Involvement of Active Cellular Mechanisms on the Disorganization of Oral Apparatus in Amicronucleate Cells in *Tetrahymena thermophila*

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**ABSTRACT.** Ciliated protozoa, a group of unicellular eukaryotes, have two kinds of nuclei, a macronucleus (somatic nucleus) and a micronucleus (germinal nucleus) in a single cell. We previously reported that amicronucleate cells of *Tetrahymena thermophila* induced by nocodazole gradually lost their oral apparatus (OA) and ciliary rows but that amacronucleate cells and empty cells did not. Since the macronucleus is responsible for the gene expression in the vegetative phase, the effects of actinomycin D and cycloheximide on the disorganization of the OA in amicronucleate cells induced by nocodazole were investigated. These inhibitors prevented the disorganization of the OA in amicronucleate cells. Amicronucleate cells did not grow even in the medium supplemented with high concentration of Fe, Cu and folinic acid which allow cells to grow without formation of food vacuoles. The results suggest that the macronucleus in the amicronucleate cells plays an active role in the induction of disorganization of the OA and malfunctions of nutrient uptake from the cell surface and/or in the fundamental cell division mechanisms, resulting in the death of amicronucleate cells.

The most remarkable aspect of ciliated protozoa is their nuclear dimorphism. *Tetrahymena thermophila*, like most ciliates, has a micronucleus and a macronucleus in a single cell. During the vegetative growth, the polygenomic macronucleus which is somatic, directs the phenotype of the cell, but the diploid micronucleus which is germinal, is believed to be genetically inert. However, functions of the micronucleus during the vegetative phase have been suggested in many ciliates such as *Paramecium, Euplotes* and *Stylonychia* (for a review see ref. 16). In many cases of these species, a cell which has no micronucleus (amicronucleate cell) can give rise to a cell line. In cells of the line, however, the oral apparatus (OA) becomes structurally abnormal (for a review see ref. 16).

In *T. thermophila*, the genic activity of the micronucleus in the vegetative growth has been detected neither by autoradiography (12) nor by a more sensitive approach using heterokaryons in which the macronucleus and micronucleus have different alleles at specific loci. The phenotypes of these heterokaryons cells always depend on the phenotype of the macronucleus and not of the micronucleus (1, 2, 4, 15). In spite of repeated attempts, however, artificial induction of an amicronucleate cell line in this species has not been successful.

Since loss of the micronucleus (the germinal nucleus) might be a serious problem for the preservation of a species, the micronucleus may have an essential role even in the vegetative phase. We recently reported that amicronucleate cells were induced artificially by treatment of dividing cells with an antitubulin drug, nocodazole (13). Main findings of previous study are as follows. Nocodazole inhibits the divisions and distributions of nuclei but as its block of cytokinesis is weak, it induces amicronucleate cells. The amicronucleate cells lost their OAs and their normal ciliary rows without cell division. We concluded that the vegetative micronucleus may play an essential role in the maintenance of the cortical structure, especially of the OA and of the ciliary rows. Nocodazole induces not only amicronucleate cells but also cells lacking a macronucleus and cells having no nuclei in which only a small fraction of the cells lacked the OA and ciliary rows. Irrespective of the fact that the macronucleus responsible for transcriptional activity was absent in amicronucleate or empty cells, delay in disintegration of the OA and ciliary rows was observed (13). These findings reported suggest that some active mechanisms are involved in the disorganization of the cortical structure.

We will report in this paper that disorganization of the OAs observed in the nocodazole-induced amicronu-
ucleate cells is mediated by an active process required for new RNA and protein synthesis. In addition, we discovered that in amicronucleate cells nutrient uptake systems may be defective, and that this defect is not caused by the disorganization of the OAs. Finally, we will discuss the similarity and the difference of apoptosis in the phenomena observed in the amicronucleate cells.

MATERIALS AND METHODS

Strains and growth conditions. The stocks used are derived from the inbred strain B of Tetrahymena thermophila. Wild-type strains used were B22942 and B22944. Cycloheximide-resistant mutant was ChB10 carrying ChxA2/ChxA2. Cells were grown axenically in PYG medium (2% proteose peptone (Difco), 1% yeast extract (Difco), 1% glucose) or enriched proteose peptone medium (EPP: 2% proteose peptone (Difco), 2 mM sodium citrate, 1 mM FeCl3, 30 μM CuSO4 and 1.7 μM folic acid, Ca salt (Sigma)) (20) at 26°C. In this EPP medium, cells were grown indefinitely without formation of food vacuoles.

