The fate of the centrosome–microtubule network in monocyte-derived giant cells

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Summary

Avian monocyte-derived giant cells in vitro, which are in many respects similar to osteoclasts, display a complex microtubule array that plays a prominent role in cell spreading. It is organized by a polygonal row of regularly spaced centrosomes surrounding an irregular cluster of nuclei. The immediate progenitor cells are binucleate cells with a single microtubule-organising center (MTOC), the result of the congregation of the two individual centrosomes. The one-to-one correspondence between numbers of centrosomes and nuclei in giant cells suggests that the centrosome of each precursor cell has been conserved through the fusion process. This is in marked contrast to the absence of centrosomes in myotubes, another example of a differentiated cell derived from the fusion of progenitor cells.

Key words: osteoclasts, centrosomes, microtubules, cell spreading.

Introduction

In most animal cells, the microtubular network is typically organized about a juxtanuclear organizing center, the centrosome. This organization has long been seen as potentially important for the definition of cell polarity (reviewed by Bornens and Karsenti, 1984; Kirschner and Mitchison, 1986) and for cell locomotion (Albrecht-Buehler and Bushnell, 1979). In support of this view, young myotubes, which neither migrate nor show antero–posterior polarity, have been shown to completely reorganize their microtubular network at the stage of fusion of competent myoblasts, involving the disappearance of centrosomes (Tassin et al. 1985a). Artificially induced syncytia possess a quite different organization, often similar to that of the precursor mononucleated cells, i.e. the individual centrosomes aggregate into a common centrosphere cluster (Wang et al. 1979; Tassin et al. 1985a). The locomotory activity of such syncytia is difficult to assess although it has been attempted recently by Lewis and Albrecht-Buehler (1987), who reported evidence of an involvement of centrosomes in the determination of the syncytium polarity associated with efficient locomotion.

Looking for a physiological system where mononucleated precursor cells would fuse into a differentiated syncytium as in myogenesis, but in which the end product, unlike myotubes, would retain active locomotory activity, we turned to monocyte-derived giant cells, which are in many respects similar to osteoclasts (Testa et al. 1981; Ibbotson et al. 1984; Zambonin-Zallone et al. 1984). We report here that these cells reorganize their centrosome-microtubular network very differently from the way myotubes do: not only do they maintain individual centrosomes but these latter display a remarkably geometric organization, which permits the establishment of a highly complex microtubular network. Comparison of these two systems could be of value in studying the specific role of centrosomes in the properties of the microtubular network.

Materials and methods

Cells

Tibias and femurs from 18-day-old chick embryos were dissected in sterile conditions. Bone marrows were flushed either in plastic culture flasks or in culture dishes on glass coverslides. Marrows were cultured in RPMI culture medium (Eurobio, Paris, France), supplemented with fetal calf serum (20%, v/v) at 37°C in an air/CO2 incubator. It is essential to preserve the bone marrow plugs from mechanical dissociation, otherwise the giant mature osteoclasts are damaged. Osteoclasts and monocytes attached to the culture surface after 24 h of incubation. Mature osteoclasts needed 3–4 days to achieve a complete spreading, depending on their size.

Generation of chicken osteoclasts in culture by fusion of monocyte precursors was carried out using a modification of the technique reported by Testa et al. (1981). The presence of
fibroblastic stromal cells in the culture was essential for the proliferation of monocytic precursors and for cell fusion; this supports the contention that in birds, as in mammals, fibroblastic stromal cells produce CSF-1, the growth factor required for monocyte progenitor proliferation (Lanotte et al. 1982) and monocytic cell fusion (McDonald et al. 1986). The extensive growth of fibroblasts impaired the observation of osteoclasts, as the latter cell type grew firmly attached to the substratum and was often covered by the fibroblastic layer. The use of high concentrations of serum (5% horse serum plus 20% fetal calf serum) frequently induced, after three weeks of culture, a spontaneous detachment of the fibroblastic monolayer, thus leaving a homogeneous population of adherent osteoclasts. Alternatively, batches of horse serum selected for their limited ability to support fibroblastic cell growth were used.

