

Phosphoproteomic Characterization of Human Eosinophils Activated with GM-CSF.

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Abstract

RATIONALE: Activated eosinophils are critically involved in the pathogenesis of allergic diseases and asthma. Little is known concerning the exact signaling mechanisms promoting eosinophil growth, migration to lung tissue, and tissue survival. Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) is involved in the up-regulation of all known markers of eosinophilic activation. The precise molecular mechanism by which GM-CSF along with other cytokines exerts its activating effects is not understood.

METHODS: Freshly harvested human eosinophils were left unstimulated or stimulated with GM-CSF for 5 min, 15 min, and 18 hr, lysed, and soluble proteins were separated by two-dimensional electrophoresis. Gels were either transferred to PVDF and probed with antiphosphotyrosine G10 antibody or stained with Pro Q Diamond phosphoprotein and Sypro Ruby total protein stain, followed by image analysis and MALDI-TOF MS/MS protein identification.

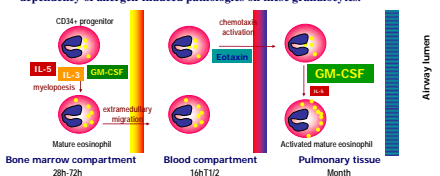
RESULTS: GM-CSF stimulation yielded a distinct phosphoproteomic pattern of labeled proteins as assessed by staining with Pro Q Diamond in freshly isolated eosinophils. Comparison of Pro Q Diamond stained protein patterns in eosinophils stimulated at different time points demonstrated the temporal nature of GM-CSF mediated phosphorylation events. Phosphorylation differences were further revealed using anti-phosphotyrosine G10 antibody. Proteomic comparison of eosinophils stimulated for 5 min, 15 min, and 18 hr revealed distinct patterns of phosphorylated and expressed proteins. Using subcellular fractionation, we identified changes in protein expression, phosphorylation and subcellular translocation occurring in GM-CSF-activated eosinophils. Identification of proteins by MALDI-MS revealed numerous proteins of interest that relate to the physiologic role of GM-CSF in cell survival, cellular metabolism, actin skeleton reorganization, and intracellular signaling in activated eosinophils.

CONCLUSIONS: Through the identification of eosinophil-specific patterns of phosphorylated proteins, our findings illustrate the potential of phosphoproteomics in the discovery of new eosinophil-specific signaling pathways and rational utilization of receptor specific therapies for modulation of eosinophilic inflammation. (Supported by NIH/NHLBI N01-HV-28184, NIH/NCI CA88317, and an ABRF travel grant).

Introduction

Eosinophils and bronchial asthma

1. Asthma is characterized by a prominent eosinophilic inflammatory infiltrate in the bronchial mucosa.
2. Increased eosinophil number in lung tissue correlates with severity of asthma symptoms.
3. Experimental models of asthma using eosinophil-deficient mice point to dependency of allergen-induced pathologies on these granulocytes.



GM-CSF and bronchial asthma

1. GM-CSF level is increased in BAL fluid and lung tissue in the course of allergic inflammation.
2. Majority of pro-survival activity toward eosinophils in blood and BAL fluid is driven by GM-CSF.
3. Genetically engineered mice with overexpressed GM-CSF gene in bronchial mucosa shows enhanced susceptibility to allergen sensitization.

Hypothesis

The phenotypic properties of activated eosinophils are dependent on transcriptional induction of protein expression and their posttranscriptional modification. Phosphorylation of signaling/effector proteins is a major posttranscriptional modification involved in signaling during cell activation. GM-CSF is responsible for the expression and modification of proteins contributing to the "activated eosinophil" phenotype. We hypothesize that GM-CSF-induced signaling events are transduced by phosphorylated proteins and phosphoproteomic analysis of GM-CSF-stimulated eosinophil may reveal novel targets for modulation of eosinophilic activation.

Specific Aims

1. Identify GM-CSF-regulated changes in eosinophil proteome using 2-dimensional gel analysis followed by fluorescent staining proteins.
2. Investigate phosphorylation of proteins in GM-CSF-stimulated eosinophils using phosphospecific fluorescent stain.
3. Determine GM-CSF-induced protein phosphorylation in subcellular fractions of GM-CSF-stimulated eosinophils.
4. Compare phosphoproteomes of eosinophils using phospho-specific prefractionation techniques.

