Inhibition of Smooth Muscle Cell Proliferation by Visible Light–Activated Psoralen

Bauer E. Sumpio, Guangdi Li, Lawrence I. Deckelbaum, Francis P. Gasparro

Abstract The present study was designed to evaluate the effect of 8-methoxypsoralen (8-MOP) activated with visible light (419 nm) on the suppression of smooth muscle cell (SMC) proliferation in vitro. We hypothesize that if visible light (VL) instead of UVA is used to photoactivate 8-MOP, cytotoxic 8-MOP–DNA cross-link formation can be minimized. Bovine aorta SMCs (2×10^5/cm²) were incubated with 8-MOP (1 µg/mL) for 30 minutes (in the dark) and exposed to a range of VL (2 to 69 J/cm²) to determine the dose of VL that inhibits SMC proliferation with minimal toxicity. The results show that 8-MOP in combination with 2 to 12 J/cm² VL reversibly inhibited SMC proliferation for up to 5 days after treatment. SMC viability was confirmed by trypan blue exclusion.

The significant incidence of restenosis after coronary angioplasty continues to dim the attractiveness of this minimally invasive therapeutic option for coronary artery disease. Although many mechanical and pharmacological remedies have been proposed, none have withstood the test of clinical trials. One of the primary reasons for this failure is the gaps in our knowledge of the mechanism of restenosis and hence the lack of a more “selective interference” in the process. Localized delivery of drug represents a logical approach to this problem, since optimum amounts of drug can be delivered with minimum concern for systemic side effects.

Psoralen phototherapy has been used in a variety of clinical settings because of its ability to interrupt cell replication. Since the process of intimal hyperplasia and restenosis represents a significant defect in the control of smooth muscle cell (SMC) migration and proliferation, several groups, including ours, have sought to determine whether psoralen phototherapy could be used as an adjunct to angioplasty. Our previous work demonstrated that 8-methoxypsoralen (8-MOP) activated with long-wavelength ultraviolet radiation (320 to 400 nm, UVA) could dose-dependently inhibit the proliferation of cultured SMCs. However, compared with UVA light, visible light (VL) would have deeper penetration into tissue, would be easier to deliver intra-arterially, and might be less mutagenic and cytotoxic for photoactivation of 8-MOP, since the level of highly toxic interstrand cross-links can theoretically be reduced by nearly an order of magnitude. Therefore, in the present study, we sought to evaluate the potential efficacy of VL for the photoactivation of 8-MOP on SMC proliferation.

Our results show that VL at 419 nm can activate 8-MOP, resulting in a dose-dependent inhibition of SMC replication. Furthermore, we observed a significant decrease in the percentage of cross-links in SMCs with VL-activated 8-MOP (VL/8-MOP) compared with SMCs with UVA-activated 8-MOP. There was also a correlation between the cytostatic effect of this protocol and the kinetics of DNA photoadduct formation and repair. Finally, in cell-cycle progression studies, we noted a specific blockade at G2.

Materials and Methods

Cell Culture

Thoracic aortas were removed aseptically from calves and transferred on ice to the tissue culture facility, and SMCs were obtained by the explant method. The vessels were opened in a laminar flow hood, and the endothelium was mechanically scraped off. Two-millimeter biopsies of the aorta media layer were obtained and placed in a culture dish with media consisting of Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, antibiotics (300 U/mL penicillin, 300 U/mL streptomycin), and 0.2 mol/L L-glutamine. The media biopsies were left at 37°C in a 5% CO2 incubator until SMC explants were detected. SMCs were differentiated from fibroblasts by their appearance during confluency; SMCs form a hill-and-valley morphology, whereas the latter produce a whorl type of appearance. In addition, SMC proliferation was inhibited by heparin (100 µg/mL), and the cells stained positively with an antibody specific for SMC actin (HHF35, Enzo Diagnostics). SMCs were subcultured with 0.01% trypsin/EDTA, and SMCs from passages 3 to 7 were used for experimentation.

Phototherapy

8-MOP (Sigma Chemical Co) was prepared in absolute ethanol, and the concentrations of all diluted solutions were...
verified either spectrophotometrically or by high-performance liquid chromatography (HPLC) analysis. Cells were exposed to VL with a photochemical reactor (Southern New England Ultraviolet Co) equipped with six equally spaced 8-W lamps emitting broadband blue light (peak λ, 419 nm; bandwidth, 34 nm) at a distance of 12.5 cm. The light doses were determined with a calibrated silicon diode UV250BQ (EG&G). The average irradiance of the lamps was 6.5 mW/cm². In other studies, cells were exposed to 2 J/cm² UVA through UVB-absorbing plate glass in a light box equipped with broadband UVA lamps (Westinghouse UVAR 05-88; 320 to 400 nm, peak emission at 355 nm). The light doses were determined by radiometry (International Light). The average irradiance of the lamps was 5 mW/cm².

