HHS Public Access

Author manuscript

Biomaterials. Author manuscript; available in PMC 2017 December 28.

Published in final edited form as:

Biomaterials. 2006 July ; 27(20): 3745–3757. doi:10.1016/j.biomaterials.2006.02.028.

Effect of surface-adsorbed proteins and phosphorylation inhibitor AG18 on intracellular protein expression in adherent macrophages

Sean T. Zuckerman[a,b] and **Weiyuan John Kao**[a,b,*]

[a]Department of Biomedical Engineering, University of Wisconsin-Madison, Madison, WI 53705, USA

[b]School of Pharmacy, University of Wisconsin-Madison, 777 Highland Ave., Madison, WI 53705, USA

Abstract

Macrophages are believed to play an important role in the host inflammatory response to implanted biomaterials. However, the mechanism of macrophage adhesion to protein-adsorbed substrates and the subsequent activation and inflammation is unresolved. Previously the effect of various surface-adsorbed proteins and increasing concentrations of phosphorylation inhibitor AG18 on intracellular protein expression levels in adherent human monocytic cell line U937 was identified using SDS-PAGE and densitometry. The protein ligands and AG18 concentrations up or down regulated the expression of a set of proteins ranging from ~200 to ~23 kDa. In the present work, HPLC coupled tandem mass spectroscopy (LC/MS) was used to identify proteins in these bands. We hypothesized that key proteins in macrophage adhesion and activation could be identified by observing protein expression resulting from various surface-adsorbed ligands and AG18 concentrations. Increasing concentrations of AG18 down or up regulate protein expression in adherent U937 on PBS-adsorbed TCPS at ~52, ~42 and ~23 kDa. AG18 concentrations had no effect surfaces on cells on albumin (Alb)-adsorbed but regulated different protein expression in adherent U937 on fibronectin (FN)-adsorbed TCPS at 40 and 80 μM AG18. Both Alb and FN regulate distinct sets of proteins in adherent cells as surface-adsorbed ligands. Based on the data from LC/MS, both surface associated ligand and increasing concentrations of AG18 modulate shifts in intracellular signaling.

Keywords

LC/MS; U937; Proteomics; Tyrosine phosphorylation; Inflammation; Fibronectin

1. Introduction

Macrophages can interact with extracellular matrix (ECM) proteins adsorbed on the biomaterial surface and adhere via integrin receptors expressed at the cell surface [1–4].

*Corresponding author. Department of Biomedical Engineering, University of Wisconsin-Madison, 777 Highland Ave., Madison, WI 53705, USA. Tel.: +1 608 263 2998; fax: +1 608 262 5345. wjkao@pharmacy.wisc.edu (W.J. Kao).

Subsequently cells may alter gene expression via intracellular signaling cascades driven by tyrosine residue phosphorylation allowing the macrophages to respond to extracellular signals [5–7]. However, the molecular mechanism underlying ECM–integrin ligation remains unresolved. Numerous ECM proteins contain the amino acid sequence arginine–glycine–aspartic acid (RGD) known to trigger cell adhesion [8,9]. We have previously employed the human monocytic cell line U937 to probe the response of macrophages to surface-adsorbed peptide or protein ligands and increasing concentrations of the phosphorylation inhibitor tyrphostin 23 (AG18). After adhering to ligand-adsorbed TCPS for 12 or 24 h, U937 were trypsinized, lysed, immunoprecipitated for tyrosine phosphorylated proteins and separated by sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE). Densitometry was performed on the gels after staining with Coomassie Brilliant Blue dye to identify bands showing up or down regulated protein expression. Various surface associated ligands and AG18 concentrations affected the expression levels of a set of proteins ranging from ~200 to ~23 kDa [10] (Table 1). Western blotting and mass spectrometry are two methods for protein identification in proteomics study [11,12]. Since several proteins in an immunoprecipitated cell lysate may possess a similar molecular weight range, Western blotting can identify a specific protein [12,13], but for complex mixtures such as cell lysates, Western blots are inefficient in identifying a large number of proteins.

A more comprehensive proteomics tool is mass spectroscopy [14–17]. The basis of mass spectrometry is detecting charged ions in the gas phase thus allowing calculation of the overall molecular weight. The two main variations of mass spec applied to proteomics are matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI). Both techniques are considered “soft” ionizations that minimize protein or peptide degradation. MALDI uses an organic matrix that absorbs energy from a laser to vaporize the peptide. MALDI does not reveal amino acid sequence directly but does allow rapid and sensitive peptide mass detection. MALDI is limited to relatively simple protein mixtures. Peptide sequence data can be obtained from a variation of MALDI using magnets to trap ions for dissociation and further detection. This variation is called Fourier transform mass spec (FT-MS). While MALDI utilizes a laser for peptide ionization, ESI relies on fluid sprayed from a fine needle to form tiny droplets that evaporate to form charged particles in the gaseous phase. ESI instruments are typically coupled to HPLC (LC/MS) allowing for resolution of complex peptide mixtures before ionization. Often an ion trap is included on the ESI to capture specific peptide ions and induce dissociation based on collision with an inert gas. This dissociation enables compilation of detailed sequence data. The determination of ion mass/charge in ESI is typically less sensitive than MALDI. There are two kinds of ESI used for proteomics: microspray and nanospray indicating the level of sensitivity (i.e. micrograms for microspray and nanograms for nanospray). In the present study, intracellular proteins from adherent U937 were identified using nanospray LC/MS. We hypothesized LC/MS could effectively survey and identify intracellular signaling proteins modulated by surface-adsorbed proteins and AG18 concentration.

