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Quantitation of Phosphotyrosine Signals in Human Prostate Cell Adhesion Sites

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ABSTRACT

A simple method is described for the quantitation of phosphotyrosine signaling in human prostate cell cultures. The phosphotyrosine signals are observed by standard immunohistochemistry techniques, and the resulting digital images are analyzed using the Scion image software program. The signals within the cell adhesion sites are quantitated using the density slice and particle analysis features of the software. The immunohistochemistry results are compared with detection of phosphotyrosine signals using a standard Western blotting procedure with whole cell lysates. The resulting data is converted into graphs using the Sigma Plot Program. This method is illustrated using damage-induced signaling within cell adhesion sites after a low dose of ionizing radiation.

INTRODUCTION

The induction of a biochemical signal within cells occurs in response to a wide variety of environmental conditions and insults, initiating cascades for cell growth, repair or cell death. The increase in phosphotyrosine signaling in particular has been observed as an early event in many of these processes (3,5,7). The detection of signaling within cells is investigated either using a whole cell lysate and observing the signal by gel electrophoresis or using intact cells and observing the phosphotyrosine signal by confocal laser scanning microscopy (CLSM). The detection of a biochemical signal by CLSM can be useful for determining the increased production of the signal within a subcellular component or tracking the movement of proteins from the cytoplasm to the nucleus (4).

In this report, a method is described for using the digital images collected

by CLSM to determine the amount of a signal when it is induced within the localized region of a cell adhesion site. The localized nature or clustering of the signal within the cells makes the image amenable to particle analysis coupled with the density-slicing feature of the software. In principle, this method could also be applied to other cell images in which the image signal is clustered, such as in the quantitation of nuclear DNA replication, transcription or repair domains (1,8). Alternatively, in tissue samples, the method could be used to determine blood vessel densities using standard immunohistochem-

istry-specific staining protocols (2,6) and applied to digitized images from many different sources.

MATERIALS AND METHODS

Normal prostate epithelial cells were obtained from Clonetics Corporation (San Diego, CA, USA) and used between passage number 3 and 5. The cells were grown on glass coverslips, irradiated with 1 Gy ^{60}Co γ rays, fixed with 100% cold methanol for 30 min at various times after irradiation and stained for phosphotyrosine signaling.

Figure 1. Adhesion site analysis using the Scion Image Program. The original image (panel A) is inverted (panel B) as described in the text. The entire image is selected, and the density slice tool is used to determine which range of pixel values can be used to quantitate the signal of interest. The red color highlights the areas to be counted (panel C). The particles within the density range are counted and labeled (panel D). The particle size range chosen here was a minimum of 4 and a maximum of 12.

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For Western analysis, cell lysates were obtained by harvesting the cells in ice-cold RIPA buffer [50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% (v/v) Triton® X-100, 1% (w/v) deoxycholate

and 0.1% (w/v) SDS] containing 2 mM PMSF, 0.5 mM sodium vanadate, 10 µg/mL aprotinin and 10 µg/mL leupeptin. Equivalent protein concentrations were analyzed using 7.5% PAGE.

Phosphotyrosine signals were detected using the mouse PY20 monoclonal antibody (1:50 dilution for the CLSM procedure or 1:1000 dilution for Western blotting; Transduction Laboratories, Lexington, KY, USA) and an affinity-purified FITC-goat anti-mouse secondary antibody (CLSM procedure, 1:20 dilution; ICN-Cappel, Costa Mesa, CA, USA) or an HRP-goat anti-mouse secondary (Western blotting procedure, 1:10000 dilution; Transduction Laboratories).

The cell images were collected for all samples using identical contrast and brightness settings on a confocal laser scanning microscope (LSM 210; Carl Zeiss, Thornwood, NY, USA), saved as TIFF files and transferred to an Iomega® Zip disk. For Western analysis, the film was scanned at 300 dpi using a flatbed scanner equipped with a transilluminator (Epson® model 1600). The software used for the image analysis was Scion Image Beta 3b Release,

Figure 2. Activation of phosphotyrosine signal in normal prostate epithelial cells using ionizing radiation. The top panel shows representative views of the staining of normal prostate epithelial cells for phosphotyrosine. The non-irradiated (control) cells and cells treated with 1 Gy ^{60}Co γ rays and incubated for 15, 30 or 90 min are shown. The images were collected at identical contrast and brightness settings. The bottom panel shows the quantitation of the phosphotyrosine signal from the four fields represented in the top panel.

