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Morphological apoptotic characteristics of the post-meiotic micronuclei in *Paramecium caudatum*

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Abstract

In a previous study, the apoptotic degeneration of meiotic products outside the paroral region of *Paramecium caudatum* was indirectly demonstrated by means of "apofluor" staining. In this experiment, conjugating pairs and exconjugants of *P. caudatum* were stained with either "apofluor" or carbol fuchsin or both to find some direct evidence to demonstrate the apoptotic characteristics of this process. As a result, asynchronous meiotic nuclear degeneration was observed. Furthermore, a number of additional meiotic nuclei were found. Disintegrating/dividing meiotic nuclei outside the paroral region were observed, which might be the origin of these additional meiotic nuclei. Condensed chromatin and disintegrated chromatin attached to the nuclear membrane were also observed in degenerating nuclei, which are the typical morphological characteristics of apoptosis. Comparison of the cells stained by the above two methods indicated that "apofluor"-stained meiotic nuclei could not be detected by carbol fuchsin in some cells, which suggests a time lag between meiotic nuclear DNA degradation and their eventual disappearance. In this study, some direct evidence was found to show that the meiotic nuclear degeneration in *P. caudatum* is of apoptotic nature, which further confirmed our previous study (Yang et al. 2007) and indicated that morphological apoptotic characteristics discovered in multicellular organisms do exist in unicellular eukaryotic ciliate protozoa.
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Keywords: *Paramecium*; Meiotic nuclear degeneration; Apoptosis

Introduction

Paramecium caudatum, a unicellular eukaryotic ciliate protozoan, has a micronucleus and a macronucleus. The former is small, diploid, transcriptionally inactive, and functions as a germinal nucleus, while the latter is large, polygenomic, transcriptionally active, and functions as a somatic nucleus. Although they differ in size, ploidy and function, both of them are derived from the synkaryon division (Fig. 1). During the conjugation of *P. caudatum*, the micronucleus undergoes meiosis. Four haploid nuclei are formed. Only one of those entering the paroral region survives and divides once more to form two gametic nuclei. The remaining three degenerate. After reciprocal exchange of gametic nuclei, a synkaryon forms which divides three times successively (Calkins and Cull 1907; Wichterman 1986). At the telophase of the 3rd synkaryon division, four of the division

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products are located in the posterior region of the cell, which all develop into macronuclear anlagen (new macronuclei) later. The other four are in the anterior region, called presumptive micronuclei, among which only one is selected as the germinal nucleus for new generation. The remaining three degenerate (Mikami 1980, 1982; Wichterman 1986). By two consecutive cell divisions after conjugation, four macronuclear anlagen are distributed into four new cells during which the selected micronucleus undergoes mitosis. As a result, new paramecia with one macronucleus and one micronucleus are formed (Wichterman 1986; Yang and Takahashi 2000). In case of maternal macronuclei, they break into several ten pieces soon after the 3rd synkaryon division. After conjugation they degenerate undergoing four to five fissions (Kimura et al. 2004; Wichterman 1986). Therefore, during the conjugation of *P. caudatum*, there are two times of nuclear selection/degeneration. The nuclei are either selected and survive or they degenerate. By nuclear elimination experiments and morphological studies, it has been suggested that nuclear selection and degeneration are two independent processes in the above two cases (Taka et al. 2006; Yanagi 1987; Yang and Takahashi 2000). By staining with ‘‘apofluor’’, a combination of two vital fluorescent dyes containing acridine orange (AO) and Hoechst 33342 (HO) (Santos et al. 2000), the degeneration process of meiotic nuclei in *P. caudatum* is demonstrated to be apoptotic (Yang et al. 2007), as what happens in *Tetrahymena* (Santos et al. 2000). Based on the observation of degenerating nuclei in exconjugants at the 3rd telophase of synkaryon division, it was concluded that meiotic nuclear degeneration is also a long process (Yang et al. 2007). The details of the meiotic nuclear degeneration remained unclear, particularly the question whether any morphological apoptotic characteristics discovered in multicellular organisms exist in unicellular ciliates. In this experiment, we focused on the details of meiotic nuclear degeneration. The conjugating pairs and the exconjugants of *P. caudatum* were stained mainly by ‘‘apofluor’’. As mentioned above, ‘‘apofluor’’ is an excellent staining technique to detect apoptotic cells/nuclei, but it is not apoptosis-specific. ‘‘Apofluor’’ can stain other structures such as food vacuoles (Yang et al. 2007), because its mechanism to detect apoptotic structures is based on the change of pH which occurs after the prospective degenerating meiotic nuclei were recognized by lysosomes and fused with them (Santos et al. 2000). Therefore, carbol fuchsin (Carr and Walker 1961), a nice nuclear staining solution was also adopted in the current study to detect nuclei. ‘‘Apofluor’’-stained cells were stained by carbol fuchsin solution consecutively in some cases in this study to confirm that the ‘‘apofluor’’-stained structures were truly nuclei (Yang et al. 2008a). We observed some interesting phenomena concerning apoptosis, such as disintegration of degenerating nuclei, which might be a similar process of the formation of apoptotic bodies, chromatin condensation, and small chromatin particles attached to the nuclear membrane. All these features are typically apoptotic characteristics of multicellular organisms (Kerr et al. 1972). This study provides direct evidence to show that meiotic nuclei degenerate in an apoptotic way in *P. caudatum*.

