Photodynamic therapy inhibits transforming growth factor β activity associated with vascular smooth muscle cell injury

Randolph G. Statius van Eps, MD, and **Glenn M. LaMuraglia, MD,**
Boston, Mass.

Purpose: **The multifunctional cytokine, transforming growth factor β1 (TGF-β), plays an important role in the development of injury-associated intimal hyperplasia (IH). Strategies to suppress local TGF-β activity may have a clinical potential to prevent restenosis caused by IH. Photodynamic therapy (PDT) involves the local generation of cytotoxic free radicals by light activation of photosensitizer dyes and has been shown to inhibit experimental IH. This study investigated whether PDT-generated free radicals can affect TGF-β activity in a biologic system using vascular smooth muscle cells (SMCs).**

Methods: **The release and activation of TGF-β by injured SMCs in culture was compared between mechanical injury and PDT. Mechanical injury was induced with a rubber policeman, and PDT was performed with the photosensitizer chloroaluminum sulfonated phthalocyanine (5 μg/ml) and 675 nm laser light at subtherapeutic 10 J/cm^2 and the in vivo therapeutic dose of 100 J/cm^2. Cell viability was assessed by the tetrazolium salt conversion assay, and active and total (active + latent) TGF-β was determined by enzyme-linked immunosorbent assay in the conditioned media of SMCs 24 hours after treatment. Functional TGF-β activity was assessed by inhibition of endothelial cell mitogenesis.**

Results: **Both forms of injury severely reduced ($p < 0.0005$) SMC viability to less than 15%. In untreated SMC conditioned media, only 14.5% of the total TGF-β was active (27.7 ± 8.7 pg per 1 × 10^5 cells). However, after mechanical injury and PDT with 10 J/cm^2, there was a significant increase ($p < 0.02$) in active TGF-β (60.1 ± 10.1 pg and 48.6 ± 21.0 pg, respectively), despite a total reduction of approximately 50%. In contrast to this result, PDT with 100 J/cm^2 did not result in increased levels of active TGF-β (8.1 ± 3.5 pg), despite having similar levels of total TGF-β. Consequently, the conditioned media of SMCs that had 100 J/cm^2 PDT did not inhibit endothelial cell mitogenesis as compared with the conditioned media of SMCs with mechanical injury and 10 J/cm^2 PDT ($p < 0.0002$).**

Conclusions: **This report describes two novel findings: (1) injury to SMCs in vitro induces the conversion of biologically latent TGF-β to active TGF-β; and (2) the therapeutic PDT dose interferes with this injury activation process. This study substantiates the concept of local cytokine inhibition by PDT in a biologic system and provides new insights into the mechanisms of PDT-mediated inhibition of experimental IH. (J Vasc Surg 1997;25:1044-53.)**

From the Division of Vascular Surgery, General Surgical Services and Wellman Laboratories of Photomedicine, Massachusetts General Hospital, Harvard Medical School.

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Reprint requests: Glenn M. LaMuraglia, MD, Division of Vascular Surgery, Massachusetts General Hospital, Boston, MA 02114.

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Cell migration and proliferation and enhanced production of extracellular matrix are important events in the biologic repair processes that restore tissue integrity and physiologic function after injury.[1] However, failure to properly terminate this response may lead to progressive fibrosis and tissue damage.[2] The repair process is to a large extent mediated by the release of cytokines and growth factors in response to injury.[1] 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.[2,3]

One such fibrotic condition is intimal hyperplasia (IH) induced by vascular injury, which is a major cause of restenosis after invasive vascular interventions.[4] TGF-β has been shown to be involved in IH after balloon injury in experimental models,[5,6] in human vascular restenosis lesions,[7] and in experimental vein graft IH.[8] 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.[5,6,9,10] Although all cells involved in IH, including smooth muscle cells (SMCs),[11] endothelial cells (ECs),[11] platelets,[12] and monocytes,[13] are known to produce TGF-β in vitro, it is thought that neointimal SMCs are the major source of this cytokine during vascular repair.[5,10]

