Quantitative and Distributive Changes in Metal Content during Myeloid Cell Differentiation

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Introduction

Physiologically, the importance of trace metals has been substantiated in numerous epidemiological studies of dietary impact on human health. Diets deficient in trace minerals such as copper or zinc are associated with failure-to-thrive pathologies such as anemia [1]. On a biochemical level, metals are cofactors or structural components of a wide variety of enzymatic or functional proteins [2]. For example, zinc is a structural component of numerous protein kinases, a regulator of matrix metalloproteinases, a component of numerous extracellular matrix proteins, and an essential activator/structural nucleus for the large family of transcription factors possessing the "zinc finger" domain [3]. Although a large body of literature substantiates the importance of trace metals at the organismal, cellular, and biochemical levels, little is known about metal homeostasis and any possible dynamic roles for trace metals in regulating any of the aforementioned plethora of processes, in part, for several reasons. First, ions such as zinc or copper exist at only "vanishingly small" levels as free ions in the cell; instead, they are almost invariably tightly bound to a protein or carrier molecules. Since many detection methods rely on detecting metals by chelation of free ions into a reactable fluorophore (e.g., Fura dyes for calcium), measurements of zinc and copper by such approaches are inherently limited. Second, there has not been an adequate source of synchrotron-generated x-ray flux to permit anything except soft x-ray fluorescent approaches for elemental mapping.

The development of third-generation sources such as the APS has provided sources of very brilliant, highly coherent x-rays. They solve both limitations to studying elements such as copper or zinc. By using a hard x-ray scanning fluorescent microprobe approach, metals can be measured and mapped, irrespective of whether they are bound to a carrier molecule. In addition, the flux requirements for performing fluorescence in the hard x-ray range are now met, and elements above the edge of soft x-rays can now be studied.

An active area of research in our laboratory has been the cellular differentiation of the human promyelocytic cell line HL-60 into monocytes, macrophages, and granulocytes and the molecular mediators of this cellular maturation [4-6]. This transformed tumor cell line can be induced to acquire a phenotypically "normal" macrophage-like endpoint by inducing the protein kinase C signal transduction cascade with the phorbol ester PMA [7]. Several lines of evidence led us to hypothesize that the regulation of trace metal quantities or distribution may play a functional role in this differentiation. First, several components of this transduction cascade that we and others have identified as critical mediators are, in and of themselves, metalloproteins [8-10]. Second, it has recently been reported that a decrease of available free zinc precedes the induction of apoptosis, a programmed cell death process, in the HL-60 cell system [11]. Myeloid cells, as part of their immune role and because of their high level of physiological turnover, are consistently subject to a decision-making process, switching between maturation and death as cells are constantly being replaced. It logically follows that a decrease in zinc levels preceding an apoptotic process may be counteracted by an increase in zinc-sensitive reactions in proliferative or differentiation processes. The present studies were therefore undertaken to examine if any quantitative or distributive changes in trace metals, particularly zinc, are associated with human myeloid differentiation.

Methods and Materials

The human promyelocytic cell line HL-60 was grown as described [4-6]. Differentiation was induced by stimulation with 10 nM phorbol 12-myristate 13-acetate (PMA). To study early signaling events associated with myeloid differentiation, cells were collected following 0, 15, 30, 60, and 120 min of PMA stimulation. Cells were then collected by centrifugation and washed in either phosphate-buffered saline (PBS) or 25 mM tris-acetate pH 7.4, 200 mM sucrose. The cells were then suspended in 50 µL of either 4% paraformaldehyde or 2.5% glutaraldehyde solved in either PBS or tris-sucrose. A portion of this suspension was spotted onto carbon-formvar gold electron microscopy (EM) grids, and the cells were allowed to sediment and fix for 15 min. The fixative was removed, and the cell-laden grids were allowed to air dry. Cells were then examined and photographed by using optical differential interference contrast (DIC) microscopy on a Leica DMRXE microscope at sector 2.

