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The Centrosome Moves out of a Nuclear Indentation in Human Lymphocytes upon Activation

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Using a β-tubulin specific antibody, centrosomes were labeled in paraformaldehyde fixed human lymphocytes. Cells were kept in suspension to preserve the three-dimensional (3D) morphology as much as possible. The centrosome was generally identified as the focus of the microtubule array. Resting (G_0) and phytohemagglutinin activated cells in G_1 stage were taken for 3D analysis of the centrosome position, using confocal microscopy and 3D analysis software. Measurements were performed in relation to the nuclear center and the periphery of the propidium iodide stained area ("nuclear envelope"). The distribution of the distances between the centrosome and the nuclear center revealed that in most resting cells the centrosome was located at the basis of a nuclear indentation. Upon activation, however, the centrosome appeared to move out of the indentation during transition from G_0 to G_1 stage.

INTRODUCTION

During mitosis, a clear relationship between the position of the centrosomes and the chromosomes exists: chromosomes align along the equatorial plane during metaphase and subsequently move toward the centrosomes in anaphase. During interphase, however, the relationship between chromatin organization and the centrosome is much less clear.

Several studies have indicated that the relative position of the centrosome is important in several cellular processes. In migrating cells the direction of movement is correlated with the position of the centrosome [1] and in cytotoxic T lymphocytes the centrosome is positioned toward the target cell [2]. Capping of surface receptors in lymphocytes occurs adjacent to the cytoplasmic region containing the centrosome. Disruption of the microtubule system still results in capping, but the location of the cap is random with respect to the centrosome position [3, 4]. A disrupted microtubule system also results in a disorganization of the Golgi complex. The scattered Golgi elements recluster near the centrosome after repolymerization of the microtubules [5]. These findings indicate that the position of the centrosome is related to that of several cytoplasmatic components during interphase, whereas other findings have led to the idea that the centrosome merely attempts to maintain a position in the centroid of the cell to maximally support a polarized cytoplasmic organization [6].

Little is known about the position of the centrosome during interphase in relation to nuclear components. With respect to resting and activated mouse splenic lymphocytes Schweitzer and Brown [7] observed that the centrosome was located "adjacent to a nuclear cleft." These studies were carried out by electron microscopy, which precludes easy acquisition of three-dimensional (3D) data. Such data are more conveniently (although at a lower resolution) obtained by confocal scanning laser microscopy [for reviews, see 8].

In this paper we present morphological and statistical data on the perinuclear position of the centrosome. Resting and mitogen activated human lymphocytes were kept in suspension and labeled with β-tubulin specific antibodies. Analysis of the 3D data sets revealed that the presumed spherical nuclei of lymphocytes contained indentations. The position of the centrosome appeared to be at the basis of such an indentation in resting cells. In activated cells, however, the distance between the nuclear center and the centrosome was significantly larger.

MATERIALS AND METHODS

Isolation, growth, and fixation of human lymphocytes. Lymphocytes were isolated from peripheral blood with lymphocyte separation medium (Organon Teknika, Durham, NC) and were either cold treated (20 min on ice) and directly fixed (20 min at room temperature (RT) in phosphate-buffered saline (PBS) with 2% (w/v) paraformaldehyde, pH 7.4, or cultured in alpha modification of Eagle's medium (Flow Laboratories, Irvine, Scotland) with 10% (v/v) fetal calf serum. Cells were stimulated to enter the cell cycle by addition of 1%

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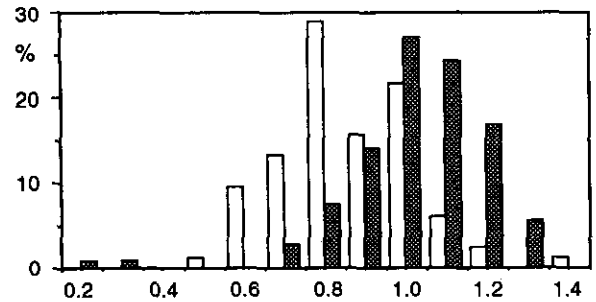

FIG. 1. Distribution of the distances from the nuclear center to the centrosome, measured in mainly resting, G_0 stage (white bars), and in activated, mainly G_1 stage (gray bars), human peripheral blood lymphocytes. The mean distance in resting cells (0.8 length units) was significantly different from that in G_1 stage cells (1.0 length units). The nuclear center was defined at 0 and the nuclear boundary at 1.

