the optical densities of the known bFGF concentrations from which the unknown sample concentrations are calculated. Color is developed by addition of hydrogen peroxide and chromogen tetramethylbenzidine and the intensity measured at 450 nm.

For cell lysate preparation, the medium was removed and the cells lysed immediately after treatment using a cell lysing reagent (Proteins International, Rochester Hills, MI) suspended in the assay diluent (R&D Systems). The cell lysate was then clarified at 2,000 rpm for 15 minutes and assayed within one hour. For the purpose of normalizing bFGF protein levels, a group of untreated SMC were seeded in seperate wells in parallel and counted at the time of PDT treatment. The cell numbers were analyzed after trypsinization using an electronic coulter counter (Multisizer) and represented the amount of cells present at the time of PDT-treatment. To exclude the possibility that immediately after PDT-treatment there was detachment and loss of cells, pilot experiments were performed to determine the number of detached cells in the supernatant after PDT-treatment. The results demonstrated that there was no difference in the number of detached cells between PDT and control cells (data not shown).

Preparation of Conditioned Media

To induce the release of cellular bFGF, PDT- treated and control SMC were injured by vigorously scraping them from the plastic substratum with a rubber policeman in the presence of low serum (1%) medium (Chapter 6). The cells were allowed to condition the medium for 30 minutes at 37°C after which the suspension was centrifuged at 2,000 rpm for 15 minutes. The supernatant was assayed for SMC growth promoting activity and the conditioned media of SMC left undisturbed in their dishes served as control. To determine whether the SMC growth promoting activity of the injured SMC-conditioned media was mediated by bFGF, a neutralizing antibody against bFGF (R&D Systems) or a non-immune control antibody (normal rabbit IgG, R&D Systems) was added to the conditioned media of mechanically disrupted SMC.

Mitogenesis Assay

To functionally evaluate cellular bFGF release associated with SMC injury, [^{3}H]-thymidine incorporation in SMC was used as an indicator of DNA replication (Klagsbrun et al., 1977).

Cells were seeded in low serum medium at a density of 6 x 10^3/cm^2 and allowed to attach for 24-hours. The mechanically disrupted SMC-conditioned media was subsequently added and incubated with the cells for 48-hours and 2.5 mCi of [^{3}H]-thymidine was included in the medium for the last 5 hours. The cells were then washed 3 times with PBS, dissolved in 0.1N NaOH and placed in Ready Gel scintillation fluid. Cell incorporated radioactivity was counted with a scintillation counter.

To directly examine the effect of PDT on the mitogenic activity of pure bFGF, PDT was performed of bFGF in solution as described above. The PDT treated bFGF solution was then supplemented with calf serum to make a 1% calf serum solution and added to SMC to evaluate SMC mitogenesis. Baseline control for these experiments included 1% calf serum medium containing 0.1% BSA and 5 µg/ml CASPc, and the positive control was a non-irradiated bFGF solution containing the photosensitizer.

Statistical Analysis

All data is expressed as mean ± standard deviation (SD). For a comparison of means between multiple groups, a one-way analysis of variance and Tukey's HSD post hoc test for multiple comparisons was applied (Statistica, Statsoft, Tulsa, OK). p-values of less than 0.05 were considered significant.

RESULTS

PDT-mediated SMC cytotoxicity

To study the relationship between PDT-mediated cytotoxicity and its effects on cell-associated bFGF, SMC viability was assessed after in vivo therapeutic (100J/cm^2) and subtherapeutic (50 and 10J/cm^2) doses of PDT (Ortu et al., 1992). Immediately after PDT-treatment with all doses, the cells remained attached to the culture plates and there were no gross changes in cell morphology as assessed by phase contrast microscopy. Approximately 1 hour after PDT-treatment, changes in the cell shape with deterioration of the normal cell membrane contour was first observed. Although most cells remained attached to the tissue culture plate, there was no evidence of SMC survival with PDT-doses of 50 and 100J/cm^2 ($p< 0.0005$) as determined by the tetrazolium salt (MTT) conversion assay (Fig. 7.1). After PDT with 10J/cm^2, SMC viability decreased to around 50% ($p< 0,0005$), whereas, exposure of SMC to either light (100J/cm^2) or photosensitizer only had no effect on cell viability (Fig. 7.1).

PDT effects on cell-associated bFGF

To determine whether PDT-mediated cytotoxicity is accompanied by specific effects on cell-associated bFGF, the concentration of bFGF in SMC lysates was quantitated by ELISA in

untreated controls and immediately after PDT treatment. PDT of SMC resulted in a dose-dependent decrease in cellular levels of bFGF (Fig. 7.2). Whereas, 10J/cm² failed to affect cellular bFGF levels, there was a significant decrease ($p < 0.0005$) after PDT with 50 and 100J/cm². The effect of PDT on cell-associated bFGF required the production of free radicals since the interaction of light or the photosensitizer alone, did not affect bFGF levels (Fig. 7.2). To exclude the possibility that cellular bFGF leaked out of the cells during laser irradiation, bFGF was measured in the medium immediately after PDT treatment. No detectable levels of bFGF could be measured in the medium of either PDT-treated SMC, or untreated cells (data not shown).

To further examine whether the PDT-effect on cell-associated bFGF was mediated through the generation of free radicals, a free radical quencher, sodium azide, was added to SMC immediately prior to irradiation. The addition of sodium azide to non-treated SMC did not affect the cellular levels of bFGF (data not shown). However, sodium azide protected cellular bFGF levels from the PDT-effect in a dose dependent manner (Fig. 7.3).

Functional consequence of PDT effects on cell-associated bFGF

To assess whether the reduced measurable levels of cell-associated bFGF after PDT had a functional significance, a SMC mitogenesis assay was used to determine bFGF growth-promot-

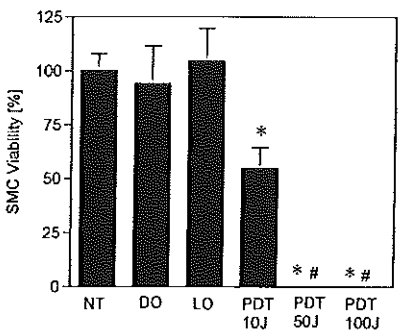


Figure 7.1.
*Effect of PDT on smooth muscle cell viability. Viability, as determined by the tetrazolium salt conversion assay, of PDT-treated SMC with total fluences of 10J/cm², 50J/cm² and 100J/cm² and SMC exposed to either the photosensitizer (DO) or light (LO) only is compared to untreated (NT) SMC viability, which represents 100% viable cells. Values are mean ± SD, * denotes $p < 0.0005$ versus NT, DO and LO, and # denotes $p < 0.0005$ versus PDT with 10J/cm2 (ANOVA, n = 6).*

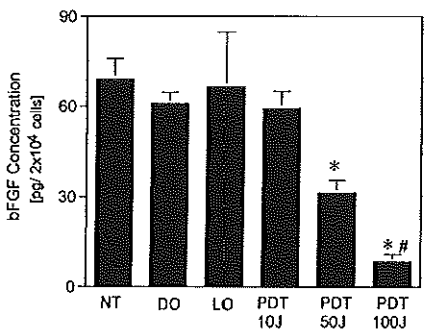


Figure 7.2.
*Effect of PDT on cell-associated levels of bFGF. The concentration of bFGF, as measured by ELISA, in SMC lysates is plotted for PDT of SMC with 10J/cm², 50J/cm² and 100J/cm² and the different controls: non-treated (NT) and exposure of SMC with the photosensitizer drug (DO) or light (LO) only. Values are mean ± SD, * denotes $p < 0.0002$ versus NT, DO, LO and PDT 10J/cm², and # denotes $p < 0.0005$ versus PDT 50J/cm² (ANOVA, n = 8 for NT, PDT 10, 50 and 100J/cm², n = 6 for LO and DO).*

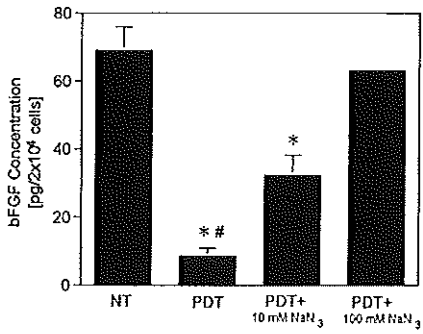


Figure 7.3.
*Reversal of the PDT-effect on cell-associated levels of bFGF by the free radical quencher, sodium azide. The concentration of bFGF in SMC lysates is plotted for non-treated SMC (NT), PDT-treated SMC with 100J/cm² (PDT) and PDT-treated SMC that received sodium azide (PDT + 10mM and 100mM NaN_3). Values are mean ± SD, * denotes p< 0.0005 versus NT and PDT + 100mM NaN_3, and # denotes p< 0.0005 versus PDT + 10mM NaN3. (ANOVA, n = 8 for NT, n = 3 for PDT + 10 and 100mM NaN_3).*

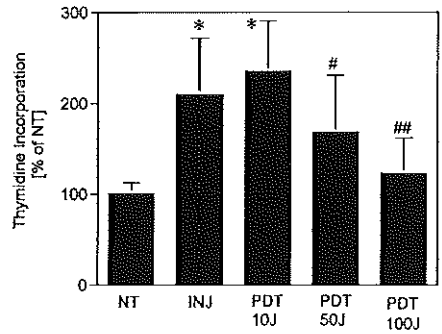


Figure 7.4.
*Effect of PDT on growth-promoting activity of injured SMC-conditioned media. The conditioned media of control non-treated (NT) SMC, mechanical injured (INJ) SMC and injured SMC that were PDT-treated with 10J/cm², 50J/cm² and 100J/cm², were used to assess its growth-promoting activity (3H-thymidine incorporation) on SMC. Values are mean ± SD, * denotes p< 0.0002 versus NT, # denotes p< 0.01 versus NT and # # denotes p< 0.001 versus INJ (ANOVA, n = 25 for NT, n = 15 for INJ and n = 9 for PDT 10, 50 and 100J/cm²).*

ing activity. For this purpose, mechanical injury of cells was utilized as a means to release cellular bFGF (McNeil et al., 1989). As expected, the conditioned media of mechanically injured SMC significantly increased SMC mitogenesis (p< 0.0005) as compared to the conditioned media of untreated SMC (Fig. 6.4). Correlating with the PDT-effects on cellular levels of bFGF, PDT of SMC prior to mechanical injury decreased the growth-promoting activity of the conditioned media in a dose dependent manner (Fig. 7.4).