In some experiments cells were treated with $10^{-6}$M-nocodazole (from Janssen, Beerse, Belgium) or $1 \mu g/ml$ cytochalasin D (from Sigma Chemical Co.; St Louis, MO) for 1h before fixation. In others, culture dishes were placed on ice for 1h before cell fixation.

**Immunofluorescence**

Cells were fixed with dry methanol at $-20°C$ for 6min after extraction with 0.1% Triton for 30s at room temperature in PHEM buffer, pH6.9 (45mM-Pipes, 45mM-Hepes, 10mM-EGTA, 5mM-MgCl$_2$, 1mM-phenylmethylsulfonyl fluoride; all chemicals from Sigma).

Centrosomes were specifically labeled in chicken cells with the human serum 5051 (Tuffanelli et al. 1982). Immunocytochemical labeling of microtubules was accomplished using mouse monoclonal anti-$\alpha$- or anti-$\beta$-tubulin antibodies (Amerham France SA, Les Ulis).

The second antibodies were goat anti-mouse or anti-human immunoglobulins labeled with fluorescein isothiocyanate or rhodamine (Cappel laboratories, Cochranville, PA), or with Texas Red (Amersham). The antibodies were diluted in PBS containing 0.1% Tween 20 and 3% bovine serum albumin. All washing steps were performed in PBS–Tween.

Double immunofluorescence was achieved by mixing, respectively, the two first antibodies and the two second antibodies. Each incubation took 30min at room temperature.

**Results**

**Microtubules in giant cells are organized by a row of regularly spaced centrosomes**

In a bone marrow culture from 18-day-old chick embryos, the formation and maintenance of osteoclast-like cells during several weeks can be observed by phase-contrast microscopy (Fig. 1). They appear as very large cells, up to 1 mm in diameter, with nuclei often clustered in one central area from which phase-dark tracks appear to radiate (Fig. 1A). May Grunwald–Giemsa staining demonstrated a dense area in the vicinity of the nuclei, whereas the cell periphery was apparently unstained and probably very thin (Fig. 1B).

Giant cells displayed a complex microtubular network characterized by a considerable number of microtubules apparently growing from centers regularly spaced along a row concentric with respect to the cluster of nuclei (Fig. 2). Each center contained a tiny dot in the corresponding picture (observed with the excitation wavelength adapted to the anti-centrosome serum). There was no exception to this rule. The size of the dots could, however, vary: in favorable cases, it was possible to identify centers with two dots, whereas others contained only one dot (see Fig. 3C). This suggested that the dots were centrosomes and that centrioles in a centrosome could occasionally be separated.

The distal ends of individual microtubules could not be localized. Each aster growing from an individual center was apparently mingled with the neighboring asters in a complex manner.

The possibility that the localization of centrosomes between the nuclear area and the cell periphery was correlated with the presence of paramarginal rows of
Fig. 2. Centrosome-microtubular organization in osteoclasts observed by double immunofluorescence with a mouse monoclonal anti-β tubulin (A,C) and the human anti-centrosome serum 5051 (B,D). Note the regular distribution of centrosomes, most often as a single row (A,B), sometimes in a more complex array (C,D). Note the unequivocal correspondence between microtubule asters and centrosomes. Bars, 20 μm.
adhesion dots is suggested by pictures such as those presented in Fig. 3A-F. Adhesion dots were not observed when the centrosomes were near the nuclear area. Images of close apposition of microtubules to the plasma membrane at the internal and external sides of the paramarginal zone of adhesion dots were very frequent (Fig. 3G-H).

Progenitor cells are binucleate and display a single microtubule-organizing center (MTOC)

The possibility that the precursors of giant cells are mono- or binucleate cells has been substantiated (Testa et al. 1981). Giant cells may be formed by continuous fusion with the immediate precursors. Alternatively, multinucleate cells may fuse, as suggested by the presence of more than one cluster of nuclei in some giant cells. We have observed both pathways, without being able to estimate precisely their relative frequency, and have documented the organization of the microtubular network in the various partners.