(v/v) phytohemagglutinin (PHA; Welcome Diagnostics; Dartford, UK).

After 65 hr of stimulation, 10 μg bisbenzimide No. 33342 (Hoechst 42; Riedel-de Haen AG, Hannover, Germany) per ml was added and the culture was incubated for 1 h at 37°C. Next, the cells were washed twice in PBS containing 10 μg Hoechst 42 per ml. Finally, 1.2 μg propidium iodide (PI) per ml was added for flow cytometric sorting of the G_0/G_1 fraction. After fixation, all cell samples were stored in 70% ethanol at −20°C.

Flow cytometric analysis and sorting. Cells were analyzed on the RELACS-2 flow cytometer [9], equipped with two lasers for dual beam flow cytometry. The first laser (Coherent Innova 90, Palo Alto, CA) was set at 488 nm (300 mW) to excite PI. PI fluorescence was detected through a BP630P10 emission filter (Corion, Holliston, MA). The second laser (Series 2000; Mountain View, CA) Spectra Physics, was set at ultraviolet wavelengths (351 and 364 nm; 150 mW) to excite Hoechst 42 that was detected through a 408 long pass plus a 450 short pass filter (Schott Glaswerke, Mainz, Germany).

The same setup was used to sort the PI negative (viable) lymphocytes of the G_0/G_1 fraction on the basis of the DNA (Hoechst 42) content. Since these cells had been activated by PHA, this sorted G_0/G_1 fraction was enriched for cells in G_1 stage of the cell cycle. After sorting, these cells were cold treated, fixed, and stored as described above.

Tubulin labeling. Cells were rehydrated in PBS for 15 min at RT and preincubated in PBS containing 1% (w/v) bovine serum albumin (PBS/BSA) (45 min at RT). Incubation for 30 min with a murine monoclonal antibody to β-tubulin (Boehringer Mannheim, Germany) diluted 1:50 in PBS/BSA was followed by two washes in PBS/BSA. Next, the cells were incubated for 1 h at RT with FITC conjugated goat polyclonal antibody to murine Ig-G light chains (Tago, Burlingame, CA) diluted 1:50 in PBS/BSA. After three washes in PBS, the cells were resuspended in VectaShield (Vector, Burlingame, CA) containing 0.5 μg PI per ml.

Confocal scanning laser microscopy. A Bio-Rad MRC 600 CSLM (Hertfordshire, UK) was used to record 35 optical *XY* sections of each cell. A 63×/1.3 oil objective (Leica, Heidelberg, Germany) was used and the zoom factor was set at 6 (1 pixel:81 nm). Each optical section (170 × 170 pixels) was separated by 300 nm in the *Z*-direction. Measurements were performed with 1% (±0.1 mW, adjusted by a gray filter) laser output of the 10 mW 488-nm argon line.

Cells were recorded in simultaneous two-color detection mode. The FITC fluorescence was separated from the PI fluorescence by a standard A2 filter block. Both detection pinholes were set at scale mark 4.

3D image analysis. Analysis of the cells was performed on a Hewlett Packard/Apollo 425 series workstation (Palo Alto, CA) with an interactive software program IMAP3D. This software program was designed at our department at the University of Amsterdam especially for measuring distances in 3D confocal data sets [10].

The dimensions of the nuclei were determined using a 3D cursor. The cursor was present in three windows on the computer screen. The central window displayed a front view (*XY*-projection) of the 3D confocal data set. A top view (*XZ*-projection) and a side view (*YZ*-projection) were displayed on two sides of the central window. First, the cursor was positioned at the center of the nucleus. Second, the *X*-, *Y*-, and *Z*-axis were elongated such that they reached the nuclear boundary. To indicate the position of the centrosome, the 3D cursor was moved to the XY-position of the centrosome spot in the front view and to the *Z*-position in the side views. The 3D coordinates were stored with the length of the three axes (in voxels) by pressing a user defined key. In order to compare the data from different cells, the nuclear dimensions were transformed to a nominated orthogonal coordinate system with the nuclear center at $(x, y, z) = (0, 0, 0)$ and the nuclear boundary at 1. The position of the centrosome was used to rotate the coordinate system such that the centrosome was on the positive *Z*-axis. Finally, the distance between the nuclear center and the centrosome was calculated.