Experimental Protocol

Bovine aortic SMCs were plated (10,000 to 20,000/cm²) on six-well plates and after a 24-hour attachment period were incubated with 1 µg/mL 8-MOP for 30 minutes in the dark. SMCs were exposed to either 0, 2, 7, 12, 23, or 69 J/cm² of 419-nm VL, and on specified days, the wells were washed with phosphate-buffered saline (PBS), 0.25% trypsin/EDTA was added, and the cell number of an aliquot was counted with a Coulter Counter (model ZM). Cell viability was determined by trypsin blue exclusion. Preliminary studies with longer-wavelength visible light (447 nm) were also performed to determine the efficiency of photoaduct formation and the effect of cross-link formation. For these studies, cells were incubated with a higher concentration of 8-MOP (20 µg/mL) (see "Discussion").

In parallel experiments, the cells were washed once with PBS for 3 minutes, and fresh medium was placed on the cells. [3H]Thymidine (2.5 µCi/mL) was added to the quiescent cells just before the conditioned medium. After a 24-hour incubation period, the medium was removed and the cells were washed three times, 0.25% trypsin was added for 10 minutes, and the well surface was scraped. The cells were precipitated with 10% trichloroacetic acid (TCA), and TCA-precipitable [3H] counts were determined.

Cell Cycle Analyses

Control or VL/8-MOP–treated SMCs were detached with 0.005% crystalline trypsin and 0.02% EDTA at 4°C and then neutralized by soybean trypsin inhibitor (4:1, wt/wt). Cells were washed, resuspended at 2x 10⁵ cells/mL in cold PBS, and fixed by dropwise addition of 2.4 mL 95% ethanol at −20°C. The fixed cells were washed with cold PBS, microscopically checked for the absence of doublets and clumping, treated with 100 µL propidium iodide (180 µg/mL) and 100 µL ribonuclease A (16 µg/mL), and mixed with 800 µL PBS for 20 minutes at room temperature. Samples were filtered through 37-μm nylon mesh (Small Parts, Inc) immediately before analysis. Flow cytometry was performed on a Becton-Dickinson FACS IV system. Single-cell DNA fluorescence measurements were done by waveform discrimination of raw fluorescence data by use of correlated area/peak measurements of DNA content histograms to exclude doublets, higher aggregates, and cell debris. Histograms of individual cell DNA content versus number of cells were used to discriminate cells in quiescence or interphase (G₀+/G₁) from cells that had completed doubling of DNA content but were not yet in mitosis and those in mitosis with double normal DNA content (G₂+/M) and a third group still making DNA but intermediate in DNA content (S phase).

HPLC Analysis of 8-MOP–DNA Photodadducts in SMCs

SMCs (10x10⁶ cells) were incubated with 1 µg/mL 8-MOP (including 100 to 250 µL of [3H]8-MOP, specific activity, 83 Ci/mmol; Amersham) for 30 minutes in the dark and then irradiated with VL as described above. The cells were then trypsinized and suspended in 500 µL of 50 mmol/L Tris/150 mmol/L NaCl/100 mmol/L EDTA (pH 8), and the DNA was isolated as previously described. DNA concentration was determined by UV spectroscopy (LKB UltrSpec II), and the molar concentration in terms of nucleotide units (base pairs, bp) was computed from the absorbance at 260 and 280 nm. The DNA was hydrolyzed enzymatically with 1 U/mL P, nuclease and 0.1 U/mL DNase I in water and then applied to an ODS reversed-phase column (Regis Rexchrom; 4.6x250 mm, 5 μm) for analysis. The [3H]8-MOP photodadducts (4',5'- monoadadducts [4',5'-MA], 3,4 monoadadducts [3,4-MA], and cross-links) were detected by scintillation analysis of HPLC fractions collected during the analysis of enzymatically hydrolyzed DNA isolated from cells. With <50 μg cellular DNA, this technique can detect <0.2 photodadducts per million bp (Mbp).

