Scanning Microscopy

Volume 6 | Number 1 Article 24

1-22-1992

Structural Artifacts and Advantages of Cytocentrifugation of Cells as Viewed by Scanning Electron Microscopy

Mário N. D. Peão
University of Porto

Artur P. Águas
University of Porto

Carlos Moreira de Sá
University of Porto

Nuno R. Grande
University of Porto

Scanning Microscopy, Vol. 6, No. 1, 1992 (Pages 281-285)
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

0891-7035/92$5.00+.00

STRUCTURAL ARTIFACTS AND ADVANTAGES OF CYTOCENTRIFUGATION OF CELLS AS VIEWED BY SCANNING ELECTRON MICROSCOPY

Mário N. D. Peão[1,2], Artur P. Águas[2,3], Carlos Moreira de Sá[3,4] and Nuno R. Grande[2,*]

Depart. of Anatomy, Abel Salazar Institute for the Biomedical Sciences, University of Porto, 4000 Porto, Portugal[1], Depart. of Morphological Sciences of the Medicine Faculty, University Agostinho Neto, Luanda, Angola,[2] Center for Experimental Cytology, University of Porto, 4100 Porto,[3] and Metallurgic and Material Sciences Center of the Engineering Faculty, University of Porto, 4100 Porto, Portugal[4].

(Received for publication October 13, 1991, and in revised form January 22, 1992)

Abstract

Cytocentrifugation of cell suspensions onto glass slides is a widely used procedure in contemporary cytology. We employed here scanning electron microscopy (SEM) to investigate putative morphological changes induced in cells submitted to cytocentrifugation. The fine structure of murine pleural exudate cells (macrophages mainly) processed by spinning was compared with that of similar cells treated without centrifugation (poly-L-lysine attachment of the cells to glass slides at 1 g). Cells of cytocentrifuged preparations showed a significant increase in diameter and smoothening of the cell surface as compared with the morphology of non-centrifuged cells. Cytocentrifugation also induced the formation of thin elongations coming out of the cellular outlines. The centrifugation-induced flattening of the pleural macrophages improved the detection of large intracellular inclusions (containing tungsten particles): these bodies were readily identified by secondary-electron imaging mode of SEM in cytospinned cells whereas their detection in non-centrifuged spherical cells required the use of the backscattered-electron imaging mode of SEM. We conclude that the cytocentrifugation methodology, on one hand, requires caution on the interpretation of the microanatomy of the cells and, on the other hand, the procedure may be an adequate method to improve the identification of large intracellular inclusions by routine (secondary-electron imaging mode) SEM.

Key Words: pleural cells, tungsten, lung, macrophages, cytocentrifugation, scanning electron microscopy.

***Address for correspondence**:
Nuno R. Grande, Department of Anatomy, Abel Salazar Institute for the Biomedical Sciences, Largo Prof. Abel Salazar, 4000 Porto, Portugal.
Phone No. (3512) 310359

Introduction

Cytocentrifugation of cell suspensions is a commonly used method to process samples for cytological screening by light microscopy[5]. This procedure involves the spinning down of cells onto the surface of glass slides, followed by fixation and staining. We have used here scanning electron microscopy (SEM) to investigate the fine morphology of cells submitted to this procedure. For that, we have harvested cellular exudates from the pleural spaces of mice that were previously injected with microparticles of calcium tungstate[1,2]. The cell suspensions were processed either by the routine cytocentrifuge procedure or, instead, attached to glass slides without cytocentrifugation (i. e., poly-L-lysine "glueing")[4]. We found that the pleural cells submitted to cytocentrifugation showed up with increased diameters and also with changes of their surface microanatomy. These artifactual modifications should be taken into consideration whenever cytological interpretations of cytospin preparations are made. In addition, we observed that the flattening of the cells improved the identification of intracellular tungsten inclusions by SEM.

Material and Methods

Animals.

Female CD-1 mice, 6-8 weeks old, weighing about 20g were obtained from the animal house of the Gulbenkian Institute of Science (Oeiras, Portugal). The animals were housed in groups of 15 and given balanced food and water *ad libitum* .