Strategies to suppress TGF-β activity may have an enormous clinical potential to inhibit IH and other fibrotic conditions that are associated with overproduction of TGF-β. In fact, antibodies against TGF-β have been shown to inhibit IH[14] and several other experimental fibrotic conditions, such as glomerulosclerosis,[15] and skin scarring.[16] 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 human beings. One important aspect of TGF-β regulation is its activation from its precursor latent form to elicit biologic 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.[13] Although it is not clear how TGF-β becomes activated in vivo, it is thought that protease cleavage by plasmin represents a physiologic mechanism of TGF-β activation.[17] Interference with the TGF-β activation process or use of LAP-like proteins that specifically bind to TGF-β are potential means to inhibit TGF-β activity.[18] However, because of the essential systemic physiologic function of TGF-β,[19] only local inhibition of TGF-β at the site of overproduction and intended inactivation may be feasible.

An approach to locally interfere with the biologic activity of important proteins, such as TGF-β, may be photodynamic therapy (PDT). This technique uses wavelength-specific light to activate photosensitizer dyes, which are otherwise relatively biologically inert, for the production of free radical species.[20] As a result of the short half-life of these 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. The PDT-generated free radicals are highly cytotoxic, which has formed the basis for the application of PDT to treat neoplastic disorders[21] and other pathologic states that are characterized by cellular proliferation, such as experimental IH.[22-25] On the other hand, it is known that free radicals can chemically react with lipids and proteins, which may cause functional disturbance of biologic molecules.[26,27] In fact, it has been recently demonstrated that the photochemical reaction induced by PDT profoundly alters the biologic characteristics of extracellular matrix deposited by ECs in vitro.[28] 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.

Because TGF-β is a key mediator of IH and other fibrotic states, this study concentrated on the effects of PDT on the biologic activity of TGF-β. Using a defined in vitro model with vascular SMCs, 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.

MATERIALS 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.[28] Cells were kept in a 37° C, 5% CO_2 incubator, refed every 42 to 72 hours with Dulbecco's modified Eagle's medium 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 (Gibco) on reaching confluence and were used for experiments between the second and sixth subpassages for ECs and between the second and fourth subpassages for SMCs.

PDT. To perform PDT of SMCs in culture, the cells were seeded in full medium at a density of 2.5 ×

10^4 per cm^2 on tissue culture plates (Falcon, Becton Dickinson, Lincoln Park, N.J.) and allowed to attach for 24 hours. The photosensitizer chloroaluminum sulfonated phthalocyanine (CASPc), at a concentration of 5 mg/ml, was subsequently added to the cells in serum-free medium and incubated for 2 hours. After two rinses with phosphate buffered saline solution (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, Calif.) tuned at 675 nm for optimal absorption. The end-fiber irradiance was set at 100 mW/cm^2 to avoid any thermal effects, and two different fluences (total light energies) were applied: a subtherapeutic dose of 10 J/cm^2 and the in vivo therapeutic dose to inhibit IH of 100 J/cm^2. To confirm that PDT cytotoxicity 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 control specimens.

Cell viability assay. SMC viability was determined 24 hours 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) by viable cells.[29] 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 dimethyl sulfoxide (Sigma) for 30 minutes. The intensity of the developed color in each well was read by an enzyme-linked immunosorbent assay (ELISA) reader at 570 nm. The optical density of untreated cells represented 100% viable cells, and background color formation of MTT with dimethyl sulfoxide added to an empty plate represented 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 SMCs that were PDT-treated or mechanically injured. Control specimens included media from untreated SMCs and SMCs that were exposed to the photosensitizer only. To induce mechanical injury, SMCs were vigorously scraped from the well with a rubber policeman.[30] Cell scraping with a rubber policeman represents a form of barotrauma, which resembles in vivo mechanical injury and causes cell membrane damage that could lead to either cell death or recuperation of cell integrity and survival.[30] This form of cell injury was used to compare an in vivo, relevant method of cell trauma with PDT-induced cytotoxicity and to compare 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). The medium was then collected and clarified by centrifugation at 2000 rpm for 15 minutes for TGF-β assay. There were two reasons why the conditioned media was collected after 24 hours. It enables cells to recover or die, and some time was needed for the lethally-injured cells to release their cell-associated factors to the environment. Preliminary experiments at earlier time points after mechanical SMC disruption (data not shown) demonstrate the presence of TGF-β in the media acutely after mechanical cell disruption. This supports the theory that there is a store of TGF-β in the cells that is released to the environment immediately after injury.

