Photodynamic Therapy Generates a Matrix Barrier to Invasive Vascular Cell Migration

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Abstract—Photodynamic therapy (PDT) inhibits experimental intimal hyperplasia. PDT results in complete vascular wall cell eradication with subsequent adventitia but minimal media repopulation. This study was designed to test the hypothesis that PDT alters the vascular wall matrix thereby inhibiting invasive cell migration, and as such, provides an important barrier mechanism to favorably alter the vascular injury response. Untreated smooth muscle cells (SMCs) and fibroblasts were seeded on control and PDT-treated (100 J/cm²; photosensitizer was chloroaluminum-sulfonated phthalocyanine, 5 µg/mL) 3-dimensional collagen matrix gels. Invasive cell migration was temporally quantified by calibrated microscopy. Zymography and ELISA assessed SMC matrix metalloproteinase levels. Molecular changes of gel proteins and their susceptibility to collagenase were analyzed by SDS-PAGE and Western blot. Limited pepsin digestion and histology were used to assess the in vivo relevance of the model, using an established rat carotid artery model at 1 and 4 weeks after balloon injury and PDT. PDT of 3-dimensional matrix of gels led to a 52% reduction of invasive SMCs and to a 59% reduction of fibroblast migration (P<0.001) but did not significantly affect secretion of matrix metalloproteinases. PDT induced collagen matrix changes, including cross-linking, which resulted in resistance to protease digestion. PDT led to a durable 45% reduction in pepsin digestion susceptibility of treated arteries (P<0.001) and inhibition of periadventitial cell migration into the media. These data suggest that PDT of matrix gels generates a barrier to invasive cell migration. This newly identified effect on matrix proteins underscores its pleiotropic actions on the vessel wall, and as such, PDT may be of considerable potential therapeutic value to inhibit restenosis. (Circ Res. 2000;86:334-340.)

Key Words: photodynamic therapy ■ restenosis ■ cell migration ■ collagen ■ metalloproteinases

Restenosis remains the major obstacle to long-term success after invasive vascular procedures. As part of the injury to the vessel wall, vascular smooth muscle cells (SMCs), adventitial myofibroblasts, and fibroblasts proliferate and migrate into the subintimal space, where they deposit extracellular matrix (ECM), thereby resulting in lumen loss. The role of cellular migration from the media and the adventitia has remained a focal point in the effort to identify an approach to inhibit vascular restenosis. Using this strategy, inhibitors of matrix metalloproteinases (MMPs), enzymes crucial for invasive SMC migration, ECM repair, and remodeling, prevented SMC migration, thus resulting in a temporary repression of experimental intimal hyperplasia (IH). This transient success reflects the complexity of vascular injury and subsequent healing and emphasizes why restenosis is a difficult problem to solve. Of the many approaches tested, only stents and ionizing irradiation have been clinically demonstrated to reduce this process.

Photodynamic therapy (PDT) is another promising approach undergoing an early clinical trial. PDT uses light to activate otherwise inert photosensitizer dyes to produce photochemical reactions through the production of free radical moieties without the generation of heat. These free radicals eradicate the entire cell population of the artery wall without inducing inflammation or structural deterioration and thus result in long-term inhibition of experimental IH. Vascular PDT has other effects, including inactivation of matrix-associated cytokines and growth factors, which result in alteration of vascular cell function. These matrix effects may influence the observed cellular repopulation of PDT-treated arteries, including reendothelialization and repopulation of the adventitia, but delayed and only sparse repopulation of the media.

This study tests the hypothesis that PDT generates a matrix barrier to cell migration through the vessel wall. This barrier would inhibit cells from the adventitia from migrating into the intima, therefore explaining, in part, the favorable effects of PDT in vivo. To this end, SMC and fibroblast-invasive migration through control and PDT-treated 3-dimensional (3D) collagen matrix gels were studied. In addition, the mechanisms by which PDT-altered matrix inhibited SMC migration were investigated. To accomplish this, MMP levels were assessed in cultures of SMCs on PDT-treated matrix gels, and changes of the molecular structure of the matrix...
after PDT were determined. To demonstrate the in vivo relevance of these data, we investigated the levels and stability of PDT-induced cross-links in the rat carotid artery and its effect on cellular migration through the artery after balloon injury and PDT.