Experimental Protocol.

The animals were anesthetized by intravenous injection of ketamin (Ketalar®, Parke-Davis Co., Barcelona, Spain; 4.0 to 8.0 mg/kg of weight) and xilazine (Rompum®, Bayer Co., Amadora, Portugal; 0.8 to 1.6 mg/kg of weight). The right side of the thorax of the mice was shaved and the skin cut in the mid-axillar line. One hundred µl of a suspension of calcium tungstate particles ($CaWO_4$; Aldrich

Chemical Company, Inc., Milwaukee, WI, USA) in saline, at a concentration of 2.5mg/ml, were injected in the pleural space through the right eighth intercostal space. The animals were killed by a lethal dose of ethyl ether at 48h after the intrapleural injection. The pleural exudates were collected by lavage of the pleural space with 2.5 ml of phosphate buffered saline (PBS), pH 7.4, containing 5% sucrose.

Light Microscopy.

The total number of cells in the pleural exudates was calculated with an automatic cell counter (Sysmex Microcellcounter CC-110, Toa Medical Electronics Co., Japan). Cytocentrifuge preparations were made using a Cytospin apparatus (Shandon Southern Products Ltd, Cheshire, England). The cell suspensions were centrifuged at 90 g for 5 min onto glass slides. This protocol is routinely used in cytology[3,6]. In order to attach cells to the glass slides without centrifugation we adopted the widely used procedure of precoating slides with 1% poly-L-lysine[4,7]. One drop of the cell suspensions was placed on the precoated slides and incubated at 37 ºC for 60 min in a humidified atmosphere. This was followed by fixation in an aldehyde mixture made up of of 4% formaldehyde, 1.25% glutaraldehyde, and 10 mM $CaCl_2$ in 0.05 M cacodylate buffer[8]. Some of the cell suspensions were fixed with the aldehyde mixture immediately after harvesting, and the fixed cells then submitted to cytocentrifugation onto glass slides.

Scanning Electron Microscopy.

Cytocentrifuge preparations of pleural cells and similar samples handled without centrifugation were both processed for SEM. The fixation of the cells was done with the same aldehyde mixture cited in the previous paragraph. All samples were dehydrated in ethanols and critical-point-dried in a Balzers apparatus using carbon dioxide as the transitional fluid. The preparations were coated by Au/Pt under vacuum and examined in a JEOL JSM-35C scanning electron microscope. The electron micrographs were derived either from secondary or backscattered imaging modes of SEM, the later mode being coupled to the detection of tungsten *in situ* by elemental particle analysis of the cell preparations.

The instrumental conditions for all the observations, SEI and BEI, are: 25kV, 0.2nA, 30s/frame (photography). In the text secondary electron imaging is used for the observations with Everhart-Thornley detector and backscattered electron imaging refers to observations using an annular semiconductor detector. The use of a 25 kV beam at high currents can damage the surface of the cells, however this was not observed at the low magnification used for observation, and this high voltage was required to allow the observation and analysis of the heavy particles of $CaWO_4$ deep inside the cells.

Figures 1 and 2. General SEM views of pleural macrophages of mice processed without centrifugation (Fig. 1; poly-L-lysine attachment to glass slides) and by cytocentrifugation onto glass slides (Fig. 2). The pleural exudates were collected 48 hours after injection of calcium tungstate into the pleural space. Cytocentrifugation resulted in flattening of the phagocytes with increase in cell diameter and induction of elongations on the cellular outlines. The SEM secondary imaging mode used here was enough to reveal the presence of intracellular tungsten inclusions (white spots, arrowheads) in the cytocentrifuged cells (Fig. 2) but failed to do so in the non-centrifuged, spherical cells (Fig. 1).