Statistical analysis. The Kolmogorov–Smirnov Test [11, 12] was used to test the hypothesis that both samples were taken from the same population. Cumulative values of the distributions were calculated and the maximum deviation (δ_{max}) was determined. The level of significance taken was $\alpha = 5\%$. The solution c of the equation $P(A \leq c) = 1 - \alpha$ was determined and the hypothesis was rejected if $\delta_{max} > c$.

RESULTS

Efficiency of Tubulin Labeling and Visualization of the Centrosome

The 3D morphology was preserved as much as possible by performing all the procedures on suspended intact cells. However, the main disadvantage of working with cell suspensions is that clumps of cells arise, thus influencing the labeling efficiency. Special attention was, therefore, paid to resuspension after each centrifugation step. Thoroughly resuspended cell suspensions resulted in relatively high labeling efficiencies. The percentages of tubulin-labeled cells varied from 70 to 82%. Lymphocytes from the PHA stimulated sample usually contained more tubulin than resting lymphocytes, as has also been reported by Waterhouse *et al.* [13].

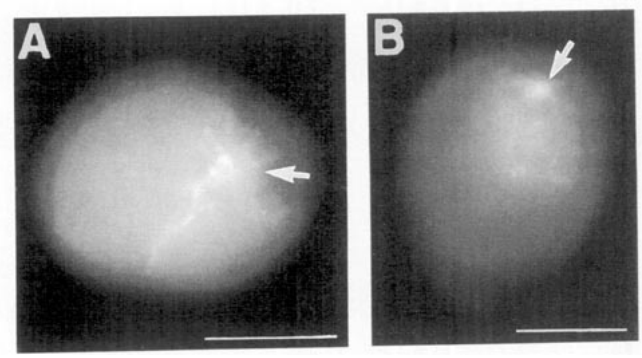

FIG. 2. Conventional fluorescence microscopy photos of a (A) resting, G_0 stage, and an (B) activated, G_1 stage, human lymphocyte. The position of the centrosome was identified as the focus of microtubules after labeling with a β-tubulin specific antibody (arrows). Nuclei were counterstained with propidium iodide to determine the nuclear periphery. Bars, 5 μm.

