Unraveling The Macrophage Response to Particulate Biomaterials: Gene-expression Clustering Using Self-organizing Maps

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INTRODUCTION

Microarray technology makes it possible to measure the mRNA gene expression levels for large numbers of genes simultaneously. Analysis of a few microarray experiments can unravel many important biological phenomena, such as patterns of gene expression over time, groups of genes regulated by the same processes, highly responsive genes, and comparisons between experimental conditions. This data analysis is a prodigious job and represents an emerging field where biochemistry, computer science, and mathematics are combining to solve clinical problems. In this study, several microarray analysis techniques, including the use of Self-Organizing Maps, were developed and used to understand gene expression changes following macrophage culture with clinically relevant ultra-high molecular weight polyethylene (UHMWPE) and titanium-aluminum-vanadium (TiAlV) particles. The expression analysis yielded not only the expected inflammatory genes, but also new potential targets for research and therapeutics.

MATERIALS AND METHODS

In vitro: Monocytes were harvested from 400ml of peripheral blood from human volunteers (n=4). After an overnight incubation, adherent cells were cultured with UHMWPE (PE), TiAlV, lipopolysaccharide (LPS) as a positive control, or medium only as a non-stimulated control (NS). Cells were harvested at 30 minutes, 4 hours, 8 hours, and 24 hours after culture, RNA extracted (Trizol, Gibco BRL, Grand Island, NY), and converted into radiolabeled cDNA using RT-PCR with ^{32}P-labeled primers specific for every gene on the array. The cDNA was then hybridized to a nylon membrane with specifically positioned probes for about 1,200 genes and analyzed by autoradiography (Atlas Human Array 1.2, Clontech, Palo Alto, CA). Gene arrays were performed on one trial and two samples and the remaining sample was used for confirmatory PCR. Preliminary analysis of cytokine expression was previously presented[1]. Macrophage conditioned media was also extracted at each of the four time points for ELISA analysis of key inflammatory mediators and growth factors (data not shown).

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In silico: In this study, a variety of clustering analyses were performed to identify interesting patterns of gene expression. Radiographic films were scanned, standardized, aligned, and contrast-adjusted (Adobe Photoshop 5.0, Adobe, San Jose, CA). Using array-specific software (Atlas Image, Clontech, Palo Alto, CA), distortions in location and background were removed, and the background was subtracted. Each condition, LPS, PE, and TiAlV, was compared to NS using a normalized ratio. While ratios greater than 1 represented up-regulation of the gene, ratios less than 1 were transformed to represent symmetric gene down-regulation.

The adjusted ratio time-courses for each gene were then ordered and clustered in "Cluster" (M. Eisen, Stanford University, Palo Alto, CA) using a self-organizing map (SOM) optimized to 7 nodes, with 1,000,000 iterations. Clustering the genes with SOMs produces not only a grouping of genes into 7 rough divisions, but also an ordering of each gene on the array that can be visualized (See Figure 1) (TreeView, M. Eisen).

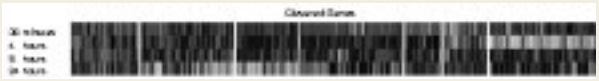


Figure 1: SOM clustered gene responses to PE

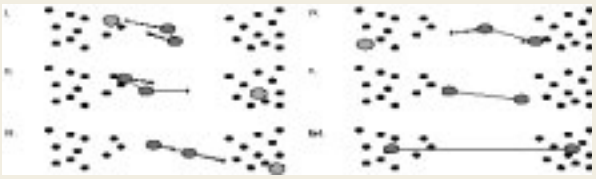
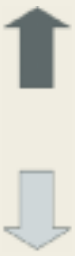


Figure 2: Nodes spread out as if attracted by clusters of points in 5 SOM iterations and the final solution

The SOM algorithm begins by laying down a pre-specified geometry of interconnected nodes. Each SOM iteration consists of randomly selecting a gene expression time-course from the data set, represented in the figure as a point, and moving the nodes toward that point according to the learning rule. The learning rule moves the nodes such that the closer a node is to the selected point, the farther that node is moved toward the selected point, and the amount of movement decreases with each iteration (See Figure 2)[2]. This procedure results in nodes spreading out as if attracted to clusters of points, hence "self-organizing." The actual points can then be collapsed onto the array of nodes to yield the one-dimensional list of genes ordered by gene expression, preserving the topology, hence a "map."