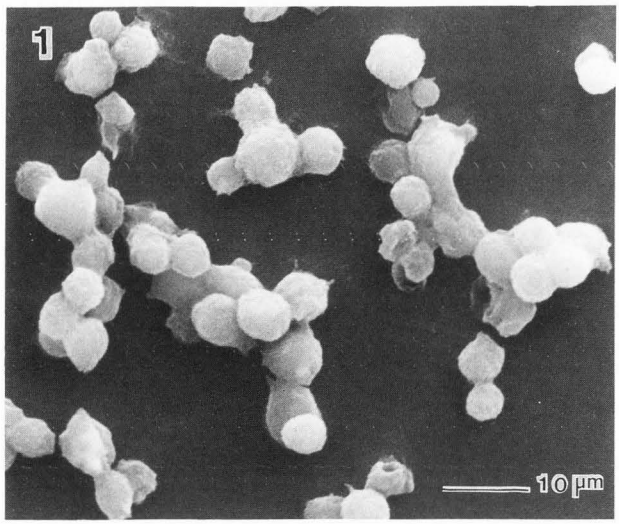
Figure 3. SEM view of murine pleural macrophages that were fixed in an aldehyde mixture before cytocentrifugation onto glass slides. Prefixation of the cells prevented the flattening induced by the cytocentrifugation procedure (compare with Fig. 2) and, therefore, resulted in a round-shaped appearance of the cells which is comparable with the morphology of pleural cells processed without centrifugation (as shown in Fig. 1).

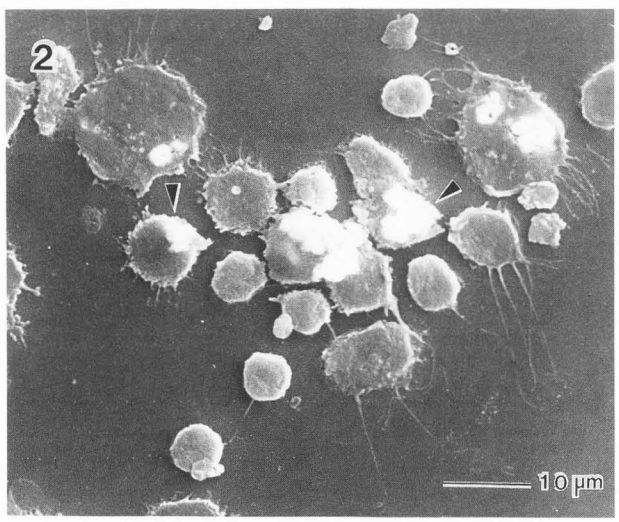
Figures 4a and 4b. High magnification SEM views of inflammatory pleural macrophages 48 hours after the injection of calcium tungstate in the pleural cavity of mice. The cell suspensions were processed without centrifugation (poly-L-lysine attachment to glass slides). 3a - SEM secondary electron imaging mode. 3b - SEM backscattering imaging mode. The latter SEM mode is required for the detection of intracellular tungsten inclusions in these cells.

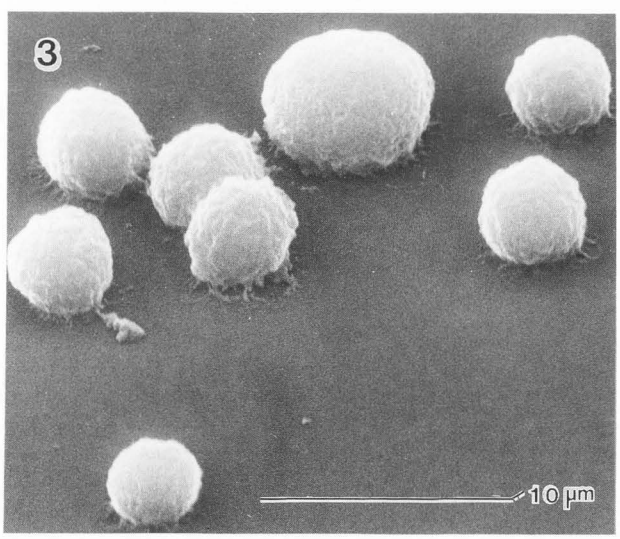
Figure 5. High magnification SEM view of the structural changes on the cell surface of murine pleural macrophages provoked by centrifugation of the cells onto glass slides (secondary electron imaging mode). The herein illustrated flattening of the cells resulted in excellent visualization of intracellular inclusion of tungsten (white spots) not requiring the backscattering SEM mode. Thin elongations coming out of the cellular outlines are also observed (arrowheads).