2. Materials and methods

2.1. Cell line and reagents

Human monocytic cell line CRL-1593.2/U-937 (American Type Culture Collection (ATCC)) [18] were used. Reagents were obtained from the following sources: glacial acetic acid (Aldrich); 10-well Ready Gel® (Tris–HCl, 10% resolving®, 4% stacking®), Coomassie Brilliant Blue (G-250), 10× Tris/Glycine/SDS buffer (BioRad); Clonetics reagent pack (trypsin/EDTA, trypsin neutralizing solution, HEPES-buffered saline solution) (Cambrex); immobilized phospho-tyrosine monoclonal antibody (mAb) P-Tyr-100 (#9419) (Cell Signaling); 1× phosphate buffered saline solution (PBS), Rosswell Park Memorial Institute (RPMI) 1640 media (CellGro); human plasma purified fibronectin (FN) (Chemicon); isopropyl alcohol (EM Sciences); HPLC grade acetonitrile (ACN), methanol (Fisher); fetal bovine serum (FBS) (HyClone); ZipTip® (C_{18}, P10 size) (Millipore); bicinchonic acid (BCA) protein quantification kit and standards, M-PER® mammalian protein extraction reagent, Halt™ Protease Inhibitor Cocktail, PepClean™ C-18 spin columns (Pierce); trypsin (modified sequence grade) (Promega); sodium orthovanadate, ammonium bicarbonate ($(NH_4)HCO_3$), dithiothreitol (DTT), iodoacetamide (IAA), trifluoroacetic acid (TFA), formic acid (FA), human purified serum albumin, tyrphostin 23 (AG18), phorbol 12-myristate 13-acetate (PMA) (Sigma).

2.2. Preparation of cell lysates and immunoprecipitation

About 1 μM solutions of albumin (Alb) or FN were adsorbed to 75 cm^2 TCPS flasks for 24 h at 37°C. Surfaces were washed once with 1 × PBS. About 1.7×10^5 cells/cm^2 U937 cells were seeded with RPMI 1640, 5% FBS, 50 ng/mL PMA and 0, 20, 40, 60 or 80 μM AG18 and were incubated for 24 h at 37°C, 5% CO_2 and ~95% humidity. Adherent cells were trypsinized, lysed with M-PER® containing 1×HALT™ protease inhibitor and 1 mM sodium orthovanadate to inhibit phosphatase activity. BCA was performed on the raw cell lysates to determine protein concentration. Antiphosphotyrosine mAb P-Tyr-100 was used to immunoprecipitate 550 μg of raw cell lysate at 4°C overnight. M-PER® was used to wash the IP beads twice before denaturing the proteins for 10 min at room temperature in 3×loading buffer containing 50 mM DTT. The IP beads and proteins were incubated at ~90°C for 10 min before SDS-PAGE on Tris–HCl gels (10% resolving, 4% stacking). The gels were run at 84 V for 30 min during the stacking gel and 129 V for 1 h 45 min through the resolving gel [10]. Under the present analysis scheme, one sample for protein analysis requires more than 25 million cells. Analyzing the 47 conditions presented in this work in duplicate (i.e. n=2) with primary monocytes would thus require a prohibitively large number of donors and introduce inherent donor variations. The data obtained from working with U937 will help us construct a framework for understanding primary cell behavior.

2.3. LC/MS

Aseptic techniques performed in a laminar flow hood were used for all LC/MS sample preparation. A total of 47 combinations of AG18 concentrations and surface-adsorbed proteins were analyzed in duplicate (n = 2). LC/MS sample preparation was performed according to a protocol established by the Biotechnology Center, University of Wisconsin-Madison. After SDS-PAGE residual SDS was removed by washing three times in Milli-Q

water for 10 min. Gels were stained with ~5 mL of Coomassie Brilliant blue for 1 h at room temperature. The gels were destained for 1 h with 20 mL of Milli-Q water. The bands of interest (Table 1) were excised from the gel using an X-acto® knife and placed into siliconized 0.65 mL Eppendorf tubes. Gel fragments were destained for 10 min with 100 mM $(NH_4)HCO_3$/50% methanol with intermittent vortexing until all the dye was removed, dehydrated twice for 10 min in 25 mM $(NH_4)HCO_3$/50% ACN and dried in a vacuum centrifuge for 10 min. About 25 mM DTT was used to rehydrate the bands and reduce the proteins for 30 min at 56°C. The gel fragments were then allowed to cool to room temperature, any residual liquid was pipetted off and reduced cysteine residues were alkylated for 30 min in the dark with fresh 55 mM IAA. Residual liquid was pipetted off and the fragments equilibrated in Milli-Q water for 15 min followed by equilibration in 25 mM $(NH_4)HCO_3$ for 10 min with intermittent vortexing. Gel fragments were dehydrated twice with 25 mM $(NH_4)HCO_3$/50%ACN for 10 min and dried on the vacuum centrifuge for 10 min. The bands were then rehydrated with 20 ng/mL trypsin in 25 mM $(NH_4)HCO_3$ for 15 min at 4°C. A minimal amount of 25 mM $(NH_4)HCO_3$ was placed on top of the bands to ensure full immersion throughout the 16–20 h incubation at 37°C. After digestion the solutions were placed into clean 0.65 mL siliconized Eppendorf tubes. Any peptides remaining in the gel were extracted using 0.1% TFA in Milli-Q water for 20 min with constant vortexing and combined with the overnight digestion solution. Peptides were extracted two additional times for 20 min each with 5% TFA/70% ACN and constant vortexing (high setting). Peptides were obtained from the peptide solution by drying in a vacuum centrifuge for ~1.5 h. The peptides were desalted using C_{18} ZipTips® or PepClean™ C-18 spin columns according to manufacturer's protocol replacing TFA with FA to prevent ion pairing during ionization. Peptides were stored at −80°C until LC/MS analysis.

Peptides were separated by an 1100 HPLC system with nano-pump flow capability coupled to an Agilent MSD ion trap mass spectrometer. Samples were initially separated on a Zorbax C-18sb trap column at 0.280 μL/min for 15 min. The trap column eluted to a 75 μm×150 mm analytical column in a 120 min gradient from 0.1% FA/10% ACN/water to 0.1% FA/60% ACN/water. Over the course of 10 min, the ACN was ramped to 95%. Afterwards the column was re-equilibrated with 10% ACN for 20 min. Peptides eluted from the analytical column directly into the ion trap via an 8 μm glass spray needle (New Objective). The ion trap was configured to prefer doubly charged ions and trigger MS/MS at 0.1% of the absolute maximum intensity as a threshold value. The actual threshold was 100,000 counts. The ion trap captured 75,000 ions per trap cycle and was set for a maximum accumulation time of 100 ms per cycle. Any peptide fragments in each cycle were resolved using Agilent proprietary software for the ion trap. Mass spectrometer data was converted to .mgf files using the Agilent ion trap software and searched against the human portion of the National Center for Biotechnology Information (NCBI) database using Mascot. Hits with a Mowse score >40 were collected [19]. The significance of the peptide hits were assessed by this Mascot score.