Material and Methods

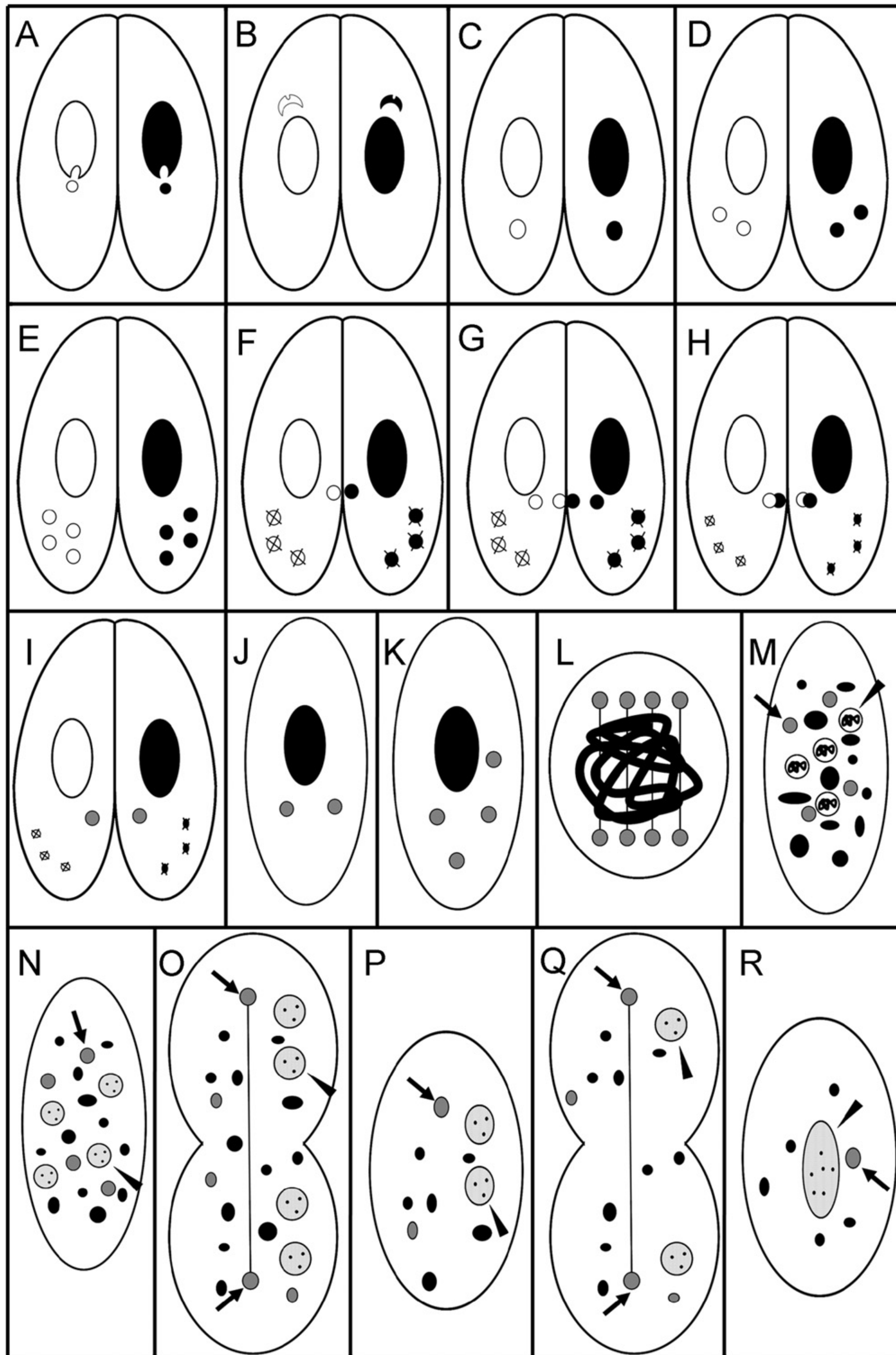
Chemicals and stock solutions

Acridine orange (AO) was purchased from Shanghai Chemical Reagent Company (China), and Hoechst 33342 (HO) from Beyotime Institute of Biotechnology (Haimen, Jiangsu, China). The other chemicals were from Hangzhou Dafang Chemical Reagent Inc (China). Preparations of stock solutions for ‘‘apofluor’’ and carbol fuchsin solution were according to previous descriptions (Carr and Walker 1961; Yang and Takahashi 1999; Yang et al. 2007).

Fig. 1. Schematic representation of major nuclear events during the conjugation of *Paramecium caudatum*. A. Shortly after the formation of a conjugating pair. Micronuclei have left the concavity of the macronuclei. B. Crescent stage of the 1st meiotic prophase. C. The 1st meiotic metaphase. D. Two nuclei formed after the 1st meiotic division. E. Four meiotic nuclei formed after the 2nd meiotic division. F. One meiotic product enters the paroral region, and the remaining three degenerate (crosses). G. The nuclei that entered the paroral region divide mitotically to form two gametic nuclei (a migratory pronucleus and a stationary pronucleus) in both conjugants (The 3rd prezygotic nuclear division), three prospective degenerating meiotic nuclei still remained (crosses). H. Reciprocal exchange of migratory pronuclei. I. Synkaryon formation. J. After the 1st synkaryon division. K. After the 2nd synkaryon division. L. 3rd synkaryon division. At this stage, cells become rounder and shorter. Four nuclei are located in the posterior region, and the other four in the anterior region of the cell. The old macronuclei show a thick skein form. M. Nuclear differentiation. Arrowhead: macronuclear anlagen (MA) having aggregated chromatin; arrow: spherical presumptive micronuclei. There are many old macronuclear fragments. N. Development of MA. Arrowhead: more developed macronuclear anlagen; arrow: presumptive micronuclei. O. The 1st exconjugant division. Four MA are going to be distributed into two daughter cells, a selected micronucleus is dividing, three unselected presumptive micronuclei still remained. P. After the 1st exconjugant division. Q. 2nd exconjugant division. Two MA are going to be distributed into two daughter cells, a micronucleus is dividing. R. After the 2nd exconjugant division. New paramecia with 1 macronucleus and 1 micronucleus are formed. There are still some old macronuclear fragments left.