To assess the importance of bFGF in the increase of SMC mitogenesis after mechanical injury, an anti–bFGF neutralizing antibody was used to inhibit its activity. Addition of the antibody to the conditioned media of mechanical wounded SMC, completely removed its growth-promoting activity (Fig. 7.5). In fact, the presence of the antibody in the conditioned media of injured SMC decreased SMC mitogenesis below the level of untreated SMC control. Addition of the antibody to control medium also significantly (p< 0.001) reduced SMC mitogenesis (58.5 ± 23.5%, n= 6) below the level of untreated SMC control. These findings may be explained by the inhibition of bFGF present in 1% calf serum medium used to prepare the conditioned media (see methods). Alternatively, the presence of the antibody during the incubation time could inhibit autocrine bFGF function in SMC (Mignatti et al., 1991).

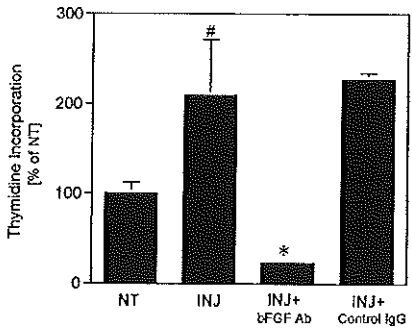


Figure 7.5.
*Neutralizing antibody against bFGF inhibits growth-promoting activity of conditioned media of injured SMC. The conditioned media of control non-treated (NT) SMC, mechanical injured (INJ) SMC and injured SMC that were pre-treated with an anti-βFGF neutralizing antibody (20 mg/ml) or a normal rabbit IgG control (20 mg/ml), were used to assess its growth-promoting activity (^{3}H-thymidine incorporation) on SMC. Values are mean ± SD, * denotes p< 0.0005 versus NT, INJ and INJ + control IgG, and # denotes p< 0.0002 versus NT. (ANOVA, n = 25 for NT, n = 15 for INJ, n = 6 for INJ + bFGF Ab and n = 3 for INJ + control IgG).*

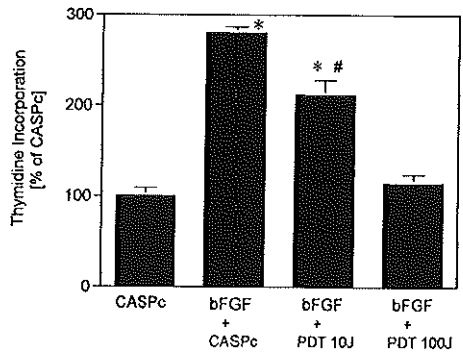


Figure 7.6.
*PDT-mediated inhibition of bFGF activity. The functional activity of pure bFGF in solution was determined with a SMC mitogenesis assay (^{3}H-thymidine incorporation). PDT of bFGF in solution (250 pg/ml) was performed with fluences of 10 and 100 J/cm². Negative control included a 5 mg/ml CASPc medium solution (CASPc), which was compared to SMC mitogenesis exposed to an untreated bFGF-CASPc solution (bFGF + CASPc), as a positive control, and light irradiated bFGF-CASPc solutions (bFGF + PDT 10 and 100J). Values are mean ± SD, * denotes p < 0.0005 versus CASPc, and bFGF + PDT 100J, and # denotes p < 0.001 versus bFGF + CASPc (ANOVA, n = 3).*

PDT effects on bFGF in solution

To confirm, that PDT can inactivate the SMC mitogenic function of bFGF, PDT was performed of pure bFGF in solution. The addition of 250 pg/ml of bFGF to SMC lead to a significant increase in SMC mitogenesis (Fig. 7.6). In a light-dose dependent way, PDT of bFGF resulted in a decrease in its mitogenic activity on SMC (Fig. 7.6). These results confirm that bFGF is sensitive to the photochemical reaction induced by PDT, which leads to inactivation of its SMC mitogenic function.

DISCUSSION

The principal finding of this study is that PDT-generated free radicals inactivate cell-associated bFGF. Consequently, the bFGF induced stimulation of SMC mitogenesis after cellular injury in vitro is inhibited by PDT. In this regard, PDT represents a unique way of cell eradication, since other means of either sublethal or lethal cell injury have been associated with bFGF release. It has been demonstrated that mechanical injury and disruption of the plasma membrane of endothelial cells resulted in release of a bFGF-like molecule (McNeil et al., 1989). In accor-

dance with these findings, a recent study showed that mechanical injury to SMC in culture caused release of bFGF that activated replication of neighboring SMCs (Calara et al., 1996). Furthermore, other forms of cell injury such as, hypoxia (Michiels et al., 1994), endotoxin (Gadjusek et al., 1989), and gamma irradiation (Witte et al., 1989) have been shown to cause cellular bFGF release. These experimental data and the fact that bFGF does not possess a signal peptide to direct its release via the "classic" secretory pathway has led to the proposal that cell death or injury are the most likely mechanisms for cellular bFGF release (Mignatti and Rifkin, 1991). The importance of bFGF in stimulating SMC mitogenesis after SMC injury was substantiated by the present study, which demonstrated complete inhibition of these effects with a neutralizing bFGF antibody.

In support of the concept that bFGF acts as a "wound hormone" to initiate tissue repair after injury, there is strong evidence to indicate that this mitogen plays an important role in the initial proliferative response of SMC after vascular injury (Lindner et al., 1991). Balloon injury and other forms of injury to the vessel wall such as, thermal injury (Douek et al., 1992), stent implantation (Bai et al., 1994) and gamma irradiation (Phillips et al., 1992), which are all associated with widespread cell death, results in an increased SMC proliferative response. The extent of SMC proliferation and subsequent neointimal formation has been found to be proportional to the degree of vascular injury (Fingerle et al., 1990; Indolfi et al., 1995). The role of bFGF in these initial injury responses has been confirmed by the finding that anti–bFGF antibodies can inhibit SMC proliferation (Lindner and Reidy, 1991) and supress neointimal lesions after experimental balloon-injury (Nguyen et al., 1994).

PDT of the vessel wall is a form of injury that results in local eradication of vascular cells, and yet unlike other forms of injury, there is an absence of a proliferative or inflammatory response. This consistent histologic finding after therapeutic doses of PDT in experimental models of IH, formed the basis of this study. It brings up the consideration that PDT-mediated cell injury does not only target cellular membranes but may also affect cell-associated bFGF and possibly other important cellular cytokines. The present study focused on the effects of PDT on cell-associated bFGF because of its well established biological importance in initiating SMC proliferation after cell injury (Reidy et al., 1992; Lindner et al., 1991). It has been demonstrated that proteins can undergo free radical-induced modification. (Adili et al., 1996; Freeman and Crapo, 1982). Free radicals react with sensitive amino acids such as, histidine, methionine, tyrosine and tryptophan, and the susceptibility of proteins to free radical damage depends mainly on their amino acid composition, the importance and location of susceptible amino acids that mediate protein conformation and activity and the cellular location (Freeman and Crapo, 1982). Whether PDT-generated free radicals can target and affect cell-associated growth factors, such as bFGF, has not been studied before. In Chapter 4, it was shown that SMC-growth was inhibited on PDT-treated ECM. Since bFGF, a powerfull SMC-mitogen, is known to be present in ECM deposited by EC another study performed by this laboratory addressed the premise

that PDT may inactivate matrix-associated bFGF. LaMuraglia et al (1997) demonstrated that inhibition of SMC-growth on ECM is at least in part mediated by inactivation of matrix-bound bFGF.

The present study demonstrated that free radicals generated in SMC can destroy cell-associated bFGF. The sensitivity of bFGF to PDT was confirmed by the finding that PDT of pure bFGF in solution inhibited its SMC mitogenic function. The requirement of PDT-produced free radicals to inactivate bFGF was demonstrated by the fact that neither the photosensitizer or the light only had any effects on bFGF. In addition, the free radical quencher sodium azide, protected bFGF from the PDT-induced reaction. Sodium azide primarily quenches singlet oxygen, but can also react with other excited states (Freeman et al., 1982). Therefore, no conclusion can be drawn as to which specific free radical pathways are involved in the inactivation of bFGF.

The effects of PDT on cell-associated bFGF was studied as a model to test the hypothesis that free radicals can react with cellular mediators and thereby modify the biological response associated with cell injury. Because of potential free radical reactions with proteins, lipids and other chemical structures, it should be emphasized that PDT does not specifically affect a biological factor. In fact, in chapter 4 and 5 it was demonstrated that PDT-produced free radicals can profoundly alter the biological characteristics of extracellular matrix and inactivate matrix-associated transforming growth factor–β. Thus, the distinct vascular healing response after PDT-induced SMC eradication is likely of a multifactorial nature, mediated by free radical cytotoxicity and potential chemical reactions with a host of cellular mediators.

Of special interest in the present study was the finding that the threshold doses for PDT-mediated cytotoxicity was lower then for cellular bFGF inactivation. Although PDT with 10J/cm^2 caused significant SMC death, it had no effect on cellular bFGF levels. Likewise, PDT with 50J/cm^2 and 100J/cm^2 equally eradicated cell viability, but there was significant less effect on cell-associated bFGF after doses of 50J/cm^2 as compared to PDT with 100J/cm^2. This observation, which correlated with the mitogenic response of SMC after different doses of PDT, may have important implications concerning the dosimetry of PDT for clinical application. Considering the importance of bFGF and other growth factors in determining the outcome of the vascular response to injury it can be conceived that mere eradication of the SMC population may not be sufficient for effective inhibition of IH. It can therefore be speculated that besides cytotoxicity, PDT-mediated inactivation of bFGF and potentially other cellular biological mediators may be imperative for its successful inhibition of experimental neointima formation. If this can be achieved in the clinical setting, PDT may prove to be a silent and effective way of eradicating the SMC population involved in neointima formation and the problem of restenosis.

