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Microsurgical Removal of Centrosomes Blocks Cell Reproduction and Centriole Generation in BSC-1 Cells

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Summary

We have removed the centrosome from cultured BSC-1 cells by microsurgery, leaving enough cytoplasm with the nucleated cell fragment (karyoplast) to ensure survival and growth. In each experiment, we followed the fate of the karyoplast as well as the anucleate cell fragment (cytoplast) containing the original pair of centrioles. Experimental karyoplasts reestablish a juxtanuclear microtubule-organizing center, an astral array of microtubules, and a compact Golgi apparatus. They enter and presumably complete S phase, and they grow beyond the size of an average BSC-1 cell. However, they do not regenerate centrioles in time periods equivalent to more than 10 cell cycles and do not undergo cell division. Control-operated cells with centrosomes left in the karyoplast progress through the cell cycle, duplicate the centrosome, and form clonal cell colonies. We conclude that the removal of centrioles uncouples cell growth from cell reproduction and impedes centriole biogenesis and centrosome duplication.

Introduction

Since the first description of centrosomes over 100 years ago (van Beneden, 1876; Boveri, 1888), generations of cell biologists became fascinated with this organelle. Being composed of, at least in animal cells, a pair of centrioles embedded in a cloud of amorphous pericentriolar material, centrosomes are involved in the generation of the bipolar spindle in mitosis and the array of microtubules in interphase (for recent reviews see Bornens and Karsenti, 1984; Vorobjev and Nadezdhina, 1987). They exert control over the number, polarity, and spatial and temporal patterns of microtubules, and thereby influence cell shape and cellular dynamics. Therefore, the centrosome may be considered a mediator between two-dimensional molecular information and three-dimensional cellular information, or "an instrument through which the cell is constructed out of the molecules commanded by the genes" (Mazia, 1987). However, despite their fundamental role in the organization of the cytoskeleton, little is known about their biochemical composition, mode of function, regulation of their activity, and their generation and inheritance. The discrepancy between the centrosome's apparent significance and our lack of knowledge about it is the reason for the aura of enigma that still surrounds this organelle.

The importance of the centrosome for the life of a cell mandates tight control over not only centrosome activity, but also their number and replication. However, it is precisely the question of centrosome replication and inheritance (in particular that of its most conspicuous constituent, the centriole) that has remained most controversial. Because of their ability to self-duplicate, they were considered to possess a certain degree of autonomy or even genetic continuity (Lwoff, 1950), or represent a structure strictly elaborated from a similar parent structure (Sonneborn, 1970; Dippell, 1968; Kochanski and Borisy, 1990; Mazia, 1984, 1987; for reviews see Fulton, 1971; Wheatley, 1982). However, there is clear evidence for the formation of centrioles independent of preexisting centrioles during the cell cycle in invertebrate eggs (Yatsu, 1905; Dirksen, 1961; Kallenbach, 1982), Naegleria (Fulton and Dingle, 1971), and mouse embryos (Maro et al., 1985; Schatten et al., 1986). Furthermore, in certain differentiating plant and animal cells, large numbers of centrioles or basal bodies may arise from amorphous bodies called blepharoplasts, proliferative elements, or generative complexes (reviewed in Hepler and Palevitz, 1974; Raff, 1979). Complicating the issue even further, there are reports of an acentriolar Drosophila cell line (Debec et al., 1982) and acentriolar poles in cranefly spermatocytes (Dietz, 1966) or PtK cells (Brenner et al., 1977). Claims that karyoplasts separated from their centrioles and 90% of their cytoplasm by enucleation may generate centrioles de novo (Zorn et al., 1979) are open to the criticism that the regenerates are the result of unsuccessful separation of the nucleus from the centrosome.

The question of a possible genetic continuity of centrioles is still unresolved. Because replication is best understood in the context of the semiconservative replication of DNA and RNA, efforts were directed toward the demonstration of a direct involvement of DNA or RNA in centriole duplication, with inconclusive results (for a review of the earlier literature, see Fulton, 1971). The controversy about this point persists until today with reports that Chlamydomonas basal bodies do (Hall et al., 1989) or do not (Johnson and Rosenbaum, 1990; Johnson and Dutcher, 1991) contain DNA. It is conceivable, in addition, that centriole inheritance is fundamentally different in early embryos and somatic cells. Thus, the nucleus, DNA, RNA, and protein synthesis are not involved in centriole reproduction in embryonic cytoplasm (Raff and Glover, 1988; Gard et al., 1990; Sluder et al., 1990), whereas in somatic cells centrioles cannot be generated without the nucleus (Kuriyama and Borisy, 1981) or in the absence of protein synthesis (Phillips and Rattner, 1976).

Our approach to the question of centrosome/centriole function and continuity has been to produce nucleated, centriole-free BSC-1 cell fragments (karyoplasts) by micro-

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surgery. This approach allows unequivocal verification of the removal of centrioles for each microsurgical operation. In each experiment, we determined whether the karyoplast could regenerate centrioles and divide to form clonal colonies. We also followed the fate of the anucleate cell fragment (the cytoplast) that contained the original pair of centrioles. We show here that centrosome-free karyoplasts regenerate large amounts of cytoplasm, reestablish a microtubule-organizing center and an astral microtubule array, reconstitute a Golgi apparatus comparable with that removed by the operation, and proceed through S phase. However, they do not regenerate centrioles, undergo normal cell division, or form clonal colonies similar to control-operated cells.