Cell culture and induction of conjugation

Two complementary mating types of *P. caudatum* collected from East Lake Campus of Zhejiang Forestry University (China) were cultivated in 2.5% fresh lettuce juice diluted with K-DS, in which *Klebsiella pneumoniae* was inoculated one day before use (Dryl 1959; Hiwatashi 1968). Conjugation was induced by mixing highly reactive cells of complementary mating types (Hiwatashi 1968), and better synchrony in conjugation was obtained by addition of iron-dextran particles (Sun et al. 2010; Vosskühler and Tiedtke 1993; Yang and Takahashi 1999). All the experiments were performed at room temperature (∼25 °C).



Staining, immobilization, observations, and photographing

"Apofluor" staining was performed by adding 10 μl each of AO and HO stock solutions to 480 μl of cell suspension (final concentration: 0.02 μg/ml of AO; 0.2 μg/ml of HO) 10 min or more before observations (Yang et al. 2007). To slow down the cell movements, cells were deciliated with 5% ethanol (final concentration) (Ogura 1981). After 3–5 min, most cells were deciliated and sedimented on the bottom of the depression slide, when the upper part of the solution was sucked out and replaced by fresh K-DS (Yang et al. 2008b). Temporary preparations were then made by the "volume-fixing" method. Ten μl of "apofluor"-stained deciliated cell suspension was transferred to the center of a piece of slide glass. Then the cell suspension was covered with a $20 \times 20\ mm^2$ glass coverslip. About 40% of the cells on the preparations were squashed and moved slowly, which were easy to observe and photograph (Lin et al. 2009; Yang et al. 2008b). In some cases, cells were stained by "apofluor" and carbol fuchsin solution consecutively (Yang et al. 2008a). After the observation and photographing, "apofluor"-stained cells were stained a second time by adding a drop of carbol fuchsin solution on one side of the coverslip and retaining 3–5 min. Afterwards they were observed and photographed under bright field microscopy. All observations and photographing were done using a Nikon 50*i* fluorescence microscope.