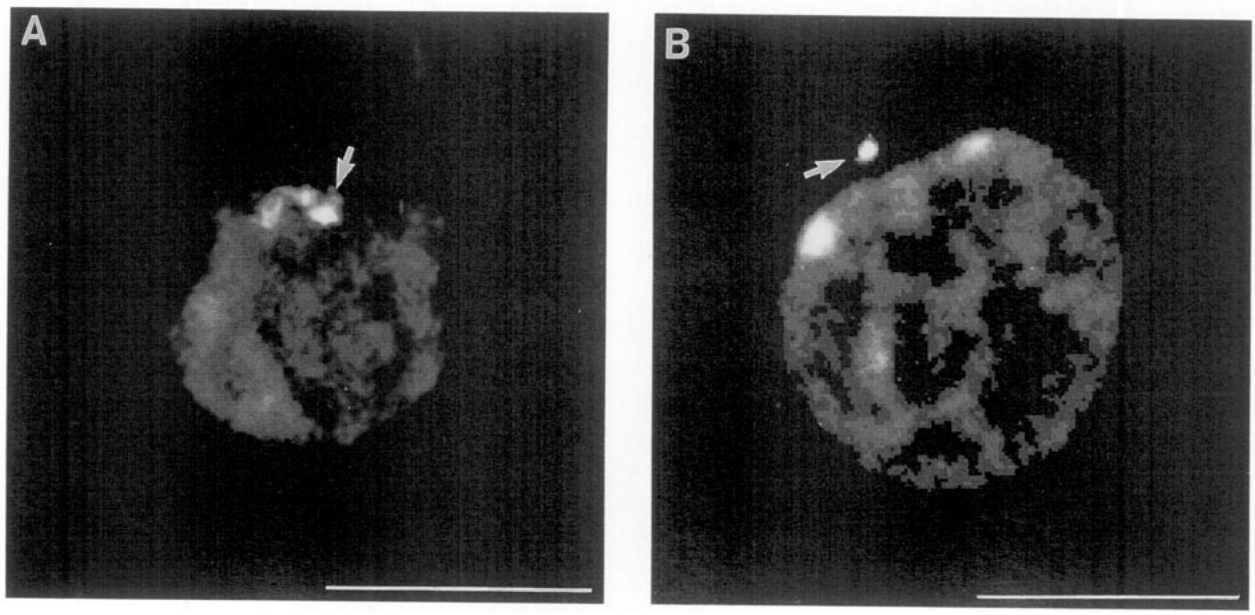

FIG. 3. Confocal microscopy *XY*-optical sections of a (A) resting and an (B) activated (G_1 stage) human lymphocyte. The centrosome (arrows) is located adjacent to a nuclear indentation in both stages, yet at a greater distance from the nuclear center in G_1 stage lymphocytes. Optical sections were recorded in two-channel detection mode. Both figures are a merged section of the FITC (centrosome) and the PI (nucleus) detection channels. Bars, 5 μm.

Generally, the focus of the microtubule array was taken as the position of the centrosome. Using fluorescence microscopy, the focus was seen as a bright fluorescence spot. However, in some cells (19–29%) the centrosome was still obscured by cytoplasmic microtubuli despite the cold treatment that is known to depolymerize labile microtubuli [14]. These cells could consequently not be used for the spatial analysis of the centrosome position.

The Perinuclear Position of the Centrosome in Resting and in Cycling Cells

The position of the centrosome was analyzed by determining its distance toward the nuclear center. In most resting cells ($n = 86$) this distance was less than 1.0 length units (mean distance 0.8), indicating that the centrosome was located within an indentation of the nucleus (Figs. 1, 2A, and 3A).

Analysis of G_1 cells ($n = 86$), however, revealed a mean distance of 1.0 length units (Fig. 1). This suggested that the centrosome was located at about the same distance from the nuclear center as the nuclear membrane would be. Yet, as could be deduced from the PI staining pattern, the nuclear indentation was still present in the region of the centrosome (Figs. 2B and 3B). A significant difference between the relative position of the centrosome in resting and in activated cells was found by comparison the distributions of the measured distances ($\delta_{max} = 0.425$; $c = 0.207$). These findings indicate that the centrosome moves out of a nuclear indentation upon entering G_1 stage (Fig. 4).

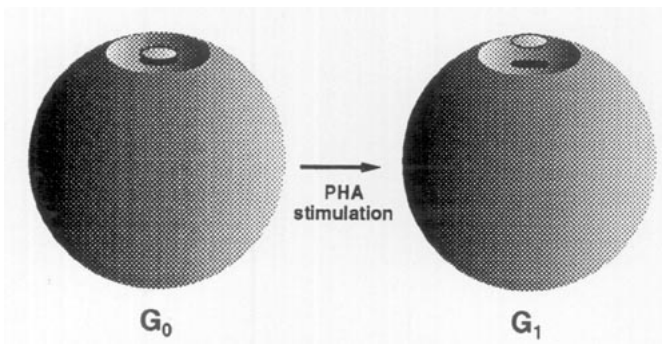

FIG. 4. Schematic representation of the results, indicating a relocation of the centrosome in human lymphocytes upon entering G_1 stage after stimulation by phytohemagglutinin (PHA).

DISCUSSION

3D analysis of resting lymphocytes revealed that the centrosome was located at a mean distance of 0.8 length units from the nuclear center. Because the boundary of the nucleus was defined at a distance of 1.0 length units from the nuclear center, we concluded that the centrosome was located at the basis of a nuclear indentation. This confirms earlier electron microscopy observations by Schweitzer and Brown [7]. A more extreme form of the nuclear indentation has been mentioned by Wilson [15] in pharyngeal epithelium of tunicate *Salpa*. In these

cells the centrosome was described to lie within a "nuclear bay." The relevance of this particular position is unclear, but might simply be a matter of available cytoplasmatic space. The small cytoplasmic volume in lymphocytes might force the centrosome to have a position within an indentation of the nucleus. One might also speculate that the nuclear indentation has been formed during the formation of the nuclear membrane. In telophase, the centromeres are located close to the centrosome, leaving a small open space between the chromatin and the centrosome. Next, the nuclear membrane is formed between the chromatin and the centrosome. The configuration of the chromosomes in telophase may thus cause the formation of an indentation in the nuclear membrane.