Results

Physical Removal of Centrosomes

Methods were sought that allow unequivocal separation of the centrosome from the rest of the cell. Our first approach was to fuse centriole-free karyoplasts (produced by centrifugation in the presence of cytochalasin B) with centriole-free cytoplasts (produced by centrifugation in the presence of both cytochalasin B and nocodazole; see Karsenti et al., 1984) to provide the karyoplasts with a larger cytoplasm and restore their viability. However, this approach posed many problems (e.g., low yield, difficulties in proving fusion, combination of harsh treatments) and was abandoned. Therefore, we resorted to microsurgical removal of the centrosome under microscopic observation. The major advantage is that the success (or failure) of each experiment can be evaluated microscopically. A disadvantage is that it is time consuming because only one experimental cell is produced per operation.

Using a micropipette drawn to a diameter of 3–5 μm, the centrosome-containing portion of the cytoplasm (cytoplast) of BSC-1 cells was separated from the nucleus-containing portion (karyoplast). BSC-1 cells are particularly well suited for these experiments because the position of the centrosome is clearly visible by phase contrast microscopy as a "centrosphere" (Wilson, 1925), around which membraneous organelles arrange in a radial array. In addition, the centrosphere is often located several micrometers away from the nucleus, particularly in daughter cells during the first few hours after division, further facilitating the microsurgical separation of centrosomes

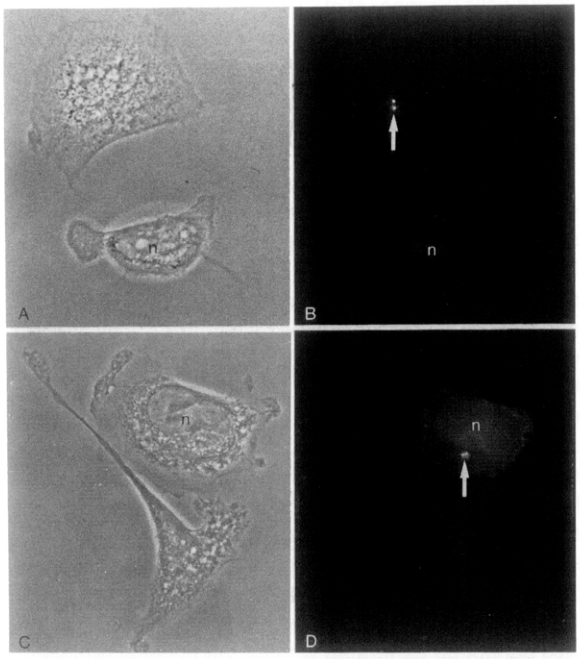

Figure 1. Centrosome (Centriole) Visualization 1 hr after Microsurgery

(A and B) Experimental operation. The centrioles (arrow) are in the cytoplast (top) that has been separated from the karyoplast.

(C and D) Control operation. Centrioles (arrow) have remained in the nucleated cell fragment (top). n = nucleus.

Magnification = 174.9×.

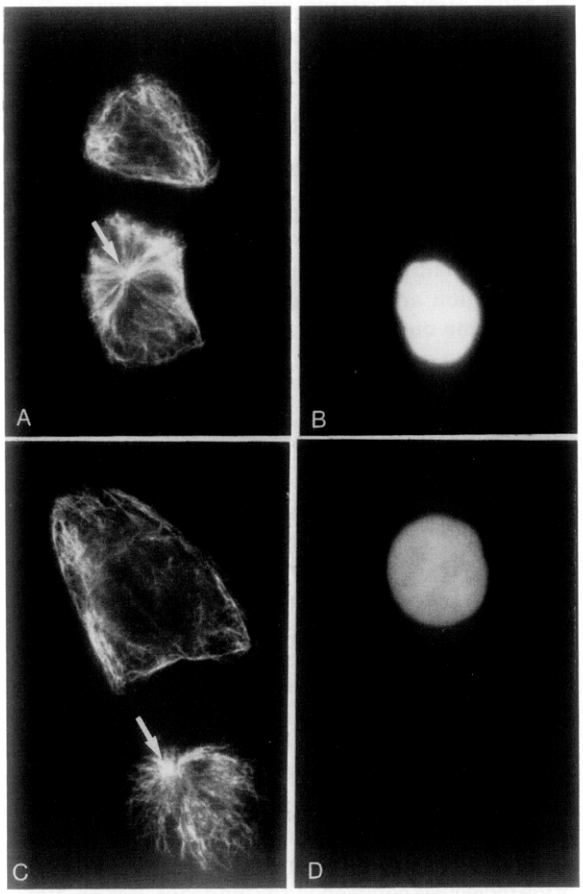

Figure 2. Microtubule Organization in Karyoplasts and Cytoplasts 1 hr after Microsurgery

Cell fragments were treated with 5 μg/ml nocodazole for 1 hr to depolymerize microtubules and allowed to recover for 5 min. Microtubules visualized with anti-tubulin (A and C); nucleus visualized with DAPI (B and D).

(A and B) Control cell. Only the karyoplast has generated an astral microtubule array (arrow).

(C and D) Experimental cell. Only the cytoplast has generated an astral microtubule array (arrow).

Magnification = 241×.

from nuclei. In control-operated cells, approximately the same amount of cytoplasm was removed except for the centrosome. Figures 1A–1D compare control and experimental cells 1 hr after operation. In these detergent-extracted cells, the centrioles are clearly visualized with the anti-centrosomal serum 5051. As outlined in detail in the Experimental Procedures, 5051 serum visualizes just the centrioles if the cells were extracted with Triton X-100 prior to antibody processing. Similar findings were also reported by Bre et al. (1987).