Results

In *P. caudatum*, four haploid nuclei are formed after meiosis. Only one of those entering the paroral region survives and divides once more to form gametic nuclei. The remaining three degenerate (Fig. 1a–f) (Calkins and Cull 1907; Wichterman 1986). By "apofluor" staining, the degeneration process of these meiotic nuclei has been indirectly demonstrated to be of apoptotic nature (compare Fig. 2A, E) (Yang et al. 2007).

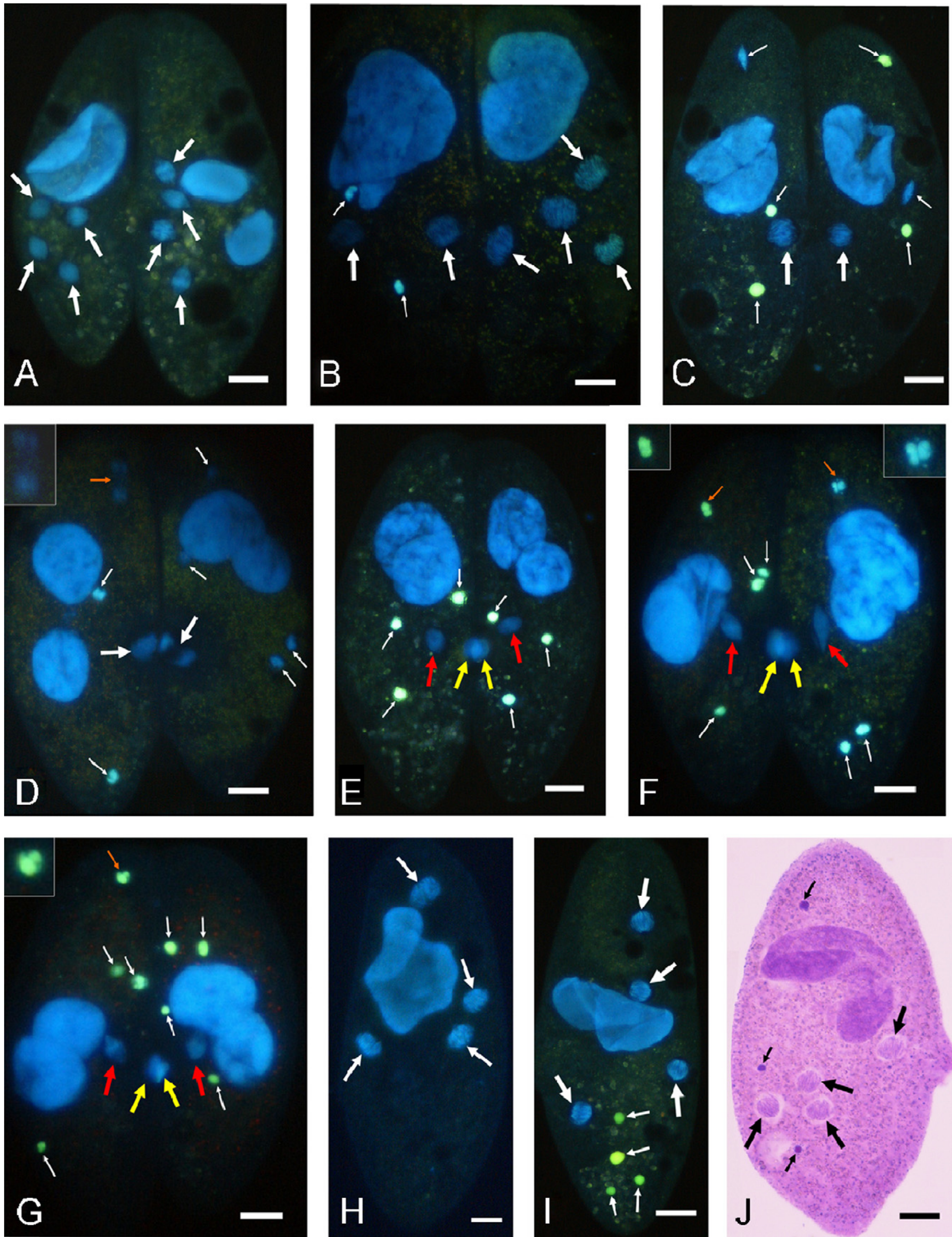
Asynchronous degeneration of meiotic nuclei