CHAPTER 8

EFFECTS OF PDT ON THE VASCULAR FIBROTIC RESPONSE

Adapted from: Statius van Eps RG, Mark L, Schiereck J, LaMuraglia GM. Photodynamic Therapy Inhibits the Injury-Induced Fibrotic Response of Vascular Smoooth Muscle Cells. *Eur J Endovasc Vasc Surg* 1999 (in press).

INTRODUCTION

Restenosis due to intimal hyperplasia (IH) limits the long-term patency of catheter-based and surgical procedures for the treatment of atherosclerotic occlusive disease (Bauters et al., 1996). IH is a local fibrotic lesion that develops in response to vascular wall injury. Sustained production and deposition of extracellular matrix (ECM) components, such as collagen, by intimal SMC are prominent features of restenosis. This connective tissue mass forms the bulk of the intimal lesion encroaching on the vessel lumen which may ultimately cause loss of patency (Davies and Hagen, 1994).

Numerous mechanical and pharmacological approaches have been used to inhibit the occurrence of IH, however none of them has been proven clinically successful in preventing the development of restenosis (Chapter 1). Among several novel experimental investigations, such as gene-therapy and ionizing irradiation, photodynamic therapy (PDT) has gained interest as an approach to inhibit injury-induced IH (Chapter 2). Inhibition of IH by PDT in balloon-injury models has been related to depletion of medial SMC at the site PDT-treatment. However, recent in vitro studies have shown that PDT-mediated changes in the ECM and its interaction with biologically active proteins may be important to provide a favorable healing response after vascular PDT.

The SMC fibroproliferative response following vascular injury is to a great extent mediated by the release and activation of cytokines from adhering platelets and damaged vascular cells. Subsequent deposition of fibroproliferative factors, such as platelet derived growth factor (PDGF), in the surrounding ECM is believed to play an important role in the sustained stimulation of cells for matrix production (Chapter 1). To examine whether PDT could interfere with the fibrotic response associated with vascular injury, the production of collagen by SMC was monitored after PDT-treatment of isolated ECM, injured SMC in culture and the polypeptide PDGF.

MATERIALS AND METHODS

Cell Culture

Primary bovine aortic SMC and endothelial cell (EC) cultures were obtained from aortas of slaughtered calves and cellular identity confirmed as previously described (Chapter 4). Both cell types were kept in a 37°C incubator in a concentration of 5 % CO_2. Cells were passed using 0.05% trypsin (Gibco) and subcultures from passages 2 through 4 were used for experiments.

Preparation of ECM

EC-derived ECM was prepared as previously described (Chapter 4). In brief, the cells were seeded at a density of 1.0 x 10^5 on twelve well plates and grown to confluence for 7 days. The cell monolayer was removed by incubation with a phosphate-buffered saline (PBS) solution containing 0.5 % Triton X-100 and 20 mmol/L NH_4OH for 30 minutes. After rinsing x3 with PBS, the resultant ECM coating the cell-culture plates was covered with 1 ml PBS and used immediately for experiments.

Photodynamic Therapy

PDT was performed on isolated ECM, SMC in culture, and pure PDGF. Careful measures were taken to minimize ambient light exposure of the preparations in all experiments. To conduct PDT of ECM, the matrix coated plates were covered with the photosensitizer chloroaluminum sulfonated phtalocyanine (CASPc) diluted in PBS (5 µg/ml) and irradiated with an argon-pumped dye laser using in vivo relevant light parameters (wavelength: 675 nm; irradiance: 100 mW/cm²; fluence: 10 and 100 J/cm²). Controls included plates without ECM, non-treated ECM, laser only irradiated or drug only exposed ECM.

To perform PDT of SMC, 2.5 x 10^5 cells were seeded on 6-well plates. After 24-hours incubation time the medium was removed and the cells were incubated for two hours with either PBS or CASPc (5 µg/ml). The cells were then irradiated as described above with fluences of 10 and 100 J/cm². Immediately after PDT-treatment, the CASPc was removed and the cells incubated in serum-free 0.1% bovine serum albumin (BSA) medium for preparation of conditioned media.

To perform PDT on pure PDGF, a solution of CASPc (5 µg/ml), 5 ng/ml of PDGF-BB (Gibco) and 0.1 % BSA was prepared. One ml of this solution was placed on 12 well-plates and irradiated as above with fluences of 10 and 100 J/cm². The solution with CASPc only served as baseline control and non-irradiated CASPc-PDGF solution as positive control.

Preparation of conditioned media

These experiments were performed to examine whether PDT of SMC could interfere with fibroproliferative factors released after cell-injury. To have an in vivo relevant positive control, SMC were mechanically injured with a rubber policeman as described (Chapter 8). For preparation of conditioned media, both PDT and untreated cells were mechanically injured immediately following PDT-treatment. Cell survival is reduced to less than 10% in all groups after cell scraping (data not shown). The cells were than incubated for 24-hours to allow the cells to disintegrate and release biologically active proteins. After this time, the medium was collected and centrifuged at 2000 rpm to remove cellular debris and the supernatant collected.

Collagen and Protein Production

The production of collagen by SMC was evaluated by incorporation of [^{3}H]-proline into insoluble cell protein, which is largely collagenous (McCaffrey et al., 1996). For assessment of total protein synthesis, [^{3}H]-leucine incorporation was determined.

For the ECM experiments, SMC were seeded at a density of 1.0 x 10^5 cells in 1% calf serum medium on the prepared matrices. Following 24 hours of incubation at 37 °C, 10 μCi of L-[2,3,4,5-^{3}H]-proline (23 Ci/mmol) or 5 μCi of L-[4,5-^{3}H]-leucine (60 Ci/mmol) (Amersham, Arlington Heights, IL) was added to the SMC for another 24 hours of incubation. For the conditioned media and PDGF experiments, the effector SMC were seeded on empty plates and after 24-hours of incubation, the media was removed and the prepared conditioned media or PDGF-solutions were added together with [^{3}H]-proline for 24-hours. In all collagen production experiments, 50 μg/ml of ascorbic acid was included in the medium.

After incubation, the medium was removed and the cell layer washed 3 times with PBS. Subsequently, 0.5 N NaOH was added to dissolve the cells. The collected material was precipitated with 20 % Trichloroacetic acid (TCA) to remove the non-incorporated radioactive amino acids, and 1 % BSA was added as a carrier. The solution was allowed to flocculate for 30 minutes and centrifuged at 14000 rpm for 30 minutes. Supernatant was then removed and 5% TCA was added twice for washing. Finally, 1N NaOH was incubated with the pellet for 24 hours at 37°C and an additional 3 hours at 60° C to dissolve the protein material. The solution was supplemented with 3.0 ml of liquid scintillation cocktail (Beckman Instrument, Inc., Fullerton, Calif.) and radioactivity was determined with an automatic radioactive counter (Beckman # LS 3801). For the purpose of normalizing protein content to cell number, a group of SMC treated similarly to the experimental group was run in parallel and the cell number analyzed by an electronic coulter counter after removal with 0.05% trypsin.

Statistics

All data is expressed as mean ± standard deviation (SD). For comparison of means between multiple groups, a one-way analysis of variance and Tukey's HSD post hoc test for multiple comparisons was applied (Statistica, Statsoft, Tulsa, OK). p-values of less than 0.05 were considered significant.

RESULTS

PDT effects on ECM-induction of SMC collagen production

ECM deposited by EC in vitro is known to contain numerous profibrotic factors, such as transforming growth factor–β and PDGF (Field et al., 1996; Taipale et al., 1995). These experiments assessed whether PDT of ECM could modify SMC production of collagen. SMC seeded on

ECM appeared healthy and there were no morphologic differences between cells grown on untreated ECM or PDT-treated ECM (figure 8.1). However, SMC grown on PDT-treated ECM demonstrated a significant decrease (62.4 ± 6.5%, p = 0.0001) in [^{3}H]- proline incorporation as compared to untreated matrix (100.0 ± 7.5%). Laser irradiation only or drug exposure only of the ECM did not have any effect (figure 8.2). As compared to untreated ECM, SMC grown on an empty plate also showed a decrease (p = 0.0001) in [^{3}H]-proline incorporation (figure 8.2).

The decrease in collagen synthesis by SMC grown on PDT-treated ECM did not appear to be specific for collagen since total protein synthesis (^{3}H-Leucine) was similarly affected. As

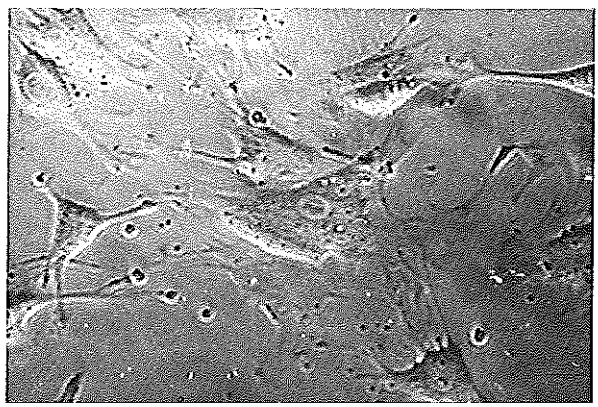
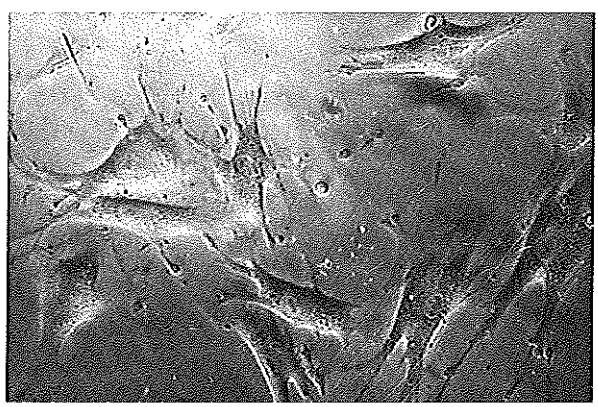


Figure 8.1.
Contrast photomicrograph of vascular SMC seeded on untreated ECM (above) and PDT-treated ECM (below). The pictures were made 24-hours after seeding to allow for proper attachment and organization of the cells. Note that there is no difference in the morphologic appearance between the SMC on the two different matrices.