Figure 3. Activation of phosphotyrosine signals detected in normal prostate epithelial cell lysates. The top panel shows the proteins (A–D) that contain phosphotyrosine as detected by a Western blot procedure. The non-irradiated cells (Con) or cells treated with 1 Gy ^{60}Co γ rays and incubated for 15, 30 or 60 min are shown. The bottom panel shows the results of proteins A–D analyzed for the relative mean pixel density values.

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downloaded at no cost from Scion Image Corporation (www.scioncorp.com). The Scion Image program is based on the NIH Image program for Macintosh® by Wayne Rasband (wayne@helix.nih.gov) at the National Institutes of Health (USA) and was modified for Windows® by the Scion Corporation.

The CLSM image analysis can be performed in six simple steps. First, open the image using the Scion Image program. Second, select the area to be analyzed using either the marquee tool (I) or the Select All command in the toolbar. Third, if analyzing a fluorescence image (black background and white signal as shown in Figure 1, panel A), invert the image using the Invert command under the Edit menu in the toolbar. The image should now have a white background and black signals as shown in Figure 1, panel B. Fourth, select the pixels to be counted, using the Density Slice or Threshold feature un-

der the Options command in the toolbar. In the example shown in Figure 1, panel C, pixel densities of 150–255 were chosen. The pixels selected for analysis at this density are shown in red. Fifth, go to the Analyze command in the toolbar and choose the Particle Analysis option. Select the particle sizes to be counted within the density range by numerically defining the minimum and maximum pixel values. Select the Label Particles option and the Include Interior Holes option. In the example shown in Figure 1, panel D, a minimum size of 4 and a maximum of 12 were selected. The info window will display the number of particles counted in the image. Sixth, record the measurements into Sigma Plot for presentation.

Western analysis was performed using the box tool and analyzing the same size area around each of the protein bands. The mean density values were recorded into Sigma Plot for presentation.

RESULTS AND DISCUSSION

The clustering of signals within a sample image can be analyzed easily if the density of the pixel values and the particle sizes are chosen for analysis. Used together, both of the features avoid counting high-density pixels outside the clustered signals at the adhesion site. In Figure 1, panel C, the density slice method will highlight in red the areas of pixel density between 155 and 255. The selected densities include pixels within the adhesion sites and pixels that are independent of the sites (e.g., the red circle within the middle of the cell). In Figure 1, panel D, the particle size was defined as a minimum of 4 pixels and a maximum of 12 pixels, and the resulting particles are labeled when counted. Defining the particle size allows the analysis of clustered pixels within the adhesion site and avoids counting the red circle within the middle of the cell.

The definition of the pixel density and the particle sizes to be counted were empirically determined. Once these parameters were set, the analysis was then applied to a series of samples within the same experiment. Figure 2 illustrates the application of this method to determine the alteration of the phosphotyrosine signal in response to 1 Gy ^{60}Co γ rays. The top panel of Figure 2 shows the representative confocal images of phosphotyrosine signaling in normal human prostate cells before and after irradiation. The individual panels were analyzed by selecting the image exclusive of the tool bar and using a density slice range of 155–255 and a particle size of 4–12. Four fields were analyzed for each time point, and the average pixel values were plotted. The quantitation of the images indicates that a significant time-dependent increase of the phosphotyrosine signal occurred and determines that approximately 50–60-fold more signal was induced. The use of this analysis will result in knowing the magnitude and duration of signals observed within the cells from images that are commonly collected for co-localization purposes. The analysis of the entire image (i.e., using the entire field of cells) using the defined parameters eliminates the need for selecting individual cells and offers an objective approach to quantitate trends or impressions of changes in signals observed by CLSM.

For comparative purposes, whole cell lysates were used to determine the amount of phosphotyrosine in the cells using a standard Western blotting procedure. Figure 3 indicates that at least four proteins (proteins A–D) are increased in the phosphotyrosine signals following irradiation. These proteins correspond to molecular weights of known focal adhesion proteins. A comparison of the quantitation of phosphotyrosine using the two methods suggests that they yield similar results. For example, Figure 2 indicates a phosphotyrosine signal of approximately 300 U at 60 min compared with a sum value of approximately 335 U (proteins A–D) at 60 min (Figure 3) using the Western blot procedure. The CLSM method can detect the phosphotyrosine signal earlier than the Western blotting method. This is presumably due to the detection of a focal adhesion signal contributed

by many proteins, while the Western blotting procedure detects individual protein phosphotyrosine signals. Both methods detect alterations in phosphotyrosine signals after irradiation.

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