Effect of various surface adsorbed proteins and phosphorylation inhibitor AG18 upon intracellular signaling proteins in adherent U937 cells identified by LC/MS

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Statement of Purpose: Adsorption of extracellular matrix (ECM) proteins mediate macrophage adhesion to biomaterials. Macrophages adhere to the ECM proteins via integrin receptors and activate the host inflammatory response. Intracellular signaling cascades depend on the receptor-ligand interaction, but the actual mechanism is unresolved and poorly understood. Previously the effect of various surface adsorbed ligands and phosphorylation inhibitor tyrphostin 23 (AG18) upon intracellular protein expression in adherent U937 cells was probed. Proteins ranging from ~200k to ~23k Da were identified as exhibiting up or down regulated protein expression[1]. The present work uses LC/MS to sequence and identify critical intracellular signaling proteins expressed by adherent human monocytic cell line U937 in response to various surface adsorbed protein ligands and AG18.

Methods: $1.7x10^5$ cells/cm^2 U937 cells were seeded in RPMI 1640 containing 5% FBS, 50ng/ml phorbol 12-myristate 13-acetate, and 0, 20, 40, 60 or 80μM AG18 on to TCPS adsorbed for 24 hours with PBS, albumin (Alb) or fibronectin (FN). Adherent cells were trypsinized after 24hrs, lysed, immunoprecipitated with P-Tyr-100 mAb against phosphorylated tyrosine proteins and separated by SDS-PAGE. Protein bands of interest were excised, destained, reduced, alkylated, digested, and desalted before sequencing on an electrospray mass spectrometer coupled to HPLC (LC/MS). The mass spectrometer's ion trap was configured to prefer doubly charged ions and switch to MS/MS with a threshold intensity of 0.1% of the absolute maximum. Peptide fragments trapped in each cycle were scanned out using Agilent proprietary software. Hits with a Mascot score >40 were collected. From these protein hits, any putative proteins, unnamed proteins, or those proteins in common with U937 cells grown in suspension were removed as non-relevant. Protein function, protein-protein interactions, and tyrosine phosphorylation state were obtained from the Human Protein Reference database (www.hprd.org) and Expert Protein Analysis System (Expasy) to help identify critical proteins in the intracellular signaling cascade.

Results / Discussion: Coupling immunoprecipitation, SDS-PAGE, and LC/MS allows many proteins to be screened quickly and efficiently for various characteristics. To illustrate the ability of this proteomic scheme, 192 peptides with ion scores >40 were detected in 26 FN-adsorbed TCPS samples. 63 peptides were removed from consideration by refining for putative, unnamed or proteins in common with U937 cells grown in suspension leaving 129 relevant peptides of interest. 46 different proteins of varying molecular weights present in U937 cells adherent to FN were identified by the 129 peptides remaining after the data filtering process.

The combined effect of AG18 and surface adsorbed ligand upon intracellular protein expression was probed using LC/MS. AG18 decreased protein expression on PBS-adsorbed TCPS at 40μM verses 0μM AG18. FN regulated a different set of 9 proteins compared to PBS at 20μM AG18 and ~52kDa while AG18 had no effect on Alb-adsorbed TCPS at 0 and 40μM AG18. At ~42kDa, 40 and 60μM AG18 decreased protein expression on PBS compared to 12 proteins found at 0μM. Mutant beta actin was found on both Alb and PBS surfaces at 0μM AG18 while FN up regulated the expression of 8 proteins at 20μM AG18 compared to PBS. At ~23kDa 60 and 80μM AG18 increased protein expression on PBS compared to 0μM. However, AG18 had no effect on protein expression on Alb-adsorbed TCPS at 0 and 40μM. FN regulated a different set of 11 proteins compared to PBS at 40μM AG18. 80μM AG18 increased expression of 8 proteins on FN-adsorbed surfaces compared to 0μM. 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 PBS-adsorbed TCPS at ~65kDa, AG18 decreased protein expression at 20 and 40μM AG18 compared to 0μM. Tyrosine phosphorylated titin was found at 20μM AG18 on PBS-adsorbed TCPS at ~65kDa. Increasing concentrations from 0 to 60μM AG18 down regulated protein expression at ~42kDa on PBS adsorbed surfaces. At ~23kDa, AG18 increased protein expression at 60 and 80μM with 5 common proteins. AG18 appears to regulate the expression of peroxiredoxin on FN-adsorbed TCPS at ~23kDa. Peroxiredoxin was found at 0, 40 and 80μM AG18 while H1 histone was found only at 0 and 80μM. Increasing concentrations of AG18 thus regulate different sets of proteins in adherent U937 cells. As a surface associated ligand, Alb and FN elicited expression of different sets of proteins at ~52k and ~42k Da.

Conclusions: Both increasing concentrations of AG18 and different surface adsorbed proteins modulate shifts in intracellular signaling pathways. Increasing concentrations of AG18 elicited expression of different sets of proteins from PBS and FN adsorbed TCPS but demonstrated no effect upon protein expression in response to Alb adsorbed surfaces. The surface ligands Alb and FN regulated expression of distinct sets of proteins at ~52k and ~42k Da compared to PBS adsorbed surfaces.

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