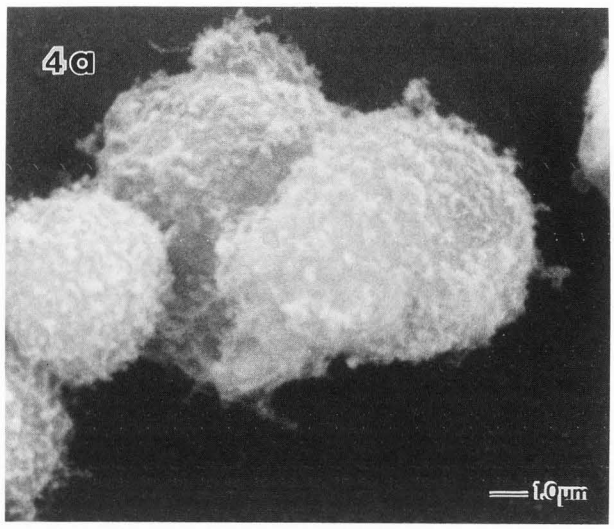
Results

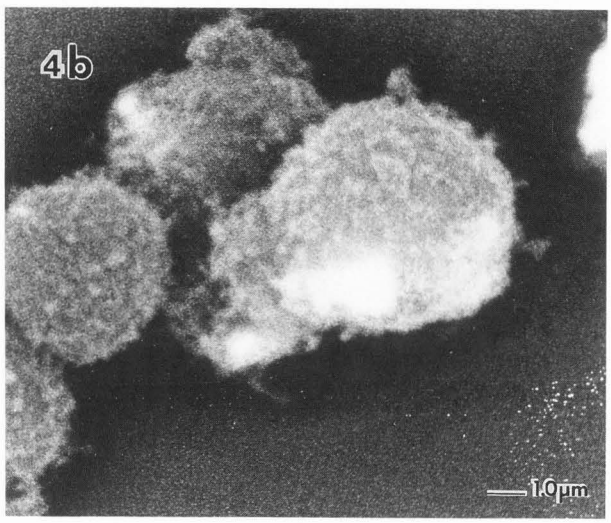
Resident and inflammatory cells of the pleural space of CD-1 mice were mostly made up of macrophages that were spherical in shape (Fig. 1). This illustrates the SEM morphology of cells processed without centrifugation. Comparison of the

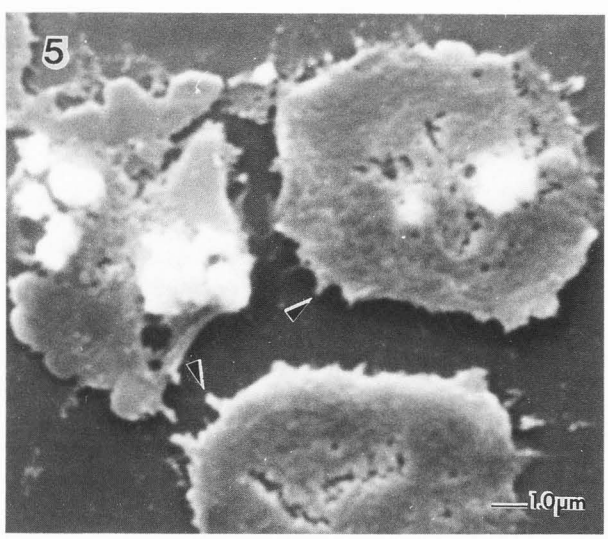

1
10 µm


2
10 µm


3
10 µm


4a
1.0µm


4b
1.0µm


5
1.0µm

SEM structure of similar samples submitted to the cytocentrifuge method showed that the procedure resulted in flattening of the cells (Fig. 2). That the flattening of the cells was due to the cytocentrifugation step was confirmed by the finding that they kept their original spherical shape on the glass slides when they were prefixed with aldehydes before cytospinning (Fig. 3). Cytocentrifugation also resulted in the smoothening of the surface of the cells. In addition, the cellular outlines were often decorated by thin elongations of the cytoplasm. Ingested particles of tungsten were readily visible by SEM inside the flattened cytocentrifuged cells by the routine, secondary electron mode of SEM.