Statistical Analysis

The data are expressed as mean±SD. The cell proliferation studies and [3H]thymidine studies were analyzed by repeated-measures ANOVA followed by post hoc testing. Values of P<.05 were considered statistically significant.

Results

Effect of VL/8-MOP on SMC Proliferation

The effect of VL/8-MOP (1 µg/mL) (doses of VL over a wide range, 2 to 69 J/cm²) on SMC proliferation is shown in Fig 1. The lowest dose of VL (2 J/cm²) had a minimal, reversible effect on SMC proliferation, whereas high doses of VL (23 and 69 J/cm²) were cytotoxic. The growth of SMCs treated with drug or light alone (at each dose) was not significantly different from control, untreated SMCs (data not shown). At intermediate doses (7 and 12 J/cm²), SMC number remained at a constant low level for 3 to 5 days after the VL/8-MOP treatment. This effect was transient and reversible, as evidenced by the increase in cell number by day 10. In contrast, our previous study showed that activation of 8-MOP (1 µg/mL) with low-dose UVA (2 J/cm²) led to cytotoxicity.4

Fig 2 shows the results of experiments to assess cell number at a longer time course and [3H]thymidine incorporation in SMCs exposed to 8-MOP (1 µg/mL)
and 12 J/cm² VL. Panel A shows that after an initial nonsignificant decrease 1 day after treatment, control cells (8-MOP without VL) showed a normal growth rate. For VL/8-MOP–treated SMCs, after a similar nonsignificant initial decrease on day 1, cell number remained constant for 7 days and then achieved a higher plateau level between days 10 and 14. Panel B shows that control SMCs had a typical [³H]thyminde incorporation into DNA, with an initial exponential phase followed by a reduced level of DNA synthesis when confluence was attained. SMCs treated with VL/8-MOP had a reduced [³H]thyminde incorporation into DNA that increased only slowly with time.

**Effect of VL/8-MOP on SMC Cell Cycle**

To determine the effect of VL/8-MOP on SMC-cycle progression, the DNA content of SMCs treated with 12 J/cm² and 1 μg/mL 8-MOP was analyzed by flow cytometry (Fig 3). Control SMCs maintained a similar profile through 10 days with 64% to 80% of cells in G₁ of the cell cycle (DNA content before DNA replication), 6% to 23% in the S phase (during DNA replication), and 9% to 21% in G₂ (with double the DNA content after DNA replication) or the M (mitosis) phase. SMCs treated with VL/8-MOP had a decrease in G₀/G₁ to 43% by day 1 that persisted to day 5, with a corresponding increase in G₂/M to 21%. This pattern reverted to control profiles by day 7.

**Quantification of 8-MOP Photoadduct Formation and Repair in VL/8-MOP–Treated SMCs**

HPLC analysis of enzymatically hydrolyzed DNA from 12 J/cm² VL–activated 8-MOP–treated SMCs is shown in Fig 4. The pattern of the peaks for the cross-link, the 3,4–MA, and 4',5'–MA corresponds to that previously reported for keratinocytes and lymphocytes.10,11 Fig 5 shows that immediately after VL/8-MOP treatment, there were 13.5 adducts (9% 3,4–MA, 78% 4',5'–MA, and 13% cross-link). By day 1, the overall number of adducts was reduced, with a proportional decrease in monoadducts, and this pattern continued until day 10, when 37% photoadducts remained. The number of cross-links, however, remained constant (~10% of the photoadducts) throughout the period tested. The initial suppression in cell number on days 3 through 5 seen in Fig 2A parallels the high adduct burden depicted in Fig 5, and the partial recovery in cell number noted on days 7 through 15 also correlates with the removal of 8-MOP photoadducts. In addition, although the cells began proliferating by day 7 after VL/8-MOP treatment, the presence of low levels of...
cross-links may have prevented the ultimate attainment of control levels of cell number.

Discussion

Despite the established role and widespread application of percutaneous transluminal coronary angioplasty, the restenosis rate is limited by several factors such as animal, autopsy, and other class of photosensitizer activated with longer-wavelength VL, have minimal toxicity and, because they have recently been reported to be effective for the reduction of SMC proliferation in an animal model, show great promise.