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, Wis.), which uses 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 1 Normal HCl, pH 1.5 to 2.5) treatment for 30 minutes before neutralization with 20 ml of 1 Normal NaOH 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 mechanically injured SMCs, an EC mitogenesis assay was used. EC mitogenesis is known to be strongly inhibited by TGF-β.[31] For this purpose, [^{3}H]-thymidine incorporation in ECs was determined as an indicator of DNA replication.[32] ECs were seeded in full medium at a density of 10×10^3 per cm^2 and were allowed to attach for 24 hours. To overcome any depletion of the 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 ECs for 24 hours. In separate experiments, the conditioned media of mechanically injured SMCs was pretreated with a neutralizing antibody against TGF-β1 (R & D Systems, Minneapolis, Minn.) or a nonimmune con-

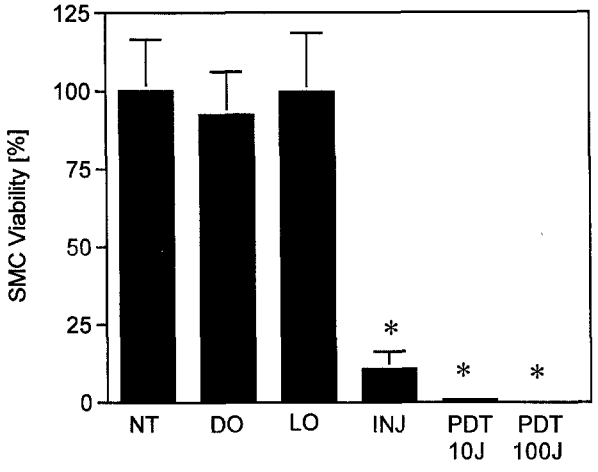

Fig. 1. Effect of PDT and mechanical injury on SMC viability. Viability (as determined by the tetrazolium salt conversion assay) of PDT-treated SMCs with total fluences of 10 J/cm^2 and 100 J/cm^2, SMCs exposed to either the photosensitizer *(DO)* or light *(LO)* only, and mechanically injured *(INJ)* SMCs are compared with nontreated *(NT)* SMC viability, which represents 100% viable cells. Values are mean ± SD. $*p < 0.0005$ versus NT, DO, and LO (analysis of variance; n = 6).

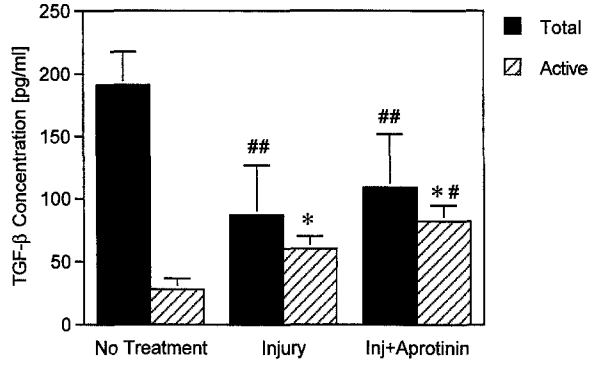

Fig. 2. Effect of mechanical SMC injury on release and activation of TGF-β. Concentration of active and total TGF-β, as measured by ELISA, in the conditioned media of SMCs is plotted for nontreated SMCs, mechanically injured SMCs *(injury)*, and SMCs that were mechanically injured in the presence of the plasmin inhibitor, aprotinin (100 mg/ml). Values are mean ± SD. $*p < 0.0005$ versus active no treatment. $\#p < 0.0005$ versus active injury. $\#\#p < 0.01$ versus total no treatment (analysis of variance; n = 9 for no treatment and injury; n = 6 for active injury and aprotinin; and n = 3 for total injury and aprotinin).