Genes are placed near each other based on similarity of their responses and clusters of genes are ordered based on similarity of average responses. To assess the biological significance of the clusters, we grouped the genes into 5 functional classifications (cell cycle, signal transduction, apoptosis, inflammation, and other).

Five custom-designed software programs calculated the average cluster responses and compared the frequency of each gene class within each cluster to the expected distribution based on class-size alone. The custom software also tabulated the genes which were among the top 25 up- and down-regulated genes under at least one condition across both replicates of the experiment. This software then related the conditions by classifying the response of each gene, selecting out genes up- or down-regulated at least twofold, and then searching for genes that differentiated each condition under both trials.



30 min	4 hours	8 hours	24 hours
MAD		IL-1 β C.E.2	integrin β2
MAD receptor 4		actin	MRP8
MARK3		MMP9	TNF & (LPS cell)
Ile-RA		HP31	MRP14
AL Kinase		β actin	CSF-R

Table 1: Top up- and down-regulated genes indicate important pathways

RESULTS

Inflammation-related genes were almost always over-represented in groups with significant up-regulation at 30 minutes and 4 hours and unchanged or slightly down-regulated at 8 and 24 hours (See Table 1). This time-course is consistent with previous RT-PCR studies[3].

At 30 minutes the up-regulated genes included matrix turnover proteins, cytokines, and anti-apoptosis proteins. Highly down-regulated genes included signal transduction machinery, gene expression repressors, and anti-activating proteins. After 4 and 8 hours, cytokines and cytokine-related proteins were common among the highly up-regulated genes (See Table 1).

After 24 hours in culture, a variety of cell signaling molecules from the IL-1/TNFα pathways were up-regulated. Simultaneously, adhesion and motility factors were down-regulated (See Table 1).

In order to find the genes that differentiate among the conditions, each gene's response was defined as "up," "down," or "unchanged" for each array. About 70% of the over 1,200 genes analyzed responded in the same category for each replicate. For these genes, the macrophage responses to LPS and PE were 95.5% similar, LPS and TiAlV were 96.9% similar, and PE and TiAlV were 95.0% similar. The majority of the gene expression similarities were due to the approximately 80% of genes with expression changes of less than twofold.

Figure 3: Major gene expression changes indicate important processes in the pathogenesis of osteolysis and aseptic loosening

Genes which responded uniquely to one stimulus, but similarly under the other two conditions, were termed "differentiators." LPS had the fewest differentiators (11), demonstrating that the macrophage response to PE and TiAlV may be an elaboration upon the more fundamental response to LPS. PE had 27 differentiators, which were primarily interleukins (IL-1,3,5,9,15). TiAlV had 23 differentiators including 7 cell-cycle genes which were down-regulated or unchanged with TiAlV and up-regulated after LPS and PE exposure.

DISCUSSION

Clustering micro-array data with SOMs imposes partial structure on the data set, summarizing the response profiles of a few thousand genes into a handful of generalized responses. Genes and clusters tend to be smoothly ordered by response and average-response, respectively[2].

The genes identified in our analysis validate and logically extend the current model of osteolysis and aseptic loosening (Figure 2)[4,5,6]. The most significant gene expression changes indicated four main categories: cytokines and inflammatory mediators, angiogenesis and vascular permeability factors, extracellular matrix remodeling, and osteoclastogenic factors. The macrophage phagocytosis of particulate biomaterials is thought to be central to the pathogenesis. Indeed, the gene expression changes in our *in vitro* model indicate processes which might explain the histology and pathology observed *in vivo*.

The macrophage response to 20th century biomaterials such as UHMWPE and TiAlV alloys has much in common with its response to a much older foe—gram-negative bacteria. This study underscores the extensive interplay between man-made implant components and the patients in which they reside. We have also introduced exciting new methods of gene expression analysis using self-organizing maps, as well as a vastly expanded list of genes with potentially important roles in aseptic loosening.

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Karl Schultz and Paul Appleton enjoy some “tubing” during a weekend off.

Drew, Sandy, Brent, and Will Ponce