Induction of the amicronucleate cells. To induce amicronucleate cells, an antitubulin drug nocodazole (methyl 5-2-thioxonylcarbonyl, 1-benzimidazole-2-yl carbamate; Sigma), was added to a dividing cell population as described previously (13). To concentrate amicronucleate cells, we modified the previous method of Haremaki et al. (13) as follows. Late-log phase cells were pelleted by hand centrifugation and an equal volume of fresh PYG medium was added to the cell pellet. This cell suspension was made as shallow as possible and kept at 26°C. Nocodazole (5 μg/ml in final concentration) was added to this cell suspension 60 min after the dilution. In this condition, almost no effects on cell divisions were observed after treatment with nocodazole, and amicronucleate cells were stabilized. Dividing cells began to appear about 1/10 minutes after the dilution, and at this time iron dextran particles (21) were added to this cell suspension. Since dividing cells cannot form food vacuoles containing iron dextran particles, they can swim freely under a magnetic force. After incubation with the iron dextran particles for 10 min, the cell suspension was subjected to a magnetic force to remove cells which formed food vacuoles. The division index after the selection was 70-80%. Then, the cell suspension. Since dividing cells cannot form food vacuoles containing iron dextran particles, they can swim freely under a magnetic force. After incubation with the iron dextran particles for 10 min, the cell suspension was subjected to a magnetic force to remove cells which formed food vacuoles. The division index after the selection was 70-80%.

Induction of the amicronucleate cells. To determine whether the process of disorganization of the OA in the amicronucleate cells involves some metabolic activity, cells treated with nocodazole were incubated at various temperatures. Samples were obtained at 7 and 24 h after removal of nocodazole, and the OAs of amicronucleate cells were observed. Disorganization of the OA in the amicronucleate cells was brought about when nocodazole treated cells were kept at 25, 30 and 35°C, while the disorganization was prevented when kept at 10 and 15°C (Fig. 2). This result suggests that the process of disorganization of the OA involves some metabolic activi-
Active killing events in *Tetrahymena* amicronucleate cells.

**Fig. 1.** Photomicrographs of nocodazole treated cells. Cells were fixed with 3% formalin and stained with Giemsa. A. Normal cell (with a micronucleus (mic) and a macronucleus (mac)). B. Amicronucleate cell (with a mac but lacking the mic). C. Amacronucleate cell (with a mic but lacking the mac). D. Empty cell (with no nuclei). Normal, amacronucleate and empty cells have the oral apparatus (OA) and ciliary rows (CR) but amicronucleate cell lacks them. Bar indicates 10 μm.
Table I. LOSS OF THE ORAL APPARATUS (OA) IN FOUR TYPES OF CELLS AFTER TREATMENT WITH NOCODAZOLE.

<table>
<thead>
<tr>
<th>Types of cells</th>
<th>Normal</th>
<th>Amic</th>
<th>Amac</th>
<th>Empty</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cells observed</td>
<td>2552</td>
<td>69</td>
<td>42</td>
<td>18</td>
</tr>
<tr>
<td>No. of cells lacking OA (%)</td>
<td>0 (0)</td>
<td>68 (98.6)</td>
<td>2 (4.8)</td>
<td>2 (11.1)</td>
</tr>
</tbody>
</table>

Cells were treated with nocodazole for one h and washed with NKC solution. Samples were observed 24 h after the end of nocodazole treatment.

* Normal indicates a cell having a micronucleus (mic) and a macronucleus (mac). Amic indicates a cell having a mac but lacking the mic. Amac indicates a cell having a mic but lacking the mac. Empty indicates a cell having no nuclei.

* Number in parentheses shows percentages of the OA lacking cells.

Effect of inhibitors on disorganization of the OA in amicronucleate cells. In terms of the metabolic activities, since neither the amicronucleate cells nor empty cells have the macronucleus and at the same time hardly lose their OAs (Table I), the macronucleus in the amicronucleate cells may have a positive role in the disorganization of the OA and ciliary rows. To determine whether transcription or translation activity of macronucleus is necessary for the disorganization of the OA, effects of inhibitors of RNA and protein synthesis on this process were examined. Cells treated with nocodazole were incubated in NKC solution containing various concentrations of actinomycin D and cycloheximide (Fig. 3). Twenty-four hours after the incubation amicronucleate cells were observed. Disorganization of the OA in about 80% of the amicronucleate cells was inhibited by treatment with 10 μg/ml of actinomycin D and 2 μg/ml of cycloheximide. Although cells were not healthy in more than 25 μg/ml of actinomycin D or 5 μg/ml of cycloheximide, no apparent effects were observed on their OAs and ciliary rows in the micronucleate, amicronucleate and empty cells. To determine whether there is a sensitive period for the inhibitors to act on the disorganization of the OA in the amicronucleate cells, cells were washed with fresh NKC solution 6 and 12 h from the beginning of the inhibitor treatment. Samples were obtained at 2 h intervals and fixed and the OAs of the