Both karyoplasts and cytoplasts are capable of organizing microtubule arrays after microsurgery, but with different patterns. When freshly operated cells were first exposed to nocodazole to depolymerize microtubules and then allowed to recover in drug-free medium, a radial microtubule array is generated only in those cell fragments that contain the centrosome/centrioles, i.e., in the experimental cytoplast (Figure 2A) and the control karyoplast (Figure 2B). The respective counterparts are characterized by a random (nonfocal) microtubule pattern.

Karyoplast and Cytoplast Behavior during the First 30 hr after Microsurgery

Roughly 20–30 hr after microsurgery, experimental karyoplasts regenerate a functional microtubule-organizing center lacking centrioles, while control-operated karyoplasts undergo cell division. Figure 3 compares control and experimental cells processed for double labeling with tubulin and centrosome antibodies about 28 hr after microsurgery. These micrographs demonstrate several important features typically observed in these experiments. In both cases, approximately the same amount of cytoplasm was separated from the karyoplast (Figures 3A and 3F). Microtubules of the aging experimental cytoplast that contains the centrioles (Figure 3E) are still concentrated around the centrosome (Figure 3D), but the pattern is no longer strictly radial. The corresponding centriole-free karyoplast has a radial microtubule array nicely focused upon a region near the nucleus where one would expect to find the centrosome (Figures 3B and 3C). After nocodazole treatment, microtubule regrowth begins in this perinuclear location (not shown). The control cytoplast (Figures 3F and 3G, center), which does not possess a pair of centrioles (Figure 3H), shows a dense but unorganized microtubule array. The original control karyoplast, however, has divided, and each of the daughter cells (Figure 3F, top and bottom) shows a radial microtubule pattern (Figure 3G) focused upon a pair of centrioles (Figure 3H). Even control cells that were operated on twice to remove more than half of the cytoplasm (but leaving the centrosome in the karyoplast) usually divided within about 30 hr.

Experiments in which karyoplasts and cytoplasts were fixed roughly every 2–3 hr after microsurgery show that the focused microtubule array in centriole-free karyoplasts

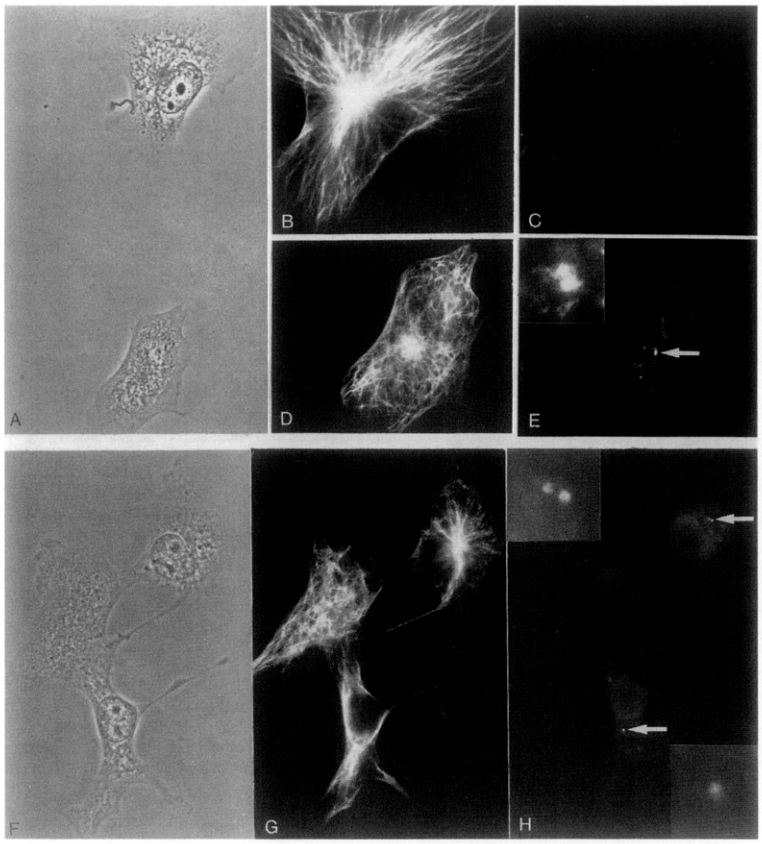

Figure 3. Centrosome Deployment and Microtubule Organization 27–28 hr after Microsurgery

(A–E) Experimental operation. The karyoplast ([A], top) possesses a striking radial microtubule pattern focused upon a region next to the nucleus (B) despite the absence of centrioles (C). The cytoplast ([A], bottom) has a more irregular microtubule pattern (D) even though it possesses the centrioles ([E], arrow); this is perhaps an initial sign of degeneration.

(F–H) Control operation. The original karyoplast has divided ([F], top and bottom). (Note: in control cells division typically occurs 25–30 hr after microsurgery.) Both daughter "karyoplasts" possess a more or less radial microtubule pattern (G) and a pair of centrioles (H). The centriole-less cytoplast exhibits a disorganized microtubule pattern ([G] and [H], center). The insets are higher magnifications of the respective pericentriolar regions.

Magnifications: (A) and (F–H) = 125.4×; (B–E) = 188.1×; insets = 3420×.

Table 1. Summary of Experimental and Control Operations (No Transfer)

	Experiment	Control
Initial centrosphere removal (0 hr)	659	56
Both cell fragments intact at 1 hr	488	56
Both cell fragments intact at 20–48 hr (time of processing varies)	194	35
Centrioles in cytoplast	194	0
Division of karyoplast	0/194	33/35

usually appears 20 to 30 hr after operation. This is the same time window in which control karyoplasts divide for the first time.

Some of the experimental karyoplasts that had regenerated a (centriole-free) centrosphere after about 30 hr may exhibit deformed, contorted, or even fragmented nuclei (not shown). However, nuclear envelope breakdown, spindle formation, or cytokinesis were not observed. The data of these experiments are summarized in Table 1.