Before further analysis the raw data from the LC/MS experiments had to be refined for relevance and significance. Novel proteins, unnamed proteins or protein hits from species other than *Homo sapiens* were not considered relevant. Proteins identified from different peptide hits were condensed into one entry noting both the maximum and minimum ion

scores. Table 2 illustrates this analysis scheme for PMA$^-$ proteins with all of those proteins that satisfy the filtering criteria highlighted in bold. Since the goal of the present work is to identify proteins expressed in adherent U937 cells, any proteins identified from PMA$^-$ cells were removed from data obtained from cells adherent to ligand-adsorbed TCPS. Table 3 illustrates the data refining methodology for U937 on Alb-adsorbed TCPS. The Human Protein Reference Database (www.hprd.org) and Expert Protein Analysis System (Expasy) were searched for the remaining proteins. Relevant data such as protein function, protein–protein interactions and if the protein is phosphorylated on tyrosine residues was recorded if available. If the protein name from Mascot could not be located in either database, the locus identification tag for the peptide was found using the NCBI accession number. The corresponding protein was then found in Expasy using the locus ID tag. The protein name from Expasy was used to locate the protein in the HPRD. Comparisons based on previous densitometry, surface-adsorbed ligand and AG18 concentration were made after protein identification.

3. Results/discussion

3.1. Comparison of nanospray LC/MS results based on previous densitometry findings

Mass spectrometry coupled to HPLC was used to identify intracellular proteins expressed by adherent U937 macrophages in response to various culture conditions. These protein identities may provide insight into the previously observed densitometric trends shown in Table 1 [10].

U937 cells on Alb- or FN-adsorbed TCPS with 40 μM AG18 showed an increased ~160 kDa protein expression compared to cells cultured without AG18 on the respective surfaces (Table 1). However, no proteins were identified at ~160 kDa from cells on Alb- or FN-adsorbed TCPS with 0 or 40 μM AG18. Cells on FN-adsorbed TCPS with 20 μM AG18 showed decreased ~130 kDa protein expression versus those without AG18. Antigen MLAA-44, alpha actinin 4 and unconventional myosin 1G valine form were found in cells with 20 μM AG18 while no relevant hits were found from cells cultured on FN-adsorbed TCPS without AG18. Table 1 shows that ~100 kDa protein expression in cells on FN-adsorbed TCPS with 20 μM AG18 decreased compared against those without AG18. DNA topoisomerase II beta and DNA-dependent protein kinase catalytic subunit were identified in cells without AG18; no significant hits were found in cells with 20 μM AG18 on FN-adsorbed TCPS at ~100 kDa. Cells on PBS-adsorbed TCPS with 40 μM AG18 showed decreased ~65/70 kDa protein expression against those without AG18 (Table 1). There were no significant hits identified from cells on PBS-adsorbed TCPS with 40 μM AG18. Six proteins were identified from cells cultured without AG18 (Table 4).

Cells on Alb-adsorbed TCPS with 40 μM AG18 showed decreased ~52 kDa expression compared to those without AG18 (Table 1). There were no proteins identified in cells treated with 40 μM AG18. Tubulin beta-4 chain and titin, which can be tyrosine phosphorylated, were identified in cells without AG18 on Alb-adsorbed TCPS. U937 on FN-adsorbed TCPS showed decreased ~52 kDa protein expression when compared to cells on PBS with 20 μM AG18. Nine proteins were found in cells on the FN-adsorbed TCPS with 20 μM AG18

samples (Table 5). No proteins were identified from cells on the PBS-adsorbed TCPS with 20 μM AG18.

Cells on PBS-adsorbed TCPS treated with 40 or 60 μM AG18 showed a decreased ~42 kDa protein expression compared against those on PBS-adsorbed TCPS without AG18 (Table 1). Twelve proteins were found in cells on PBS-adsorbed samples without AG18 (Table 6). No proteins were identified from cells on PBS-adsorbed TCPS with 40 or 60 μM AG18. Cells on Alb-adsorbed TCPS without AG18 showed decreased ~42 kDa protein expression versus those on PBS-adsorbed TCPS without AG18 (Table 1). Mutant beta actin was identified from cells on both Alb-and PBS-adsorbed TCPS samples without AG18. Plasminogen activator inhibitor type 2 was also identified from cells on Alb-adsorbed samples without AG18. Eleven unique proteins were identified in U937 on PBS-adsorbed TCPS without AG18 (Table 6). Cells on FN-adsorbed TCPS with 20 μM AG18 showed decreased protein expression versus those on PBS-adsorbed TCPS with 20 μM AG18 (Table 1). No proteins were identified from cells on PBS-adsorbed samples; however, eight proteins were identified from those on FN-adsorbed TCPS (Table 6).

Previous densitometry showed that cells on PBS-adsorbed TCPS treated with 60 or 80 μM AG18 exhibited increased ~23 kDa protein expression compared to those on PBS-adsorbed TCPS without AG18 (Table 1). No proteins were identified from cells on PBS-adsorbed samples without AG18. Twelve and eight proteins were identified from cells on PBS-adsorbed samples with 60 or 80 μM AG18, respectively (Table 7). Cells on Alb-adsorbed TCPS with 40 or 80 μM AG18 were compared against those on Alb-adsorbed TCPS without AG18 (Table 1). No proteins were identified from cells on Alb-adsorbed samples with 0, 40 or 80 μM AG18. Cells on FN-adsorbed TCPS exhibited increased ~23 kDa protein expression versus those on PBS-adsorbed TCPS with 40 μM AG18 (Table 1). No proteins were identified from cells on PBS-adsorbed samples cultured with 40 μM AG18, but 11 proteins were identified from cells on FN-adsorbed TCPS (Table 7). Cells on FN-adsorbed TCPS with 80 μM AG18 showed decreased ~23 kDa expression compared to those without AG18 (Table 1). Peroxiredoxin 1 and histone H1.4 were found in cells with 0 or 80 μM AG18. Testicular H1 histone was also found in cells without AG18 while eight unique proteins were found in cells with 80 μM AG18 (Table 7). There is no consistent trend in protein expression elicited by protein-adsorbed TCPS and increasing concentrations of AG18 as revealed by nanospray LC/MS.

