GLYCOSYLATION OF MINI-COLLAGENS MODULATES MELANOMA CELL ADHESION, SPREADING, AND METALLOPROTEINASE PRODUCTION

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Introduction: Three-dimensional interactions between receptors and their ligands can modulate cell behavior. We have previously identified a region from type IV collagen, α1(IV)1263–1277, which promotes human melanoma cell adhesion and spreading in a conformationally-dependent manner [1]. These studies were expanded (a) using an alternative triple-helical construct and (b) incorporating a glycosylated hydroxysine in position 1265. Melanoma cells were plated on the glycosylated and non-glycosylated triple-helical α1(IV)1263–1277 “mini-collagens” and subsequently tested for the promotion of adhesion and spreading, and regulation of proteolytic enzymes.

The linear version of α1(IV)1263–1277 is known to bind CD44 chondroitin-sulfate proteoglycan [2]. We have performed similar studies on the triple-helical version of α1(IV)1263–1277 to determine if ligand three-dimensional structure modulates receptor recognition. The studies utilize sub-populations of M14 melanoma cells that are either enriched or deficient in cell surface CD44.

The α1(IV)1263–1277 sequence was originally synthesized as a branched triple-helical peptide (THP) as described previously [1]. This construct utilizes a C-terminal branch and N-terminal 6 Gly-Pro-Hyp repeats to ensure a stable triple-helix. More recently we have described the synthesis of “peptide-amphiphiles” (PA) that incorporate an N-terminal lipid moiety that works in conjunction with N- and C-terminal Gly-Pro-Hyp repeats to form a stable triple-helix [3]. Both constructs can be used to create a triple-helix that is stable at physiological temperatures, but the later mini-collagen allows for greater flexibility in controlling Tg, and enhanced ability to work on solid surfaces. The construction of tumor cell receptor ligands with their native three-dimensional structure and post-translational modifications permits a more realistic evaluation of the in vivo mechanisms of tumor cell interaction with and invasion of the basement membrane.

Materials/Methods: The α1(IV)1263-1277 protein sequence contains a glycosylated Hyl residue in position 1265. A 6-O-glycosylated 9-fluorenylmethoxycarbonyl-hydroxysine (Fmoc-Hyl) derivative was prepared by introduction of a β-D-galactopyranosyl group to copper-complexed Hyl-[ε-tetra-tert-butyloxy carbonyl (Boc)]ε, followed by decomposition of the copper complex and addition of an Fmoc group to the ε-amino group [4]. Fmoc-Hyl-[ε-Boc, O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)] was used in the solid-phase synthesis of α1(IV)1263-1277 PA.

Triple-helical peptide substrates were assembled on an Applied Biosystems 433A peptide synthesizer using standard Fmoc solid-phase methodology. Peptides were purified by RP-HPLC and characterized by analytical RP-HPLC, MALDI-TOF mass spectrometry, and Edman degradation sequence analysis. Peptides were also examined on a Jaros J-600 spectropolarimeter to verify the degree of triple-helicity of the prepared “mini-collagens”, with readings collected in the range of λ = 190-225 nm. Melting temperatures were calculated by monitoring molar ellipticity at λ = 225 nm while changing the temperature from 5-85 °C and taking the first derivative of the curve.

PA was passively adsorbed to a 96-well plate at various concentrations, followed by an incubation with BSA. Melanoma cells were labelled with carboxyfluorescein diacetate and allowed to incubate at 37 °C. Unbound cells were removed and the remaining cells were either lysed and analyzed on a Molecular Devices Gemini Spectrafluorometer or fixed, stained, and analyzed for cell area.

For protease expression and production assays, melanoma cells were cultured in serum-free media, and seeded into 6-well culture dishes previously coated with the α1(IV)1263-1277 PA. Aliquots of conditioned media were harvested at regular intervals. Total RNA was isolated with the Perfect RNA™ Eukaryotic RNA isolation kit (Eppendorf). Total RNA was first treated with DNA-Free™ (Ambion) to remove genomic DNA, followed by reverse transcription using oligo(dT)12-18 primers. PCR was then performed using primers for GAPDH as well as various MMPs. PCR products were electrophoresed and stained with SYBR® Green I. Images were captured on a Fluor-S™ Multimager (BioRad) and analyzed with Quantity One® v4.2.2 software package.

Results: Adhesion to the non-glycosylated PA plateaued at [PA] = 10-25 µM, ~60% relative adhesion, in comparison to ~45% relative adhesion to type IV collagen. The E50 is in the range of 1-2 µM. In comparison, the glycosylated PA supported only about 30% adhesion at 25 µM, in comparison to ~10% to BSA coated wells. Cell spreading mirrored adhesion, with the non-glycosylated PA promoting greater spreading than the type IV collagen control. The glycosylated PA supported very little spreading, but the cells were better spread than the BSA control.

Canonical drop RT-PCR was the primary quantitative methodology used to evaluate the relative MMP expression levels. Relative to the peptide-free control, non-glycosylated PA stimulated expression of MMP-1 nearly 2-fold maximal stimulation at 0.5 h. Stimulation of expression could be inhibited at early time points in the presence of 2 mM p-nitrophenyl-β-D-xylpyranoside, an alkyloid used to passivate cell surface proteoglycan receptors, such as CD44. For MMP-14 expression, maximal levels were achieved at 5 h, in which a 1.6-fold stimulation was observed. As with MMP-1, MMP-14 expression was inhibited by -xyloside treatment. These studies will be extended to include MMP expression in response to the glycosylated α1(IV)1263–1277 sequence as well as zymographic analysis to monitor for active proteolytic enzymes produced in response to both ligands.

Initial affinity chromatography results indicate the triple-helical α1(IV)1263–1277 sequence binds chondroitin sulfate A at levels similar to type IV collagen. These studies will be extended to various other glycosaminoglycans as well as melanoma cell lysates.

Conclusions: In the present study we are able to correlate cell binding to a triple-helical site within type IV collagen with production of specific proteolytic enzymes, namely MMP-1 and -14. We are also able to study how post-translational modifications modulate binding events as well as downstream signaling, i.e. MMP production. Our system allows us to mimic the three-dimensional structure of type IV collagen, yet trigger single receptor types allowing us to definitively link receptor binding to discrete cellular responses. Ultimately, we will better understand the tumor cells’ ability to efficiently bind to, degrade, and move through the basement membrane during metastasis.

References:

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