MICROTUBULE-ORGANIZING CENTRES IN BINUCLEATE CELLS AND HOMOSYNKARYONS

FIONA M. WATT, E. SIDEBOTTOM AND H. HARRIS

Sir William Dunn School of Pathology, South Parks Road, Oxford OX1 3RE, England

SUMMARY

Immunofluorescence studies showed that most binucleate Vero cells formed by virus-induced fusion or by inhibition of cytokinesis had a single microtubule-organizing centre (MTOC) when examined during the reassembly of microtubules after chilling, but two or more organizing centres when examined after exposure to colcemid. These findings suggest that although binucleate cells initially contain more MTOC than mononucleate cells, the extra MTOC are normally aggregated, so that the number of MTOC in binucleate cells tends to be reduced very quickly to that in mononucleate cells.

INTRODUCTION

In the preceding paper, we used immunofluorescence microscopy to observe microtubule-organizing centres (MTOC) in cultured cells under different experimental conditions (Watt & Harris, 1980). In the present paper we use the same techniques to study MTOC in binucleate cells produced by virus-induced fusion or by inhibition of cytokinesis. Such cells, of course, initially contain more MTOC than normal mononucleate cells, and our aim was to discover what happens to these extra organizing centres. In addition, the number of MTOC in mononucleate homosynkaryons was compared with the number in unfused mononucleate cells.

MATERIALS AND METHODS

Cells and cell culture

The method of culture and source of Vero cells are given in the preceding paper (Watt & Harris, 1980).

Cells were fused in suspension by inactivated Sendai virus, essentially as described by Harris & Watkins (1965). Vero homokaryons were formed by mixing 2-4 x 10^6 cells with 40-50 haemagglutinating units of inactivated Sendai virus in a volume of 1 ml. Immediately after fusion, the cells were allowed to attach and spread on 11-mm diameter round glass coverslips: 20-30% of the cells had more than one nucleus, and about half of these were binucleate. The proportion of binucleate cells in an unfused population was approximately 0.3-0.8%.

At different times after fusion, cells were either chilled on ice or incubated with 0.5 μg/ml colcemid, and then allowed to recover partially so that MTOC could be visualized by immunofluorescence microscopy. The precise techniques are given in the preceding paper.

* Present address: Room 56-543, Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, U.S.A.
Counting microtubule-organizing centres

The method used for counting MTOC in cells on coverslips is given in the preceding paper. MTOC were only counted in cells with an interphase nuclei. Each set of results was derived from at least 3 experiments involving different populations of cells. Replicate experiments did not show significant variation by $x^2$ analysis.

Calculating the expected number of microtubule-organizing centres in binucleate cells resulting from cell fusion

After fusion, a binucleate cell should contain the sum of the number of MTOC in the 2 cells from which it was derived. Assuming that 3 cells at any stage of the cell cycle can fuse (Yamanaka & Okada, 1966; Johnson & Harris, 1969), the expected distribution of organizing centres in cells with a nuclei can be calculated as follows. Let

$P_x = \text{probability that a single cell has } x \text{ MTOC}$

and

$B_{px} = \text{probability that a binucleate cell has } x \text{ MTOC}.$

Since all unfused Vero cells have at least 1 MTOC and none has more than 4 (Watt & Harris, 1980):

$B_p1 = 0,$

$B_p2 = P_1^2,$

$B_p3 = 2P_1P_2,$

$B_p4 = P_1^3 + 3P_1P_2P_3.$

Only binucleate cells with both nuclei in interphase are considered.

In the preceding paper we showed that, after 1 h in 0.5 μg/ml colcemid followed by 30 min recovery in fresh medium, 57.4 % of interphase Vero cells had 1 MTOC, 42.0 % had 2 MTOC, 0.4 % had 3 MTOC and 0.2 % had 4 MTOC. Thus for colcemid-treated cells:

$P_1 = 0.574,$

$P_2 = 0.420,$

$P_4 = 0.002.$

After chilling on ice for 30 min and 30 s recovery at 37 °C:

$P_1 = 0.895,$

$P_2 = 0.105,$

$P_4 = 0.000.$

RESULTS

Microtubule-organizing centres

Our previous experiments with normal mononucleate cells suggested that chilling was the gentlest, and probably the most reliable, way to reveal MTOC. This method was therefore used first to study MTOC in binucleate cells. The cells were placed on ice for 30 min to disrupt microtubules, transferred to medium at 37 °C for 30 s, and then fixed and stained with antibodies (Fig. 1).