4. Effect of AG18 concentration

Table 8 shows the culture conditions from Table 1 arranged by ligand and molecular weight. This comparison will reveal the effect of increasing concentrations of AG18 on U937 adherent to surface-adsorbed proteins. The effect of increasing concentrations of AG18 on the expression of ~65/70, ~42 and ~23 kDa proteins in U937 adherent on PBS-adsorbed TCPS was elucidated (Table 9). At ~65/70 kDa, five proteins were identified in cells without AG18. Only titin, which can be tyrosine phosphorylated, was found in cells with 20 μM AG18. No proteins were identified from cells cultured with 40 μM AG18. At ~42 kDa there were 12 proteins identified from cells without AG18. No proteins were identified in cells with 20, 40 or 60 μM AG18. Thus AG18 down regulates the expression of 12 proteins found

in cells adherent to PBS treated TCPS without AG18. The opposite phenomenon was observed at ~23 kDa where no proteins were identified from cells treated with 0, 20 or 40 μM AG18. However, 12 and 8 proteins were identified in cells with 60 and 80 μM AG18, respectively. Five proteins were found in both the 60 and 80 μM AG18 samples. Therefore at ~23 kDa, AG18 appears to up regulate the expression of proteins in cells with 60 or 80 μM that are not present in cells with 0, 20, or 40 μM while at ~42 and ~65/70 kDa AG18 appears to down regulate intracellular protein expression.

The effect of AG18 concentration upon intracellular protein expression of U937 adherent to Alb-adsorbed TCPS could not be resolved at ~23 kDa because no proteins were identified from cells at 0, 40 or 80 μM AG18.

The effect of increasing AG18 concentration was identified in cells on FN-adsorbed TCPS (Table 10). Peroxiredoxin 1 was identified in cells treated with 0, 40 or 80 μM AG18. Testicular H1 histone was identified in cells with 0 and 80 μM AG18 but not in cells with 40 μM AG18. Histone H1.4 was unique to cells without AG18. Ten unique proteins were identified from cells with 40 μM AG18. Eight proteins were identified only in cells with 80 μM AG18. Four proteins were common to cells with 40 and 80 μM AG18 but not cells without AG18. These proteins were hnRNP protein A2, high mobility group protein 2, 60S ribosomal protein L15 and an Ig fragment. Therefore AG18 at 0, 40 and 80 μM appears to affect different sets of ~23 kDa proteins expressed in cells on FN-adsorbed TCPS. Overall increasing concentrations of AG18 down or up regulate a unique set of proteins in U937 adhered on a given protein-adsorbed surface.

5. Effect of surface-adsorbed ligands

Cells adherent to Alb- and FN-adsorbed TCPS were compared to cells on PBS-adsorbed TCPS to determine the effect different ligand–receptor interactions have upon intracellular protein expression (Table 11). The effect of adsorbed Alb on ~160, ~52, ~42 and ~23 kDa protein expression in adherent U937 without AG18 was elucidated (Table 12). No ~160 kDa proteins were identified from cells on Alb-adsorbed TCPS without AG18. DNA-dependent protein kinase catalytic subunit was found in cells on PBS-adsorbed TCPS at ~160 kDa. Titin and tubulin 5-beta were found in cells on Alb-adsorbed TCPS while five different proteins were found in U937 on PBS-adsorbed TCPS at ~52 kDa (Table 12). Two of those five proteins were Ig fragments. At ~42 kDa mutant beta actin was identified in cells on PBS- and Alb-adsorbed TCPS samples. Plasminogen activator inhibitor type 2 precursor was also identified in cells on Alb-adsorbed TCPS without AG18. Eleven unique proteins were identified from cells on PBS-adsorbed TCPS at ~42 kDa. There were no proteins identified from cells on PBS- or Alb-adsorbed samples at ~23 kDa. Thus Alb appears to regulate the expression of a unique set of U937 proteins when compared to those on PBS treated TCPS at ~52 and ~42 kDa.

The effect of adsorbed FN on intracellular protein expression was compared against adsorbed PBS at six molecular weights ranging from ~160 to ~23 kDa (Table 13). No proteins were identified from cells on FN-adsorbed TCPS at ~160 kDa. DNA-dependent protein kinase catalytic subunit was found in cells on PBS-adsorbed TCPS, however. DNA

topoiosmerase II beta and dedicator of cytokinesis protein 2 (DOCK 2) were found in cells on PBS-adsorbed TCPS at ~130 kDa. No proteins were found in cells on FN-adsorbed TCPS at ~130 kDa. DNA topoisomerase II beta and DNA-dependent protein kinase catalytic subunit were found in cells on FN-adsorbed TCPS at ~100 kDa. No proteins were identified from cells on PBS-adsorbed TCPS at ~100 kDa. At ~52 kDa growth-regulated nuclear 68 protein and two Ig fragments were found in cells on both PBS- and FN-adsorbed TCPS (Table 13). Vimentin and mitochondrial ATP synthase beta subunit (AA 1-312) were identified only in cells adherent to FN treated TCPS. HLA-B associated transcript 1 was found in cells adherent to PBS-adsorbed TCPS at ~52 kDa. At ~42 kDa vimentin, NCL protein and eukaryotic translation elongation factor 1 gamma were detected in cells adhered to both PBS- and FN-treated TCPS. Six unique proteins were identified in cells on the FN samples at ~42 kDa; nine proteins were identified only in cells on PBS-adsorbed TCPS at ~42 kDa. Peroxiredoxin 1, histone H1.4 and testicular H1 histone were found in the ~23 kDa samples from cells on FN-adsorbed TCPS. No proteins were identified from cells adherent to PBS-adsorbed TCPS at ~23 kDa. Based on the LC/MS data, surface-adsorbed FN appears to regulate a unique set of proteins in adherent U937 cells compared against PBS at molecular weights ranging from ~160 to ~23 kDa. This data appears to show a change in cellular signaling pathway mediated by surface-adsorbed ligand.