Materials and Methods

In Vitro PDT Effects on Collagen Type I Cross-Linking and Inhibition of Cellular Migration

Cell Culture

Bovine aortic SMCs and adventitial fibroblasts were obtained and cultured as described before.14,15

3D Collagen Matrix Gel

3D collagen type I matrix gels (Vitrogen100 Collagen Biomaterials) were prepared as described,16 and for PDT or photosensitizer-only control, chloroaluminum-sulfonated phthalocyanine (5 μg/mL, Novartis) was added. Control groups included albumin instead of calf serum (CS), to control for growth factor-independent migration, and D-ribose (4 mg/mL, 4 days), known to induce glycation-dependent cross-links.17

PDT Treatment

After gelation, the matrix gels were irradiated with in vivo effective laser light dosimetry (100 J/cm²; 100 mW/cm²; λ=660 nm).

Cell Migration Assay

Untreated SMCs and fibroblasts were seeded on the matrix gel surface at 8×10⁴ cells/well. Migration was assessed at 4, 8, and 11 days18 by calibrated phase-contrast microscopy (Zeiss IM35). Cells were counted at 0.8-mm depth and in a cylindrical field through the entire depth of the gels. SMC morphology was assessed at ×400 magnification.

Zymography

Gelatinzymography assessed MMP-2 and MMP-9 secreted by SMCs at day 8. SDS–polyacrylamide gels with copolymerized 0.2% gelatin (Sigma) were used.19

ELISA

No specific test for bovine MMP-1 is available. MMP-1 levels were determined using a human MMP-1 ELISA (Calbiochem), given that human and bovine MMP-1 are 87% homologous.20 This assay does not cross-react with MMP-2, MMP-3, or MMP-9.

Gel Electrophoresis

The resistance of collagen type I matrix gel solution (1.5 mg/mL) to collagenase was assessed by digesting matrix gel solutions with clostridial collagenase (600 to 1200 μg/mL; Gibco) followed by SDS-PAGE (5%).21

Figure 1. SMC and fibroblast migration into PDT-treated, control, and D-ribose–treated matrix gels. Data are mean±SD. A, Total SMC number migrating into matrix gels per microscopic field. Total SMC migration was measured after 4, 8, and 11 days. *P<0.001 at all time points in PDT vs controls vs D-ribose. n=15 for all groups. B, SMC number migrating into the matrix gels at a specific depth (0.8 mm) per microscopic field. Total SMC number at 0.8-mm depth was measured after 4, 8, and 11 days. *P<0.01 at all time points in PDT vs controls vs D-ribose. n=15 for all groups. C, Fibroblast migration (total number). Total fibroblast number migrating into matrix gels per microscopic field. *P<0.001 at all time points in PDT vs controls vs D-ribose. n=15 for all groups. D, Fibroblast number migrating into the matrix gels at a specific depth (0.8 mm) per microscopic field. *P<0.001 in PDT vs controls vs D-ribose; +P<0.01, D-ribose vs controls at 4 days. n=15 for all groups. E, Dose-dependent SMC migration (total number). Total SMC number migrating into matrix gels per microscopic field. *P<0.001 at 11 days, 50 J/cm² vs 100 J/cm²; +P<0.05 at 11 days, 100 J/cm² vs 200 J/cm². n=15 for all groups.
**Western Blot**
Collagen matrix changes were revealed with rabbit anti-bovine collagen type I antibody (diluted 1:160, Biodesign), followed by an anti-rabbit IgG (diluted 1:1000).

**In Vivo PDT Effects on Matrix Cross-Linking and Cellular Migration**

**Animal Model**
Animal care was in compliance with Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals (NIH publication No. 80-23, revised 1985) and approved by the institutional animal care committee.

Rats (Charles River) were anesthetized with ketamine (35 mg/kg), atropine (40 µg/kg), and xylazine (5 mg/kg). Photosensitizer application, balloon injury of the carotid artery, and laser irradiation were performed as described.12 Animals were euthanized at 1 hour (n=3) and at 1 (n=3) and 4 (n=3) weeks after PDT (n=3).