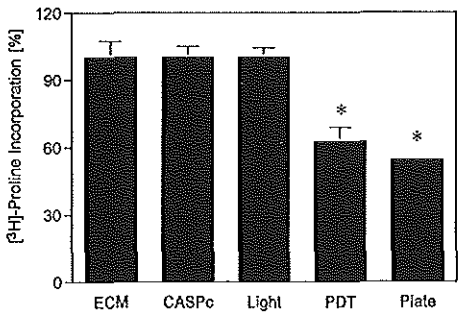


Figure 8.2.
*Inhibition of SMC collagen production grown on PDT-treated ECM. Collagen production, measured by [³H] proline incorporation into cell associated proteins, by SMC seeded on untreated ECM and compared with collagen production by SMC on ECM treated with CASPc only (CASPc) or light only (light), PDT-treated ECM (PDT) and plates not coverd by ECM (plate). Values are mean ± SD expressed as a percentage of baseline control (untreated ECM), * denotes p < 0.0005 versus ECM, n = 9 for ECM, n = 6 for PDT and CASPc only, and n = 3 for light only and culture plate.*

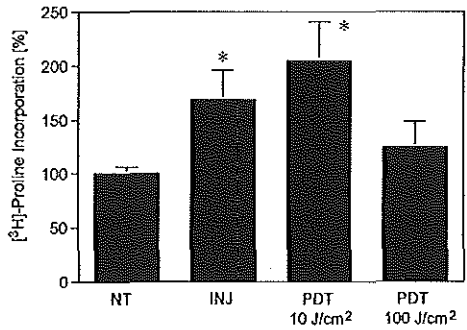


Figure 8.3.
*PDT inhibits SMC-injury associated profibrotic response. The conditioned media of control non-treated (NT) SMC, mechanical injured (INJ) SMC and injured SMC that were PDT-treated with 10 J/cm² and 100 J/cm², were used to asses its activity on SMC production of collagenous proteins ([³H] proline incorporation). Values are mean ± SD expressed as a percentage of baseline NT control, * denotes p < 0.0005 vs. NT, n = 6 in all groups except n = 9 in NT.*

compared to total protein synthesis by SMC grown on untreated matrix (100 ± 4.9%) there was a reduction to 59.1 ± 6.6% (p = 0.0002) by SMC grown on PDT-treated matrix and to 67.1 ± 2.6% (p = 0.0002) by SMC grown on an empty cell culture plate.

Effect of PDT on injury-associated stimulation of collagen production

SMC are known to contain cytokines that are released upon cell-injury. These experiments confirmed that mechanically injured cells could induce a fibrotic response and that PDT could interfere with this process (figure 8.3). As compared to the conditioned media of untreated SMC, the conditioned media of mechanically injured SMC caused a significant increase (168.5 ± 27.6%, p = 0.0004) in collagen production. Whereas PDT of SMC with 10 J/cm² prior to mechanical injury did not affect (204.9 ± 36.2%, p = 0.0002 vs. untreated) this fibrotic response, PDT with 100 J/cm² significantly reduced (124 ± 24%, p = 0.03 versus injured SMC) the collagen production associated with SMC injury and was similar to uninjured cells (100,0 ± 6.5%, p = 0.26 vs. untreated).

Effect of PDT on PDGF-stimulation of collagen production

To evaluate whether PDT-mediated decrease in collagen production could be mediated

by inactivation of profibrotic factors, these experiments directly examined the effects of PDT on PDGF. PDGF-mediated stimulation of SMC resulted in a significant increase (181.2 ± 19.8%, p = 0.0002) in collagen production versus control (100,0 ± 3.9%). In a dose dependent way, PDT of PDGF inhibited this profibrotic response. At 100 J/cm², there was a significant decrease (124.3 ±

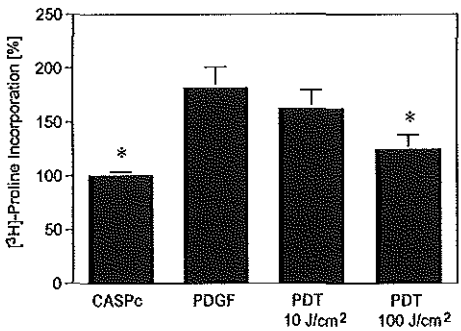


Figure 8.4.
*PDT inhibits the profibrotic ativity of PDGF. A solution of PDGF (5 mg/ml) containing the photosenstizer CASPc (5 mg/ml) was directly irradiated with 675 nm light for and incubated with SMC. Collagen production ([³H] proline incorporation) of SMC incubated with CASPc without PDGF served as baseline control and compared with the CASPc-PDGF solution as positive control and PDT with 10 and 100 J/cm². Values are mean ± SD, expressed as a percentage of CASPc only control * denotes p < 0.005 vs. PDGF, n = 6 for all groups.*

13.7%, p = 0.0002) in SMC collagen production as compared to non-irradiated PDGF (Fig. 8.4).

DISCUSSION

Restenosis develops as the result of an aberrant wound healing process which involves an excessive fibroproliferative response associated with injury to the vessel wall. Vascular PDT is a local experimental approach to inhibit IH based on the acute depletion of cells at the site of treatment. However, the ultimate outcome of the vascular healing process following injury and subsequent PDT is also dependent on the biological behavior of surviving vascular cells, namely at the borders of the PDT-treatment field (Chapter 3).

Proliferation and migration of SMC are early events in the vascular injury response, but sustained production of connective tissue proteins by SMC in the intima is responsible for the bulk of the intimal lesion (MacLeod et al., 1994). Although it is not clear why SMC continue to produce excessive amounts of matrix proteins, it has been suggested that matrix-associated cytokines could be a source of long term stimulation of SMC matrix production. Release of several factors, such as PDGF and TGF–β, from adhering platelets and injured SMC represent an important pathway for deposition of these bioactive molecules in the ECM (Reidy et al., 1992; Schwartz et al., 1995). The present study was designed to examine whether PDT could interfere with these factors that regulate the fibrotic response associated with vascular injury. An in vitro model was used which enabled the evaluation of PDT-effects on sepa-

rate elements involved in SMC production of collagen and protein.

The findings show that as compared to SMC seeded on empty cell-culture plates, there is a significant increase in collagen and total protein production by SMC seeded on EC-derived ECM. This increase in collagen production is also noted when cells are incubated with the conditioned media of injured SMC. These results confirm the presence of matrix and cell-associated factors that stimulate SMC production of collagen and other proteins. It is known from the literature that matrix deposited by EC in culture contains many cell growth regulators, including PDGF, TGF–β and basic fibroblast growth factor (bFGF) (Rogelj et al., 1989). In addition, SMC in culture are known to contain several growth regulatory peptides and it has been shown that mechanical injury results in growth factor release (Calara et al., 1989). Some of these growth factors are also potent profibrotic factors for different cell types including SMC. Both TGF–β and PDGF have been shown to stimulate matrix production in SMC and are believed to play an important role in the fibrotic process after vascular injury (Nabel et al., 1993; Bendeck et al., 1996).

The present data show that SMC grown on PDT-treated ECM results in a significant reduction in collagen and protein synthesis. This inhibition of collagen production is also observed with the conditioned media of PDT-treated SMC. These findings implicate that PDT affects matrix and cell-associated profibrotic factors. It is known, that PDT-generated free radicals can chemically react with certain amino acids which may result in structural alterations and dysfunction of proteins (Freeman et al., 1982). It is therefore likely that certain profibrotic factors in the matrix and SMC are affected by the photochemical reaction induced by PDT. Considering the presence of several cell and matrix-associated factors that could potentially affect collagen production, this study did not attempt to pinpoint which specific factors were inactivated by PDT. Furthermore, since free radical-sensitive amino-acids are present in numerous polypeptides, it is highly likely that various growth factors and cytokines are affected by PDT.

To directly examine the effects of PDT on a biological relevant fibroproliferative factor, PDT on pure PDGF was performed. The results clearly demonstrated that in a dose dependent way, PDT inhibited the profibrotic properties of PDGF. These findings complement the results of recent studies from this laboratory which demonstrated that both TGF–β and bFGF are sensitive to the photochemical reaction induced by PDT (Chapters 5-8). It is important to note that all these studies used the photosensitizer CASPc, which is known to bind to albumin and other proteins. Since free radicals travel a very short distance, proximity of the photosensitizer to the protein may be important in eliciting these effects. It is unknown if lipophilic photosensitizers, such as photofrin, could react with biological molecules involved in the pathogenesis of IH.

The findings in this study provide further support to the concept that besides cytotoxicity, PDT can inactivate cellular mediators involved in the vascular healing response. These effects may be of pivotal importance in order to suppress the injury-induced SMC fibroproliferative reaction that underlie the development of IH. Better understanding of PDT-effects on the vascular wall are necessary for further development of this novel approach to prevent clinical restenosis.

CHAPTER 9

GENERAL DISCUSSION

GENERAL DISCUSSION

The treatment of obstructive vascular disease with surgical and endovascular procedures is limited by the development of restenosis. Among the several processes that can cause restenosis including vascular recoil, thrombosis and chronic remodeling, intimal hyperplasia (IH) plays an important role. The procedure-related mechanical trauma to the vascular wall encites an healing response involving migration and proliferation of smooth muscle cells in the intimal space with excessive deposition of extracellular matrix. This fibroproliferative response can result in an intimal mass wich encroaches on the vessel lumen causing luminal narrowing.

