Optimization of Targeted Therapies to Inhibit Smooth Muscle Cell Invasion In Vitro

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Abstract-**Vascular smooth muscle cell (VSMC) proliferation and migration has been correlated with intimal hyperplasia (IH) after vascular interventions such as angioplasty, stenting, and vascular graft surgery. Therefore, therapies targeting inhibition of VSMC migration may lead to higher patency rates in vascular grafts and reduced IH in other vascular interventions. This study tested three targeted therapies and their combinations: hyperfunctional $\alpha_v\beta_3$ integrin expression, cyclic-RGD release and tissue inhibitor of metalloprotease (TIMP)-1 release. It was found that a combination of 1.0 mM cyclic-RGD and 10 ng/mL TIMP-1 maximally inhibited smooth muscle cell invasion over individual or other combinations of treatments over 72 hours.**

***Keywords*---Hyperplasia, smooth muscle cells, invasion, migration, integrin, peptide.**

I. INTRODUCTION

Cardiovascular surgical procedures, such as angioplasty, stenting and vascular grafts, have a high incidence of intimal hyperplasia, which often leads to restenosis or occlusion of the artery. Intimal hyperplasia is characterized by a hyperplastic response of arterial smooth muscle cells (SMCs) [1]. In a non-pathologic response, the SMCs heal a damaged artery wall and return to a quiescent state. In small diameter arteries and grafts (ID < 6 mm), this response tends to be pathologic when the SMCs proliferate, invade to the arterial lumen and deposit extracellular matrix (ECM) proteins in excess (hyperplasia) leading to reduced flow or occlusion [2].

Many proposed therapies targeting different mechanisms of SMC invasion are being studied, though few studies focus on quantitatively comparing and combining therapies to engineer a clinical solution [3-7]. This study focuses on choosing which therapy or combination of therapies provide a superior solution to SMC hyperplastic behavior. The three potential therapies examined in the study are: tissue inhibitor of metalloproteinase 1 (TIMP-1) release, cyclic-RGD release and over expression of hyperfunctional $\alpha_v\beta_3$ integrins (Hyp-int) using a gene therapy delivery vehicle. The experiments modeled 1-D invasion behavior through the arterial basement membrane.

Invasive behavior is not only affected by the factors involved in surface behavior, but also by ECM density and composition, proteinase activity and chemoattractor concentration gradients. All three potential therapies were quantitatively compared using an optimally designed fluorescent invasion assay to test for SMC invasion using platelet derived growth factor-BB (PDGF-BB) as a potent chemoattractor. TIMP-1/C-RGD and TIMP-1/ Hyp-β_3 combinations were also analyzed for synergistic, constructive or destructive interactions using general factorial design models.

II. METHODOLOGY

A. Optimal Invasion Well Preparation

The FluoroBlok™ assay comes in a 24 well plate configuration and efficiently blocks wavelength transmissions from 490-700 nm. The membranes' raw and corrected mean fluorescence blocking efficiency (FBU), with 8μm pore sizes, for Oregon Green 514 is 99.85% and 99.87%, respectively (Becton, Dickinson and Co., Franklin Lakes, NJ). For all the studies, we modified the use of the standard membrane FluoroBlok™ well inserts with upper and lower reservoirs. The detailed protocol is outlined in Appendix H: Protocols and Procedures-SMC Invasion 24-well preparation. Prior to the experiments, we added a dilute thin layer of Matrigel® (38.75 mg/100 mL/well, determined from previous section results) and let dehydrate at room temperature in the sterile hood for 48 hours with the lid slightly ajar to promote slow dehydration. The plates were then frozen at -20°C until experiment day. On experiment day, the plate was rehydrated for 2 hours at 37°C and 5% CO2 in air with 100mL and 900 mL serum free media (Medium 231 + 0.25 wt% BSA + 1% ABAM) above and below the membrane, respectively.