The mean distance to the nuclear center found in G_1 cells is in agreement with the generally accepted perinuclear position of the centrosome. Nevertheless, when compared to its position in resting cells, obviously, a relocation of the centrosome to a position away from the nuclear center has occurred upon activation. A displacement of the centrosome has also been reported by Brown *et al.* [16]. In this study, mouse splenic lymphocytes were treated with taxol, resulting in a displacement of the centrosome toward the plasma membrane. This taxol-induced displacement was found in both activated and resting cells.

Data from both the nonstimualted and the PHA stimulated sample showed centrosome positions at the basis as well as at the top of the nuclear indentation. This phenomenon may indicate that the nonstimulated sample contained some cells in G_1 stage. Furthermore, PHA stimulation affects only a certain percentage of lymphocytes, depending on the amount of PHA and the sensitivity of the donor's cells. The stimulated sample, therefore, contained also cells in G_0 stage which might explain why some data from the activated sample show a centrosome position within the nuclear indentation. However, the difference in the mean values between the two samples clearly indicates a change in position upon activation of the cells.

The change in position of the centrosome after stimulation may be necessary to enable duplication of the centrosome. Electron microscopy studies on serial sections have shown that the centrosome increases in size upon cell activation [7]. Duplication of this large centrosome may be impossible within the relatively small nuclear indentation, thus forcing the centrosome out of the indentation. Furthermore, the nuclear indentation could indicate a reference point in the movement of the centrosomes after duplication. In the mitotic spindle the centrosomes have opposite positions. In order to reach this state the centrosomes can migrate in two possible ways, as is shown in Fig. 5. In Fig. 5A, both centrosomes migrate relative to the perinuclear position, resulting in a change of cell polarity of 90°. In Fig. 5B, only one centrosome migrates relative to its perinuclear position. The nuclear indentation may function as a reference point for the centrosome to stop its migration at the point at which it reaches the opposite position. The idea that the centrosome is always positioned adjacent to a nuclear indentation favors the mechanism in which only one centrosome migrates while the other stays at the indentation. In addition, Omura and Fukui [17] reported that in Dictyostelium the centrosome is linked to the nucleus, supporting the idea of a fixed perinuclear position of the centrosome.

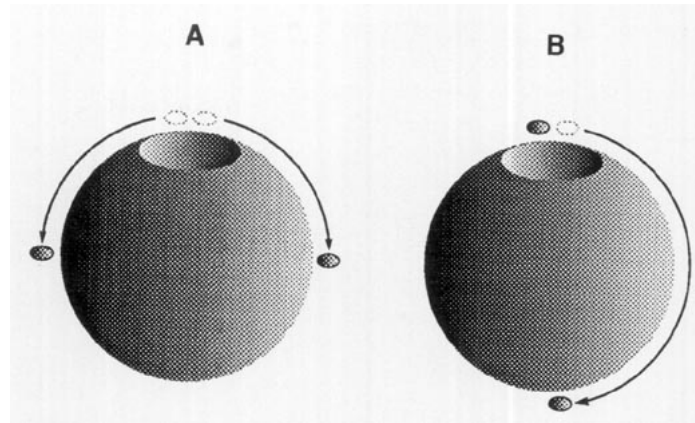

FIG. 5. Models for migration of the centrosome in late G_2/early prophase. (A) Both centrosomes migrate relative to the nuclear indentation; (B) one centrosome migrates while the other stays at the indentation.

Our findings are in agreement with the general idea that the centrosome plays a central role in the mechanical and topological organization of the cell. Moreover, the position of the centrosome appeared to be related to a nuclear indentation during early interphase. The morphological and functional changes that appear in the cell upon activation are preceded by a relocation of the centrosome, indicating a function-dependent position of the centrosome.

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