The lack of an effective strategy to prevent restenosis has stimulated a great deal of research in this field, but to date all clinical studies have fallen short to achieve this goal. It is not entirely clear why certain drugs such as ACE-inhibitors, which were effective in the basic animal models to inhibit IH, failed to prevent restenosis in the clinical setting. This failure contested the role of small animal models that cannot mimic the human situation in which complex atherosclerotic lesions are treated. In addition, the failure of antiproliferative agents have questioned the importance of proliferation in the pathogenesis of angioplasty-related restenosis (Schwartz et al., 1995). Although very important considerations, it should also be emphasized that the reason of failure in these clinical studies were not well investigated and it is not known whether there was indeed effective inhibition of intimal mass (Pratt and Dzau, 1996). New theories on the etiology of clinical restenosis in the human situation are emerging. There has recently been much attention on the role of vascular remodeling which basically suggest that the vascular wall is able to adapt its size to maintain sufficient lumen caliber in the setting of a growing intimal mass. Lack of compensatory enlargement may be of great importance in causing restenosis after angioplasty (Gibbons and Dzau, 1994). It is clear that more knowledge is needed to better understand the mechanisms of remodeling and its significance in restenosis, but the participation of an intimal mass in contributing to restenosis seems undisputed. This is substantiated by the problem of restenosis after stent-placement in which the vessel wall is mechanically enlarged and IH develops through the stentwires to cause luminal narrowing. Further development and application of intravascular ultrasound seems a promising tool to address the role of hyperplastic mass and vascular remodeling in the pathogenesis of restenosis and to examine the effects of novel therapeutic strategies (van Urk, 1998).

Vascular PDT is a novel strategy being investigated to supress intimal hyperplasia asso-

ciated with vascular and endovasclar procedures. The motivation behind this approach has been that free radicals produced at the site of treatment could eradicate the SMC-population responsible for the proliferative process. Principally, this is an illogical biological reasoning considering the basic concept that IH develops as the result of cell death and damage to the vessel wall as demonstrated in several experimental vessel wall injury models (Reidy et al., 1992). With sufficient dosimetry, PDT of the vascular wall results in massive vascular cell eradication at the site of treatment. However, the healing response of the uninjured rat carotid artery subjected to PDT is characterized by the lack of an intimal hyperplastic response (chapter 3). Instead, the eradication of vascular cells is followed by reendothelialization and adventitial repopulation but a lack of medial SMC ingrowth. This favorable healing response can also be achieved if vascular PDT is preceded by balloon injury and in this way IH is effectively inhibited (chapter 2). The enthusiasm for vascular PDT was stimulated by these histological observations, with the premise that if this can be achieved in humans, the hyperplastic mass invoved in restenosis can be inhibted. However, the mechanisms underlying the characteristic healing process after PDT in the described animal models are not understood and therefore it is not known which factors are critical to achieve this favorable response.

The studies described in this thesis were undertaken to gain better understanding in the vascular healing process after PDT. The main hypothesis was that mere PDT-mediated cytotoxicity cannot explain the distinctive histological aspects seen after vascular PDT. In an in vitro model which enabled the study of PDT-effects on several biological factors involved in vascular healing, it was demonstrated that free radicals produced by PDT can alter extracellular matrix and react with important cellular mediators. In a series of experiments, the consequence of this photochemical reaction for vascular SMC and EC function was explored. The principle outcome of these studies was that, besides cytotoxicity, PDT using the photosensitizer CASPc can inactivate biologically active proteins such as, TGF-β, bFGF and PDGF. These polypeptides are known to be released and activated after vascular injury and thereby regulate the healing process. Overproduction and dysregulation of these mediators during the repair process is believed to contribute to the formation of an exaggerated intimal mass (Wolf et al., 1994; Lindner et al., 1991; Bendeck et al., 1996). The simultaneous acute eradication of vascular cells and inactivation of biologically active proteins by PDT, radically alters the normally occuring hyperplastic injury response. It can be reasoned that PDT-induced reduction of the cytokine load could result in a more appropriate healing process and mask the initial injury. Thus, whereas mechanical and other forms of injury results in vascular cell damage and cytokine elaboration, PDT can induce cytotoxicity and cytokine inactivation. This principle difference is well illustrated in the studies described in chapter 3 which demonstrated how IH develops in a PDT treated artery if there is mechanical injured vascular tissue near the PDT-treated area.

The nature and extent of PDT-mediated cytotoxicity and cytokine inactivation depends on a multitude of factors, e.g.; photosensitizer concentration, cellular and extracellular distribution of the photosensitizer, protein binding characteristics, presence and activation state of cytokines, oxygen-status and light fluence. PDT is a technique in which the treatment-parameters can be adjusted in various ways. There are several photosensitizers available and the light-doses and drug concentrations can be altered. In addition, the timing, route of photosensitizer administration and irradiation, and treatment field are impotant changeable options. The experiments described herein, used only one photosensitizer at a set concentration and the light-doses were changed. The importance of mere light dosimetry was well demonstrated in the TGF–β activity studies. Whereas PDT with 10 and 100 J/cm² caused the same degree of cytotoxicity, after PDT with 10 J/cm² there was significant TGF–β activity. This increase in TGF–β activity was also seen after mechanical injury, which illustrates how the cascade of cytokine action is initiated from the moment of cell injury. Inadequete PDT may be comparable to mechanical injury in this perspective, which may result in the trigger of events that lead to IH. Similar results were found in the cellular–bFGF experiments: more light was needed to significantly diminish cell-associated bFGF concentrations. These observations have important implications for the dosimetry of PDT for in vivo application. Considering the importance of these proteins in determining the outcome of the vascular response to injury, it can be conceived that mere eradication of the SMC population may not be sufficient to effectively inhibit IH. Moreover, histologic examination of PDT arteries with subtherapeutic doses of PDT have shown complete local eradication of SMC but with subsequent delayed IH development (Ortu et al., 1992 and Adili et al., 1999).

Initiated by the novel mechanistic studies presented in this thesis, more research is needed to enhance the odds for effective clinical PDT. The presented experimental studies have opened a new line of investigation to further explore the effects of PDT on vascular healing. The majority of vascular PDT studies were performed to test this new approach in the well described rat balloon-injury model. In these experimenets, the carotid was externally irradiated with a laser source. To prevent restenosis after angioplasty or stenting, PDT will serve as an adjunctive method after the endovascular procedure and endoluminal irradiation is applied. The effects of endoluminal PDT on the vascular healing process have not been studied to a great extent (Gonschior et al., 1998; Jenkins et al., 1998). As compared to the external irradiation model, the treated artery cannot be isolated using the endoluminal approach. In this way, the direct environment of the treated artery will receive some light and be subjected to PDT-effects and as demonstrated by the current studies, suboptimal PDT results in cytotoxicity and cytokine activation and release. To minimize these effects, appropriate timing of irradiation after photosenzitation will be of major importance. Alternatively, local drug delivery can be an option (Adili et al., 1998; Gonschior et al., 1998). A recent study presented histological data on endovascular PDT

in a non-injured pig-model (Jenkins et al., 1998). The main outcome of this study was that intra-arterial PDT can be performed safe without inflammation, thrombosis and IH development. However, an important feature of this study was that, although there was a significant reduction in the number of medial SMC at 3 and 14 days, there was not complete eradication of these cells. In addition, since it is not known how cellular mediators have been affected with this PDT-regime, the biological behavior of the surviving cells and the outcome of the healing process cannot be anticipated. The importance of the extent of cell eradication and matrix effects is well illustrated by a recent study from Adili et al in the rat model (Adili et al., 1999) that suggested a classificaion of effects on the artery. After subtherapeutic dosis of PDT, which resulted in incomplete cell eradication, there was significant IH at two weeks. Furthermore, there was a second level of PDT which resulted in complete cell eradication but with subsequent development IH and a higher PDT dose was needed to result in complete cell eradication and inhibition of IH. These results correspond with some of the in vitro data presented in this thesis describing PDT-mediated cytotoxicty in relation to cytokone effects.

In conclusion, vascular PDT affects the injury-induced healing process in a multifactorial way. Before vascular PDT can be effectively applied in patients to prevent restenosis, more fundamental research is needed. At the Department of Surgery, Erasmus University in Rotterdam a research program was initiated in 1998 to proceed with these experimental investigations. The main objectives of this program is to develop a model to further examine endovascular PDT. The distribution of several photosensitizers in the vascular wall will be studied in detail in order to choose appropriate timing of light irradiation. Histological effects of PDT will be correlated with PDT-mediated effects on cytokines to further analyze the in vivo significance of photochemical-cytokine interaction and vascular healing. At the Department of Vascular Surgery, Wellman Laboratories of Photomedicine, Massachusetts General Hospital, Boston further research is undergoing to develop a clinical application of PDT by endoluminal irradiation. The mechanisms of how PDT causes its effect on the artery wall and affects smooth muscle cell functions such as migration and proliferation are also being further examined.

CHAPTER 10

SUMMARY/ SAMENVATTING

SUMMARY

1. Introduction.

The development of restenosis after invasive vascular interventions remains a significant clinical problem. The pathophysiology of renarrowing of the vessel lumen involves a fibroproliferaive healing response which leads to intimal thickening. In addition, other vascular remodeling mechanisms play a crucial role in angioplasty-related restenosis. The vascular wall is able to adapt its size in response to a growing intimal mass and failure of this response may promote luminal narrowing. Up to date, there is no effective means of preventing restenosis to occur. It is clear that multiple factors are involved in the vascular healing process and it seems logical that the problem of restenosis should be adressed in a multifactorial way. Vascular photodynamic therapy (PDT) is a novel strategy to inhibit the formation of experimental intimal hyperplasia (IH). PDT utilizes the combination of light and a photosensitive dye to locally generate cytotoxic free radicals. Being able to cause localized tissue destruction, PDT has been developed and applied to treat malignant disorders and is currently used for treatment or as a palliative method for several cancers. More recently, PDT has been tested as a method to treat other disorders in which cell proliferation is prominent such as psoriasis, arthritis and IH.