B. Smooth Muscle Cell Fluorescence and Preparation

Human coronary artery smooth muscle cells (HCASMCs) in serum free media (Medium 231 + 0.25 wt% BSA + 1% ABAM) are labeled with 10 μM CellTracker™ Green in media (ex: 485nm, em: 530 nm wavelengths) cytoplasmic fluorescent tag (CellTracker™ Green: MP #C-7025; Molecular Probes, Eugene, OR) and incubated for 30 minutes at 37°C and 5% CO_2 in air (See Appendix H: Protocols & Procedures-Matrix Migration Assay Protocol). The staining media is aspirated and serum free media is added. The cells incubated for another 30 minutes at 37°C and 5% CO_2 in air to conformationally change the fluorescent tag causing the cells to retain the dye long term. Cells are then detached using 0.05% trypsin, 0.53 mM EDTA and seeded at a concentration of $5.0x10^4$ cells/100 μL/well over the Matrigel™ barrier.

C. Hyperfunctional Integrin MOI Preparations

For this study, Hyp-int lysate was added to serum free media (Medium 231 + 0.25 wt% BSA + 1% ABAM) to create (3X) concentration values of 0, 5, 10 and 20 MOI. The control vector, β-gal, was also mixed in serum free

media at (3X) concentration of 20 MOI. The concentrated solutions were diluted 1:3 in the upper well insert to achieve the indicated (1X) concentrations for experimentation.

D. Matrix Invasion Assay Experimental Setup

The matrix invasion assay experiment was set up according to the Matrix Invasion Assay Protocol in Appendix H: Protocols and Procedures. In summary, 100 µL of fluorescently stained cells (~50,000 cells) were added to the upper inserts of the rehydrated Matrigel invasion wells already containing 100 µL of serum free media. 100 µL at 3X concentration of appropriate inhibitor: cyclic-RGD, TIMP-1 or Hyp-int. was added to the upper well inserts to obtain the desired 1X concentrations under study. Lastly, PDGF-BB at (10X) concentration was added to the bottom well containing 900 µL serum free media to obtain the 100 ng/mL concentration. This method eliminated errors or variability due to aspiration of matrix or gel observed during optimization experiments. These volumes minimized expensive protein and peptide quantities used and eliminated hydrostatic pressure effects between the upper and lower wells according to the manufactures specifications (BD Biosciences Discovery Labware, Bedford, MA).

E. Fluorescent Microscopic Imaging

SMC migration was visually quantified by digital capture of inverted fluorescent microscopy pictures at 0, 24, 48 or 72 hours, depending upon the experiment (See Figure 7.2). Up to five non-overlapping fields of view could be achieved by geometry alone, however out-of-plane auto-fluorescing debris would not guarantee quantitative low noise data from all five fields. Therefore, three random non-overlapping pictures per well were taken with a 5X objective with four replicate wells for a total of n =12 independent pictures. Images were digitally captured using a Magnifire camera and saved as 8-bit TIFF files for later image processing. Invasion of fluorescent cells through the bottom membrane was quantified via their total cell count and total cell area per frame.

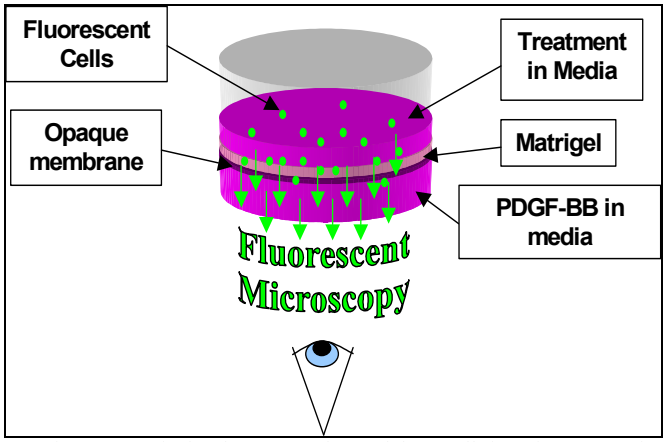

Fig. 1. Matrigel® layer on FluoroBlok™ cell invasion assay for comparison of invasion and migration therapies. Used with permission [8].

F. Statistical Analysis of Results

The raw cell count and total area ratio data was averaged and normalized to each 24-well plate's average positive control data. Dividing the raw average by the positive control average and multiplying by 100 obtained the resulting percent of positive value. This normalization allowed for comparison of results between different plates and thus different inhibitors and their combinations.