6. Conclusions

Based on the proteins identified by LC/MS from adherent U937 cells, there appears to be a shift in intracellular signaling mediated by different surface-adsorbed ligands interacting with macrophages. This shift appears to be modulated by a complex combination of surface-adsorbed ligand such as Alb or FN and increasing concentrations of AG18. Macrophage activation may be altered by this shift in intracellular signaling in response to surface-adsorbed ligands therefore modulating the host response by activating different genes. However, more work resolving the actual signaling cascades is needed to fully understand any signaling shifts and their implications regarding the host inflammatory response.

Acknowledgments

This work was funded partly by NIH HL077825. The author would like to acknowledge Jim Brown for help with LC/MS.

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Biomaterials. Author manuscript; available in PMC 2017 December 28.

Table 1

Differential expression of various intracellular proteins in adherent U937 on protein-modified TCPS treated with or without AG18 as assayed by previous study [10]

MW (kDa)	Ligand-adsorbed surface	[AG18] (μM)	Trend[a,b]	Ligand-adsorbed control surface	[AG18] (μM)
~160	Albumin	40	>	Albumin	0
	Fibronectin	40	>	Fibronectin	0
~130	Fibronectin	20	<	Fibronectin	0
~100	Fibronectin	20	<	Fibronectin	0
~65	Phosphate buffer	40	<	Phosphate buffer	0
~52	Albumin	40	<	Albumin	0
	Fibronectin	20	<	Phosphate buffer	20
~42	Phosphate buffer	40	<	Phosphate buffer	0
	Phosphate buffer	60	<	Phosphate buffer	0
	Albumin	0	<	Phosphate buffer	0
	Fibronectin	20	<	Phosphate buffer	20
~23	Phosphate buffer	60	>	Phosphate buffer	0
	Phosphate buffer	80	>	Phosphate buffer	0
	Albumin	40	>	Albumin	0
	Albumin	80	>	Albumin	0
	Fibronectin	40	>	Phosphate buffer	40
	Fibronectin	80	>	Fibronectin	0

[a] > is up regulated.

[b] < is down regulated.

Table 2

Raw LC/MS data for U937 cells in suspsension (PMA)[a]

Protein MW	Peptide sequence identified	Mascot score	Protein ID
264,161	AVSLKALPDFSNVEIK	45	Predicted: similar to myocyte nuclear factor
13,444	**MIAPILDEIADEYQGK**	**69**	**Chain B, Ige Fv Spe7 complexed with a recombinant thioredoxin**
26,759	VLEGNEQFINAAK	67	Unnamed protein product
67,751	**TSRPENAIIYNNNEDFQVGQAK**	**62**	**Transketolase**
67,751	**SVPTSTVFYPSDGVATEK**	**46**	**Transketolase**
46,957	**AAVPSGASTGIYEALELR**	**66**	**Muscle specific enolase**
95,277	**ARPFPDGLAEDIDKGEVSAR**	**56**	**Human elongation factor 2**
187,771	**LLYNNVSNFGR**	**46**	**CLTC protein**
59,492	GSLGGGFSSGGFSGGSFSR	125	Unnamed protein product
59,492	ELTTEIDNNIEQISSYK	94	Unnamed protein product
59,492	ALEESNYELEGK	85	Unnamed protein product
59,492	LENEIQTYR	71	Unnamed protein product
59,492	VLDELTLTK	65	Unnamed protein product
59,492	QSLEASLAETEGR	58	Unnamed protein product
49,640	**LAVNMVPFPR**	**55**	**Tubulin, beta polypeptide**
76,104	MFCYDPSHNMWLKCVSLK	48	Unnamed protein product
59,772	**ILGADTSVDLEETGR**	**95**	**ATP synthase alpha**
64,542	**TILPAAAQDVYYR**	**50**	**Similar to ribophorin I**
84,621	**GVVDSEDLPLNISR**	**72**	**Heat shock protein HSP 90-alpha (HSP 86)**
84,621	**ADLINNLGTIAK**	**45**	**Heat shock protein HSP 90-alpha (HSP 86)**
101,495	**YVVVTGITPTPLGEGK**	**51**	**C-1-tetrahydrofolate synthase, cytoplasmic (C1THF synthase)**

[a]Proteins in bold are relevant hits used for data comparison with those from adherent cells.

Table 3

Raw LC/MS data for adherent U937 cells on Alb-adsorbed TCPS[a]

Protein MW	Peptide sequence identified	Mascot score	Protein ID
59,492	GSLGGGFSSGGFSGGSFSR	71	Unnamed protein product
59,492	ELTTEIDNNIEQISSYK	66	Unnamed protein product
59,492	GSLGGGFSSGGFSGGSFSR	97	Unnamed protein product
59,492	GSLGGGFSSGGFSGGSFSR	101	Unnamed protein product
59,492	SLLEGEGSSGGGGR	72	Unnamed protein product
59,492	SQYEQLAEQNR	77	Unnamed protein product
59,492	ALEESNYELEGK	75	Unnamed protein product
59,492	GSLGGGFSSGGFSGGSFSR	103	Unnamed protein product
39,195	NLDLDSIIAEVK	56	Unnamed protein product
39,195	ADTLTDEINFLR	64	Unnamed protein product
2,991,589	**LEPPELILDANMAR**	**41**	**Titin**[b]
59,772	ILGADTSVDLEETGR	87	ATP synthase alpha[c]
59,492	GSLGGGFSSGGFSGGSFSR	135	Unnamed protein product
49,599	**LAVNMVPFPR**	**48**	**Tubulin 5-beta**
49,599	**IMNTFSVVPSPK**	**56**	**Tubulin 5-beta**
59,492	GSLGGGFSSGGFSGGSFSR	119	Unnamed protein product
59,492	ELTTEIDNNIEQISSYK	62	Unnamed protein product
39,195	NLDLDSIIAEVK	59	Unnamed protein product
46,597	**GSYPDAILQAQAADK**	**88**	**Plasminogen activator inhibitor type 2 precursor**
46,597	**IPNLLPEGSVDGDTR**	**42**	**Plasminogen activator inhibitor type 2 precursor**
59,492	GSLGGGFSSGGFSGGSFSR	121	Unnamed protein product
59,492	NVSTGDVNVEMNAAPGVDLTQLLNNMR	45	Unnamed protein product
41,786	**SYELPDGQVITIGNER**	**56**	**Mutant beta-actin (beta'-actin)**
59,492	GSLGGGFSSGGFSGGSFSR	81	Unnamed protein product

[a] Proteins in bold are those identified after data analysis.