Results

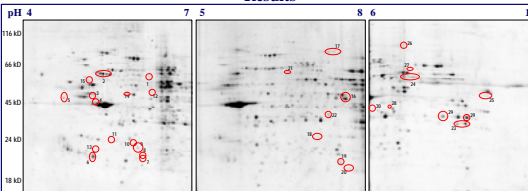


Figure 1: Systematic analysis of eosinophil proteins provides a map from which to observe changes in the eosinophil proteome. Proteins are spread across several pH ranges to increase visualization of spots. pH 4-7 and 5-8 contain ~1200 spots, while pH 6-11 contains ~700, ~400 spots have been identified to date by MALDI-TOF MS/MS.

Table 1: Selected proteins identified in eosinophils by MALDI-TOF MS/MS

pH 2 ID #	Protein Name	GI Number	Expectation Value	Change	pH 10 ID #	Protein Name	GI Number	Expectation Value	Change
1	Chaperone containing TDP1 subunit 1 isoform 1	2423705	3.0E-4	Green	1	phosphoglucomannan debranchase	4050216	2.0E-2	Red
2	Leukocyte cell adhesion protein 1 isoform 1	4594965	8.0E-05	Green	17	Grp94 protein	3719047	5.0E-38	Red
3	Interleukin-25 receptor, K170x4, 4	3053003	6.0E-05	Green	18	proteasome activator complex subunit 1	7362811	1.2E-05	Red
4	IL-5 receptor protein	3053000	6.10E-11	Green	19	Chain A, Chaperone-assisted Cysteine Protease	1764263	1.80E-03	Red
5	Chain D, Interleukin-5 receptor-Alpha/Argonin Comp	3820219	7.0E-20	Red	20	Min, epoxide hydrolase domain 1 precursor	8770235	3.70E-05	Red
6	Pro-Q Diaporphosphatase inhibitor (DPI) isoform 1	1410762	1.20E-11	Green	21	actin	2022263	3.0E-11	Red
7	RAB11B, member RAS oncogene family	1410644	2.2E-01	Green	22	Fucosyl 1,6-N-acetylglucosaminyl 1	5710355	3.5E-05	Red
8	SPPL2 Protein	4706527	4.0E-10	Green	23	Interleukin-5 receptor (IL5R) subunit A2/B1	4050447	1.0E-05	Red
9	Interleukin-5 receptor (IL5R) subunit 1 isoform 1	3820219	1.40E-03	Red	24	Adenovirus G protein-associated protein	1452556	2.0E-11	Red
10	Chain H, Chaperone D	580201	1.40E-20	Red	25	membrane-associated phosphatase	1149400	6.70E-04	Red
11	Interleukin-5 receptor subunit 2	4717000	1.40E-20	Red	26	adenovirus membrane-associated protein 2	4050452	1.40E-04	Red
12	Ras GTPase-activating protein 1	4707766	3.0E-20	Red	27	actin	2022263	1.50E-04	Red
13	Ras-related protein Rab 27A	3448706	1.00E-04	Red	28	membrane-associated phosphatase	4050355	1.70E-10	Red
14	phosphotyrosine phosphatase	4204162	6.00E-03	Red	29	Unc-119A, Cysteine protease	8074	6.0E-06	Red
15	serpinorexin 3 isoform 1	3240207	1.40E-02	Red	30	adenoviral capsid hexon 4 (Ad5) subunit 1, unspliced	3082843	6.80E-11	Red

* Low stringency (10³ qE²), whereas * is the probability that the observed match is a random event. Expectation values <10⁻⁵ are significant (p<0.05).
 † Indicated by a red color, where P is increased or decreased > 2-fold in GM-CSF stimulated cells either due to phosphorylation (Pro Q Diamond stain), or other expression changes (immunoblotting modification (Sypro Ruby)).