Karyoplast Transfer for Long-Term Observation

The BSC-1 cytoplasts generated by microsurgery have a limited lifespan, ranging from roughly 20 to 50 hr. Since the evaluation of the success or failure of each experiment critically depends on the cytoplast, long-term experiments are not possible unless karyoplast and cytoplast are separated for independent processing. This allows the cytoplast to be examined for the presence of centrioles before it degenerates and the karyoplast to be cultured for long time periods. These operations were done on one of a pair of daughter cells early in G1, a time when centriole replication had not yet occurred. In 20 successful experiments (10 control, 10 experimental), the karyoplast was transferred 15 min after microsurgery. The corresponding cytoplast was examined for the presence of centrioles, while the karyoplast was permitted to regenerate for longer time periods. Figure 4 shows one example. One of a pair of daughter cells was operated (Figure 4A), and the karyoplast was transferred soon thereafter (Figure 4B) and allowed to recover for 38 hr (Figure 4C). The cytoplast as well as the unoperated daughter cell both possessed a microtubule aster focusing upon a pair of centrioles (Figures 4D and 4E). The 38 hr transferred karyoplast also had a radial microtubule array (Figure 4F), but no centrioles (Figure 4G). The transfer experiments are summarized in Table 2. Even when observed for up to 2 weeks, none of the centriole-free karyoplasts underwent bona fide cell division or formed cell colonies, while all the control karyoplasts divided and formed cell colonies of up to several hundred cells in the long-term experiments. Some of the long-term centriole-free karyoplasts became very large, as shown in Figure 5.

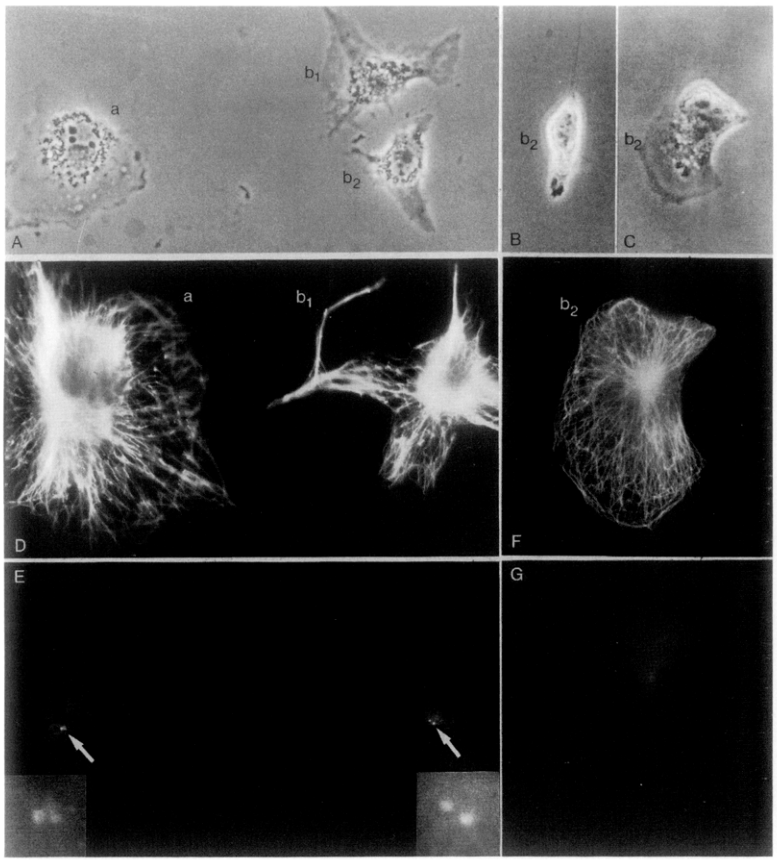

Figure 4. Karyoplast Transfer Experiment

Of two recent daughter cells a and b, cell b was microsurgically separated into cytoplast, b_1, and karyoplast, b_2 (A). One hour after surgery, the karyoplast (b_2) was transferred to a separate dish ([B], shown 15 min after transfer) and kept for 38 hr (C). Several hours after transfer of the karyoplast, the cytoplast (b_1) and the unoperated cell (a) were processed for immunofluorescence with anti-tubulin (D) and 5051 antiserum (E) to assay for microtubule organization and the presence of centrioles, respectively. Indeed, both possessed a pair of centrioles ([E], arrows and insets). The transferred karyoplast (b_2) was fixed after 38 hr and likewise processed for immunofluorescence (F and G). While it, too, exhibited a nice radial microtubule array (F), it lacked a pair of centrioles (G).

Magnifications: (A–C) = 118.8×; (D–G) = 226.8×; insets = 3240×.

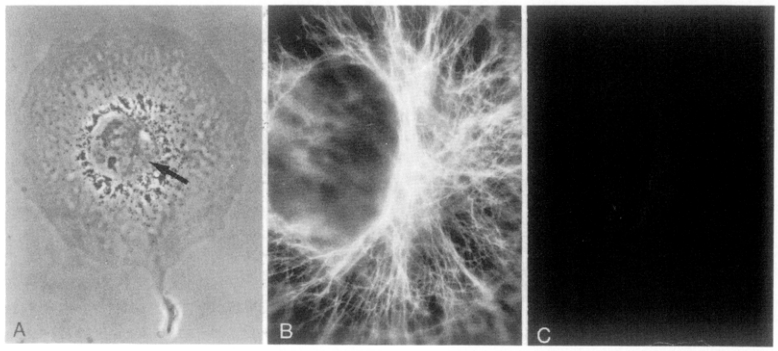

Figure 5. Transferred Karyoplast 96 hr after Microsurgery

(A) Phase contrast micrograph demonstrating large cell size. A centrosphere-like region is located to the left of the nucleus (arrow).