The advantages of photoactivated 8-MOP for prevention of restenosis include the ability to target therapy to the angioplasty site, the absence of cytotoxicity, and the reversibility of inhibition. The rapid clearance of 8-MOP minimizes clinical phototoxicity. In addition, its common use in the treatment of dermatologic lesions has demonstrated its long-term safety and thus suggests that it may also be an excellent agent for photochemotherapeutic inhibition of SMC proliferation. Since restenosis is probably a manifestation of the general wound healing response of vascular tissue subjected to angioplasty, potent localized therapy will probably be necessary for efficacy with minimal systemic effects. UVA/8-MOP therapy can be directed to a distinct site on the vessel either by local drug and/or local light delivery by currently available technology. Although 8-MOP has been administered systemically (orally and intravenously), it could also be administered through an intraluminal catheter to achieve high local concentrations. Intra-arterial light irradiation of a portion of the arterial wall is feasible with a recently described fiber-optic balloon catheter that has been evaluated clinically for simultaneous balloon dilatation and arterial wall irradiation.

In a previous study, we showed that exposure of cultured SMCs to UVA-activated 8-MOP led to the inhibition of cell proliferation. Scintillation analysis of HPLC fractions collected during the analysis of enzymatically hydrolyzed DNA isolated from SMCs indicated that a large number of 8-MOP photoadducts were formed in SMCs on exposure to 1 μg/mL 8-MOP and 2 J/cm² UVA (day 0, 45% cross-links and 55% monoaducts; 88 adducts/Mbp). We computed an integrated extinction coefficient (IEC) from the overlap of the 8-MOP absorption spectrum and the output of the UVA and 419-nm lamps and preliminary data obtained with a 447-nm lamp (see Table 1). The relative IEC was used to estimate doses of VL and 8-MOP concentration that would induce levels of adduct formation that would reversibly inhibit SMC proliferation. Table 2 shows that the number of photoadducts/Mbp demonstrated a linear correlation with the product of incident VL dose, drug concentration, and the relative IEC at a particular wavelength. Table 2 also shows the adduct distribution for DNA isolated from SMCs treated with 8-MOP and VL.

In the present study, we make the novel observation that 8-MOP activation with VL resulted in a partially reversible, dose-dependent inhibition of SMC replication. The required dose of VL, with 8-MOP at a concentration 10 times higher than with UVA, was initially estimated on the basis of the IEC. A time-course study of the cell distribution during the cell cycle by flow cytometry demonstrated that at a dose of VL/8-MOP that reversibly inhibits SMC proliferation,

### Table 1. Integrated Extinction Coefficients

<table>
<thead>
<tr>
<th>Lamp*</th>
<th>Range (nm)</th>
<th>ε, M⁻¹·cm⁻¹</th>
<th>εIntegrated (a.u.)</th>
<th>εIntegrated (relative)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UVA (355)</td>
<td>320-400</td>
<td>1650</td>
<td>48 180</td>
<td>1.0</td>
</tr>
<tr>
<td>(419)</td>
<td>385-445</td>
<td>0.66</td>
<td>730</td>
<td>0.015</td>
</tr>
<tr>
<td>(447)</td>
<td>400-465</td>
<td>0.22</td>
<td>46</td>
<td>0.0010</td>
</tr>
</tbody>
</table>

*λmax, nm, indicated in parentheses.
the cells were arrested at the G₂ phase during the interval of maximal SMC inhibition. There was a concomitant accumulation of cells in the S phase and a decrease in the number in the G₁ phase. However, the possibility that there may be abnormalities in the progression of cells from early G₁ to the late G₂ phase cannot be excluded. Likewise, the mechanism of VL/8-MOP arrest of cells at G₂ has not been defined and cannot be determined from our study. However, this G₂-specific block in the cell cycle has also been reported in cells exposed to x-rays,22 some cancer chemotherapeutics,23 and previous studies reporting cell cycle-specific interference by 8-MOP activated by UVA,5 although our study is the first to confirm this phenomenon in SMCs with VL activation of psoralen. Some investigators have suggested that this shift to G₂ may result from cell aggregation or adsorption of DNA from the lysed cells. Furthermore, since with this method, the migration of the cells through the cell cycle was detected by the change in DNA content of each cell, the halt and recovery of the migration of the cells should mirror the change in status of DNA replication of the cells. This is corroborated by the studies of [³H]thymidine incorporation. There was a decrease in DNA replication immediately after treatment, which after 5 days slowly recovered.