**Histology**
To assess cell migration after 1 and 4 weeks, the PDT-treated artery was fixed with formalin, and cross-sections were stained with hematoxylin and eosin for light microscopy.

**Limited Pepsin Digestion**
To determine matrix cross-linking, the unfixed artery and the untreated contralateral artery were harvested for pepsin digestion.

**Statistical Analysis**
Results were expressed as mean ±SD. A 1-way ANOVA and Tukey post hoc test were applied. Differences between controls and PDT in zymography, ELISA, and pepsin digestion experiments were analyzed with the t test for independent variables by means of the Statistica software (Statsoft). A P-value <0.05 was considered significant.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

**Results**

**In Vitro PDT Effects**

**Cell Migration Assay**
Migration of untreated SMCs and fibroblasts was assayed on control and PDT-treated matrix gels. In all groups, cells formed a homogenous monolayer on the 3D gel surface within 48 hours. PDT of the matrix gel decreased the total number of SMCs that migrated into the 3D gel by 52% at 11 days (P<0.001, Figure 1A). In addition, the depth of migration was significantly reduced in PDT-treated gels (61% reduction, P<0.01, Figure 1B). PDT also decreased the total number of fibroblasts that migrated into the 3D gel by 59% at 11 days (P<0.001, Figure 1C), as well as the depth of migration (77% reduction, n=15, P<0.001, Figure 1D).

Adding D-ribose, known to induce glycation-dependent protein cross-links to the 3D gel, decreased cell migration of both cell types similarly to the decrease observed in PDT-treated gels. Cells on control gels (laser-only, photosensitizer-only, and albumin instead of CS matrix gel) had migration patterns similar to those on untreated gels (data not shown).

Invasive SMC migration was PDT dose-dependent. At 50 J/cm², the total number of cells migrating into the gel at 11 days was 33% higher compared with 100 J/cm² (P<0.001) and at 100 J/cm², 20% higher compared with 200 J/cm² (P<0.05, Figure 1E).

**Zymography**
Zymography determined the levels of MMP-2 and MMP-9 secreted by SMCs cultured in the differently treated matrix gels (Figure 3). Densitometry analysis revealed no significant differences (P=0.7) in MMP levels between SMCs cultured on untreated or PDT-treated matrix gel.

**Vascular Cell Morphology**
SMC morphology at the surface of control and PDT-treated matrix gels did not differ. Cells were flat with thin, well-spread filopodia following the main cell axis, adopting a stellate shape. Cells migrating into control gels were similar, whereas cells migrating into PDT-treated gels appeared cylindrical with an apparently reduced cytoplasm, loss of the stellate shape, and decreased spreading filopodia (Figure 2). Fibroblasts migrating into PDT-treated gels showed a similar change in morphology (data not shown).

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**Figure 2.** Photomicrograph of SMCs in matrix gels (phase-contrast microscopy). Upper panel represents SMCs in untreated gels; lower panel, SMCs in PDT-treated gels. Original magnification, ×400. Bar=5 µm.

**Figure 3.** Detection of MMP-2 and MMP-9 by gelatin zymography. Conditioned media from cultured SMCs 8 days after seeding on untreated (lane A) and PDT-treated (lane B) matrix gels were run on a gelatin zymography. CS only served as a positive control (lane C). Area of white bands indicates metalloproteinase digestion. MMP-9 and MMP-2 are shown as latent (Pro-MMPs) and activated (MMPs) forms.
ELISA
An ELISA was performed to assess differences in the levels of MMP-1, which specifically degrades collagen type I. SMCs plated on PDT-treated gels had higher MMP-1 levels compared with SMCs plated on control matrix gels (106.16±4.9 versus 91.04±2.6 ng/mL, n=6, P=0.06).