Specimens were then examined by using the hard x-ray fluorescence microprobe at the 2-ID-E beamline as described [12]. This beamline is one of two hard x-ray microprobes in sector 2 at the APS. Both microprobes are operated simultaneously by employing a beam-splitting Si

monochromator to divide the incident undulator beam. The 2-ID-E microprobe uses Fresnel zone plates for focusing 7- to 14-keV x-rays onto a small spot on the specimen. Two zone plates, one with 150-nm and the other with 250-nm outermost zone widths, with corresponding focal lengths of 10 and 40 cm, can be employed. The sample is mounted on a kinematic mount within a helium-filled sample box, which is moveable for scanning purposes by picomotor x and y motors. X-ray fluorescence is detected by using an energy-dispersive three-element Germanium detector. Typical configurations generate sufficient focused incident flux to permit 1-s dwell times/pixel at submicrometer horizontal and vertical spatial resolution for data capture. Quantitation of fluorescent signals is achieved by internal standardization against National Bureau of Standards (NBS) thin film standards NBS1832 and NBS1833.

Results

Many existing protocols for the fixation of cells have been derived for purposes other than metal fluorescence studies. Most techniques are designed to either preserve cellular microarchitecture at the expense of biochemical composition (EM techniques) or fix and denature proteins and allow access to large macromolecules (immunofluorescence techniques). The desired goal of metal mapping in cellular systems required a fixation scheme that preserved the cellular architecture and also maintained cellular salt and metal compositions and distributions. Extensive studies were conducted comparing various buffers and chemical fixatives. The conclusion was that fixing cells in a slightly hypertonic solution consisting of either 4% formaldehyde or 2.5% glutaraldehyde in either PBS or tris-sucrose preserved cellular structure (as assessed by optical light and fluorescent microscopy) and maintained adequate metal composition and distribution (as assessed by the x-ray microprobe). A typical integrated spectrum from one such experiment is shown in Fig. 1. At the left is a log-scale plot of the integrated spectra (black line) of a 31×31 pixel scan of an HL-60 cell. The 2-D scan can be separated into high-count and low-count regions algorithmically (Fig. 1, upper right panel), and integrated spectra of high-count (left panel, green trace) and low-count (left panel, red trace) regions can be obtained.

These preliminary experiments demonstrate the feasibility of examining metal distributions in intact cells by hard x-ray scanning microprobe approaches. The results shown in Fig. 1 display the high signal-to-noise ratio obtained by this instrumentation. In addition, at least at the morphological level, the cellular microarchitecture appears to be well maintained. This is most clearly demonstrated by the high degree of correlation between the structures observable by optical microscopy and the integrated spectral maps obtained by the microprobe.

To use this system to examine metal distributions during biological phenomena, the HL-60 cell system was chosen. By stimulation with the PKC activator PMA, these cells are induced to acquire a macrophage phenotype over a period of several days (as assessed by biochemical, immunophenotypic, and functional assays) [7]. To examine changes over time, cells were collected and fixed to grids following 0, 15, 30, 60, and 120 min of exposure to PMA. Optical images of individual cells were