In the round-shaped, non-centrifuged cells the intracellular tungsten inclusions could not be detected by the routine, secondary electron mode of SEM. The identification of tungsten bodies in these cells required the use of the backscattered mode of SEM (compare Fig. 4a, secondary electron mode, with Fig. 4b, backscattered mode of the same cells). Higher magnifications of the two types of preparations (centrifuged and non-centrifuged) documented with detail the two major structural changes, general smoothening and elongations at the cellular outline, that the cell surfaces underwent as a result of the centrifugation procedure (compare Fig. 4 with Fig. 5).

Discussion

We have investigated here the structural alterations suffered by cell suspensions submitted to a routine protocol to obtain cytocentrifuge preparations. Our goal was to identify putative artifacts produced by this procedure, a technical question of some importance because cytocentrifuge preparations are widely used today in diagnostic and research cytology[3,6]. We used cell suspensions of murine pleural exudates that are made up mostly of round-shaped macrophages. We compared the SEM morphology of pleural exudate cells processed by cytocentrifugation or attached to glass slides without centrifugation (poly-L-lysine "glueing")[4,7]. We found that the morphology of the pleural cells was drastically modified by the cytocentrifuge method. The major cellular changes were on cell diameter, smoothening of the cell surface, and presence of elongations on the cell outline. The diameter of cells centrifuged onto one glass slides was significantly increased. This increase was in between 1.4 and 2.3 times the original diameter of cells. These values of enhancement of cell diameter are in accordance with the mathematical calculation of the area that results from the flattening of a sphere (see appendix).

We also found that the flattening of the centrifuged cells was advantageous since the tungsten particles became readily detectable by routine SEM (secondary electron imaging mode) in cytocentrifuged cells which was not the case for the majority of tungsten bodies in non-centrifuged, round-shaped cells. In the latter samples, the use of the backscattered electron mode (coupled to X-ray microanalysis) was required to visualize all of the intracellular tungsten bodies. This means that the heavy particles can be seen with a low energy electron beam in flattened cells and that the observation of the same particles deep inside well preserved (non-flattened) cells, requires the use of an higher energy beam and BE imaging, to get Z contrast information from greater dept, as can be seen in Fig. 4. Even in this case small particles may prove hard to see. The difference indicated that the visualization of large intracellular inclusions by routine SEM may be improved if cells are previously flattened by centrifugation.

Centrifugation-induced flattening of cells was associated with an enhanced smoothening of their surface. The finding illustrates the plasticity of the cell surface of pleural macrophages. Taken together, our findings suggest that: (a) caution must be observed in the interpretation of the morphology of cells submitted to cytocentrifugation because of the several important artifacts that may be produced by the methodology; and (b) flattening of cells may be advantageous for the identification of large intracellular inclusions by SEM.

Appendix

Mathematical calculation of the radius of a flattened sphere.

I. Deduced from the area of a sphere.

S = surface R = radius

$S = 4 \pi R_o^2$; 2 faces = $2x\pi R1^2$;

$4\pi R_o^2 = 2\pi R_1^2$;

$\sqrt{2}R_o = R_1$;

$\underline{1,40R_o = R_1}$ (Flattening results in 1.4 x increase of radius)

or

II. Deduced from the volume of a sphere.

V = volume R = radius

$V = 4/3 \pi R_o^3$;

$V_o = \pi R_1^2 \times 1/10R_1$;

$4/3\pi R_o^3 = \pi/10R_1^3$;

$40/3R_o^3 = R_1^3$;

$\sqrt[3]{40/3}R_o = R_1$;

$\underline{2,37R_o = R_1}$ (Flattening results in 2.37 x increase of radius).

Acknowledgments

We are grateful to Prof. Manuel T. Silva for support and suggestions throughout the course of this study. We thank the excellent technical help of Mr. Emanuel Carvalho, Mr. António Costa e Silva and the photographic assistance of Mr. José Aurélio Mexedo. This work was supported by grants from the

JNICT (Portuguese Research Council). Mário Peão is a M.D. working at the University of Luanda and also a Ph.D. student at the University of Porto.