trol antibody (normal rabbit immunoglobulin G, R & D Systems) to determine whether 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, Mass.) was included in the medium. The cells were then washed three times with PBS, dissolved in 0.5 N NaOH, and then placed in ready gel scintillation fluid (Beckman Instrument, Inc., Fullerton, Calif.). The cell-incorporated radioactivity of the SMCs was counted with a scintillation counter (Beckman Instrument).

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

RESULTS

SMC 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/cm^2 (0.9% ± 1.2% and 0.0 ± 1.8%, respectively), whereas exposure of SMC to either light (100 J/cm^2) or photosensitizer only did not affect cell viability (Fig. 1). Mechanical SMC disruption was also associated with a substantial decrease in SMC viability to 10.9% ± 5.6% (Fig. 1).

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 mechanically injured SMCs (Fig. 2). The level of active TGF-β in the conditioned media of untreated SMCs was low (27.7 ± 8.7 pg per 1 × 10^5 cells) as compared with the total amount (191.1 ± 26.7 pg per 1 × 10^5 cells). Although mechanical injury of SMCs resulted in a decrease in the total amount of TGF-β released (86.9 ± 39.97 pg per 1 × 10^5 cells), this was associated with a significant ($p < 0.001$) increase in the level of active TGF-β (60.1 ± 10.1 pg per 1 × 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 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 with mechanical SMC injury without aprotinin (Fig. 2).

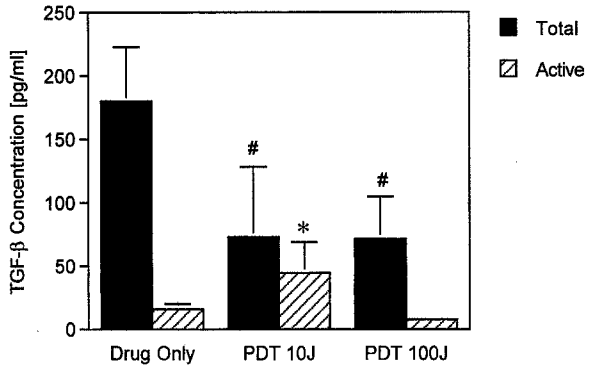

Fig. 3. Effect of PDT on SMC release and activation of TGF-β. Concentration of active and total TGF-β, as measured by ELISA, in the conditioned media of SMCs is plotted for SMCs that were treated with the photosensitizer drug only and PDT-treated SMCs with total fluences of 10 J/cm² and 100 J/cm². Values are mean ± SD. $*p < 0.02$ versus active drug only and active PDT 100 J/cm². $\#p < 0.005$ versus total drug only (analysis of variance; n = 6).