Table 1 gives the expected number of MTOC in binucleate cells (see Materials and Methods), and the actual number observed at different times after cell fusion. The cells required 2.5 h in which to attach and spread on the coverslips, and this is therefore the earliest time after fusion when MTOC could be counted.

The majority of unfused Vero cells had a single microtubule-organizing centre during recovery from cold treatment (see preceding paper), and one would therefore expect most binucleate cells to have two. However, 2.5 h after fusion over 90 % of the binucleate cells had only one MTOC. The number of organizing centres remained fairly constant during the first 2 days after fusion, although the proportion of cells
Microtubule-organizing centres in fused cells

Fig. 1. Binucleate Vero cell with 1 MTOC after cold treatment for 30 min followed by 30 s recovery at 37 °C. × 1000.

Fig. 2. Microtubules in a binucleate Vero cell, 2·5 h after fusion. × 1000.

with more than 1 MTOC did increase slightly. The display of microtubules in untreated binucleate cells 2·5 h after fusion resembled the pattern characteristic of mononucleate cells. Microtubules were most dense in the perinuclear region and spread from there throughout the cytoplasm to the cell periphery (Fig. 2).

The observed reduction in the number of microtubule-organizing centres in binucleate cells could be brought about in two ways: the extra MTOC might be destroyed, or they might aggregate and function as a single organizing centre. We were able to distinguish between these two possibilities by making use of the discovery
Table 1. MTOC in Vero cells after recovery from cold treatment

<table>
<thead>
<tr>
<th></th>
<th>MTOC per cell (%)</th>
<th>Total no. cells counted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Unfused cells*</td>
<td>89.5</td>
<td>10.5</td>
</tr>
<tr>
<td>Binucleate cells: expected number</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Binucleate cells: observed number at different times after fusion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 h</td>
<td>93.1</td>
<td>6.2</td>
</tr>
<tr>
<td>1 day</td>
<td>85.0</td>
<td>9.3</td>
</tr>
<tr>
<td>2 days</td>
<td>76.0</td>
<td>15.0</td>
</tr>
</tbody>
</table>

* Data from preceding paper.
† After 2 days the number of binucleate cells in culture had declined and the sample size was therefore smaller.

Table 2. MTOC in Vero cells after colcemid treatment

<table>
<thead>
<tr>
<th></th>
<th>MTOC per cell (%)</th>
<th>Total no. cells counted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Unfused cells*</td>
<td>57.4</td>
<td>42.0</td>
</tr>
<tr>
<td>Binucleate cells: expected number</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Binucleate cells: observed number at different times after fusion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 day</td>
<td>10.0</td>
<td>24.0</td>
</tr>
<tr>
<td>2 days</td>
<td>6.7</td>
<td>20.0</td>
</tr>
</tbody>
</table>

* Data from preceding paper.

that colcemid increases the number of MTOC in cells probably by causing aggregated organizing centres to separate (see preceding paper). If the extra organizing centres in binucleate cells are destroyed, then the number of MTOC in colcemid-treated binucleate cells should be the same as the number in mononucleate cells exposed to colcemid. However, if the extra MTOC aggregate, the number seen after colcemid treatment should more closely resemble the sum of the numbers seen in two single cells.

Two and a half hours after fusion, Vero cells were exposed to 0.5 μg/ml colcemid for 1 h and placed in medium lacking colcemid for 30 min, so that the number of MTOC in binucleate cells could be determined (Fig. 3). Table 2 shows the actual and the expected distribution of organizing centres. As expected, the majority of binucleate cells 2.5 h after fusion had 2, 3 or 4 MTOC, whereas most mononucleate cells had 1 or 2 MTOC (see preceding paper). The extra MTOC in binucleate cells were not therefore destroyed after cell fusion, but, in the absence of colcemid, were
apparently aggregated together. At later times, the number of binucleate cells with more than 4 MTOC increased slightly. About 10% of the binucleate cells still had a single MTOC after colcemid treatment. This suggests that not all the organizing centres that had aggregated were separated by this treatment.

The difference between the number of MTOC seen after exposure to low temperature and the number seen after colcemid treatment was even more marked in cells with 3 or more nuclei. In chilled cells the nuclei were often arranged round 1 large organizing centre (Fig. 4), whereas in colcemid-treated cells many MTOC were distributed among the nuclei or further away in the cytoplasm (Fig. 5).