[b] Tyrosine phosphorylated.

[c] Also found in PMA cells.

Table 4

Comparison of ~65/70 kDa proteins from adherent U937 on PBS treated TCPS with 0 or 40 μM AG18

[AG18] (μM)	Protein	Molecular wt. (Da)
0	ATP-dependent DNA helicase II, 70 kDa subunit	69,799
	DNA helicase Q1	73,366
	Heat shock-related 70 kDa protein 2 (heat shock protein)	69,952
	Lymphocyte cytosolic protein 1 (L-plastin polypeptide)	63,839
	ATP synthase beta (chain) (ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide)	54,460
40	N/D[a]	—

[a]None detected.

Table 5

Comparison of ~52 kDa protein band from adherent U937 on FN treated TCPS compared to PBS treated TCPS with 20 μM AG18

Surface ligand	Protein	Molecular wt. (Da)
PBS	N/D[a]	—
FN	Myeloid cell nuclear differentiation antigen	45,807
	Anti-colorectal carcinoma heavy chain	50,570
	Vimentin	53,653
	Eukaryotic peptide chain release factor subunit 1 (TB3-1)	47,965
	Growth regulated nuclear 68 protein	66,881
	Elongation factor 1-alpha	35,205
	Protein disulfide isomerase-related protein 5 (protein disulfide isomerase A5)	46,170
	ATP synthase beta (ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide)	56,525
	Matrix metalloproteinase 1 (interstitial collagenase)	27,105

[a]None detected.

Table 6

Comparison of ~42 kDa protein band from adherent U937[a]

[AG18] (μM)	Protein	Molecular wt. (Da)
Comparison of PBS-adsorbed TCPS with 0 or 40 μM AG18		
0	Centrosome protein cep920 (CTCL tumor antigen se2-2)	88,383
	Mutant beta-actin (beta'-actin)	41,786
	Desmoglein (type 1)	113,644
	Glyceraldehyde-3-phosphate dehydrogenase	36,031
	Alpha enolase	47,079
	Hqp0256 protein	31,162
	Apolipoprotein B precursor	187,126
	Sulfide:quinone oxidoreductase, mitochondrial	49,917
	Vimentin	53,653
	Ribosomal protein L3	45,440
	NCL protein	50,920
	Eukaryotic translation elongation factor 1 gamma	50,115
40	N/D[b]	—
Comparison of PBS-adsorbed TCPS with 0 or 60 μM AG18		
0	Centrosome protein cep920 (CTCL tumor antigen se2-2)	88,383
	Mutant beta-actin (beta'-actin)	41,786
	Desmoglein (type 1)	113,644
	Glyceraldehyde-3-phosphate dehydrogenase	36,031
	Alpha enolase	47,079
	Hqp0256 protein	31,162
	Apolipoprotein B precursor	187,126
	Sulfide:quinone oxidoreductase, mitochondrial	49,917
	Vimentin	53,653
	Ribosomal protein L3	45,440
	NCL protein	50,920
	Eukaryotic translation elongation factor 1 gamma	50,115
60	N/D[b]	—
Surface ligand	Protein	Molecular wt. (Da)
Comparison of Alb- or PBS-adsorbed TCPS without AG18		
Alb	**Mutant beta-actin (beta'-actin)**	**41,786**
	Plasminogen activator inhibitor type 2 precursor	46,597
PBS	Centrosome protein cep920 (CTCL tumor antigen se2-2)	88,383
	Mutant beta-actin (beta'-actin)	**41,786**
	Desmoglein (type 1)	113,644
	Glyceraldehyde-3-phosphate dehydrogenase	36,031
	Alpha enolase	47,079
	Hqp0256 protein	31,162

[AG18] (μM)	Protein	Molecular wt. (Da)
	Apolipoprotein B precursor	187,126
	Sulfide:quinone oxidoreductase, mitochondrial	49,917
	Vimentin	53,653
	Ribosomal protein L3	45,440
	NCL protein	50,920
	Eukaryotic translation elongation factor 1 gamma	50,115
Comparison of FN- or PBS-adsorbed TCPS with 20 μM AG18		
FN	Alpha enolase	47,079
	Beta actin variant	41,738
	Sulfide:quinone oxidoreductase, mitochondrial (CGI-44 protein)	49,917
	Vimentin	53,653
	Laminin-binding protein	31,774
	NCL protein	50,920
	Eukaryotic translation elongation factor 1 gamma	50,115
	Plasminogen activator inhibitor 2	46,615
PBS	N/D[b]	—

[a] Common proteins are in bold text.

[b] None detected.

Table 7

Comparison of ~23 kDa protein band from adherent U937[a]

[AG18] (μM)	Protein	Molecular wt. (Da)
Comparison of PBS-adsorbed TCPS with 0 or 60 μM AG18		
0	N/D[b]	—
60	Glutathione *S*-transferase A1	23,159
	peroxiredoxin 1	22,096
	40S ribosomal protein S5	22,763
	Ribosomal protein L10	9,389
	Histone H1.4 (histone 1, H1b)	22,566
	Ribosomal protein L13a	16,720
	Histone H1	21,352
	Chain A, structure of Lamin AC GLOBULAR DOMAIN	13,360
	Ribosomal protein L18	21,637
	High Mobility Group protein 2 (HMGB2 protein)	22,268
	60S ribosomal protein L13	24,247
	Chain L, crystal structure of the Fab Fragment of the monoclonal antibody mak33	23,438
Comparison of PBS-adsorbed TCPS with 0 or 80 μM AG18		
0	N/D[b]	—
80	40S ribosomal protein S5	22,763
	Histone H2A.o (histone H2A.2)	13,899
	(60S) ribosomal protein L10	9,389
	Histone H1	21,352
	Chain A, structure of lamin AC GLOBULAR DOMAIN	13,360
	Ribosomal protein L18	21,637
	60S ribosomal protein L14 (CAG-ISL 7)	23,275
	Ribosomal protein L15	24,071
Surface ligand	Protein	Molecular wt. (Da)
Comparison of PBS- or FN-adsorbed TCPS with 40 μm AG18		
PBS	N/D[b]	—
FN	HDCMB21P	12,533
	Heterogeneous ribonuclear protein A2 (hnRNP protein A2)	35,984
	Nonhistone chromosomal protein HMG-1	24,968
	Peroxiredoxin 1	22,096
	(60S) ribosomal protein L10	23,903
	Histone H1.2	21,352
	60S ribosomal protein L14 (CAG-ISL 7)	23,275
	High Mobility Group protein 2 (HMGB2 protein)	22,268
	Caspase recruitment domain protein 5	21,613
	60S ribosomal protein L15	24,145
	Chain L, crystal structure of the fab fragment of the monoclonal antibody mak33	23,438