All statistics were performed on the normalized data using Stat Ease statistical software. Statistical significance for multiple factors was determined using ANOVA general factorial model with significance defined as $p < 0.05$. Significance between means of a single factor was determined using two tailed, student t-tests assuming unequal variances with significance defined as $p < 0.05$. D-optimization methods applied to the ANOVA general factorial models were used to quantitatively determine optimal solutions for individual and combinatorial treatments. For further information on these methods, see Montgomery, Design and Analysis of Experiments [9].

III. RESULTS

A. Transient Inhibition of HCASMC Invasion by Combining TIMP-1 and Hyperfunctional β_3 Integrin Subunit Expression

All normalized area ratio data is reported as a percentage of the positive control. Figure 2 shows the normalized area ratio data results.

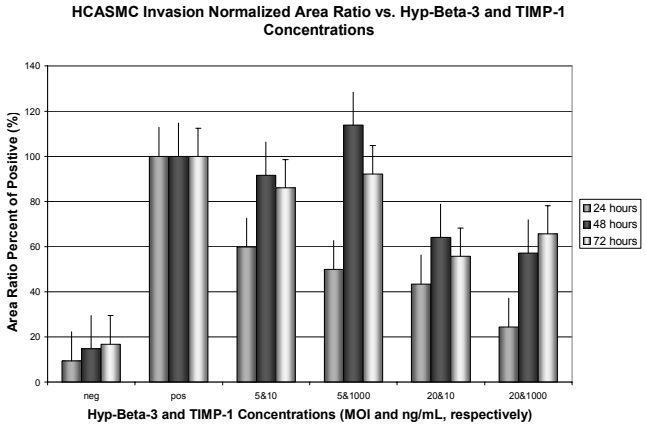

Fig. 2. Normalized area ratio results for TIMP-1 and hyperfunctional β_3 integrin subunit expression. The values are expressed as percent means ± S.E., n = 12, relative to the positive control without treatment. Used with permission [8].

The design of the experiment was a 2-level factorial with Hyp-int vector infection varied from a high of 20 MOI to a low of 5 MOI. TIMP-1 varied from a high of 1000 ng/mL to a low of and 10 ng/mL. At 24 hours, all combinations significantly inhibited HCASMC invasion with the most significant inhibition (~76%, $p = 0.0065$) at an MOI of 20 and TIMP-1 concentration of 1000 ng/mL. However, the high level of inhibition appears to be temporary when

examining the 48 and 72 hour data. At 48 and 72 hours the level of inhibition jumps to ~43% and 34%, respectively at the same combination. This result supports the earlier individual result of the hyperfunctional β_3 integrin expression showing a transient inhibition followed by possible invasion promotion.

B. Synergistic Inhibition of HCASMC Invasion by Combining TIMP-1 with Cyclic-RGD

All normalized area ratio data is reported as a percentage of the positive control. Figure 3 shows the normalized area ratio data results. The design of the experiment was a 2-level factorial with cyclic-RGD varied from a high of 1.0 mM to a low of 0.25 mM. TIMP-1 varied from a high of 1000 ng/mL to a low of 10 ng/mL. Significant SMC invasion inhibition with respect to the positive control at all times (24, 48 and 72 hours) and concentration combinations were observed ($p < 0.0001$).

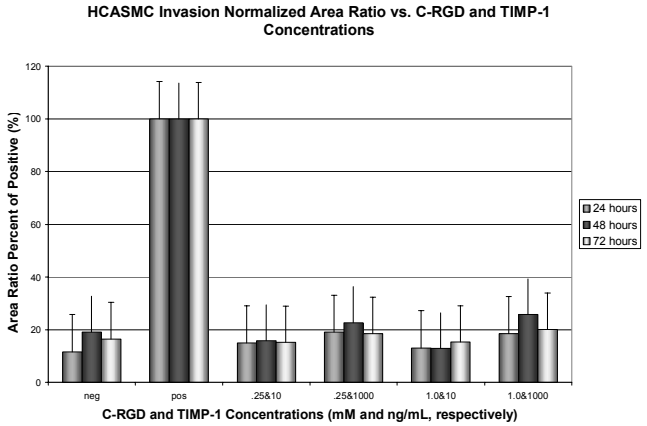

Fig. 3. Normalized area ratio results for TIMP-1 and cyclic-RGD release effect on HCASMC invasion. The values are expressed as percent means ± S.E., n = 12, relative to the positive control without treatment. Used with permission [8].