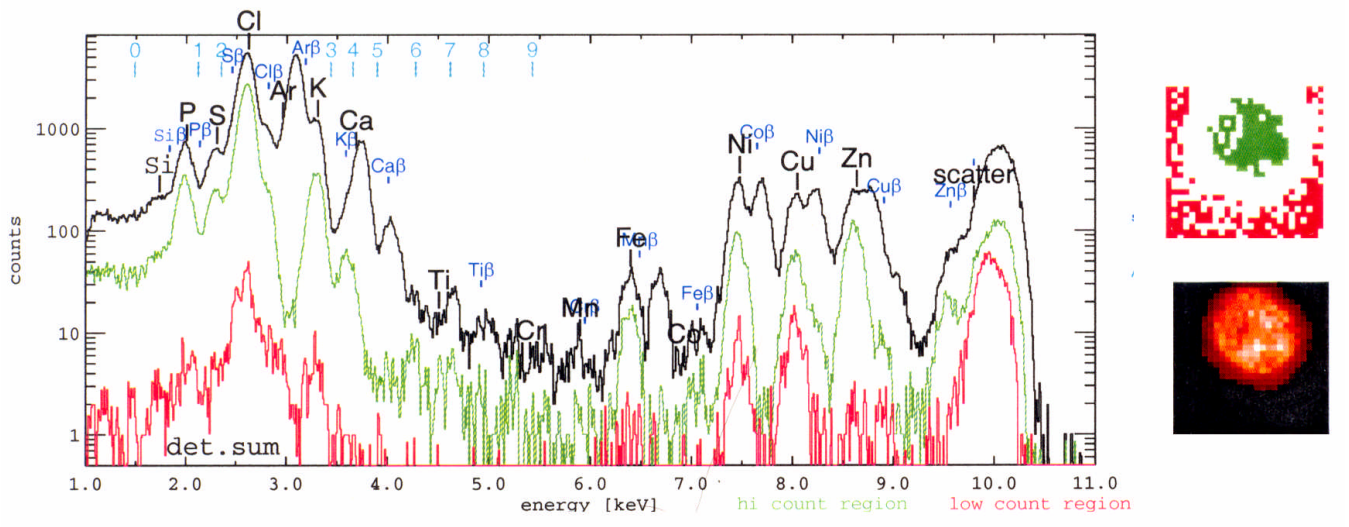

FIG. 1. Spectral output from the microprobe. A typical spectrum generated from the integrated pixel spectra of a 2-D scan is shown at the left The 2-D scan is then separated into high-count (green) and low-count (red) regions, shown in the upper right panel. Integrated spectra from high- and low-count regions (green and red traces at left) can then be compared. A 2-D integrated spectral map of the scan can also be produced (lower right panel).

then obtained, and these cells were used in the x-ray fluorescent microprobe at beamline 2-ID-E. At least seven cells per time point were examined during each run. The raw results of a typical series of scans are shown in Fig. 2. Elemental maps for phosphorous (P), sulphur (S), and potassium (K) show little variability over time and reflect large-scale macromolecular structures such as overall protein, nucleic acid, and phospholipid content. Major distributive changes for copper (Cu) and zinc (Zn) are, however, consistently evident. In untreated cells, these ions appear to be more or less uniformly distributed throughout the cell. Within 15 to 30 min of PMA stimulation, there appeared to be a consolidation of these ions and inverse compartmentalization of zinc to the nucleus and of copper to the cytoplasm. Nuclei in HL-60 cells are relatively large, making up well over 50% of the cells' 2-D space, but discernible by both elemental composition and optical microscopy. Between 60 and 120 min of stimulation, the distributions return to a state similar to those in untreated cells. By using NBS thin film emulsions as internal calibration standards, quantitative results have also been acquired. These data (not shown) demonstrate that although there may be some intake of zinc from the extracellular milieu, the changes seen in zinc elemental maps are largely distributive rather than being a result of large-scale metal uptake.

Discussion

These studies demonstrate the utility of hard x-ray scanning microprobe approaches for studying biological processes at the APS. Such an approach allows for not only investigations of not only cellular structure, as do other microprobe approaches, but also speciation of metals in intact cells. Other approaches (e.g., atomic mass absorption) for studying high-energy metals such as zinc or copper do not allow for elemental mapping of cellular substructure. This approach therefore is optimal in that it combines the speciation available from other spectroscopic methods with the spatial elemental resolution associated with scanning microprobe approaches.