[AG18] (μM)	Protein	Molecular wt. (Da)
[AG18] (μM)	Protein	Molecular wt. (Da)
Comparison of FN-adsorbed TCPS with 0 or 80 μM AG18		
0	**Peroxiredoxin 1**	**22,096**
	Histone H1.4 (HPRD: histone 1 H1E) (histone H1b)	**21,721**
	Testicular H1 histone	22,020
80	26S proteasome non-ATPase regulatory subunit 5	8,216
	Peroxiredoxin 1	**22,096**
	Myeloid cell nuclear differentiation antigen	45,807
	Heterogeneous ribonuclear protein A2 (hnRNP protein A2)	35,984
	Ost-I	102,111
	Ribosomal protein L13a	16,720
	Testicular H1 histone	**22,020**
	Ribosomal protein L15	24,071
	High Mobility Group protein 2 (HMGB2 protein)	22,268
	Chain L, crystal structure of the fab fragment of the monoclonal antibody mak33	23,438

[a] Common proteins are in bold text.

[b] None detected.

Table 8

Summary of conditions for elucidating the effect of varying AG18 concentrations

	Surface ligand	**PBS**					**Alb**			**FN**			
	[AG18] (μM)	**0**	**20**	**40**	**60**	**80**	**0**	**40**	**80**	**0**	**20**	**40**	**80**
Molecular wt. (kDa)	200	x											
	160	x					x	x		x		x	
	130	x								x	x		
	100/90	x								x	x		
	70/65	x	x	x									
	52	x	x				x	x		x	x		
	42	x	x	x	x		x			x	x		
	23	x	x	x	x	x	x	x	x	x		x	x

x—Samples obtained.

Table 9

Effect of varying concentrations of AG18 upon protein expression in adherent U937 cells on PBS-adsorbed TCPS[a]

[AG18] (μM)	Protein	Molecular wt. (Da)
Protein expression at 65 kDa		
0	ATP-dependent DNA helicase II, 70 kDa subunit (thyroid autoantigen 70 kDa (Ku antigen)	69,799
	DNA helicase Q1	73,366
	Heat shock-related 70 kDa protein 2 (heat shock protein)	69,952
	Lymphocyte cytosolic protein 1 (L-plastin polypeptide)	63,839
	ATP synthase beta (chain) (ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide)	54,460
20	Titin[b]	2,991,589
40	N/D[c]	—
Protein expression at ~42 kDa		
0	Centrosome protein cep920 (CTCL tumor antigen se2-2)	88,383
	Mutant beta-actin (beta'-actin)	41,786
	Desmoglein (type 1)	113,644
	Glyceraldehyde-3-phosphate dehydrogenase	36,031
	Alpha enolase (2-phosphopyruvate-hydratase alpha-enolase; carbonate dehydratase)	47,079
	Hqp0256 protein	31,162
	Apolipoprotein B precursor	187,126
	Plasminogen activator inhibitor 2 (urokinase inhibitor)	49,917
	Vimentin	53,653
	Ribosomal protein L3	45,440
	NCL protein	50,920
	Eukaryotic translation elongation factor 1 gamma	50,115
20	N/D[c]	—
40	N/D[c]	—
60	N/D[c]	—
Protein expression at ~23 kDa		
0	N/D[c]	—
20	N/D[c]	—
40	N/D[c]	—
60	Glutathione *S*-transferase A1 (glutathione transferase (EC 2.5.1.18)/fatty-acyl-ethyl-ester synthase)	23,159
	Peroxiredoxin 1	22,096
	40S ribosomal protein S5	**22,763**
	60S ribosomal protein L10	**9389**
	Histone H1.4 (histone 1, H1b)	22,566
	Ribosomal protein L13a	16,720
	Histone H1	**21,352**
	Chain A, structure of Lamin AC GLOBULAR DOMAIN	**13,360**

[AG18] (μM)	Protein	Molecular wt. (Da)
	Ribosomal protein L18	**21,637**
	High Mobility Group protein 2 (HMGB2 protein)	22,268
	60S ribosomal protein L13	24,247
	Chain L, crystal structure of the fab fragment of the monoclonal antibody mak33	23,438
80	**40S ribosomal protein S5**	**22,763**
	Histone H2A.o (histone H2A.2)	13,899
	60S ribosomal protein L10	**9389**
	Histone H1	**21,352**
	Chain A, structure of Lamin AC GLOBULAR DOMAIN	**13,360**
	Ribosomal protein L18	**21,637**
	60S ribosomal protein L14 (CAG-ISL 7)	23,275
	Ribosomal protein L15	24,071

[a] Common proteins are highlighted in bold text.

[b] Tyrosine phosphorylated.

[c] None detected.

Table 10

Effect of varying concentrations of AG18 upon protein expression in adherent U937 cells on FN-adsorbed TCPS[a]

[AG18] (μM)	Protein	Molecular wt. (Da)
Protein expression at 23 kDa		
0	Peroxiredoxin 1	22,096
	Histone H1.4 (HPRD: histone 1 H1E) (histone H1b)	21,721
	Testicular H1 histone	22,020
40	HDCMB21P	12,533
	Heterogeneous ribonuclear protein A2 (hnRNP protein A2)	35,984
	Nonhistone chromosomal protein HMG-1	24,968
	Peroxiredoxin 1	**22,096**
	(60S) ribosomal protein L10	23,903
	histone H1.2	21,352
	60S ribosomal protein L14 (CAG-ISL 7)	23,275
	High Mobility Group protein 2 (HMGB2 protein)	22,268
	Caspase recruitment domain protein 5	21,613
	60S ribosomal protein L15	24,145
	Chain L, crystal structure of the fab fragment of the monoclonal antibody mak33	23,438
80	26S proteasome non-ATPase regulatory subunit 5 (proteasome (prosome, macropain) 26S subunit, non-ATPase, 5)	8216
	Peroxiredoxin 1	**22,096**
	Myeloid cell nuclear differentiation antigen	45,807
	Heterogeneous ribonuclear protein A2 (hnRNP protein A2)	35,984
	Ost-I	102,111
	Ribosomal protein L13a	16,720
	Testicular H1 histone	**22,020**
	60S ribosomal protein L15	24,145
	High Mobility Group protein 2 (HMGB2 protein)	22,268
	Chain L, Crystal structure of the fab fragment of the monoclonal antibody mak33	23,438

[a]Proteins common to 0 μM AG18 are highlighted in bold text.