*Microtubule-organizing centres in binucleate cells produced by inhibition of cytokinesis*

When cytokinesis is inhibited, the binucleate cells formed should contain the same number of MTOC as pairs of daughter cells. It was of interest to see whether the extra MTOC in such cells were aggregated, as occurred in fused cells. Cytokinesis of Vero cells was therefore prevented by exposure to 1 μg/ml cytochalasin B (supplied by Aldrich Chemical Co., Milwaukee, Wisconsin, U.S.A.). This mould metabolite appears to inhibit the separation of daughter cells after nuclear division by interfering with microfilaments in the cleavage furrow (Schroeder, 1969, 1970). Table 3 shows the number of nuclei in Vero cells cultured in cytochalasin B for up to 7 days.

Cells incubated in cytochalasin B for different lengths of time were chilled or treated with colcemid in order to determine the number of MTOC in binucleate cells with two interphase nuclei (Tables 4, 5). After 24 h in cytochalasin B most binucleate cells had a single organizing centre during recovery from cold treatment,
Fig. 4. Tetranucleate Vero cell with 1 MTOC after cold treatment for 30 min followed by 30 s recovery at 37 °C. × 1000.

Fig. 5. Trinucleate Vero cell with 6 MTOC after 1 h in 0.5 μg/ml colcemid and 30 min recovery. × 1000.
Microtubule-organizing centres in fused cells

Table 3. Effect of exposure to cytochalasin B on number of nuclei in Vero cells

<table>
<thead>
<tr>
<th>Duration of exposure to 1 μg/ml cytochalasin B</th>
<th>Nuclei per cell (%)</th>
<th>Total no. cells counted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1276</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4. MTOC in binucleate Vero cells after recovery from cold treatment

<table>
<thead>
<tr>
<th>Duration of exposure to cytochalasin B</th>
<th>MTOC per cell (%)</th>
<th>Total no. cells counted</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>685</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5. MTOC in binucleate Vero cells after colcemid treatment

<table>
<thead>
<tr>
<th>Duration of exposure to cytochalasin B</th>
<th>MTOC per cell (%)</th>
<th>Total no. cells counted</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>690</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

but 2 or more after exposure to colcemid. This finding supports the view that in binucleate cells, whether they are formed by failure of cytokinesis or by virus-induced fusion, the number of functional organizing centres is approximately the same as that in mononucleate cells, because the extra organizing centres aggregate.

Binucleate cells formed by virus-induced fusion divide or are overgrown by unfused cells in a relatively short time. However, after 3 days in cytochalasin B, the number of binucleate Vero cells remains fairly constant; further nuclear division occurs in only a small proportion of cells (Table 3). Thus, MTOC can be studied in binucleate cells which persist for several days.

Tables 4 and 5 show that after 2 days in cytochalasin B, the number of MTOC in binucleate cells tended to increase (Fig. 6). By 7 days, 36% of the colcemid-treated cells had 5 or more MTOC, whereas no cells had this number of MTOC after 24 h. After cold treatment, the proportion of cells with more than 2 MTOC rose from 3% after 24 h to 16% after 7 days.
Fig. 6. Six MTOC in a binucleate Vero cell grown in 1 µg/ml cytochalasin B for 3 days, then incubated in 0.5 µg/ml colcemid for 1 h and allowed to recover for 30 min. × 1000.

Fig. 7. Two MTOC in a Vero cell containing five 1.6-µm diameter latex beads after 1 h in 0.5 µg/ml colcemid and 30 min recovery. × 1000.
The persistence of binucleate cells in cultures kept in cytochalasin B for 7 days suggests that the nuclei are incapable of further division. Such cells increase in size and resemble irradiated 'giant' cells (Puck & Marcus, 1956). It is possible that the observed increase in the number of organizing centres in these cells is due to persistence of the centriolar cycle in the presence of prolonged inhibition of nuclear division.

Microtubule-organizing centres in mononucleate homosynkaryons

The experiments which have been described suggest that the number of active microtubule-organizing centres in binucleate cells is strictly controlled. It was therefore of interest to see whether mononucleate daughters of cells with 2 or more nuclei (homosynkaryons) contain the same number of organizing centres as normal mononucleate cells.

Homosynkaryons were identified by the presence in the one cell of 2 different kinds of fluorescent latex bead (obtained from Polysciences Inc., Warrington, Pennsylvania, U.S.A.). The cells to be fused were grown in medium containing either 0.8-μm or 1.6-μm diameter beads. The smaller beads showed green fluorescence and the larger ones yellow-orange fluorescence when exposed to light of the wavelength optimal for fluorescein excitation.