Most of the immediate precursor cells were binucleate (Fig. 4). As a rule, these cells possessed only one MTOC, close to and equidistant from both nuclei. This MTOC was probably the result of the congregation of the two centrosomes of mononucleate cells as strongly suggested by the comparative size of the centrosome-specific staining in mono- and binucleate cells (see Fig. 4E) and by that of the asters of microtubules in both cases (Fig. 4B). We could not determine whether binucleate cells were the product of fusion between mononucleate cells or of a mitosis without cytokinesis.

Fusions between binucleate cells were often observed, as judged by the mixing of the two microtubular networks and the disappearance of phase-contrasting cellular limits (Fig. 4A,D,G). Occasionally, two fusing binucleate cells were observed one of which was also fusing with a large multinucleate cell (Fig. 4B,E,H). Binucleate cells also fused with giant cells from above (Fig. 4C,F,I).

Fusion between multinucleate cells could be observed only for cells with a moderate number of nuclei. An example is shown in Fig. 5. In this case centrosomes were close to the nuclei, as in binucleate cells, and displayed an almost regular polygonal distribution.

The spreading of giant cells is microtubule-dependent

In an attempt to gain information on the mechanisms sustaining the regular distribution of centrosomes in giant cells, we investigated the stability of microtubules after cold or nocodazole treatment. Unexpectedly, microtubules from giant cells were specifically cold-resistant (not shown), whereas they were virtually all disrupted after 1h of treatment with $10^{-6}$ M-nocodazole (Fig. 6A-F). More surprisingly, depolymerisation of microtubules induced the retraction of giant cells,
Fig. 4. Immediate progenitor cells of osteoclasts are binucleate cells with one MTOC. A,B,C, anti-tubulin; D,E,F, 5051 serum; G,H,I, phase-contrast. Fusion between cells was diagnosed by the mixing of microtubular networks from neighboring cells (zone between convergent arrows in A,B) and by the disappearance of phase-contrast limits of cells in contact (zone between arrows in G,H; see also I). Fusions of binucleate cells with other binucleate cells (A,D,G) and with giant cells (B,E,H) were observed. Note that the two types of fusion can occur simultaneously (B,E,H). Note also that the MTOC of binucleate cells (circled in B,D,E,G,H) appears as the congregation of two centrosomes compared to mononucleate cells (indicated by squares in E) as judged by 5051 serum staining and by the size of microtubule asters. Fusion of binucleate cells with a giant cell can take place on the top of the latter, but always close to an area containing the row of centrosomes (small circles in C,F,I); the large circle indicates the unique MTOC of the precursor cell in this case. The precursor cell is apparently fusing along a line on the top of the row of centrosomes from the giant cell, as indicated by the interruption of the phase-contrast outline of the precursor cell in I. Bars, 20 μm.
suggesting that the thin and extended periphery of these cells was solely due to microtubules. Centrosomes were then all located in the thick cytoplasm together with the nuclei, and without any apparent order. Most of them nucleated very short microtubules (Fig. 6A,D). Treatment with 1 μg ml⁻¹ cytochalasin D, however, also induced cell retraction but in a less dramatic way, as if microtubules were preventing cell collapse (Fig. 6G–H).

**Discussion**

Bone marrow cultures have been shown in several species to permit the formation and maintenance of cells with the characteristics of osteoclasts (Testa et al. 1981; Ibbotson et al. 1984; McDonald et al. 1986). These differentiated cells are derived from monocytic progenitor cells. Looking at the fate of the microtubular network, we show in the present work that centrosomes are maintained in the osteoclasts and that they are not in the vicinity of the nuclei, which are clustered within an area where most of the cytoplasm is apparently confined. They are regularly distributed in a row within the huge and very thin peripheral cytoplasm of fully differentiated osteoclasts, strongly suggesting that this distribution of centrosomes determines a network of microtubules that is instrumental in the establishment of such a peculiar shape. We further observed that the regular polygonal distribution of centrosomes was apparently microtubule-dependent: upon microtubule depolymerization, all centrosomes were relocated in the vicinity of the nuclei. However, the whole cellular periphery was also pulled back into the perinuclear cytoplasm in the absence of microtubules. This was an unexpected result, as cell spreading in many cells is primarily actin-dependent. The reason for this was probably the specific mode of cell–substratum adhesion of osteoclasts, mediated by ventral circular protrusions exclusively distributed in the paramarginal area (Marchisio et al. 1984). Such adhesion is apparently weak enough to be overcome by the retraction induced by microtubule disruption, whereas in other cell types adhesion plaques, which are both larger and spread all over the ventral side, can resist retraction. These particular adhesion structures were also sensitive to the microfilament-disrupting drug cytochalasin D, as expected from the fact that actin filaments abut onto them (Marchisio et al. 1984). However, cell retraction was less dramatic than when microtubules were disrupted, the latter being so abundant that they appear to fill collapsing cells. The general picture is therefore not fundamentally different from what was reported in other cells for the respective roles of actin and tubulin systems (see, for example, Schliwa et al. 1982), except that microtubules are particularly abundant and appear as the main structures involved in flattening the cell periphery.