Gel Electrophoresis
Control and PDT-treated matrix gel solutions (collagen type I, 10% CS, and DMEM) were analyzed by SDS-PAGE to detect molecular weight differences (Figure 4). Analysis revealed new high molecular weight protein oligomers in the stacking gel. In addition, new bands in the γ-chain range with molecular mass from 170 to 250 kDa were detected after PDT. Furthermore, 2 new protein bands migrated below the β1,2 and β1,1 band, and the original β1,2 band disappeared. An additional protein band was found in the PDT-treated samples above the α1 chain, and bands below the α chains fused into a single band (Figure 4A). Densitometry analysis confirmed the generation of proteins with different molecular weights in PDT-treated 3D gels (Figure 4B). In addition, SDS-PAGE was performed to detect specific cross-links in PDT-treated matrix solutions containing collagen alone, albumin alone, or both collagen and albumin. SDS-PAGE of PDT-treated collagen solution alone showed a distinct band of high molecular weight in the stacking gel and a faded band above the γ chain, with a loss of all lower molecular weight bands seen in controls (Figure 4C, lanes A and B). Albumin alone presented the typical albumin band without changes after PDT (Figure 4C, lanes E and F). Solutions containing both collagen and albumin showed a shift from lower to higher molecular weight after PDT with loss of the original bands and a new distinct band in the stacking gel (Figure 4C, lanes C and D). Resistance of control and PDT-treated collagen matrix solutions to digestion were investigated after incubation with collagenase. In the control groups, all higher molecular weight bands and part of the lower molecular weight bands were digested in a dose-dependent fashion, whereas PDT treatment resulted in resistance to digestion at all doses of collagenase used (Figure 4D).

Western Blot Analysis
Western blot using an anti-collagen type I antibody to specifically identify new bands in matrix gel solutions after PDT confirmed that the newly generated cross-links contained collagen (Figure 5). Western blot analysis with the SDS-PAGE results (Figure 4, lanes C and D) revealed the PDT-induced interactions between collagen and CS. This

resulted in a shift of lower (60 to 100 kDa) to higher (100 to 350 kDa) molecular weight bands with a noticeable change to \( \alpha, \beta, \) and \( \gamma \) chains in controls. Nonspecific antibody binding in the low molecular weight range appeared only in pure untreated CS, whereas after PDT, diffuse nonspecific binding of the whole lane was observed (Figure 5, lanes C1 and C2).

**In Vivo PDT Effects**

**Limited Pepsin Digestion**

PDT-treated arteries showed a significantly lower susceptibility to pepsin digestion at all time points measured (1 hour, 44%; 1 week, 36%; 4 weeks, 44%; \( P < 0.0001 \), Figure 6).

**Histology**

No IH was found at 1 and 4 weeks after balloon injury and PDT. At 1 week, no endothelium but occasional platelets adhering to the internal elastic lamina were present. In contrast, at 4 weeks, the artery was reendothelialized. At both time points, the adventitia was repopulated with cells; however, cells were unable to migrate into the media (Figure 7).

**Discussion**

In this study, PDT modified proteins in a collagen type I matrix gel and in an artery to create a barrier to invasive vascular cell migration. Evidence supports that cellular migration through the vascular wall into the subintimal space is a consequence of vessel injury and plays a critical role in the development and progression of IH.\(^{23}\) PDT inhibits IH by acute cell eradication, which is followed by cell repopulation of the adventitia but not the media.\(^{12}\) Because it is hypothesized that inhibition of cellular migration through the vessel wall may be an important factor in altering the postinterventional wound-healing response, the effect of PDT on invasive
cell migration was investigated. A 3D matrix gel model was established, because the interactions between migratory cells and the surrounding matrix are of paramount significance in vascular biology. A matrix gel of collagen type I was selected because it is one of the major connective tissue components of the arterial wall and surrounds SMCs forming a lattice network within the media. In addition to soluble collagen type I, this matrix also contains collagen type III and partially denatured collagen, as well as proteins deposited by the SMCs after plating. Because this model does not fully reflect the composition of vascular ECM in vivo, the relevance of the method was compared with PDT-induced matrix effects on in vivo migration using a balloon-injured artery model.

Next to SMCs, myofibroblast and fibroblasts are known to play a role in the vascular wound-healing response. Because myofibroblasts are not morphologically and functionally well defined, SMCs, which take on a secretory phenotype in vitro similar to injured SMCs in vivo, and adventitial fibroblasts, were used for the in vitro experiments. SMCs and fibroblasts themselves were not PDT treated, so they could simulate the nontreated, adjacent cell populations that repopulate the vessel wall after the complete cell eradication by PDT in vivo.