Although it has been described that three meiotic products outside the paroral region degenerate, there is no clear description whether all three degenerate synchronously. By "apofluor" staining, asynchronous degeneration of meiotic products was observed in both conjugating pairs and exconjugants (compare Fig. 2E, B, C, and H, I, J). As shown in Fig. 2B, the left cell has two pycnotic meiotic nuclei, which are stained yellow-green; the right cell has four meiotic products showing similar morphology and being stained blue in spite of their locations in the cell. In the conjugating pair shown in Fig. 2C, both cells have three pycnotic nuclei, two of which are stained yellow, the remaining one stained blue-green. Among 60 conjugating pairs, a total of 120 conjugants observed, the number of cells with asynchronously degenerating nuclei was 86. Asynchronous degeneration between two mating partners was observed in 40 pairs. In exconjugants at the metaphase of the 3rd synkaryon division, pycnotic nuclei completely disappeared in some cells (Fig. 2H), while some pycnotic nuclei still existed in other cells (Fig. 2I, J). These results indicated that not only three meiotic nuclei in one cell, but also the meiotic nuclei in two partner cells of one conjugating pair degenerate asynchronously. In short, the degeneration process of meiotic nuclei happens asynchronously both in their initiation and accomplishment.

Additional degenerating meiotic nuclei

Not only asynchronous meiotic nuclear degeneration, but also a number of additional meiotic nuclei were

Fig. 2. Asynchronous degeneration and additional prospectively degenerating meiotic nuclei in *Paramecium caudatum*. Except the cell in J stained with carbol fuchsin solution, all other cells were stained with "apofluor". A. A conjugating pair soon after the 2nd meiotic division. None of the four haploid meiotic nuclei entered the paroral region and all were stained blue (thick arrows). B. Four meiotic nuclei with similar morphology were stained blue in the right cell (thick arrows), while two of the four nuclei were pycnotic and stained blue-green in the left cell (thin arrows), the remaining two were stained blue. C. Conjugating pairs soon after the 2nd meiotic division. Two of the three prospectively degenerating meiotic nuclei in each cell (thin arrows) were stained yellow, the remaining one blue-green. D. A conjugating pair at the stage of the 3rd prezygotic division. Four prospective degenerating meiotic nuclei in the right cell and three in the left one were observed. One of them in the left cell was dividing (orange arrow), which was magnified in the framed area. E. A conjugating pair soon after the 3rd prezygotic division. Three prospective degenerating meiotic nuclei in each cell were stained yellow (white thin arrows), while the stationary pronuclei (red arrows) and the migratory pronuclei (yellow arrows) were stained blue. Two migratory pronuclei partially overlapped. F & G: Conjugating pairs soon after the 3rd prezygotic division. A number of additional prospectively degenerating nuclei (thin arrows) were observed, and some of them were disintegrating (orange arrows). The framed portions at the corners in each picture show the magnified disintegrating nuclei. H–J. Exconjugants at the metaphase of the 3rd postzygotic division. H. Four synkaryon division products were stained blue, but no prospectively degenerating nuclei had remained. I. Except four metaphase synkaryon division products (thick arrows), there were four yellow-stained structures (thin arrows). J. Four metaphase synkaryon division products were stained pink (thick arrows), and three degenerating nuclei were stained dark-pink (thin arrows). Bars = 20 μm.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observed during the examination of several conjugating pairs and exconjugants (compare Fig. 2E and F, G). In "apofluor"-stained cells, 46 out of 114 around the synkaryon stage were with more than three degenerating meiotic nuclei. In the case of carbol fuchsin solution staining, 25 out of 76 were with more degenerating nuclei. In some cells, degenerating meiotic nuclei were breaking (Fig. 2F, G) or dividing (Fig. 2D). Therefore, it appears that cells with additional degenerating meiotic nuclei were derived from the fragmentation and division of the original meiotic nuclei.