Fig. 2. Disorganization of the OA in amicronucleate cells after incubation at various temperatures. Cells were treated with nocodazole and transferred to NKC solution and incubated at 10, 15, 20, 25, 30 and 35°C. Samples were obtained 7 (a) and 24 (b) h after incubation. Vertical bars indicate 95% confidence limits for percentages.

Fig. 3. Effects of actinomycin D and cycloheximide on disorganization of the OA in amicronucleate cells. Cells were treated with nocodazole and transferred to NKC solution. Just after the transfer, cells were exposed to various concentrations of actinomycin D (A) or cycloheximide (B). Samples were obtained 24 h after the transfer to NKC solution. Horizontal axis; concentration of inhibitors (μg/ml). Vertical axis; the percentage of amicronucleate cells lacking the OA. Vertical bars indicate 95% confidence limits for percentages.
Amicronucleate cells were observed. During exposure to inhibitors, amicronucleate cells did not lose their OAs, but, soon after the end of the exposure to actinomycin D and cycloheximide, the amicronucleate cells began to lose the OAs (Fig. 4). No sensitive period of the inhibitors on the disorganization of the OA in the amicronucleate cells was observed. Furthermore, to determine when the disorganization of the OA in the amicronucleate cells is initiated, the treatment with inhibitors was started at various points after the end of the nocodazole treatment (Fig. 5). In the cycloheximide treatment, the percentage of amicronucleate cells lacking the OA 24 h after the end of the nocodazole treatment did not increase comparing with the samples when the cycloheximide was added. This result suggests that the cycloheximide prevents the disorganization of the OA in the amicronucleate cells immediately after the addition of the inhibitor. In contrast to the case of cycloheximide, the treatment with actinomycin D induced a little more disorganization of the OA in the amicronucleate cells than the samples when the treatment was started. This may suggest that the transcription for the disorganization of the OA in the amicronucleate cells is initiated about 1–2 hours prior to the appearance of the OA-lacking cells.

A cycloheximide-resistant mutant (strain ChB10) was also examined. When the amicronucleate cells of ChB10 induced with nocodazole were treated with 5 μg/ml of cycloheximide, they lost their OAs (the control in Fig. 6). But the disorganization of the OAs was blocked by the treatment with actinomycin D. This result shows that the block of disorganization of the OA with cycloheximide is not due to nonspecific effects of cycloheximide but that it is due to the inhibition of translation because the cycloheximide-resistant mutant used here is known to have a mutational site in the ribosome systems (3, 19).

Growth of the amicronucleate cells in EPP medium. Usually, cells of *Tetrahymena* take up nutrients from the OA into the food vacuoles, and the formation of the food vacuoles is stimulated by particulate nutrients. So, if the OA becomes defective, cells would be unable to proliferate due to starvation. To determine whether

**Fig. 4.** Increase of amicronucleate cells lacking the OA after removal of actinomycin D and cycloheximide. Cells were treated with nocodazole for 1 h and transferred to NKC solution. After the transfer to NKC solution, cells were exposed to 10 μg/ml of actinomycin D or 5 μg/ml of cycloheximide for 6 h (□) and 12 h (■) or continuously for 28 h (x; only shown at 28 h) and then washed with NKC solution to remove these drugs (arrows). Samples were obtained at 2 h intervals after the end of the nocodazole treatment. The control was obtained from nocodazole treated cells without exposure to actinomycin D or cycloheximide. Horizontal axis; time (h) after the end of the nocodazole treatment. Vertical axis; the percentage of amicronucleate cells lacking the OA. Vertical bars indicate 95% confidence limits for percentages.