2. Vascular PDT and aims of the studies.

To perform vascular PDT, a photosensitizer is administered and the area of interest is illuminated with wavelength-specific thermo-neutral laserlight. In the rat carotid balloon injury model, the histological effects of PDT were previously described. PDT resulted in acute local depletion of vascular cells throughout all layers of the vessel wall, without thrombosis or inflammation. Follow-up histological examination demonstrated reendothelialization of the intima and repopulation of the adventitial layer. Interestingly, the healing process was characterized by a lack of intimal or medial SMC ingrowth, even at 16 weeks. From these experimental studies it was learned that these effects can only be achieved within a certain dose-realated PDT frame; suboptimal PDT results in insufficient cell-eradication and subsequent IH, whereas to much PDT can cause acute vascular thrombosis. The mechanisms underlying the characteristic healing response after PDT are not understood. In fact, it seems paradoxical to find inhibtion of IH after PDT-mediated cytotoxicity with the knowledge that this fibroploriferative disorder is directly related to cell injury. Since vascular healing following injury is regulated by a complex process

of cell-interaction with extracellular matrix and biological mediators, this project tested the hypothesis that PDT could interfere with this cascade of events. To accomplish this, experimental investigations were performed to study the effects of PDT on several biological factors involved in vascular healing and how this intercation affects vascular cell function.

3. Importance of the PDT treatment field.

To address the premise that with adequate parameters, PDT-induced cytotoxicity itself does not result in IH, it is necessary to study the effects of PDT on the normal vessel wall. In previous studies, IH induced by segmental balloon injury of the rat carotid, could be prevented by PDT. Since mechanical injury to the vessel wall does result in IH, it is necessary to define how important the extent of the PDT treatment field is to effectively control IH. Therefore, this study examined the repair process of a PDT-treated balloon-injured artery in which the PDT-treatment field did not target the entire injured area. Uninjured carotid arteries were subjected to PDT after administration of the photosensitizer CASPc and external light irradiation with a total fluence of 100 J/ cm^2. In the second experimental group, the entire rat common carotid artery was balloon-injured to induce IH, whereas only the cervical segment below the bifurcation was subjected to PDT. Light-irradiation of injured arteries without photosensitizer served as control. PDT of uninjured arteries resulted in complete local irradication of vascular cells but did not result in IH. The healing response demonstrated rapid reendothelialization and adventitial repopulation but a lack of medial cellular ingrowth. Balloon-injured arteries without PDT displayed rapid IH development with a peak at two weeks. PDT of balloon-injured arteries resulted in complete local depletion of medial SMC, which was associated with a lack of IH until 2 weeks. However, at 4 and 16 weeks there was significant IH in PDT-treated arteries despite a lack of medial SMC repopulation. A wave of IH progression over the acellular media was observed in these arteries, migrating from the injured non-PDT treated area. Delayed IH development after PDT of injured vessels can result from IH progression from an injured site not included in the treatment field. Together with previous results and the determination that PDT itself does not induce IH, it can be reasoned that inclusion of the whole injured artery or a section of an uninjured margin in the treatment field is essential for effective PDT-prevention of IH.

4. PDT of extracellular matrix.

PDT has been demonstrated to inhibit experimental IH and to lead to expedient reendothelialization but negligible repopulation of the vessel media. The mechanism that underlies the differential ingrowth of cells into PDT-treated vessel segments is not understood. Because the extracellular matrix (ECM) is known to modulate specific cell functions, this study was designed to determine whether PDT of isolated ECM affects the function of ECs and SMCs. PDT of bovine aortic EC-ECM was performed with CASPc and 675-nm laser light. Control specimens included untreated ECM,

ECM-free plates, and ECM exposed to either light or photosensitizer only. Cell function was characterized by attachment, proliferation, and migration of ECs and SMCs that were plated onto identically treated matrixes. SMC attachment, proliferation, and migration were significantly inhibited after PDT of ECM when compared with untreated ECM (all $p < 0.001$). In contrast, PDT of ECM significantly enhanced EC proliferation ($p < 0.03$) and migration, but did not affect attachment. This report establishes PDT-induced changes in the ECM with a result of inhibition of SMCs and stimulation of ECs functions. It provides insight into how PDT-treated arteries can develop favorable EC repopulation without SMC-derived intimal hyperplasia.

5. PDT inhibits matrix-associated TGF–β.

Local eradication of vascular cells with PDT in vivo is followed by expedient reendothelialization and PDT of extracellular matrix (ECM) in vitro stimulates EC growth. This study explored one possible mechanism underlying these findings by investigating the effects of PDT on matrix-associated TGF–β, a potent inhibitor of EC growth. The ECM deposited by EC on tissue culture plates contained 85.4 ± 10.2 pg/10 cm² of TGF–β as measured by an ELISA. In contrast, after PDT of ECM barely detectable levels of TGF–β could be detected (0.2 ± 0.5 pg/10 cm²). The functional consequence of this observation was demonstrated by the finding that PDT of plates coated with a fibronectin-TGF–β complex stimulated EC mitogenesis ($p< 0.0005$) as compared to the untreated contol. SDS-PAGE analysis of PDT-treated TGF–β in solution demonstrated that PDT-mediated loss of TGF–β activity was not associated with changes in its molecular weight. These data demonstrate that increased EC proliferation on PDT treated matrix is, at least in part, mediated by inactivation of TGF–β. PDT-removal of this EC growth inhibitor in the intima provides a mechanism by which PDT of the vascular wall could potentiate endothelial regrowth.

6. Effects of PDT on TGF–β activity associated with vascular SMC-injury.

The multifunctional cytokine, TGF–β, plays an important role in the development of injury-associated IH. This study investigated whether PDT generated free radicals can affect TGF–β activity in a biologic system using vascular SMC. The release and activation of TGF–β by injured SMC in culture was compared between mechanical injury and PDT. Mechanical injury was induced with a rubber policemen and PDT was performed at a subtherapeutic 10 J/cm² and the in vivo therapeutic dose of 100 J/cm². Cell viability was assessed by the tetrazolium salt conversion assay and active and total (active + latent) TGF–β was determined by ELISA in the conditioned media (CM) of SMC 24-hours after treatment. Functional TGF–β activity was assessed by inhibition of endothelial cell (EC) mitogenesis. Both forms of injury severely reduced ($p< 0.0005$) SMC viability to below 15%. In untreated SMC conditioned media, only 14.5% of the total TGF–β was active TGF–β. However, after mechanical injury and PDT with 10 J/cm² there was a significant increase ($p<0.02$) in

active TGF–β, despite a total reduction of approximately 50%. In contrast to this, PDT with 100 J/cm² did not result in increased levels of active TGF–β, despite having similar levels of total TGF–β. Consequently, the CM of 100 J/cm² PDT, did not inhibit EC mitogenesis as compared to the CM of mechanical injured and 10 J/cm² PDT ($p < 0.0002$). This report describes two novel findings: 1) injury to SMC in vitro induces the conversion of biologically latent to active TGF–β 2) therapeutic PDT-dose interferes with this injury activation process. This study substantiates the concept of local cytokine inhibition by PDT in a biologic system.

7. PDT inactivates smooth muscle cell-associated bFGF.

Procedurally related vascular injury results in a SMC proliferative response which is in part initiated by SMC release of mitogens, including basic fibroblast growth factor (bFGF). PDT-mediated SMC eradication does not induce an inflammatory or proliferative response in the vessel wall. This study investigated whether PDT-generated free radicals could inactivate cell-associated bFGF normally released with cell injury. PDT of bovine SMC was performed in vitro using three different fluences: 10, 50, and 100 J/cm². After PDT, SMC viability was determined with the tetrazolium salt (MTT) assay and cell-associated bFGF was quantitated by ELISA. A SMC mitogenesis assay was utilized to detect cell-associated bFGF activity released with SMC injury. In a dose dependent manner, PDT-generated free radicals reduced cell-associated bFGF levels. After PDT with 100 J/cm², cell-associated bFGF content was reduced by 88% ($p < 0.0002$). Of special interest was the finding that PDT with 10 J/cm² significantly ($p < 0.0002$) reduced cell viability to around 50%, without affecting cellular bFGF levels. Both mechanical injury and PDT with 10 J/cm² resulted in stimulation of SMC mitogenesis. A higher PDT dose (100 J/cm²) was needed to significantly ($p < 0.001$) inhibit the SMC mitogenic response associated with SMC injury. These results provide a mechanism to explain how unlike mechanical or other forms of SMC injury, optimal doses of PDT can locally eradicate medial vascular SMC without resulting in a bFGF-induced initiation of cell proliferation.

8. Effects of PDT on the vascular fibrotic response.

Excessive deposition of extracellular matrix (ECM) proteins play a key role in the intervention-related vascular fibroproliferative response, resulting IH. Cytokines, such as PDGF, released after vascular injury and deposited in the ECM are known to stimulate production of matrix proteins. This in vitro study examined whether PDT can inhibit the fibrotic response of vascular SMC. The effect of PDT on important pro-fibrotic factors was determined by performing PDT of isolated ECM, injured SMC and pure PDGF. SMC production of collagen was monitored by cellular [³H]-proline incorporation. SMC seeded on ECM demonstrated an increase of 50% in collagen production ($p < 0.0001$) as compared to SMC on an empty plate. This increase was also seen when SMC was incubated with the conditioned media of mechanical injured SMC, or pure PDGF. However, after PDT of:

ECM, injured SMC or PDGF, there was an inhibition of 40% ($p< 0.05$) in SMC collagen production. These findings indicate that PDT can interfere with factors that lead to the vascular fibrotic response. In this way, PDT, with its cytotoxic and extracellular effects, can promote healing of the vessel wall without the stimulus of fibrosis that can lead to restenosis.