References

1. Águas AP, Grande NR, Carvalho E (1991) Inflammatory macrophages in the dog contain high amounts of intravesicular ferritin and are associated with pouches of connective tissue fibers. Am J Anat **190**: 89-96

2. Grande NR, Sá CM, Águas AP, Carvalho E, Soares M (1990) Time course and distribution of tungsten-laden macrophages in the hilar limph nodes of the dog lung after experimantal instillation of calcium tungstate into the left apical bronchus. Lymphology **23**: 171-182.

3. Hopper KE (1986) Kinetics of macrophage recruitment and turnover in peritoneal inflammatory exudates induced by salmonella or thioglycollate broth. J Leucocyte Biol **39**: 435-446.

4. Jacobson BS, Branton D (1977) Plasma membrane: Rapid isolation and exposure of the cytoplasmic surface by use of positively charged beads. Science **195**: 302-304.

5. Johnston WW (1985) Perspectives in cytological diagnosis. In: The Pleura in Health and Disease (Chretien J, Bignon J, Hirsch A, eds.) Marcel Dekker, New York, 761-785.

6. Meltzer MS (1981) Use of Wright's stain and cytocentrifuge preparations. In: Methods For Studying Mononuclear Phagocytes (Adams DO, Edelson PJ, Koren HS, eds.) Academic Press, New York, 345-347.

7. Murphy JA, (1982) Considerations, materials, and procedures for specimen mounting prior to scanning electron microscopic examination. Scanning Electron Microsc II: 657-696.

8. Silva MT, Appelberg R, Silva MNT, Macedo PM (1987) In vivo killing and degradation of *Mycobacterium aurum* within mouse peritoneal macrophages. Infect Immun **55**: 2006-2016.

Discussion with Reviewers

W.C. de Bruijn: The proposed technical aspect is not an improvement with respect to cell preservation. Flattening of these cells might also be acquired on cultivating the cells for 24 hrs.

Authors: We agree with the reviewer in that we are not reporting an improvement in cell preservation and in that flattening of the cells could be obtained by methods other than cytocentrifugation, e. g., by 24 hrs cell culture as suggested by the reviewer. The main goal of our paper was not to introduce a new treatment to enhance cell preservation but rather to show that a 5 min procedure (cytocentrifugation) makes possible, due to cell flattening, the identification of large intracellular particles by routine SEM which would not be detectable in non-flattened cells, unless a higher energy electron beam is used (backscattered electron imaging). We believe that this easy and fast procedure is advantageous over cell culture both in financial and time-consuming requirements.

T. D. Allen: The 'flattening' that the authors infer is due to 90g for 5 mins in a cytocentrifuge is, in fact, due to air-drying during centrifugation. It is the passage of the air-liquid interface that has 1. 'spread' the cells, and 2. flattened their surface morphology. Furthermore, the non-centrifuged cells fixed as a living cell suspension by poly-l-lysine, although they appear not to have done so - was there really any poly-l-lysine there - have they tried with clean glass alone? The whole idea of poly-l-lysine collection is that the cells should be fixed in suspension before harvesting onto a poly-l-lysine coated surface.

Authors: We believe that the cell flattening can not be attributed solely to air-drying: the cytocentrifuge procedure that we used here does not involve complete drying out of the cell samples. Instead the cells are still immersed in a small volume of buffer when they are recovered on the glass slides. They were then immediately fixed with aldehyde (that is, before complete drying of the sample has occurred). Thus, we see the flattening effect as derived primarily from physical alteration of the cells because of their spinning against the flat, hard surface of the glass. We show that this flattening effect is prevented if the cells are fixed before centrifugation. As the reviewer states, we did not found important ultrastructural changes on pleural cells in samples that were attached to poly-l-lysine coated glass slides. Thus, the use of poly-l-lysine coated glass slides appeared to enhance (when compared with uncoated glass slides) the attachment to the glass surface of cells in suspension without causing important morphological modifications on them.