LC/MS identification of adherent U937 intracellular signaling proteins mediated by various surface adsorbed peptides and phosphorylation inhibitor AG18

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Statement of Purpose: Macrophages play a key role in the host inflammatory response and can adhere to biomaterials via adsorbed extracellular matrix (ECM) proteins. The mechanism of integrin receptor ligation with ECM proteins and the resulting effect upon intracellular protein expression is unresolved and poorly understood. Previously a set of proteins ranging from ~200k to ~23k Da were identified as expressing up or down regulated protein expression in response to various ligand adsorbed surfaces and phosphorylation inhibitor[1]. The present work focuses on identifying critical intracellular signaling proteins in adherent human monocytic cell line U937 cells in response to various surface adsorbed peptide ligands and phosphorylation inhibitor tyrphostin 23 (AG18) using LC/MS.

Methods: Tissue culture polystyrene was adsorbed with G_6, G_3RGDG, or G_3PHSRNG. U937 cells were seeded on the ligand adsorbed TCPS surfaces at $1.7x10^5$ cells/cm^2 in RPMI 1640 with 5% FBS, 50ng/ml phorbol 12-myristate 13-acetate, and 0, 20, 40, 60, or 80μM AG18. After 24 hours, adherent cells were trypsinized and lysed. Cell lysate proteins were immunoprecipitated using P-Tyr-100 monoclonal antibodies, separated by SDS-PAGE, and stained. Bands of interest were excised, destained, reduced, alkylated, digested, and desalted. Digested peptides were sequenced using HPLC coupled to a mass spectrometer (LC/MS). The ion trap was configured to prefer doubly charged ions and switch to MS/MS with the threshold intensity of 0.1% of the absolute maximum. Agilent proprietary software was then used to scan out fragments of peptides trapped in each cycle. The data was searched against the NCBI database via Mascot. Relevant hits with a Mascot (Mowse) score >40 were collected. Any unnamed and putative proteins or those in common with U937 cells grown in suspension were considered not relevant and significant. The Human Protein Reference database (www.hprd.org) and Expert Protein Analysis System (Expasy) were searched for known protein function, protein-protein interactions, and tyrosine phosphorylation state to help identify critical proteins in the intracellular signaling cascade.

Results / Discussion: Our proteomic approach can effectively survey and identify a large number of proteins of interest. For example, LC/MS analysis of cell lysates from G_3RGDG-adsorbed samples identified 117 peptides with ion scores >40. Any non-human, unnamed or hypothetical proteins were considered not significant or relevant. Proteins present in both adherent and suspended U937 cells were also considered not relevant. The number of significant and relevant peptides after data refining was 70. These 70 peptides represented 41 different proteins of varying molecular weights present in U937 cells adherent to G_3RGDG.

The combined effect of AG18 and surface adsorbed ligand upon intracellular protein expression was probed using LC/MS. At 80μM AG18 and ~110kDa on G_3RGDG-adsorbed TCPS, four proteins were identified verses none at 0μM. At ~70kDa, G_3RGDG surface associated ligand appeared to affect distinct sets of proteins at 0, 20, 60, and 80μM AG18. Only moesin was identified common to all conditions. One tyrosine phosphorylated protein, titin, was identified from the PBS adsorbed sample at ~70kDa and 20μM AG18. At ~52kDa, different sets of proteins were identified at 0, 40 and 60μM AG18. At ~42kDa six proteins were found in both G_3RGDG at 0 and 20μM AG18. Eef1a2 was found at 0μM AG18 on G_3RGDG and is tyrosine phosphorylated. Three proteins were found at 40μM AG18 on G_6 adsorbed surfaces while no proteins were found on PBS adsorbed TCPS. At ~23kDa only hnRNP A2, a ribosomal protein, and an Ig fragment were found in both G_3RGDG 0 and 80μM AG18. hnRNP A2 and ribosomal protein L7(a) were found in both G_3PHSRNG 0 and 60μM AG18 at ~23kDa. The combination of AG18 and surface ligand had no consistent effect upon intracellular protein. The effects of AG18 and surface adsorbed ligand were isolated. On G_3RGDG-adsorbed TCPS, increasing concentrations of AG18 regulated expression of moesin, which was found in 0, 20, 60 and 80μM AG18 at ~70kDa. At ~52kDa AG18 elicited different proteins at 0, 40 and 60μM on G_3PHSRNG adsorbed TCPS including alpha tubulin at 0μM that is tyrosine phosphorylated. At ~23kDa hnRNP A2 was found at 0 and 60μM but not 20μM AG18 on G_3PHSRNG-adsorbed surfaces. Increasing concentrations of AG18 thus regulate different sets of proteins in adherent U937 cells. As a surface associated ligand, G_3RGDG elicited expression of different sets of proteins from ~200k to ~23k Da. Different proteins were also found at ~52k and ~23k Da for adsorbed G_3PHSRNG.

Conclusions: Both increasing concentrations of AG18 and different surface adsorbed peptides modulate shifts in intracellular signaling pathways. Increasing concentrations of AG18 elicited expression of different sets of proteins from both G_3RGDG and G_3PHSRNG adsorbed TCPS. The surface ligand G_3RGDG or G_3PHSRNG regulated expression of distinct sets of proteins ranging from 200k to 23k Da compared to PBS adsorbed surfaces.

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References: [1]Chen XX. Biomat. 2005;26:873-882.