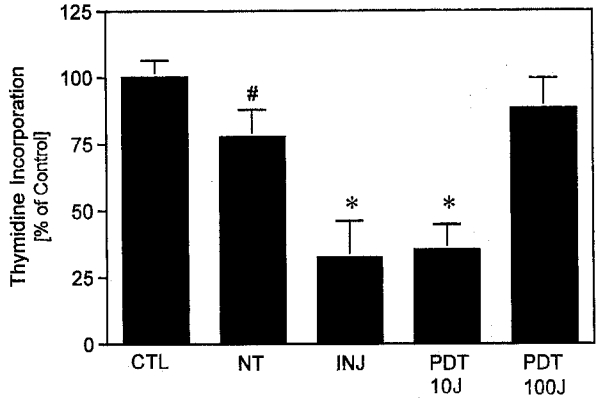

Fig. 4. Inhibition of EC mitogenesis by SMC injury-associated increase in active TGF-β: reversal by PDT with 100 J/cm². Nontreated *(NT)* SMCs, mechanically injured *(INJ)* SMCs, and SMCs that were PDT-treated with 10 J/cm² and 100 J/cm² were allowed to condition their media for 24 hours. This conditioned media was used to assess TGF-β growth-inhibitory effect (^{3}H-thymidine incorporation) on ECs. Medium with 10% calf serum served as control *(CTL)*. Values are mean ± SD. $*p < 0.0002$ versus CTL, NT, and PDT 100 J/cm². $\#p < 0.005$ versus CTL (analysis of variance; n = 12 for CTL; n = 10 for INJ; and n = 6 for NT, PDT 10 J/cm² and 100 J/cm²).

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 SMCs. Exposure of SMCs with CASPc only, which did not affect SMC viability, served as a 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 ± 22.4 pg per 1×10^5 cells), despite a decrease in the total amount (Fig. 3). In contrast, at higher doses of PDT (100 J/cm²), there was no increased level of active TGF-β (8.1 ± 3.5 pg per 1×10^5 cells) despite an equivalent level of total TGF-β (Fig. 3).

Functional effect of cell injury-associated increase in active TGF-β. The biologic activity of TGF-β in the conditioned media was determined using an EC mitogenesis assay (Fig. 4). For this purpose, EC mitogenesis incubated with 10% calf serum medium served as control. The conditioned media of untreated SMCs resulted in a significant decrease (77.6% ± 10.3%; $p < 0.005$) in EC mitogenesis as compared with control (100% ± 6.4%). However, there was significantly ($p < 0.0002$) more inhibition of EC mitogenesis with the conditioned media of mechanically injured SMCs (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 mechanically injured SMCs. 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. 5), whereas presence of the antibody in control medium had little effect on EC mitogenesis (110.7% ± 13%; n = 4).

Because 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 Fig. 4, the SMC-conditioned media of PDT with 100 J/cm² did not significantly affect EC mitogenesis (88.1% ± 11.4%), as compared with control media. This finding strongly indicate that

with this dose, PDT-mediated cytotoxicity does not result in increased TGF-β activity.

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.[22-25] The rationale of PDT as a means to prevent IH has been the local eradication of SMCs in the vessel wall responsible for the fibroproliferative process. The free radicals produced by PDT are highly cytotoxic, thus on 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 SMCs 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 a response of the vessel wall to many forms of injury.[4] 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.[33,34] With appropriate doses of PDT, there is eradication of SMCs in the vessel wall, and yet this is not followed by an inflammatory or fibroproliferative response.[22-25] Instead, the vascular healing response after PDT is characterized by rapid and complete EC regrowth but minimal repopulation of the medial layer with SMC.[23] 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 biologic 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,[35] 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,

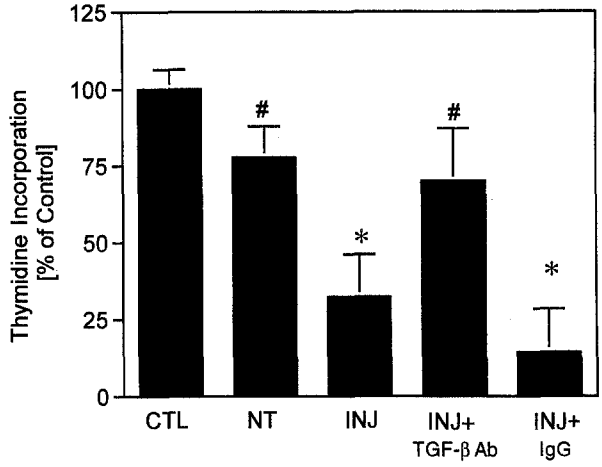

Fig. 5. Effect of neutralizing antibody against TGF-β on SMC injury–associated TGF-β activity. The conditioned media of mechanically injured SMCs *(INJ)* was pretreated with an anti–active TGF-β neutralizing antibody (90 mg/ml) or a normal rabbit immunoglobulin G control (90 mg/ml) and was used to assess the TGF-β growth-inhibitory effect (^{3}H-thymidine incorporation) on ECs. *CTL* and *NT* as in Fig. 4. Values are mean ± SD. *$p < 0.0005$ versus CTL, NT, and INJ + TGF-β Ab. #$p < 0.005$ versus CTL (analysis of variance; n = 4 for antibody groups).