The formation of 8-MOP photoadducts in SMCs after VL irradiation was confirmed by HPLC analysis, which revealed that monoadducts were formed to a much greater extent than cross-links. Other investigators have examined the mutagenicity of monoadducts and cross-links in yeast by use of VL alone to induce predominantly monoadducts or VL plus UVA to enrich cells in cross-links.24 These investigators have shown that the change in the monoadduct/cross-link ratio has a minimal impact on cell survival despite a sixfold increase in mutagenesis. Thus, the reduction in intrstrand cross-links seen in our study suggests that psoralen photoactivation by VL may be less mutagenic and cytotoxic in SMCs than UVA. The recovery in SMC number with time after VL/8-MOP coincides with the resumption (day 3 to 5) of the cell cycle through the G₂ phase and maximal level of photoadduct removal. It has been shown that DNA repair occurs with a distinct hierarchy. Adducts on transcribed strands are repaired first, followed by other adducts in nonessential regions of the DNA sequence. Furthermore, monoadducts can escape repair and be incorporated into daughter strands.25 The SMC recovery may have occurred once the adducts in a crucial “proliferation” gene had been removed. The detection of adducts at days 7 to 10, when significant cell growth had occurred, suggests that the apparent reduction in adduct number may actually be due to new SMC overgrowth and the presence of additional DNA, which would be consistent with the persistence of adducts in daughter strands.

Our studies on the time course of monoadduct and cross-link formation are consistent with this hypothesis. Repair of these photoadducts, especially the cross-links when present at higher percentages, has been postulated to be important in the recovery of cell function.8,11,12 Despite prolonged culture after VL/8-MOP treatment, the recovery of SMC number never attained control values when the VL dose was above a certain level (>7 J/cm²). Whether this can be attributed to a crucial threshold of DNA damage that cannot be corrected is unknown, as is the issue of whether a longer duration of experimentation would uncover a “rebound” effect. It also cannot be determined whether cell recovery represents a homogeneous process or simply the selection of a subset of SMCs that are more resistant to phototherapy. Likewise, the effect on SMC function, and hence other DNA synthesis pathways other than cell replication, still needs to be determined.

The clinical impact of these findings is related to the availability of VL lasers, the low level of cross-links, and, by extension, the easy and quick recovery from the psoralen phototherapy. The cytostatic effect of VL/8-MOP therapy without cytotoxicity is theoretically preferable to cytotoxic therapies for restenosis. However, in vivo studies would need to be performed to corroborate the beneficial effect of VL/8-MOP seen in our in vitro study. Nonetheless, the failure of cytotoxic chemical26 or physical27 agents to prevent restenosis has been demonstrated repeatedly. SMC necrosis could structurally weaken the arterial wall and stimulate a secondary inflammatory response. The former could result in aneurysm formation, whereas the latter could exacerbate cellular proliferation. Our results demonstrate that photochemotherapy with VL/8-MOP is reversibly cytostatic and not cytotoxic in vitro. In contrast, high-dose 8-MOP/UVa therapy can be cytotoxic.4 The arterial injury of angioplasty stimulates an acute cellular proliferative response that peaks at 2 weeks and diminishes in intensity over time.28 Therefore, transient SMC inhibition as achievable with VL/8-MOP may be optimal. The DNA repair that allows for the eventual return of proliferative capacity may restore SMC functionality at a time when the stimulus to proliferate has waned.

In conclusion, VL/8-MOP can reversibly inhibit SMC proliferation in vitro in a dose-dependent fashion that parallels 8-MOP monoadduct and cross-link formation. Since present technology is capable of delivering both light and drug intra-arterially, this approach may be the basis for a novel local treatment of the intimal hyper-

<table>
<thead>
<tr>
<th>Wavelength (dose), nm (J/cm²)</th>
<th>[8-MOP], μg/mL</th>
<th>Total Adducts per Mbp</th>
<th>4',5'-MA, %</th>
<th>3,4-MA, %</th>
<th>XL, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>UVA (2)</td>
<td>1.0</td>
<td>88</td>
<td>41</td>
<td>12</td>
<td>47</td>
</tr>
<tr>
<td>419 (7)</td>
<td>1.0</td>
<td>9.0</td>
<td>84</td>
<td>13</td>
<td>3</td>
</tr>
<tr>
<td>419 (12)</td>
<td>1.0</td>
<td>13.5</td>
<td>78</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>447 (12)</td>
<td>20.0</td>
<td>22.4</td>
<td>90</td>
<td>9</td>
<td>1</td>
</tr>
</tbody>
</table>

8-MOP indicates 8-methoxypsoralen; MA, monoadducts; and XL, cross-links.
plasia or restenosis that frequently occurs after angioplasty.

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