(B and C) Higher magnification of the centrosphere region after double labeling with tubulin antibodies (B) and 5051 antiserum (C). While the cell possesses a more or less radially arranged microtubule system (B), centrioles are not present (C); these were found in the nontransferred cytoplast processed for immunofluorescence shortly after microsurgery (not shown).

Magnifications: (A) = 83.2×; (B and C) = 256×.

BrdU Labeling of Karyoplasts

The experiments described so far strongly suggest that centriole-free karyoplasts are incapable of cell duplication. To determine whether these karyoplasts proceed through the nuclear cycle, they were cultured in the presence of BrdU for up to 26 hr. As summarized in Table 3 and shown in Figure 6, most centriole-free karyoplasts do indeed incorporate label in their nuclei. This finding demonstrates that these karyoplasts do at least enter, and likely complete, S phase, based on a qualitative comparison of the staining pattern and intensity with nuclei in control cells. The fact that not all nuclei are labeled even after over 20 hr of incubation in BrdU might suggest that they were operated on after they had already gone through S phase. It may also be due to variations in the length of the cell cycle in BSC-1 cells, since even in control cultures not all nuclei are labeled after 24 hr of incubation in BrdU (not shown), despite an average cell cycle time of 18–24 hr. Whether operated karyoplasts complete several rounds of DNA synthesis in the long-term experiments has not been determined. Judging from the large size of the nucleus (Figure 5), this is conceivable.

Golgi Apparatus Regeneration in Karyoplasts

In BSC-1 cells, as in many other cell types (Kreis, 1990), the Golgi apparatus is located in the vicinity of the centrosome (Figures 7A and 7B). Microsurgery of the type performed here is therefore likely to remove not only the centrosome, but also a major portion of the Golgi apparatus. An important question to address, therefore, is whether the (partial) removal of the Golgi apparatus has prevented cells from centriole regeneration and/or cell duplication by impairing Golgi-associated functions. Using NBD ceramide labeling of live cells to visualize the Golgi complex (Lipsky and Pagano, 1985), followed by immunofluorescence microscopy with anti-centrosomal serum, we examined the organization of the Golgi apparatus and the distribution of centrioles in experimental karyoplasts and cytoplasts. As shown in Figures 7C–7E, microsurgical removal of the centrosphere may indeed separate the vast majority of the ceramide-stainable material from the karyoplast. It is not possible to say, however, whether the entire Golgi system was removed. Ten cytoplast/karyoplast pairs examined 20–25 hr later demonstrate the recurrence of a prominent Golgi apparatus in all the karyoplasts (for an example, see Figures 7F–7L; for a summary, see Table 4). These observations show that whether or not most of the Golgi apparatus was initially removed, a Golgi apparatus of normal proportions is regenerated in experimental karyoplasts within the first 20 hr after microsurgery.

Table 2. Summary of Karyoplast Transfer Experiments

	Experiment	Control
Number of operations	30	12
Successful transfers	10	10
Centrosome in cytoplast (1–4 hr)	10	0
Centrosome in karyoplast (2–14 days)	0	10
Karyoplast divides and forms cell colony	No	Yes

Table 3. Summary of BrdU Incorporation Experiments

Number of operations	20
Both fragments viable at 1 hr	14
26 hr	14
Centrioles in cytoplast at 26 hr	14
BrdU incorporation in nucleus of karyoplast	11/14

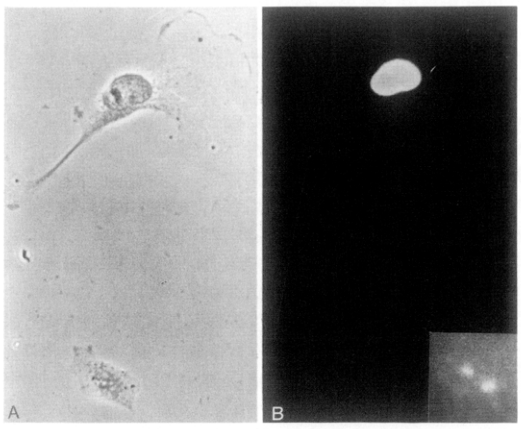

Figure 6. BrdU Incorporation

(A) Karyoplast (top) and cytoplast (bottom) 24 hr after microsurgery and culture in the continuous presence of 2 μg/ml BrdU.

(B) Anti-BrdU immunofluorescence, showing incorporation of BrdU into the nucleus. Inset: higher magnification of 5051 labeling in the cytoplast, demonstrating the centriole pair.

Magnification = 121×.