because TGF-β has been implicated to have an inhibitory effect on EC regrowth,[12,36] PDT inhibition of TGF-β activity may be a mechanism to explain the rapid EC recovery observed after experimental vascular PDT. This conjecture is supported by the functional assay performed in this study that demonstrated 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 that showed that the extent of EC recoverage after denudation injury is dependent on the degree of medial wall injury and SMC necrosis.[37] 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 100 J/cm^2 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 SMCs with the subtherapeutic dose of 10 J/cm^2, which reduced cell viability to the same extent as PDT with therapeutic 100 J/cm^2, was associated with a significant increase in active TGF-β. Likewise, vigorous mechanical SMC injury consider-

ably affected cell viability, which was also accompanied by an increase in TGF-β activity. The finding in the present study, and supported by others,[11,38] that untreated bovine SMCs 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[39] that could cleave the latent complex and liberate active TGF-β. Because plasmin is known to activate the latent TGF-β propeptide,[17] 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. Because after PDT of SMCs with 100 J/cm^2 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, because it has been demonstrated that in a dose-dependent way PDT inactivates several enzymes, including plasmin, lysozyme, and pepsin.[27]

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. Because 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 and is released after cytotoxic injury. This notion is supported by a recent in vivo study that demonstrated histochemically that there is some TGF-β present in untreated medial SMCs of the rat carotid artery.[5] 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. 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 SMCs 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 effect.[26] This study examined the effects of PDT on TGF-β activity in a biologic system with vascular SMCs in an attempt to mimic the in vivo situation in which SMCs are eradicated by PDT. Because free radicals travel a short distance of only nanometers to micrometers, the chemical characteristics and cellular distribution of photosensitizers and the laser light parameters will intrinsically determine whether certain biologic molecules will be affected by the PDT effect.[20] The present study examined the effects of PDT with the photosensitizer CASPc, which is known to bind to proteins,[40] 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 biologic 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. This may have contributed to the clinical failure of several pharmacologic attempts to prevent restenosis, monotherapies, that primarily affect one component involved in the pathogenesis of restenosis, such as anticoagulants, platelet antagonists, and angiotensin-converting enzyme (ACE)-inhibitors.[41] Vascular PDT represents a multifactorial approach in that, besides

eliminating the effector cells responsible for IH, it may affect other critical biologic mediators that regulate the excessive healing response associated with vascular injury. Furthermore, a recent in vitro study demonstrated that PDT-mediated changes of the extracellular matrix results in differential effects on vascular cell function in contact with the matrix, resulting in inhibition of SMCs but enhancement of EC proliferation and migration.[28] 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 all the sequence of events that lead to injury-associated IH. This finding has important implications 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 SMCs, but with subsequent delayed IH development.[22] 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 human beings to prevent restenosis.

CONCLUSION

This study describes PDT-effects on the biologic activity of TGF-β associated with SMC injury. Whereas mechanical SMC injury or subtherapeutic doses of PDT results in increased TGF-β activity, with optimal PDT dosimetry, this injury-associated increase in TGF-β activity does not occur. Besides providing novel insights into possible mechanisms by which PDT inhibits experimental IH and promotes intimal EC regrowth, this study offers a new concept for the application of PDT as a means to locally interfere with the aberrant activity of biologic peptides involved in the development of other fibrotic conditions, such as chronic arthritis and skin scarring.

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DISCUSSION

Dr. Gary Nackman (New Brunswick, N.J.). I enjoyed your talk very much. When I was at Dartmouth, we too were very interested in the role of TGF-β in the interactions between ECs and SMCs. I have some questions about the methods in your paper.