A time lag between meiotic nuclear disappearance and their DNA degradation

As described in our previous study (Yang et al. 2007), meiotic degenerating nuclei could be observed in exconjugants even at the 3rd telophase of synkaryon division. To clarify whether any DNA still remained in these degenerating nuclei, the exconjugants around the telophase of the 3rd synkaryon division were stained by "apofluor" firstly. Then the same cells were stained by carbol fuchsin solution (Yang et al. 2008a). The observations showed that the exconjugants could be grouped into three types (Fig. 3). One is that the "apofluor"-stained meiotic nuclei were also stained by carbol fuchsin solution (compare Fig. 3A, A′). The apoptotic nuclei indicated by carbol fuchsin (Fig. 3A′) were smaller than those indicated by "apoflour" staining (Fig. 3A). The chromatin in apoptotic nuclei was more pycnotic than the normally functioning nuclei including synkaryon division products and skein-formed maternal macronucleus, which indicated the chromatin condensation in apoptotic nuclei (Fig. 3A′). Secondly, the "apofluor"-stained nuclei were detectable by carbol fuchsin solution (Compare Fig. 3B, B′). Some chromatin granules distributed peripherally were observed (Fig. 3B′), which indicated chromatin disintegration and attachment to the nuclear membrane. In the third case, the "apofluor"-stained meiotic nuclei could not be detected by carbol fuchsin solution (compare Fig. 3C, C′). These results indicated that some of the "apofluor"-stained nuclei were merely membrane-bound vesicles without any detectable chromatin in them. This suggests that the disappearance of the apoptotic nuclei was later than the disintegration of their DNA, indicating a time lag between these two events, which is also consistent with the suggestion of asynchronous degeneration of apoptotic meiotic nuclei.

Discussion

By "apofluor" and carbol fuchsin solution staining, asynchronous degeneration with additional degenerating meiotic nuclei, and a time lag between meiotic nuclear disappearance and their inner DNA disintegration were found in *P. caudatum* (Figs. 2, 3). Not only in one cell, but also in the two partner cells of one conjugating pair, three meiotic nuclei degenerated asynchronously (Fig. 2B, C, H–J). The disintegrating or dividing meiotic products outside the paroral region were observed (Fig. 2D, F, G), both of which might be the origin of additional meiotic nuclei. Also in some of the cells, membrane-bound vesicles were observed either with condensed chromatin (Fig. 3A, A′), or without centre-located chromatin but with small disintegrated chromatin particles attached to the nuclear membrane (Fig. 3B, B′), or without any detectable chromatin (Fig. 3C, C′).

In the experiment of eliminating the prospective surviving nuclei at paroral stage, some of the operated cells developed into micronucleate cells; the remaining ones did not (Yanagi 1987). This result might indicate the different potential of the prospective degenerating meiotic nuclei to replace the removed prospective surviving nuclei in different cells. In other words, some irreversible changes in the prospective degenerating meiotic nuclei might happen asynchronously in different cells, which is consistent with our suggestion of asynchronous meiotic nuclear degeneration in the current study. In fact, asynchronous nuclear degeneration also happens during the selection of germinal nuclei in exconjugants of *P. caudatum* (Yang and Takahashi 2000; Taka et al. 2006). Usually, four anterior nuclei at the telophase of the 3rd synkaryon division called presumptive micronuclei, retain the germinal nuclear characteristics at the beginning, while only one is eventually selected as the germinal nucleus for new generation. The remaining three degenerate (Mikami 1982; Yang and Takahashi 2000). In most of the exconjugants of *P. caudatum*, there are one to four detectable presumptive micronuclei instead of a constant number of four (Yang and Takahashi 2000). In the experiment of eliminating the selected presumptive micronuclei, some of the operated cells developed into micronucleate cells, the remaining ones developed into amicronucleates (Taka et al. 2006). The degeneration of the extra-presumptive micronuclei seemed to be quite similar with meiotic nuclear degeneration from the aspect of asynchronous degeneration. This asynchronous degeneration might have a special biological significance to increase the frequencies to produce more offspring with germinal nuclei, who have the ability to undergo meiosis and produce new generations. Except micronuclear degeneration, maternal macronuclear degeneration also exists in *P. caudatum*, which delay till four to five fissions after conjugation. This delay was considered to be functioning during the new macronuclear maturation (Kimura et al., 2004).