The hard x-ray microprobe has allowed us to determine that there is a large-scale redistribution of zinc within a promyelocytic cell during early signaling events of myeloid differentiation. This translocation may explain the linkage of dietary zinc insufficiency with anemic physiologies as well as the evidence, albeit controversial, that zinc may be immunostimulatory [13]. Sufficient

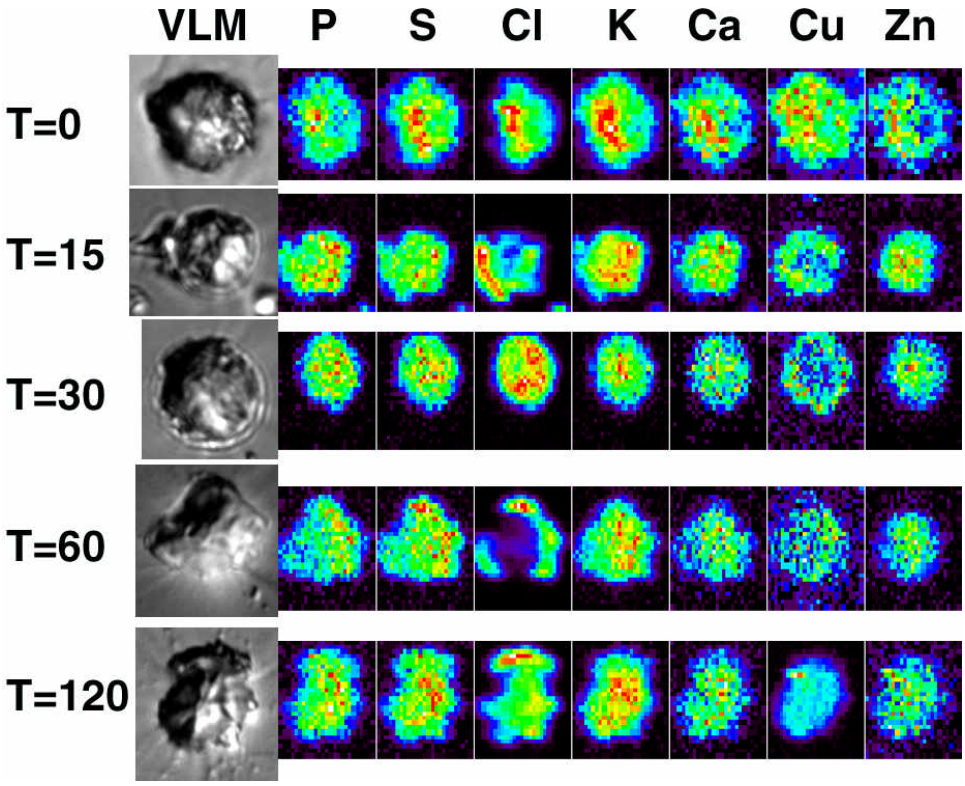

FIG. 2. Time course analysis of metal distributions following PMA stimulation of HL-60 cells. Cells were treated with PMA for the time shown at left (in minutes). Optical micrographs (visible light microscopy or VLM) of each cell, as well as elemental maps for the seven listed ions, are shown for a representative cell at each of the time points.

dietary zinc may provide enough substrate to allow this translocation to fully occur, resulting in sufficient *in vivo* macrophage maturation. Although zinc has been demonstrated to be a structural component or co-factor in a tremendous variety of proteins or reactions, little literature is available on the possibility that zinc access to these reactions may be limiting. It has largely been assumed, perhaps because of the limitations to studying zinc prior to the availability of hard x-ray microprobe approaches, that there is sufficient zinc available to bind to all proteins. Our results imply that this may not be the case. It is attractive to hypothesize that zinc finger transcription factors, because of their nuclear localization, may be inactive in proliferating cells and are charged with zinc during maturation by this nuclear translocation of zinc that we have demonstrated, resulting in transcription of differentiation-specific genes.

Future plans include expanding our studies to other hematopoietic lineages. Further discrimination between nuclear and cytoplasmic compartments will be approached by using cluster analysis and nuclear-specific stains. In addition, through a combination of molecular biological and x-ray approaches, we are interested in determining the specific mediators and targets of zinc regulation during cellular differentiation.

Acknowledgments

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