IV. Model Optimization and Discussion

A. Optimum Synergistic Inhibition of HCASMC Invasion by Combining TIMP-1 with Cyclic-RGD

Statistical analysis using ANOVA revealed that TIMP-1 alone at 1000 ng/mL inhibited HCASMC invasion better than any of the Hyp-int./ TIMP-1 combinations. However, combinations of cyclic-RGD/ TIMP-1 inhibited HCASMC invasion better than any single therapy. Therefore, optimization techniques were run on these combinations to find the optimum.

The previous analysis yielded good results at the test points (i.e. near the high and low values of each factor); however, far from the test points, standard error increases yielding unreliable predictions on performance. To rectify this "lack of fit" away from the test points a balanced 2-level factorial model is applied to the cyclic-RGD/TIMP-1 combination. Since time did not significantly vary the normalized area ratio for these combinations, the optimum combination desired should be valid for all times tested. Therefore, time is transformed into 3 blocks and the data for each combination is pooled as blocked replicates, (i.e. n = 36, blocked at 24, 48 and 72 hours). The resulting model surface contour is plotted in Figure 4.

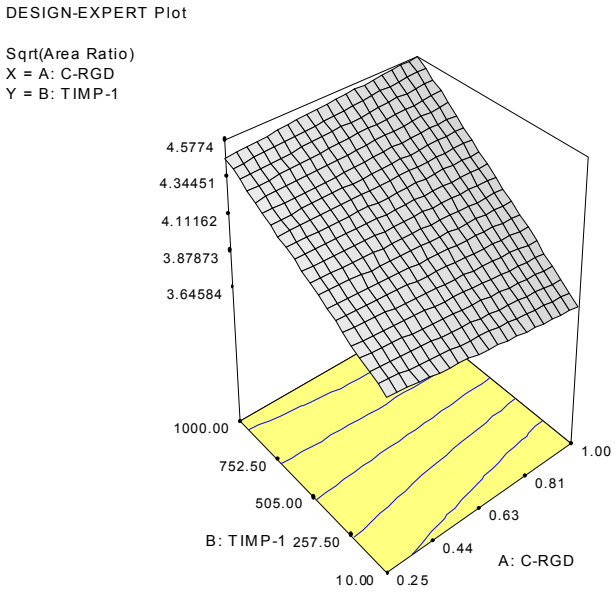

Fig. 4. Predicted square root (normalized are ratio) 3-D contour plot against factor concentrations. Used with permission [8].

Upon inspection of the contour plot, the optimum concentration combination within the boundaries under study occurs at concentrations of 1.0 mM cyclic-RGD and 10 ng/mL TIMP-1. Using the numerical optimization of the desirability value, the optimum concentration is confirmed. This level predicts that the amount level of normalized area ratio at this point would be 3.6462 = 13.29%. The observed average at all time points (See Figure 3, above) for this point is 13.74%. This is in excellent agreement with the observed values.

B. Discussion

The model shows an optimum for the ranges tested. A global optimum may be outside these ranges and would require further investigation. The model does however give direction to the query by showing the slope of the best performance. Continued investigations should look in the direction of higher than 1.0 mM cyclic-RGD concentration and lower than 10 ng/mL TIMP-1 concentration. Of course optimizing the parameter does not take into account physiological or cytotoxic limits. Therefore, based upon previous studies and knowledge of these factors the local solution is the best reliable solution. More studies outside this range would also require cytotoxic and physiologic analyses to verify safe levels of these factors.

This is no means an exhaustive study on possible factor combinations. Future works should include more potential factors, such as TIMP-2, PAI-1, etc., in combinations of 2 or more. As long as the experiment was unchanged, a data base

of results could be archived and more complex optimization models could be run to determine multiple factor optimums. Other optimization methods could also be tried.

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