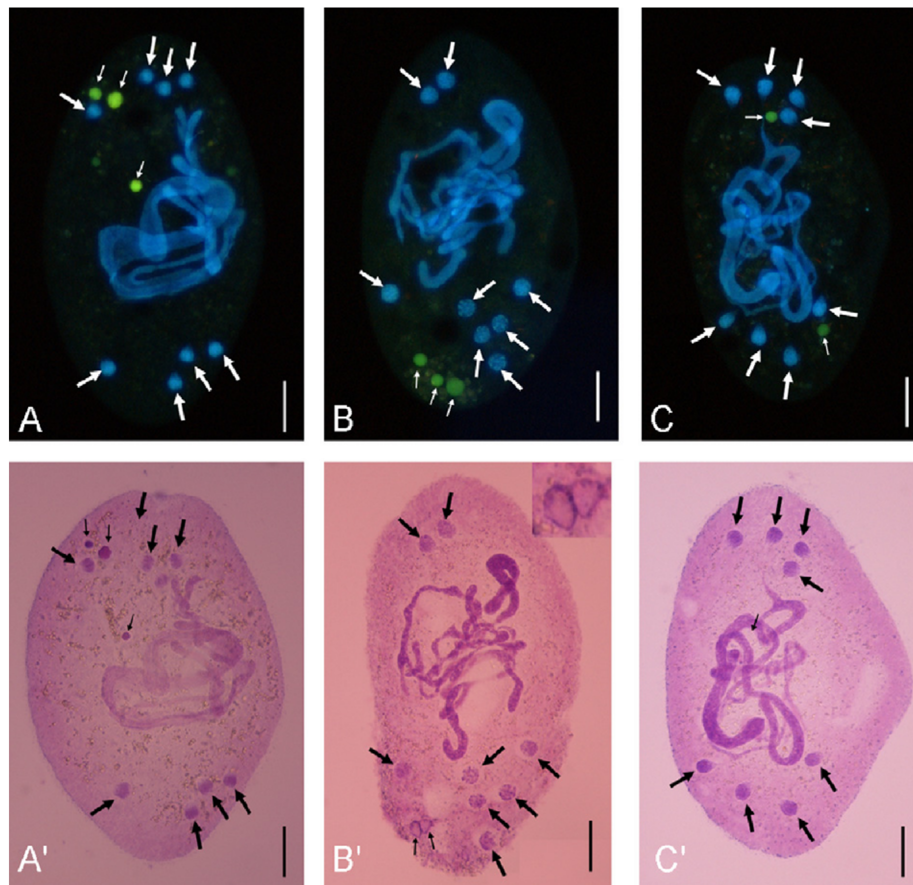

Fig. 3. Behaviour comparison of the prospective meiotic nuclei in exconjugants of *P. caudatum* stained by two different nuclear stains, "apofluor" and carbol fuchsin. A, B, C. "Apofluor" staining. A′, B′, C′. Carbol fuchsin solution staining. A, B, C are the same cells of A′, B′, C′, respectively. The cells were at the 3rd telophase of synkaryon division (A, C) or soon after nuclear determination (B). Thick arrows: eight synkaryon division products. Thin arrows: prospective degenerating meiotic nuclei. A, A': "apofluor"-stained degenerating nuclei were also stained by carbol fuchsin; B, B′: two out of three "apofluor"-stained degenerating nuclei were detectable by carbol fuchsin, whose chromatin was disintegrated into many particles and attached to the nuclear membrane, the remaining one was undetectable; C, C′: 2 "apofluor"-stained degenerating nuclei could not be detected by carbol fuchsin. Bars = 20 μm.

With regard to the number of additional degenerating meiotic nuclei, condensed chromatin, disintegrated chromatin particles attached to the nuclear membrane observed in *P. caudatum*, it can be stated that all features fit the characteristics of apoptosis, which occur in multicellular organisms (Kerr et al. 1972). In fact, apoptotic cells are fragmented into membrane-bound small particles, the so-called apoptotic bodies at the late stage of apoptosis (Cotter et al. 1992; Levee et al. 1996). Some of the extra-number of degenerating meiotic nuclei observed in this study might correspond to apoptotic bodies. This study provided some direct evidence to demonstrate that meiotic nuclei degenerate in an apoptotic way in *P. caudatum*, and it supports our previous observations (Yang et al. 2007). This is the first case to report that morphological apoptotic characteristics of multicellular organisms also exist in unicellular eukaryotic ciliate protozoa.

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