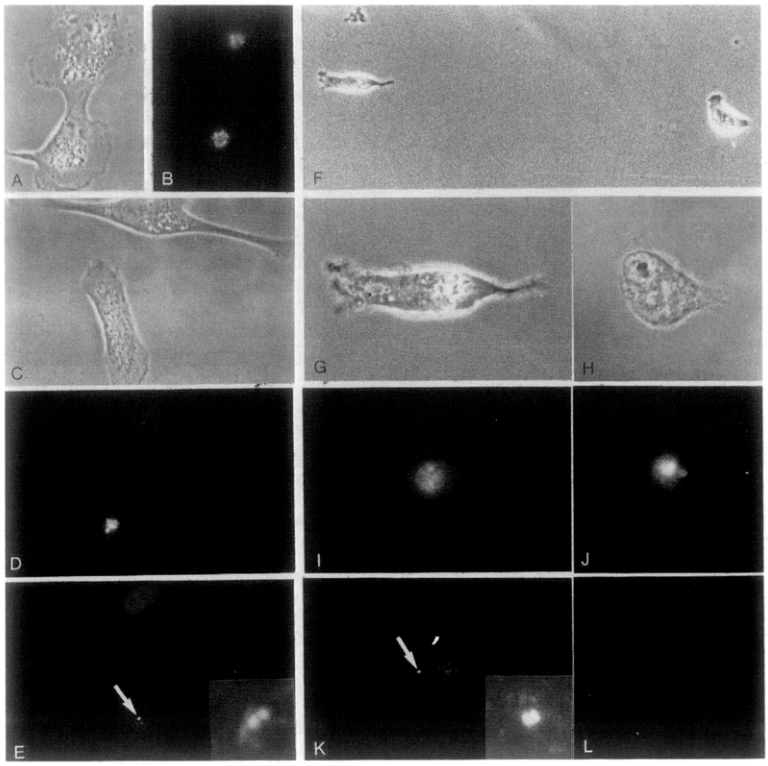

Figure 7. Distribution of Golgi Elements and Centrioles

(A and B) Location of the Golgi apparatus in a pair of BSC-1 daughter cells. Phase contrast micrograph (A) and NBD ceramide labeling (B). The Golgi apparatus is located in the vicinity of the nucleus. Magnification = 86.4 ×.

(C–E) Phase contrast (C), NBD ceramide (D), and 5051 antiserum labeling (E) of karyoplast (top) and cytoplast (bottom) 30 min after microsurgical separation. In this experiment, most of the Golgi elements ended up in the cytoplast, along with the centrosome. Magnification = 86.4 ×. Inset: higher magnification of the centrioles (3240 ×).

(F–L) Phase contrast, NBD ceramide, and 5051 antiserum labeling of a karyoplast–cytoplast pair 22 hr after microsurgical separation. (F) shows the live cytoplast (left) and karyoplast (right). At this time the NBD ceramide images (I and J) were taken. The cells were then permeabilized and fixed (G and H) and processed for immunofluorescence microscopy with 5051 antiserum (K and L). Both the cytoplast and karyoplast possess a Golgi apparatus, while the centrioles are present in the cytoplast only (K). Inset: higher magnification of the centrioles (3240 ×). Magnifications: (F) = 40.5 ×; (G–L) = 108 ×.

Discussion

The generation of two daughter cells from a parent cell encompasses both growth and reproductive events. Within the reproductive cycle, the duplication of the centrosome and, with it, the centrioles, is a key event because it insures the establishment of bipolarity in the forthcoming division. How the cell controls this precise doubling is not understood.

Here we address the question of centrosome/centriole function in a novel experimental system, namely, microsurgically severed somatic cells. This approach generates centriole-free karyoplasts with amounts of cytoplasm sufficient to ensure growth and survival. The fate of both karyoplast and cytoplast can be followed independently. We find that centriole-free karyoplasts restore a focused microtubule array and a focused Golgi apparatus; centriole-free karyoplasts do not regenerate centrioles, even after long periods of time; centriole-free karyoplasts do not exhibit mitosis, even though they enter and presumably complete S phase and grow beyond the size of an average BSC-1 cell; control operations that remove large portions of the cytoplasm but leave the centrosome in the karyoplast slightly delay, but do not block, cell cycle progression, centrosome duplication, and subsequent mitoses, indicating that the failure of centrosome-free karyoplasts to reproduce is not an artifact of the microsurgical procedure. From these observations we conclude that centriole elimination uncouples the growth cycle from the reproductive cycle; cell reproduction requires the presence of centrioles; centriole reproduction requires a preexisting centriole; pericentriolar material alone is unable to split apart and establish two autonomous poles; and the cytoplasmic microtubule complex is capable of self-organization into an astral array.

Table 4. Summary of NBD Ceramide Labeling Experiments

Number of operations	18
Both cell fragments viable at 1 hr	14
GA staining at 1 hr in cytoplast	4
karyoplast	0
GA staining at 20–25 hr in cytoplast	10
karyoplast	10
Centrioles in cytoplast at 20–25 hr	10

Note: Of 14 successful experiments, 4 were processed at 1 hr, the rest at 20–25 hr.

Centriole Duplication Requires a Preexisting Centriole

As a rule, centrioles are observed to arise in the vicinity of a preexisting centriole—with a few noted exceptions (see Introduction). That this spatial relationship is strictly required (at least in those cell types that always possess centrioles) has not been demonstrated experimentally. A significant observation of the present study is that centriole-free karyoplasts do not regenerate centrioles, even if given a timespan sufficient for untreated BSC-1 cells to complete several cell cycles. This finding suggests that some essential piece of molecular or structural information required for a centriole to duplicate is contained within, or closely associated with, a preexisting centriole. This

information apparently cannot be retrieved from elsewhere in the cytoplasm, nor is it provided by the nucleus alone. The lack of centrioles, or the lack of the information needed to reproduce this organelle, also leads to the failure of the microtubule-organizing center to establish two functional poles for mitosis.

Our observations are consistent with the concept put forth by Mazia et al. (1960) and Sluder and colleagues (Sluder and Begg, 1985; Sluder and Rieder, 1985; Sluder et al., 1989) that the reproduction of centrosomes requires a "seed" or "polar organizer," and that the behavior of the polar organizer appears to be coincident with the behavior of the centriole (Sluder, 1990). There is a correlation between the reproductive capacity of a centrosome and the number of centrioles it contains. For example, the pericentriolar material of sea urchin eggs that is separated from the centrosome either by microsurgery (Sluder, 1990) or by fragmentation before syngamy (Sluder et al., 1989) is incapable of duplication.

