Phthalimide Neovascular Factor 1 (PNF1) Modulates Endothelial MT1-MMP Activity

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Statement of Purpose: Vascular invasion will be essential for neovascularization of tissue-engineered implants. We are creating synthetic pharmaceuticals with potential to promote endothelial invasion and angiogenesis. Phthalimide neovascular factor 1 (PNF1) has been previously shown to exhibit significant *in vitro* potency in stimulating proliferation, migration, and Matrigel invasion in human microvascular endothelial cells (HMVEC); investigation of drug mechanism through transcriptional profiling has elucidated membrane type 1 metalloprotease (MT1-MMP) as a potential downstream effector. Here, we investigated the modulation of MT1-MMP activity in HMVEC by PNF1 at its most potent *in vitro* concentration (30 μM).

Methods: Matrix metalloproteinase (MMP) substrate FS-6 has been used to quantify MMP activity (including MT1-MMP), in cell cultures (Neumann U. Anal Biochem 2004;328:166-73). Confluent HMVEC in 96-well plates were supplemented with 100 μL cell media with vehicle control or 30 μM PNF1. Human recombinant tissue inhibitor of metalloproteinase-1 or -2 (TIMP-1 or TIMP-2) was added at 2.5 mg/mL, and FS-6 at 5 μM. After 2 h incubation, fluorescence was detected (320 nm ex, 400 nm em). Type I rat tail collagen gels (2.5 mg/mL) were polymerized in 96-well plates (Bayless KJ. Biochem Biophys Res Commun 2002;312:903-13). Subsequently, 100 μL volumes of HMVEC (15,000 cells/well) were added with 30 μM PNF1 or vehicle control, as well as TIMP-1 or TIMP-2, and cultured for 24 h. Cultures were then analyzed for number of cell-cell associations per well. Data are reported using mean ± standard error.

Results: Samples supplemented with vehicle control and FS-6 for 2 h served to elucidate background MMP activity. Control cultures also treated with 2.5 mg/mL TIMP-1 or TIMP-2 exhibited 64% and 74% of the fluorescence activity, respectively (Figure 1). TIMP-1 is in inhibitor of soluble MMP signaling, while TIMP-2 inhibitory activity includes that of membrane-bound MMPs, like MT1-MMP. When 30 μM PNF1 was present in cell media, cultures treated with TIMP-1 demonstrated 65% of the fluorescent activity, similar to vehicle control-treated samples. Therefore, PNF1 has no relative effect on soluble MMP signaling. However, cells supplemented with PNF1 + TIMP-2 decreased to 29% of PNF1-treated samples ($p = 0.05$), differing greatly from the 74% observed in samples treated with control + TIMP-2. As TIMP-2 inhibits both soluble MMP and transmembrane MT1-MMP signals, these data taken together suggest that PNF1 targets MT1-MMP-specifically in endothelial cells. PNF1 stimulation of MT1-MMP activity, critical for endothelial invasion via extracellular matrix (ECM) degradation, also was validated with type I collagen gel invasion assays over 24 h. These gels mimic ECM, allowing for three-dimensional culture and supporting cell-cell associations between endothelial cells. Increase in the numbers of elongated HMVEC and cell-cell associations observed with PNF1 stimulation, compared with vehicle control. PNF1-related cell invasion was not significantly inhibited by 2.5 mg/mL TIMP-1; in contrast, the number of cell-cell associations significantly decreased ($p < 0.05$) with TIMP-2, at the same concentration (Fig. 2). Significant rounding of cells and lack of cell-cell associations was seen in cultures treated with PNF1 + TIMP-2, suggesting that MT1-MMP is critical for PNF1-stimulated endothelial invasion through type I collagen.

Conclusions: MT1-MMP regulation by PNF1 was validated through the use of two *in vitro* assays. Elucidation of MT1-MMP activity as critical for PNF-induced effects introduces exciting new opportunities for clinical use of the compoundfor translational biomaterials and tissue engineering research, including regulation of circulating progenitor cells and leukocyte transmigration (Sithu SD. J Biol Chem 2007;282:25010-9) and stromal cell trafficking (Neth P. Stem Cell 2007;3:18-29).

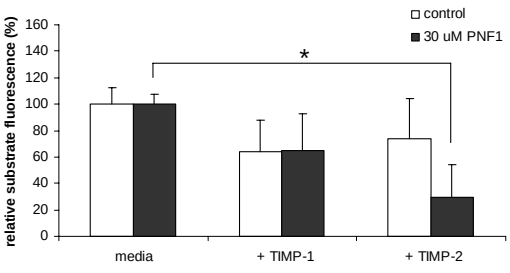

Figure 1. Relative MMP activity measured by FS-6 fluorescence after 2 h (* $p < 0.05$).

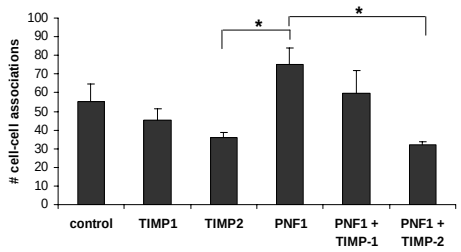

Figure 2. MMP activity measured by endothelial invasion in type I collagen after 24 h (* $p < 0.05$).