Although we did not formally demonstrate at the ultrastructural level that individual MTOCs were centrosomes, this was strongly suggested by the size and the paired configuration of the centrosome-specific staining and by the specific decoration of centrioles observed with anti-Glu α-tubulin after blocking microtubules with the anti-Tyr α-tubulin antibodies (not shown; see Gundersen and Bulinski, 1986). The polycentric origin of microtubules, which had already been suspected (Zamponin-Zallone et al. 1983), is therefore due to the presence of the centrosomes of the progenitor cells. The latter conclusion is also supported by the one-to-one correspondence observed between centrosomes and nuclei. Slight variations in this ratio were apparently due to the congregation of two centrosomes, the separation of two...
Fig. 6. The spreading of giant cells is microtubule- and microfilament-dependent. Microtubule disruption by $10^{-6}$ M-nocodazole induces the partial (A–C; arrows point to the cell margin) or total retraction of the thin peripheral cytoplasm (D–F). In both cases, centrosomes are gathered in the vicinity of the nuclei: they nucleate very short microtubules (anti-tubulin in A,D), are labeled with serum 5051 in B,E and are indicated by circled dots in phase-contrast (C,F). Giant cells are also retracted upon treatment with cytochalasin D (G–H). G. Anti-tubulin; H, nuclear staining with the Hoechst dye. Bars, 20 μm.

The striking feature of the arrays of centrosomes in osteoclasts was their highly regular spacing in a polygonal array at some distance between the nuclei and the cell margin. We could not decide if this was achieved by microtubules directly or via the actin system, as the disassembly of microtubules led to cell collapse. Evidence for an involvement of actin in the positioning and motility 

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of centrosomes has been reported in human polymorphonuclear leukocytes (Schliwa and Euteneuer, 1985). We documented the organization of the immediate progenitor cells: as a rule, they were binucleate, with a single MTOC, the result of the aggregation of the two individual centrosomes. This last feature, together with the osteoclast is derived from more precursor cells, suggests a critical role for centrosomes in the internal control of cell organization during and after fusion.

We observed a quite similar organization of the centrosome-microtubular network in human osteoclasts derived from monocytes in cultures from umbilical cord (not shown). The possibility that a similar distribution of MTOCs exists also in a malignant choriocarcinoma cell line has been documented (Ockleford et al. 1984).

The absence of centrosomes in myotubes (Tassin et al. 1985a,b) and the presence of an ordered array of centrosomes in osteoclasts demonstrated in this work represent two extreme types of microtubular organization in differentiated cells derived from the fusion of progenitor cells. Osteoclasts maintain an important locomotory activity (Zambonin-Zallone et al. 1983), and are capable of a polarized activity for bone resorption (Baron et al. 1985). Myotubes do not locomote nor do they manifest evident antero-posterior polarity. A contribution of the centrosome to the definition of cell polarity/motility has often been suggested (for a discussion, see Bornens and Karsenti, 1984; see also Lewis and Albrecht-Buehler, 1987). An attempt to demonstrate it directly in the motile newt leukocyte has produced convincing evidence (Koonce et al. 1984). The study of fish epidermal cells, or cytoplasmic fragments, which show persistent directional motility in the absence of microtubules, had led Euteneuer and Schliwa (1984) to conclude differently. Osteoclasts, with their remarkable arrays of centrosomes, could provide a favorable system for the study of the centrosome in situ, using scanning confocal microscopy and video recording.

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References


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