The findings reported here are also consistent with the observation by Kochanski and Borisy (1990) that centriole distribution to daughter cells occurs in a semi-conservative fashion. If there is nothing, as in our experimental karyoplasts, no "something" can be generated. Whether this semi-conservative distribution also implies a semi-conservative replication step of a nontubulin component (e.g., the "polar organizer") remains to be shown.

Our observations do not speak directly to the question of the existence of an informational template (DNA?, RNA?) in centrioles, a question that is as controversial today (Hall et al., 1989; Johnson and Rosenbaum, 1990; Sluder et al., 1990; Gard et al., 1990; Johnson and Dutcher, 1991) as it was 20 years ago (Fulton, 1971). However, they put some constraints on possible answers and may suggest avenues for further experimentation. Our observations argue against the possibility that the nucleus can direct de novo centriole formation. They are, however, consistent with the existence of nuclear information or "signals" (Kuriyama and Borisy, 1981) for centriole duplication, which strictly require the presence of a structural template (a preexisting centriole) to be effective. Our findings are also consistent with the idea of a nucleic acid that is part of the centriole and involved in duplication; when the centriole is lost from the cell, the ability to reproduce this organelle would be irretrievably lost as well. Ultimately, however, it is of secondary importance where the information for centriolar proteins is encoded (nucleus or centriole) because translation and assembly do most likely take place in the cytoplasm (Kochanski and Borisy, 1990). We favor the idea that in these somatic cells, centriole formation requires a preexisting centriole that provides some essential structural information for the assembly of another centriole. Since in other cell types, notably mouse embryos (Calarco-Gillam et al., 1983; Schatten et al., 1986) and the amoebo-flagellate Naegleria (Fulton and Dingle, 1971), centrioles may form in an apparent de novo fashion, generalizations about the process of centriole formation and the nature of this piece of structural information should presently be avoided.

Centriole Removal Interferes with Cell Reproduction

The cell cycle encompasses two major subcycles, the growth cycle and the reproductive cycle (which includes the chromosome cycle and the centrosome cycle) (Mazia 1978, 1987). In growing cells the two major cycles are geared together, whereas in developing embryos, the reproductive cycle may run freely without cell growth. The interdependency of these two cycles in growing cells has been demonstrated in an elegant experiment on amoebae (Prescott, 1956), where portions of the cytoplasm were cut off repeatedly, causing the cell to remain in interphase and to regenerate its cytoplasm continuously. In this experiment, the reproductive cycle was "waiting" for the interrupted growth cycle to complete. In a sense, we have performed here a complementary experiment: in removing the centrosome we have interrupted the reproductive cycle. In this case, however, cell growth did not "wait" and the cell increased in size, leading to the formation of giant cells from which portions of the cytoplasm were spun off in a process reminiscent of "traction-mediated fission" (De Lozanne and Spudich, 1987).

The key observation of the present study is that cell reproduction critically depends on the presence of centrioles. The presence of a microtubule-organizing center (presumably composed of pericentriolar material), which is regenerated within 20–30 hr (Figures 3B, 4F, and 6B), is insufficient for an experimental karyoplast to generate bipolarity and a mitotic spindle. Thus, centriole removal, in essence, prevents the onset of "two-ness" (Mazia, 1978). The pericentriolar material alone is unable to separate and establish two independent entities, suggesting that it lacks polar organizing function. This cannot be generalized, however, since there exists a mutant Drosophila cell line that lacks centrioles (Debec et al., 1982). The nature of the mutation in this cell line is unknown, but it seems to have caused the polar organizer function to become dissociated from the centrioles. In the cell type used here, it appears that there exists a causal link between centriole reproduction and cell duplication. Thus, centrioles are not mere passengers that "go along for a ride" during mitosis (Pickett-Heaps, 1969) but play a more active role than previously assumed.

The coordination of the eukaryotic cell division cycle involves regulatory proteins that bring about the proper timing of the diverse steps in cell cycle progression. A number of these regulators have been identified (reviewed by Lewin, 1990) that all seem to exert their control function via protein phosphorylation/dephosphorylation. Several of the mediators as well as targets of this regulatory machinery have been localized to the centrosome where they may affect microtubule-nucleating activity (e.g., Vandré et al., 1984; Nigg et al., 1985; Kuriyama, 1989; Bailly et al., 1989). It is conceivable that some of these components are closely associated with the centrioles. The removal of these organelles would interrupt the chain of regulatory events. One could envision that in the absence of a complete centrosome, the M phase activity of cdc2 kinase is not induced, preventing chromosome condensation and

spindle formation. Picard et al. (1987) have reported that microinjection of centrosomes into starfish oocytes releases them from interphase arrest and supports many cleavages. Thus, the centrosome may be required for the activation of cdc2 kinase and the initiation of the structural and functional changes associated with mitosis (Lamb et al., 1990; Lüscher et al., 1991; Dessev et al., 1991; for a review see Nurse, 1990).