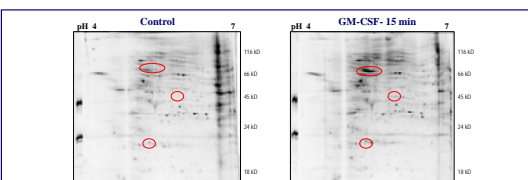


Figure 2: Eosinophil lysates were stained with Pro Q Diamond stain, revealing changes in the phosphoproteome of GM-CSF stimulated proteins (examples highlighted in red, and shown in figure 3).

Results

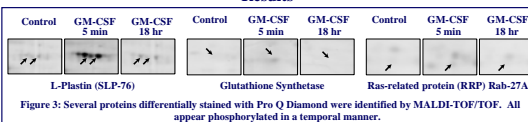


Figure 3: Several proteins differentially stained with Pro Q Diamond were identified by MALDI-TOF/TOF. All appear phosphorylated in a temporal manner.

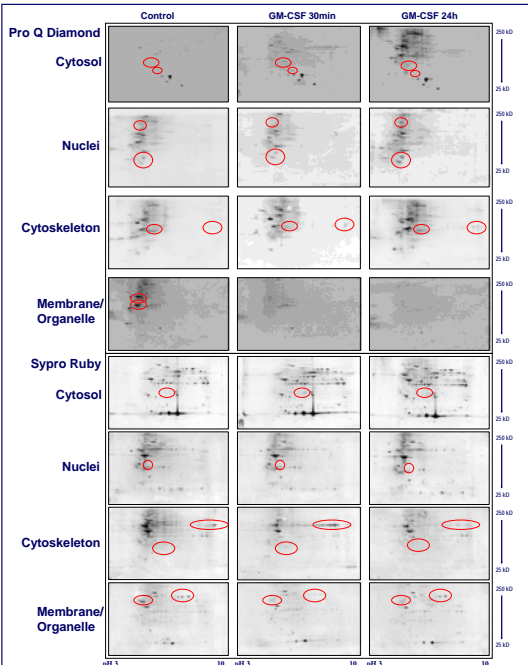


Figure 4: Freshly harvested eosinophils were subfractionated using a Proteoextract subfractionation kit from Calbiochem. Cellular subfractions were separated on pH 3-10 gradients and separated on 8-16% acrylamide gradient gels. Several regions of interest that demonstrate changes are highlight in red.

Results

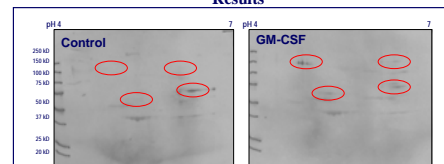


Figure 5: Images of the GM-CSF inducible phosphotyrosine proteome of human eosinophils as assessed by Western blotting with antiphosphotyrosine monoclonal antibody (4G10). Some changes to tyrosine phosphorylation are shown in red.

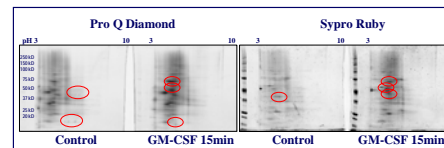


Figure 6: Preliminary results of Qiagen phosphoprotein column pull down of control and GM-CSF treated eosinophils. Examples of proteins to be identified are highlighted in red.

Conclusions

1. Stimulation with GM-CSF induces phosphorylation of a number of proteins, as determined by Pro Q Diamond Staining, antiphosphotyrosine staining, and phosphoprotein pull down.
2. Distinct and unique patterns of phosphorylation are detectable in eosinophils for short time periods in a temporal manner.
3. Subcellular fractionation reveals GM-CSF induced redistribution of eosinophil protein and enhances detectability of low abundant, effector proteins in eosinophil samples.

Future Directions

1. Identify all visible spots in eosinophil 2-D proteome
2. Identify the maximum number of phosphoproteins spots from Pro Q Diamond stained gels.
3. Compare 2-D antiphosphotyrosine immunoblots of eosinophils treated with GM-CSF with 2-D proteome map to determine ID of proteins of interest.
4. Further employ strategies of phosphoprotein enrichment, such as phosphatase antibody immunoprecipitation and metal affinity columns
5. Guide the discovery of new eosinophil specific signaling pathways and rational utilization of cell signaling specific therapies for modulation of eosinophilic/allergic inflammation.