After 2 days about 90% of the cells contained beads; that they had, indeed, been ingested was confirmed by electron microscopy (Watt, 1979). Many small beads were taken up but the number of large beads per cell was often less than 10 (Fig. 7). The presence of the beads did not affect the number of MTOC seen in single or binucleate cells.

Cells containing either large or small beads were fused after extensive washing to remove beads that had not been ingested. One or two days later, the cells were exposed to 0.5 μg/ml colcemid for 1 h and allowed to recover for 30 min, so that MTOC could be counted. Control experiments, in which cells containing one or other type of bead were grown together in culture, indicated that cells with only one nucleus but 2 or more beads of each type could only have arisen by cell fusion. The number of homosynkaryons that could thus be positively identified was, however, low, partly because of the small number of 1.6-μm diameter beads originally ingested and partly because of the low plating density necessary to prevent overgrowth by unfused cells.

Ninety-five positively identified homosynkaryons were counted in 5 separate fusion experiments. Thirty-eight of these had 1 MTOC; 38 had 2 MTOC; 14 had 3 MTOC; 4 had 4 MTOC; and 1 had 6 MTOC. The majority of homosynkaryons thus had 1 or 2 MTOC, but the proportion with 3 or more MTOC was significantly higher than in normal mononucleate cells (see preceding paper).

DISCUSSION

Most binucleate cells formed by virus-induced fusion or by inhibition of cytokinesis have a single MTOC during microtubule regrowth after chilling, but two or more organizing centres after exposure to colcemid. Our interpretation of these results is
that although binucleate cells initially contain more MTOC than single cells, the extra MTOC are normally aggregated and function as a single centre.

The aggregation of MTOC in binucleate cells could ensure that the three-dimensional array of microtubules is the same as in cells with a single nucleus, and, in addition, it might influence the events of mitosis. When spontaneous or artificially induced binucleate cells enter mitosis the chromosomes of both nuclei are commonly associated with a single mitotic spindle. The spindle formed is sometimes bipolar, but multipolar spindles are also common (Fell & Hughes, 1949; Oftebro & Wolf, 1967; Oftebro, 1968). If the aggregation of MTOC observed in binucleate Vero cells is a general phenomenon, it might ensure that a single spindle is formed. Multipolar spindles might arise from incomplete aggregation of MTOC. Evidence for aggregation of MTOC in a different situation comes from the work of Spiegelman, Lopata & Kirschner (1979). They found that undifferentiated neuroblastoma cells have an average of 12 MTOC close to the nucleus, but when neurite formation is stimulated the MTOC aggregate and form a single large organizing centre at the point where neurite outgrowth occurs.

The percentage of mononucleate Vero homosynkaryons with 3 or more MTOC is higher than the percentage seen in unfused Vero cells. In view of the small sample size, it is difficult to assess the significance of this difference, but even if it were a general phenomenon the difference is probably not large enough to account for the failure of many synkaryons to divide (Yamanaka & Okada, 1968; Gordon & Cohn, 1970) and yield clones. There is one interesting report of a permanent alteration in the number of MTOC following cell fusion (Shay, Peters & Fuseler, 1978). Cytoplasts of one cell line were fused with whole cells from another. Both parental lines and most of the cybrid clones had only one MTOC per cell during interphase, but all the cells in one of the cybrid clones had 2 MTOC.

Many features of MTOC remain to be investigated. It would, for example, be interesting to discover whether the aggregation of MTOC found in Vero homosynkaryons is a general feature of multinucleate cells. Holmes & Choppin (1968) have described syncytia of BHK-21 cells in which the nuclei are aligned in rows by microtubules, and this might reflect a different distribution of MTOC. Our studies of cells cultured in cytochalasin B suggest that prolonged inhibition of nuclear division may affect the number of MTOC per cell. Finally, if centrioles are aggregated in multinucleate cells, this would provide further evidence for a relationship between centrioles and MTOC.

F.M.W. was a recipient of a Medical Research Council Studentship for training in research methods.

REFERENCES

Microtubule-organizing centres in fused cells


YAMANAKA, T. & OKADA, Y. (1968). Cultivation of fused cells resulting from treatment of cells with HVJ. II. Division of binucleated cells resulting from fusion of two KB cells by HVJ. Expl Cell Res. 49, 461–469.

(Received 11 February 1980)