Microtubules Self-Organize into a Focal Array

A comparison of microtubule distribution in centrosome-free karyoplasts shortly after microsurgery (Figure 2C) and 26 hr later (Figure 3B) clearly demonstrates the reestablishment of a monastral microtubule pattern focused upon a region near the nucleus. This pattern of microtubule distribution is indistinguishable from that of untreated cells. It has evolved from an essentially random array (Figure 2C). This remarkable process suggests the existence of a powerful capacity for self-organization of the cytoplasmic microtubule complex. The phenomenon is not entirely unfamiliar since microtubule self-organization into focused arrays has also been observed in severed fish melanophore processes (McNiven and Porter, 1988), brain microtubule protein preparations in vitro (Weisenberg and Celati, 1984), and taxol-induced microtubule asters in Xenopus extracts (Verde et al., 1991). Its mechanism is unknown and merits further study. In BSC-1 cells, it may correlate with the replenishing of the pool of pericentriolar material, most (if not all) of which was partitioned, along with the centrioles, into the cytoplast upon microsurgery. Thus, the resurgence of this material and the "asterization" of the microtubule system may go hand in hand. The opposite is observed in cytoplasts where the initially strongly focused microtubule system (Figure 2C) becomes more random (Figure 3D). This would be consistent with a gradual loss of microtubule-nucleating sites, owing to the complete absence of transcriptional, and waning translational, activity in these cytoplasts. Our findings suggest that the pericentriolar-nucleating sites are capable of self-aggregation into a compact organizing center. Whether this process depends on the presence of microtubules, microtubule motors, or other macromolecular complexes remains to be demonstrated.

Experimental Procedures

Cell Culture

African green monkey kidney cells (strain BSC-1) were used for the experiments described here. They were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum (GIBCO) and 5% NU serum (Collaborative Research). The pH of the complete medium was 7.2–7.4. The large size of the cell and clear visibility of the centrosphere greatly facilitated the microsurgical experiments. RAT-1 cells, NIH 3T3 fibroblasts, mouse L-9 cells, and chicken embryo fibroblasts proved too small and fragile for the microsurgery to work consistently.

For microsurgery, cells were seeded at very low density onto 22 mm coverslips, on which a recognizable pattern had been etched to facilitate cell relocalization. Plastic culture dishes (60 mm) filled with 10 ml of medium were used for all microsurgery experiments.

Microsurgery

A Zeiss inverted microscope and a Leitz micromanipulator, both placed on a vibration isolation table, were used for the microsurgery experiments. A micropipette drawn to a diameter of 3–5 μm was pressed down on the cell between the centrosphere and the nucleus. After separation of the two cell halves, the pipette was moved back and forth between the cell fragments several times to insure complete separation. Pipette shape, length, and flexibility were of critical importance for the success of the operation. The temperature of the culture medium was less critical, though temperatures above 30°C accelerated the process of cell separation. Following microsurgery the cell fragments were returned to the incubator and allowed to recover for at least 30–60 min.

Karyoplast Transfer

Because of the limited lifespan of the cytoplast, the karyoplast had to be moved away from the cytoplast for long-term observation and independent processing. To achieve this, the karyoplast was gently removed from the coverslip after completion of microsurgery using a glass needle and transferred to another coverslip placed in the same 60 mm culture dish. There the karyoplast was allowed to attach under visual inspection. This coverslip was then transferred to a separate 60 mm or 35 mm culture dish for long-term observation. The corresponding cytoplast still attached to the original coverslip could now be processed independent of the karyoplast to verify the absence of the centrosome/centrioles.

Centrosome, Centriole, and Microtubule Visualization

The autoimmune serum 5051 (a kind gift of Dr. Tim Mitchison, UCSF) was routinely used for centrosome and centriole visualization. In extensive control experiments it was determined that 5051 serum can be used as a reliable marker for centrioles if cells are extracted with detergent prior to processing for immunofluorescence microscopy. If this is done, two 5051-positive dots are visualized in virtually every BSC-1 cell except prophase to metaphase cells where two dots are seen at both spindle poles. The identification of these dots as centrioles is based on three lines of evidence: double-labeling experiments with antibodies against tubulin after depolymerization of the cytoplasmic microtubules with nocodazole or low temperature, where the position of the 5051-positive dots exactly corresponds to the position of the two nocodazole or cold-resistant dots representing the stable centriolar tubulin (not shown); regrowth of microtubule asters during the recovery from nocodazole treatment, which begins at the 5051-positive dots; and correlative light and electron microscopy. In collaboration with Dr. U. Euteneuer, five experimental karyoplast–cytoplast pairs were processed for serial section electron microscopy, and centrioles were shown to be present in the cytoplasts. Bre et al. (1987) also employed double labeling and electron microscopy to demonstrate that 5051 serum localizes centrioles in MDCK cells. In our study, therefore, electron microscopy was not used routinely because it was considered to be counterproductive in light of the reliability of the staining procedure employing 5051 serum.

For immunofluorescence microscopy, cells or cell fragments were lysed with 1% Triton X-100 in PHEM buffer (Schliwa and van Blerkom, 1980) and then fixed in cold methanol for 5 min. Further processing with either 5051 serum or tubulin antibodies was as described (Karsenti et al., 1984).

It is important to note that all experimental and control cells were processed along with nonoperated cells, preferably on the same coverslip, to verify the success of the staining procedure. Such a positive control is mandatory, particularly for the transferred karyoplasts that lack centrioles.

Other Procedures

To visualize the nucleus, DAPI (SIGMA) was applied 5 min before mounting of the coverslip at a dilution of 1:1000. NBD ceramide (a kind gift of Dr. H.-P. Moore, University of California, Berkeley) was applied to live cells at a concentration of 4 μg/ml for 30 min and back exchanged for 10 min (Cooper et al., 1990). Cells were photographed in the fluorescence microscope using a 0.9 ND filter. BrdU (Sigma) was applied to live cells at a concentration of 2 μg/ml for up to 26 hr after microsurgery and was visualized after fixation in either methanol or 1% glutaraldehyde with an antibody against BrdU (a kind gift of Dr. D. Weisblat, University of California, Berkeley).

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