Did you normalize the amount of active TGF-β to the amount of viable DNA present at the end of the 24-hour period after intervention? And did you consider measuring some of the other described activators of TGF-β such as cathepsin D which is present in SMCs that might be released, or thrombospondin, which has been described as an additional method of a nonproteolytic activation of the TGF-β?

Dr. Randolph G. Statius van Eps. Thank you, Dr. Nackman. We performed the mitogenesis assay 24 hours after adding the condition medium, and we did not correct for DNA content for EC mitogenesis, but other experiments from our laboratory have shown that there is no difference at this time point in cell number.

Thank you for your comments regarding the other pathways that could be involved in the activation after SMC injury. Until now we have not performed these experiments. We only looked at plasmin, and it does not seem that plasmin is indeed the factor involved in the activation of TGF-β in these experiments, but other factors such as thrombospondin might well be involved because this molecule is indeed associated with SMCs and can activate TGF-β.

Dr. K. Craig Kent (Boston, Mass.). I want to congratulate you on your work in this study. It is very exciting. In fact, I have followed with interest over the last several years the many manuscripts about PDT that have emanated from your laboratory.

I have three questions. The first is a question about

your technique. You compared PDT with an injury model that you created in vitro, but your injury model was one in which you used a rubber policeman to scrape the bottom of a plastic dish. Now some people do that just to remove their cells from the dish. How do you know that you have injured and not completely eliminated the cells with this technique?

Second, do you have any insight as to why PDT, which I think is a cytotoxic therapy, kills cells in a different way than does your injury model. Because both methods are cytotoxic, why does just one of them reduce the amount of active TGF-β and the other not?

My third question is about the application of this technique. I have been very impressed from what I have read. It sounds like photodynamic therapy has great potential, at least in animals. Has this technique been applied to human beings? Is this the next step?

Dr. Statius van Eps. Thank you, Dr. Kent. The answer to the first question, we use mechanical injury to SMCs because it has been described by a research group from the Children's Hospital that mechanical injuries to SMCs mimic the injury that occurs in cells in vivo. There is disruption of plasma membrane, and if severe, it will cause lethal injury. So it was based on a method that was used in the literature to injure cells, but I presume that other ways of injuring the cells could also be applied.

The second question is regarding the mechanism by which we think PDT might be influencing growth factors. Well, PDT generates free radicals, and free radicals are highly reactive molecules. Besides free radical effects on cells that yields cytotoxicity, we hypothesize that free radicals may also chemically react with proteins. This might be possible, that factors that are involved in the activation of TGF-β might be affected. It may also be the case that free radicals alter TGF-β itself; however, this was not addressed directly in this study.

To address your last question, PDT has not yet been used in human beings to inhibit restenosis. There have been some investigations with larger animals, with variable results, and we are moving towards larger animal studies. We believe that really understanding how we affect the vascular wall with PDT in a little more detail could improve the PDT parameters that we are going to use in larger animals and in human beings, that is, the class of photosensitizer, the amount of light that we have to give, and how we are going to give the light.

Dr. Richard Powell (New Haven, Conn.). Could you comment on the TGF-β that you are measuring? Do you think it is from increased SMC expression or an increase in release from the extracellular matrix?

Dr. Statius van Eps. We measured TGF-β 24 hours after treatment. After PDT and, actually, also after mechanical injury there is almost no cell viability, so we were actually surprised to still be able to measure TGF-β after 24 hours when all cells died. So we now think that a large portion of the TGF-β we are measuring is coming directly from the cells when the cells are injured and killed. In other words, we believe that there is some TGF-β stored in the cells that gets released after the cell membrane has been damaged, so we do not think that this has to do with expression of TGF-β. That might explain the decrease in the total amount after injury as compared with nontreated cells, because the nontreated cells are still able to make their TGF-β, whereas the injured cells are dead and cannot produce TGF-β.