9. General Discussion.

Vascular PDT represents one experimental method in the search of the magic bullet to deal with restenosis. The characteristic healing response of the vessel wall after applying this cytotoxic method to the vascular wall could not be explained by mere SMC destruction. The studies described in this thesis examined how PDT can erradicate vascular cells and promote a favorable healing response without IH. In chapter 3 it was shown that PDT of a non-injured carotid artery resulted in cel eradcation through all layers of the vessel wall without causing IH. Furthermore, it was demonstrated that if there is mechanical injured tissue next to the PDT-treated area IH develops. This finding is important for the application of vascular PDT because it indicates that a margin of non-injured tissue should be included in the PDT-field to achieve the favorable PDT response. This favorable healing response is characterized by a lack of inflammation and thrombosis and a swift reendothelialization. In chapter 4 and 5 it was demonstrated that PDT can affect the extracellular environment by changing the ECM. PDT-induced inactivation of the endothelial cell inhibtor TGF–β promoted endothelial cell growth. In chapter 6 and 7 it was shown that PDT also had a direct effect on the cytokine response associated with cell injury. It was demonstrated that mechanical injury which occurs after dilatation of the vascular wall, resulted in TGF–β activation and bFGF release. This response was also seen after suboptimal doses of PDT, whereas with adequate light-dose there was a significant reduction in TGF–β and bFGF activity. These findings highlight the importance of dosimetry, since suboptimal PDT can initiate the cascade of events that lead to IH. Therefore, besides cytotoxicity, a reduction in the cytokine response should be effectuated to perform optimal PDT to inhibit IH. In chapter 8 it was demonstrated that PDT-mediated inhibition of cellular mediators also affected the fibrotic response. After PDT of ECM, SMC and the pro-fibrotic factor PDGF, there was a reduction in collagen production. The extracellular effects described in this study were achieved using one photosensitizer in a simple biological in vitro and in vivo system. It is therefore by no means clear whether these effects can be achieved in more complex situations, such as the human atherosclerotic plaque. In addition, besides the inhibition of IH, it is not known how PDT affects other remodeling processes. Although the studies described herein are the beginning of some understanding of PDT effects on the vascular wall, they represent an exciting platform for future investigations. The multifactorial aspect of PDT-induced photochemical reactions makes it a promising tool to challange restenosis.

SAMENVATTING

1. Introductie.

Vaatvernauwing als gevolg van atherosclerose leidt tot extremiteit en orgaanschade en is de belangrijkste doodsoorzaak in de Westerse bevolking. Het optreden van een hervernauwing (restenose) bij de behandeling van stenoserend vaatlijden beperkt de lange termijn resultaten van vasculaire en endovasculaire ingrepen. Restenose ontstaat als gevolg van een proliferatieve genezingsreactie in de vaatwand op de schade van de invasieve behandeling. Hierbij gaan gladde spiercellen van de middelste laag (media) van de vaatwand delen en migreren naar de binnenste laag (intima) waar een grote hoeveelheid extracellulaire matrix wordt gemaakt. Bij dit genezingsproces ontstaat een verdikking van de intima die intima hyperplasie (IH) wordt genoemd. Naast IH spelen andere mechanismen een rol bij het ontstaan van restenose. Bij toename van de intimadikte kan het vat compensatoir vergroten om het lumenkaliber constant te houden. Het falen van dit mechanisme lijkt ook van belang voor het ontstaan van restenose. Tot op heden is er geen effectieve methode gevonden om het ontstaan van restenose te voorkomen. Hoewel experimentele studies het inzicht in de pathogenese van restenose hebben vergroot heeft de klinische toepassing van experimentele strategieën teleurstellende resultaten opgeleverd. Het is evident dat multipele factoren een rol spelen in de pathogenese van restenose en het lijkt logisch om dit complex probleem op een multifactoriële wijze aan te pakken.
Vasculaire Photodynamische Therapie (PDT) is een nieuwe experimentele strategie om IH te voorkomen. PDT maakt gebruik van licht om een lichtgevoelige stof (photosensitizer) te activeren waardoor cytotoxische vrije radicalen worden gevormd. Deze techniek wordt gebruikt om op een lokale wijze weefseldestructie te induceren en wordt momenteel toegepast bij de behandeling van verschillende kwaadaardige tumoren. Recent onderzoek heeft aangetoond dat PDT ook gebruikt kan worden voor de behandeling van benigne aandoeningen waarbij celdeling op de voorgrond staat, zoals psoriasis, arthritis en IH.

2. Vasculaire PDT en doel van de studie.

Om PDT op de vaatwand toe te passen wordt een photosensitizer systemisch of lokaal toegediend en het behandelde vaatsegment belicht met laserlicht van een specifieke golflengte. In een rattenmodel waarbij met behulp van ballondilatatie IH wordt geïnduceerd zijn de effecten van PDT bestudeerd. Hieruit bleek dat na PDT massale celdood door alle lagen van de vaatwand optreedt. Kenmerkend voor de genezingsreactie na PDT is dat er geen ontstekingsreactie of thrombusvorming ontstaat. Daarnaast treedt er repopulatie van de intima en de adventitia op, terwijl zelfs na 16 weken geen ingroei van de media wordt waargenomen. Hoewel de remming van IH na PDT wordt toegeschreven aan de uitschakeling van gladde spiercellen in de media, kan het karakteristieke genezingspatroon hiermee niet worden verklaard. Het is bekend dat gladde spiercel-beschadiging juist een belangrijke stimulus is voor het ontstaan van IH. De genezingsreactie van de vaatwand na beschadiging

wordt gereguleerd door een complexe interactie van cellen, de extracellulaire matrix en verschillende mediatoren. De effecten van PDT op deze interactie is niet bekend. Het doel van de studies beschreven in dit proefschrift was om na te gaan of PDT deze biologische factoren kan beïnvloeden. Om dit te onderzoeken werd nader gekeken naar de effecten PDT op de vaatwand in vivo waarbij de genezing na PDT werd vergeleken met de genezing na mechanische beschadiging. Vervolgens werd een experimenteel model ontwikkeld om de effecten van PDT op verschillende factoren die een rol spelen bij de vaatgenezing te bestuderen.

3. Het belang van het behandelingsgebied.

Het effect van PDT op een niet beschadigd vat is nog niet goed onderzocht. Om na te gaan of PDT geen IH veroorzaakt werd allereerst gekeken naar de genezingsreactie van de vaatwand na alleen PDT. In voorgaande experimenten werd een segment van de carotis beschadigd en PDT van het hele beshadigde gebied uitgevoerd. Aangezien het bekend is dat mechanische beschadiging door ballon-dilatatie IH induceert, moet worden nagegaan wat er gebeurt als er mechanisch beschadigd weefsel bestaat naaast het PDT-segment. Hiervoor werd in deze studie gekeken naar het genezingsproces indien een segment binnen het beschadigde gebied met PDT werd behandeld. De carotis communis van de rat werd met PDT behandeld na systemische toediening van de photosensitizer CASPc en uitwendige belichting met een totale energie van 100 J/cm^2. In de tweede experimentele groep werd de hele carotis met de ballon gedilateerd, terwijl alleen het cervicale carotissegment met PDT werd behandeld. Controles voor beide groepen waren belichte vaten zonder vooraf toedienen van de photosensitizer. PDT van de normale carotis resulteerde in een volledige uitroeiing van alle cellen van het behandelde gebied zonder IH te induceren. Na PDT trad reëndothelialisatie en repopulatie van de adventitia snel op. Ballon gedilateerde vaten zonder PDT resulteerde in IH met een piek bij twee weken. Na PDT van ballon gedilateerde vaten was er een remming van IH tot twee weken. Bij 4 en 16 weken ontstond er significante IH terwijl geen repopulatie van de medialaag werd waargenomen. Een golf van IH migreerde vanuit de beschadigde gebieden die niet met PDT waren behandeld. Deze studie laat zien dat PDT met de juiste dosering geen IH induceert ondanks de massale celdood in de vaatwand. Aangezien IH ontstaat als er mechanisch beschadigd weefsel bestaat naast het met PDT behandeld gebied, moet een niet beschadigd segment in het PDT behandelingsgebied worden geïncludeerd om IH na ballon-beschadiging effectief te remmen.

4. PDT van de extracellulaire matrix.

Na PDT van de vaatwand repopuleert de intima met endotheelcellen terwijl geen ingroei optreedt van de media met gladde spiercellen. Het mechanisme van deze differentiële ingroei van cellen na PDT van de vaatwand is niet bekend. Omdat de extracellulaire matrix een belangrijke rol speelt in de regulatie van verschillende celfuncties ging deze studie na of PDT de matrix kan

veranderen. In een in vitro model werd PDT uitgevoerd op geïsoleerde door rund endotheelcel gemaakte matrix. Controles waren: niet behandelde matrix, alleen belichte matrix, matrix met alleen de photosensitizer CASPc en een kweekbodem zonder matrix. Van de gladde spiercellen en endotheelcellen gekweekt op de verschillende kweekbodems werd proliferatie, migratie en adhesie bestudeerd. De adhesie, migratie en proliferatie van gladde spiercellen waren verminderd na PDT van de matrix in vergelijking met niet behandelde matrix. Deze vermindering in functie werd ook gezien op een lege kweekbodem. In tegenstelling tot deze bevinding werd juist een stimulate van endotheelcel migratie en proliferatie waargenomen na PDT van de matrix. Deze studie toont aan dat PDT de extracellulaire matrix verandert met een verschillend effect op de groei van gladde- en endotheelcellen. Deze waarneming geeft inzicht in de effecten van PDT op de vaatwand waarbij IH wordt geremd en een snelle repopulatie van de endotheelcellaag optreedt.

5. PDT inactiveert matrix-gebonden TGF–β.

Na PDT van de vaatwand treedt snelle repopulatie van de endotheelcellaag op en PDT van matrix in vitro stimuleert endotheelcel groei. Deze studie onderzocht een mechanisme om deze bevindingen te verklaren door naar de effecten van PDT op de endotheelcel remmende factor Transforming Growth Factor–β (TGF–β) te kijken. Matrix gemaakt door endotheelcellen in vitro bevatte 85.4 ± 10.2 pg/10 cm² TGF–β gemeten door middel van ELISA. Na PDT van de matrix was deze waarde gereduceerd tot 0.2 ± 0.5 pg/10 cm². Het functionele gevolg van deze waarneming werd gedemonstreerd door PDT te behandelen van TGF–β gebonden aan fibronectine. PDT resulteerde in een significante stimulatie van endotheelcel proliferatie in vergelijking met de niet behandelde TGF–β - fibronectine complex. SDS-PAGE analyse van met PDT behandelde TGF–β liet zien dat de functionele inactivatie niet gepaard ging met een verandering in het moleculaire gewicht. Dit impliceert dat de moleculaire structuur van TGF–β is veranderd na PDT. Deze studie toont aan dat inactivatie van matrix-gebonden TGF–β door PDT een rol speelt bij de stimulatie van EC-groei na PDT van ECM. Inactivatie van deze EC-remmer in de vaatwand kan een gunstig effect hebben op de vaatgenezing door bevordering van de endotheelbekleding.