Table 11

Summary of conditions for analyzing the effect of surface-adsorbed ligands

	Surface ligand [AG18] (μM)	**PBS 0**	**Alb 0**	**FN 0**
Molecular wt. (kDa)	200	x		
	160	x	x	x
	130	x		x
	100/90	x		x
	70/65	x		
	52	x	x	x
	42	x	x	x
	23	x	x	x

x—Samples obtained.

Table 12

The effect of Alb-adsorbed TCPS upon protein expression in adherent U937 cells[a]

Surface ligand	Protein	Molecular wt. (Da)
Comparison of ~160 kDa proteins from cells on PBS- or Alb-adsorbed TCPS without AG18		
PBS	DNA-dependent protein kinase catalytic subunit (DNA-dependent protein kinase)	99,816
Alb	N/D[b]	—
Comparison of ~52 kDa proteins from cells on PBS- or Alb-adsorbed TCPS without AG18		
PBS	ATP synthase beta	54,460
	TPO autoantibody immunoglobulin heavy chain, V-region (TR1.41)	13,367
	Anti-colorectal carcinoma heavy chain	50,570
	HLA-B-associated transcript 1 (BAT1 gene product)	33,121
	Growth regulated nuclear 68 protein	66,881
Alb	Titin[c]	2,991,589
	Tubulin 5-beta	49,599
Comparison of ~42 kDa proteins from cells on PBS- or Alb-adsorbed TCPS without AG18		
PBS	Centrosome protein cep920 (CTCL tumor antigen se2-2)	88,383
	Mutant beta-actin (beta'-actin)	**41,786**
	Desmoglein (type 1)	113,644
	Glyceraldehyde-3-phosphate dehydrogenase	36,031
	Alpha enolase	47,079
	Hqp0256 protein	31,162
	Apolipoprotein B precursor	187,126
	Sulfide:quinone oxidoreductase, mitochondrial	49,917
	Vimentin	53,653
	Ribosomal protein L3	45,440
	NCL protein	50,920
	Eukaryotic translation elongation factor 1 gamma	50,115
Alb	**Mutant beta-actin (beta'-actin)**	**41,786**
	Plasminogen activator inhibitor type 2 precursor	46,597

[a] Common proteins are in bold text.

[b] None Detected.

[c] Tyrosine phosphorylated.

Table 13

The effect of FN-adsorbed TCPS upon protein expression in adherent U937 cells[a]

Surface ligand	Protein	Molecular wt. (Da)
Comparison of ~160 kDa proteins from cells on PBS- or FN-adsorbed TCPS without AG18		
PBS	DNA dependent protein kinase catalytic subunit (DNA-dependent protein kinase)	99,816
FN	N/D[b]	—
Comparison of ~130 kDa proteins from cells on PBS- or FN-adsorbed TCPS without AG18		
PBS	DNA topoisomerase II beta	180,501
	Dedicator of cytokinesis protein 2 (DOCK2 protein)	38,436
FN	N/D[b]	—
Comparison of ~100 kDa proteins from cells on PBS- or FN-adsporbed TCPS without AG18		
PBS	N/D[b]	—
FN	DNA topoisomerase II beta	182,578
	DNA dependent protein kinase catalytic subunit	465,266
Comparison of ~52 kDa proteins from cells on PBS- or FN-adsorbed TCPS without AG18		
PBS	**TPO autoantibody immunoglobulin heavy chain, V-region (TR1.41)**	**13,367**
	Anti-colorectal carcinoma heavy chain	**50,570**
	HLA-B-associated transcript 1 (BAT1 gene product)	33,121
	Growth regulated nuclear 68 protein	**66,881**
FN[b]	**TPO autoantibody immunoglobulin heavy chain, V-region (TR1.41)**	**13,367**
	Anti-colorectal carcinoma heavy chain	**50,570**
	Vimentin	53,653
	Mitochondrial ATP synthase beta chain	34,026
	Growth regulated nuclear 68 protein	**66,881**
Comparison of ~42 kDa proteins from cells on PBS- or FN-adsorbed TCPS without AG18		
PBS	CTCL tumor antigen se2-2	88,383
	Mutant beta-actin (beta'-actin)	41,786
	Desmoglein type 1	113,644
	Glyceraldehyde-3-phosphate dehydrogenase	36,031
	Alpha enolase	47,079
	Hqp0256 protein	31,162
	Apolipoprotein B precursor	187,126
	Sulfide:quinone oxidoreductase, Mitochondrial	49,917
	Vimentin	**53,653**
	Ribosomal protein L3	45,440
	NCL protein	**50,920**
	Eukaryotic translation elongation factor 1 gamma	**50,115**
FN	Beta actin variant	41,738
	Lamin A/C	53,219
	Vimentin	**53,653**
	40S ribosomal protein SA (laminin-binding protein)	31,774

Surface ligand	Protein	Molecular wt. (Da)
	NCL protein	**50,920**
	Muscle specific enolase	46,957
	Eukaryotic translation initiation factor 2, subunit 3 gamma, 52 kDa	51,077
	Eukaryotic translation elongation factor 1 gamma	**50,115**
	Plasminogen activator inhibitor 2	46,615
Comparison of ~23 kDa proteins from cells on PBS- or FN-adsorbed TCPS without AG18		
PBS	N/D[b]	—
FN	Peroxiredoxin 1	22,096
	Histone H1.4 (histone H1b)	21,721
	Testicular H1 histone	22,020

[a] Common proteins are in bold text.

[b] None detected.