**Fig. 5.** Loss of the OA in amicronucleate cells when treatment with actinomycin D and cycloheximide was started at various times after the transfer to NKC solution. Cells were treated with nocodazole for 1 h and transferred to NKC solution. After the transfer to NKC solution, the treatment with 10 μg/ml of actinomycin D (□) or 5 μg/ml of cycloheximide (■) was started. The percentages of amicronucleate cells lacking the OA 24 h after the end of the nocodazole treatments are plotted to the start time (h) of the treatment with each inhibitor. At each time the percentage of amicronucleate cells lacking the OA in the samples is shown as +. Vertical bars indicate 95% confidence limits for percentages.
Fig. 6. Effects of inhibitors on the formation of amicronucleate cells lacking the OA in cycloheximide-resistant mutant. Cycloheximide-resistant cells (CUb10) were treated with nocodazole and transferred to NKC solution. Just after the transfer, cells were exposed to 5 µg/ml of cycloheximide or 10 µg/ml of actinomycin D. Samples were obtained at 24 h after transfer to NKC solution. The control was obtained from nocodazole treated mutant cells without exposure to actinomycin D or cycloheximide. Vertical axis; the percentage of amicronucleate cells lacking the OA. Vertical bars indicate 95% confidence limits for percentages.

The nocodazol-induced amicronucleate cells’ inability to grow due to starvation brought about by the defective OA, the nocodazole-treated cells were cultured in EPP medium (containing high concentration of Fe, Cu and folinic acid, see Materials and Methods). In the EPP medium, cells can take up nutrients through cell surfaces without forming food vacuoles (17, 20). If the amicronucleate cells cannot grow because of the inability of nutrient uptake through the defective OA, they should be able to grow in the EPP medium. In the experiment, dividing dumbbell-shaped cells in nocodazole-containing PYG medium were isolated in a drop of EPP medium without nocodazole. Within 30 min, divided cells were reisolated in a drop of fresh EPP medium and kept for 48 h at 25°C. The results are shown in Table II. Among 136 clones, two types of cell proliferation were observed. In one type, a cell shows multiple cell divisions (‘multiple dividers’, producing more than 16 cells) but the other shows only a few divisions (‘few dividers’ producing less than 6 cells, mostly 3). All of the few dividers were the amicronucleate cells lacking their OAs and with disintegrated ciliary rows. On the other hand, all cells in the multiple dividers were quite normal, having both the macro- and micronuclei, and normal OAs and ciliary rows. These features were not significantly different from those of the cells isolated in the PYG medium. Thus, the amicronucleate cells were unable to proliferate even in the EPP medium.

These results suggest that some metabolic changes brought about by the amicronucleate conditions not only destroys the OA but also the surface nutrient uptake system and/or some mechanisms in cell division.

DNA-strand breaks were not detected in the amicronucleate cells. The results so far obtained seem to show that when a Tetrahymena cell loses the micronucleus, that is the germinal nucleus, the cell triggers a process to kill itself and that this process is controlled by the macronucleus. These features are something like the apoptotic processes. The most common hallmark of apoptosis is a DNA-cleavage into oligonucleosomal fragments (for a review see ref. 14). In T. thermophila, the old macronucleus is eliminated during conjugation. It is reported that this elimination process is accompanied by the degradation of the old macronuclear DNA into oligonucleosome-sized fragments (6). To determine whether the DNA fragmentation occurs in the macronucleus of the amicronucleate cells as in the old macronuclear DNA during conjugation, we employed the TUNEL technique which detects DNA-strand breaks (11). Amicronucleate cells induced by nocodazole were fixed 24 h after the nocodazole treatment and analyzed by the TUNEL technique. As a positive control, conjugating cells having pycnotic old macronucleus in the late conjugating stage were also analyzed. Although signals were detected in the old macronucleus in conjugating cells, no signals of the DNA-cleavage were detected, however, in the macronucleus of the amicronucleate cells which lost their OAs and normal ciliary rows (data not shown). Thus, no DNA-strand breaks were found to occur in the macronucleus of the amicronucleate cells.

**DISCUSSION**

Nocodazole treatment induces not only amicronucle-
Active killing events in *Tetrahymena* amicronucleate cells.

...ate cells but also amacronucleate cells and empty cells. The amicronucleate cells lose their OA immediately but amacronucleate cells and empty cells hardly lose their OAs (13, Table 1). The results obtained in this study strongly suggests that the cells are killed actively by the macronucleus when the cell loses its micronucleus. We think that the amicronucleate cells are thus excluded from the population by active cellular mechanisms controlled by the macronucleus. In the following discussion we will explain from what evidence the above conclusion is derived.