6. Effecten van PDT op TGF–β activiteit bij gladde spiercelbeschadiging.

De multifunctionele cytokine TGF–β speelt een belangrijke rol in de pathogenese van IH. Onderdrukking van TGF–β activiteit in de vaatwand kan een methode zijn om IH te remmen. Deze studie bestudeerde de effecten van PDT op de activiteit van TGF–β in een biologisch kweekmodel met gladde spiercellen. De uitscheiding en activatie van TGF–β door gladde spiercellen werden onderzocht na PDT en mechanische beschadiging. Mechanische beschadiging werd uitgevoerd met een rubber stokje. PDT werd verricht met CASPc en toepassing van twee belichtingsdoses: een subtherapeutische (10 J/cm²) en een in vivo therapeutische (100 J/cm²) dosering. Cel overleving werd

gemeten met behulp van de tetrazolium zout conversie assay (MTT). De totale (latent + actief) en actieve TGF–β concentraties in het geconditioneerde medium werden door middel van ELISA gekwantificeerd. Beide vormen van celbeschadiging resulteerden in een celoverleving van minder dan 15%. In het medium van onbehandelde gladde spiercellen was 14.5% van de TGF–β actief. Na mechanische beschadiging en PDT met 10J/cm² trad er een significante stijging op in het percentage actieve TGF–β terwijl de totale concentratie was verminderd met 50%. In tegenstelling tot deze bevinding resulteerde PDT met 100 J/cm² niet in een stijging van actieve TGF–β terwijl de totale concentratie gelijk bleef. Deze bevindingen werden bevestigd met een endotheelcel proliferatie studie waarbij het medium van mechanisch beschadigde cellen en PDT met 10J/cm² een significante remming veroorzaakten in endotheelcel proliferatie terwijl het medium van PDT met 100 J/cm² geen effect had op de endotheelcel proliferatie. Deze studie beschrijft voor het eerst dat gladde spiercel beschadiging gepaard gaat met een stijging in actieve TGF–β concentratie. Met een adequate PDT dosering kan dit activatie-proces geremd worden. Deze bevindingen ondersteunen de hypothese dat PDT kan interfereren met de toegenomen cytokine activiteit na beschadiging en hiermee de genezingsreactie gunstig kan beïnvloeden.

7. PDT inactiveert intracellulaire bFGF.

Gladde spiercel proliferatie na mechanische beschadiging van de vaatwand wordt geïnitieerd door de acute uitscheiding van mitogenen. Een belangrijke groeifactor voor gladde spiercellen die vrijkomt na gladde spiercel beschadiging is basic Fibroblast Growth Factor (bFGF). De massale celdood na adequate PDT van de vaatwand wordt niet gevolgd door een proliferatieve reactie. Deze studie ging na of vrije radicalen gevormd door PDT de groeifactor bFGF geassocieerd met gladde spiercellen kan inactiveren. PDT werd uitgevoerd op rund gladde spiercellen in vitro na incubatie met de photosensitizer CASPc en belichting met 675 nm licht gebruik makende van verschillende doses. Cel overleving werd gemeten met behulp van de MTT assay en cel-geassocieerde bFGF werd gekwantificeerd door middel van een ELISA. Proliferatie van gladde spiercellen werd bepaald met een mitogenese assay om de functionele effecten te evalueren. De effecten van PDT op de concentratie van cel-geassocieerde bFGF waren dosis afhankelijk. Na PDT met 100 J/cm² was de bFGF concentratie gereduceerd met 88%. Alhoewel PDT met 10 J/cm² de cel overleving reduceerde met 50%, had deze lichtdosering geen effect op de intracellulaire bFGF concentratie. Het geconditioneerde medium van mechanisch beschadigde gladde spiercellen en cellen behandeld met 10J/cm² resulteerde ook in een stimulatie van gladde spiercel proliferatie. Een hogere lichtdosis (100 J/cm²) was nodig om de stimulatie van gladde spiercellen geassocieerd met celbeschadiging significant te remmen. Deze resultaten tonen aan dat in tegenstelling tot mechanische beschadiging en suboptimale PDT van gladde spiercellen, effectieve PDT gepaard gaat met cytotoxicitiet zonder de bFGF geïnduceerde stimulatie van cel proliferatie.

8. Effecten van PDT op de vasculaire fibrotische reactie.

De excessieve afzetting van matrix moleculen in de intima speelt een belangrijke rol bij het ontstaan van IH. Cytokinen zoals Platelet Derived Growth Factor (PDGF) die vrijkomen na celbeschadiging in de omgevende matrix zijn verantwoordelijk voor de fibrotische reactie. Deze studie evalueerde of PDT het fibrotisch proces kan remmen. Het effect van PDT op belangrijke pro-fibrotische factoren werd nagegaan door PDT uit te voeren op een geïsoleerde matrix, beschadigde gladde spiercellen en PDGF. Productie van collageen door gladde spiercellen werd gemeten aan de hand van de incorporatie van [^{3}H]-proline. Gladde spiercellen gekweekt op ECM demonstreerde een 50% toename in collageen productie. Deze toename werd ook gezien als gladde spiercellen werden geïncubeerd met het geconditioneerde medium van beschadigde gladde spiercellen en PDGF. PDT van deze pro-fibrotische factoren resulteerde in een reductie van 40% in collageen productie. Deze resultaten tonen aan dat de photochemische reactie van PDT bepaalde factoren kan uitschakelen die belangrijk zijn voor de fibrotische reactie na vaatbeschadiging. Op deze manier kan PDT toegepast op vaten, ondanks de cytotoxiciteit,, IH remmen.

9. Discussie.

Vasculaire PDT is een nieuwe experimentele methode in de zoektocht naar de "magic bullet" voor de preventie van restenose. Het basisprincipe is de lokale productie van cytotoxische vrije radicalen door de combinatie van licht en een photosensitizer. Experimentele studies hebben aangestoond dat PDT intima hyperplasie geïnduceerd door ballondilatatie kan remmen maar het is niet bekend hoe PDT de vaatwand beïnvloedt en welke factoren van belang zijn om effectief PDT toe te passen. In hoofdstuk 3 werd aangetoond dat ondanks de massale celdood in de vaatwand, PDT met de juiste dosering niet tot een fibroproliferatieve reactie en IH leidt. Dit is merkwaardig gezien het feit dat IH ontstaat als gevolg van celbeschadiging in de vaatwand. Deze studie liet ook zien dat IH ontstaat als er mechanisch beschadigd weefsel bestaat naast het met PDT behandelde segment. Voor de toepassing van PDT is deze waarneming van groot belang omdat het benadrukt dat een marge van onbeschadigd weefsel in het PDT-veld geïncludeerd moet worden om de gunstige effecten van PDT te bereiken. Deze gunstige genezingsreactie na PDT bestaat uit de afwezigheid van ontsteking en thrombose en een snelle bekleding van de endotheelcellaag en repopulatie van de adventitia. Hoofdstukken 4 en 5 toonden aan dat PDT de omgeving van de cel beïnvloedt door de biologische functie van de extracellulaire matrix te veranderen. Door bijvoorbeeld de endotheelcel remmende factor TGF–β die gebonden is aan matrix uit te schakelen kan PDT endotheelcel groei bevorderen. De resultaten in hoofdstukken 6 en 7 lieten zien dat PDT ook een directe effect had op het cytokine respons na celbeschadiging. Deze studies toonden aan na mechanische celbeschadiging, zoals optreedt na ballondilatatie van de vaatwand, TGF–β wordt geactiveerd en bFGF vrijkomt. Dit cytokine respons werd ook gezien na suboptimale PDT, terwijl een significante reductie werd

waargenomen met adequate PDT-dosering. Het belang van de juiste PDT dosering kwam hierbij aan het licht aangezien suboptimale PDT de fibroploriferatieve cascade kan activeren en IH veroorzaken. Deze studies demonstreerden dat om effectief PDT uit te voeren, niet alleen de gladde spiercellen maar ook bepaalde mediatoren moeten worden uitgeschakeld. De remming van het cytokine respons had niet alleen een effect op proliferatie en migratie van cellen maar ook op de fibrotische reactie. In hoofdstuk 8 werd de reductie van collageen productie na PDT van matrix, gladde spiercelllen en de profibrotische factor PDGF aangetoond. De extracellulaire effecten van PDT zoals aangetoond in deze studie zijn in simpele in vivo en in vitro systemen aangetoond. Het is niet bekend hoe deze effecten het genezingsproces zullen beïnvloeden in meer complexe situaties, zoals in een atherosclerotische plaque. Daarnaast zijn de effecten van PDT op het proces van remodelering niet bekend. Hoewel deze studie een licht heeft geworpen op de manier waarop PDT de vaatgenezing beïnvloedt, lijkt meer onderzoek zinvol voordat deze techniek klinisch kan worden toegepast. Het multifactoriële aspect van PDT maakt het een veelbelovende strategie om restenose te bestrijden.

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List of Abbreviations

bFGF	basic fibroblast growth factor
CASPc	chloroaluminum sulfonated phthalocyanine
CM	conditioned media
EC	endothelial cells
ECM	extracellular matrix
ELISA	enzyme linked immunosorbent assay
IH	intimal hyperplasia
PDT	photodynamic therapy
SMC	smooth muscle cells
TGF-β	transfroming growth factor-beta
PBS	phosphate buffered saline
PDGF	platelet derived growth factor

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