Under physiological conditions, SMCs are quiescent and embedded in ECM. MMPs, produced by vascular SMCs, are upregulated after arterial wall injury and are necessary for cell migration during the development of IH. In this study, collagenase was unable to digest the PDT-treated matrix to permit adequate migration. However, this is not the only MMP involved in collagen type I degradation. Other proteinases, such as MMP-2, MMP-13, and membrane type 1 MMP have also been shown to degrade collagen type I and play important roles in the migration of vascular cells. In this study, activated MMP-2 was indeed present in the conditioned media, which may explain in part why migration was significantly reduced but not abolished.

After PDT, resistance of the matrix to collagenase might not only be important in inhibiting cellular migration from the adventitia but also be crucial in maintaining the mechanical integrity of the vessel wall. This concurs with previous findings of protein cross-link resistance to enzymatic digestion.

Inhibitors of MMPs, in particular tissue inhibitor of metalloproteinase (TIMP)-3, which is known to be deposited into the ECM, could play an important role in modulating the cellular repopulation of the media after PDT. However, PDT has been demonstrated to inactivate biologically active matrix-associated proteins. Therefore, it seems unlikely that PDT could augment the biological effect of a matrix-associated protein such as TIMP-3.

Differences in depth and number of cells migrating into PDT-treated matrix gels were dose-dependent, emphasizing the importance of PDT dosimetry to inhibit IH in vivo. Cells exhibited a different morphology in matrix gels as compared with controls. This change in cell morphology, in which the cells appeared to be attempting to insinuate themselves through small spaces in the matrix gels, supported the hypothesis that the cross-linked and otherwise altered proteins did not permit the cells to digest the matrix with MMPs and expand to their regular configuration. Cell shape and migration are interconnected through the interactions between integrins and the ECM. It is conceivable that PDT, by modifying integrin binding sites on the matrix, not only affects the ability of the cell to migrate but also the cytoskeletal organization of the cell.

To further elucidate the mechanism by which PDT inhibits vascular cell migration, changes in the structure of collagen were investigated. PDT has been shown to alter matrix-associated proteins. This effect appears to be principally mediated by free radical interactions with amino acids, which lead to conformational and other chemical changes that modify biologically active or specific binding sites of these proteins. This study identified that PDT of collagen type I generated high molecular weight complexes, suggesting cross-linking with increased thermal and mechanical stability.

PDT treatment of control matrix gel solution containing collagen alone induced distinct collagen-to-collagen cross-links. However, the protein cross-links in this specific 3D model, which contained collagen type I and serum, did not only involve collagen-to-collagen interactions. Albumin, the major protein component of serum, by itself did not form cross-links, but in the presence of collagen, it formed heterotypic cross-links different from those noted in the pure collagen solution. These interactions between different molecules suggest that PDT of the vessel wall, which is composed of various proteins, including elastin and fibronectin, can also generate homotypic and heterotypic cross-links. The importance of these observations is underscored by the findings that PDT of an artery resulted in a reduced susceptibility to pepsin digestion. This suggested the in vivo formation of cross-links similar to the in vitro matrix gel data presented and resulting in a barrier for vascular cells to migrate into the media. These data thus provide a novel link between the induction of protein cross-links and the inhibition of cellular migration in the vessel wall, thereby explaining the PDT-induced inhibition of IH.

Invasive cellular migration, which is one of the key factors in the vascular wound-healing response, is modulated by a variety of mechanisms. Using this knowledge, experimental approaches to inhibit IH by using MMP inhibitors or integrin binding inhibitors only resulted in short-term inhibition. These data underline the complexity of events leading to restenosis and suggest that inhibition of cellular migration by disrupting a single pathway may not be sufficient in ultimately preventing restenosis. This study identified yet another PDT effect on the vessel wall: the inhibition of vascular cell migration by stabilization of the matrix, rendering it resistant to collagenase degradation, and the possible alteration of integrin binding sites. These newly identified PDT matrix effects on invasive vascular cell migration, in conjunction with other known effects on the vascular wall, such as complete inactivation of cell- and matrix-associated cytokines and growth factors, could all be major and necessary targets for the observed long-term PDT-mediated inhibition of IH. Thus, because of its multiple effects, PDT is a unique therapeutic approach for inhibiting vascular restenosis and
provides a strong theoretical basis for its successful clinical application.

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