Exposure to low temperature prevented the process of disorganization of the cortical structure in amicronucleate cells (Fig. 2). Low temperature naturally suppresses cellular metabolisms, which suggests that the process of cortical disorganization requires some type of metabolic activity. The process was also prevented by treatment with actinomycin D and cycloheximide (Fig. 3, 4, 5). During exposure to either actinomycin D or cycloheximide, disorganization of the cortical structure in amicronucleate cells was prevented but soon after removal of these drugs, their cortical structure began to be disorganized. If the cells were seriously damaged by these inhibitors, re-initiation of the disorganization process would not be observed. Our results showed that the effects of these inhibitors were not simple toxic effects of chemicals. Furthermore, in the amicronucleate cells induced in cycloheximide-resistant mutant their cortical structure was disorganized even by the treatment with cycloheximide, but the disorganization in this mutant was prevented by actinomycin D (Fig. 6). These results confirm that the effects of the inhibitors were not nonspecific chemical effects but inhibition of transcription and translation. Thus, our results show that the cortical disorganization process involves some active mechanisms which require transcription and translation. As to what kinds of regulation of gene expression are at work here, since the process began to work after the loss of the micronucleus, the mechanism may be triggered by the loss of it. The mechanism began to work even after 12 h of inhibition. This suggests that there is no time restriction for the mechanism to be initiated within 12 h after the loss of the micronucleus. Once the mechanism was triggered, it may continue to work. The mode of prevention of disorganization of the cortical structure by inhibitors is different between actinomycin D and cycloheximide. Cycloheximide can prevent it at any time during the process. In most of the cells treated with actinomycin D, however, the results were the alternatives of either having normal OA or complete disappearance of the OA (data not shown). This suggests that both RNA and protein synthesis are required for the initiation of the mechanism, and that the RNA is not short-lived. Continuous synthesis of new protein is required for the process to continue.

There are many reports on the effects of inhibitors of RNA, protein synthesis and metabolism on the cortical development in *Tetrahymena* (8, 9, 10), *Paramecium* (9, 18) or *Stentor* (5, 9). They suggested that de novo syntheses of protein and RNA are required for the cortical development. Our results showed that they were also necessary for the process of disorganization of the cortical structure in the amicronucleate cells.

There are dual ways of nutrient uptake in *Tetrahymena*. One is the oral uptake system and the other is the surface uptake system. Studies of temperature-sensitive mutants of the development of the OA showed that in the medium supplemented with high concentration of iron, copper and folic acid cells with non-functional OAs can grow without formation of food vacuoles (17, 20). Our results showed that the amicronucleate cells did not grow even in EPP medium which can sustain cells with defective OAs (Table 2). There are two possible explanations. First, not only the oral nutrient uptake system but also the other system, the surface nutrient uptake system, cannot work. Second, the surface nutrient uptake system is working but the fundamental cell division system does not work. The fact that significant increments of cell volume were not detected in the amicronucleate cells (data not shown) seems to favor the first possibility.

The amicronucleate cells lose their OAs and thus are prevented from proliferation and eventually die out in a week or two. This phenomenon is much similar to programmed cell death or apoptosis, because it would be purposeful for the cell population and also it needs gene expression. When the cell loses its micronucleus, the death program begins to work. The most dramatic features of the apoptotic process are condensation of the nucleus, degradation of the chromatin and pronounced blebbing of the plasma membrane. Degradation of the chromatin is usually detected as DNA-strand breaks by the TUNEL technique. Though the DNA-strand breaks were not detected by the TUNEL technique in the macronucleus of the amicronucleate cells lacking their OAs and abnormal ciliary rows, we cannot conclude that the process is not apoptosis, because not all apoptotic processes require DNA-strand breaks. Recently, evidence has shown that the central components of apoptosis are localized in the cytosol rather than in the nucleus and suggest that protease is a good candidate of the component (14). It will be interesting to determine whether certain proteases are activated in the amicronucleate cells. This problem remains for further study.

The role of the micronucleus in the vegetative phase is of interest and importance in the context of the evolution of nuclear dimorphism of ciliates, because data on the role of the micronucleus in the vegetative phase should lead to an understanding of how nuclear dimorphism was established. If the cells do not have a mecha-
nism to exclude the presence of amicronucleate cells, the population may eventually become amicronucleate, because for only proliferation, cells with directly dividing macronucleus seem more advantageous than those with both directly dividing macronucleus and mitotically-dividing micronucleus. However, since amicronucleate cells cannot perform genetic recombinations, they would eventually perish. For this reason, the positive mechanism to exclude amicronucleate cells reported in this paper is advantageous for the cell population and thus must lead to the establishment of nuclear dimorphism.

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