FINE STRUCTURAL AND BIOCHEMICAL STUDIES ON CELL DEATH OF THE DEVELOPING *BAR* EYE DISCS IN *DROSOPHILA MELANOGASTER*¹⁾

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The cell death is an important process in the development and differentiation of multicellular organisms (Saunders *et al.* 1962; O'Connor and Wyttenbach 1974; Coward *et al.* 1974). In insects, cell death is generally observed in the destruction of larval tissues during metamorphosis, and attending to an increase in hydrolytic enzyme activity (Lockshin and Williams 1965; Mulherkar *et al.* 1972; Henrikson and Clever 1972).

In contrast to these, in some of growing tissues, such as in the imaginal discs of *D. melanogaster*, the cell death has been also investigated (Fristrom 1968, 1969; Poodry and Schneiderman 1970; Spreij 1971). Recent evidences indicated that the cell death appears in the specific regions of the mutant *Bar* eye discs, i.e., in the anterior portion of the presumptive ommatidial forming regions (Fristrom 1969, 1972; Michinomae and Kaji 1973; Michinomae 1974). As pointed out elsewhere, (Michinomae 1974), the characteristic figures in which the activity of acid phosphatase reaction were detected by histochemical procedure, were found in the regions of cell death. The structures that showed positive reaction of acid phosphatase might be defined as lysosomes. On the other hand, Kaji (1954, 1960) pointed out that the size and shape of the *Bar* eye can be changed into a "wild type like" by treatment with acid amides, such as acetamide and lactamide.

Moreover, in the electron microscopic observation, the *Bar* larvae showed a marked decrease in the lysosome activity when treated with acetamide. From these results, Michinomae (1974) suggested a hypothesis that the lysosomes might act to break down cell organelles. In addition, the acetamide acts to inhibit the formation of lysosomes during cytodifferentiation of the *Bar* eye discs.

In this report, further investigations has been made on the degenerating process of cell organelles, and the relationship between the degenerating process and changes in acid phosphatase activity during the development of the *Bar* eye discs.

MATERIALS AND METHODS

Drosophila strains

The mutant *Bar* and wild type (Oregon-R) strains were used in this work. They

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were grown in the usual yeast-molasses medium at $25 \pm 0.5^\circ\text{C}$. The developmental stages were determined by measuring the time after larval hatching. Details of rearing and staging procedures of larvae were described elsewhere (Michinomae 1974). The *Bar* larvae used for the acetamide treatment were reared in the normal medium for 42 hours after hatching, and then transferred to the medium containing 1.5% acetamide until they reached the specific stages according to our definition.

Microscopic observations

The experimental materials for microscopic observations were prepared in the procedure as previously described (Michinomae 1974). The eye discs were dissected in Schneider's culture medium (Schneider 1964) and fixed twice in 2.5% glutaraldehyde containing 0.05 M sucrose and 1% osmium at 4°C . They were then dehydrated in ethanol and embedded in Epon. Tissues were sectioned, and stained with uranyl acetate and lead citrate for electron microscopy (Reynolds 1963).

For histochemical examination, sodium β -glycerophosphate was used as substrate to demonstrate the acid phosphatase reaction (Holt and Hicks 1961).

In order to calculate the relative frequencies of lysosomes in eye discs, its cells, lysosomes and lysosome derivatives were counted in 300 electron micrographs at low magnification ($\times 4500$). These electron micrographs were randomly selected from among 1000 electron micrographs which were horizontally sectioned through the eye discs.

Enzyme assay

Larvae or pupae at required developmental stage were used regardless of their sex. They were washed in distilled water, and then in insect Tyrode's solution. After washing, a known wet weight of larvae was completely homogenized by a teflon homogenizer at 0°C in solution containing 0.01 M sucrose, 1.0 mM EDTA, and 0.1% Triton X-100 (homogenizing solution). Homogenates were treated with the freezing-thawing process five times, and centrifuged for ten minutes at 3000 rpm. The membrane-bounded enzymes are released to the supernatant by this manipulation (Shibko *et al.* 1965). The supernatants were employed for assay of the total enzymes. For assay of eye-antennal discs, the larvae were dissected in insect Tyrode's solution after washing in distilled water. Subsequently, the removed discs were washed twice in the same saline solution, a total of 100-400 discs was collected and homogenized in the homogenizing solution, and then enzymes were fractionated by the same procedure.

For acid phosphatase assay, Robinson's method (1972) was employed with a slight modification, e.g., using alpha-naphthyl acid phosphate (α -NAP) as substrate. The reaction mixture was prepared as follows: 0.05 M sodium acetate-acetic acid buffer pH 5.0, 0.1 mM α -NAP and an aliquot of supernatant were mixed to bring to a final volume of 0.5 ml. The mixture was incubated at 37°C for 15 minutes, before adding 0.5 ml coupling solution which was made by adding 4.0 g sodium dodecyl sulfate and 200 mg Fast Red ITR to 100 ml of 0.05 M sodium acetate-acetic acid buffer at pH 5.0. Standard run and control were carried out simultaneously by the same procedure. The red color resulted from hydrolytic cleavage was measured by a spectrophotometer at 545 m μ . The amount of protein in the homogenate was determined by method described by Lowry *et al.* (1951). The enzyme activity was indicated as specific activity per unit

Fig. 1. Electron micrograph of a 40-hour *Bar* eye disc. The cells are typically embryonic form with numerous free ribosomes and a few poor organelles; Golgi complex, endoplasmic reticulum, mitochondria (m) and oval-shaped nuclei (N) with nucleoli (Nu). The simple vesicular figures (arrows) are detected in nuclei. $\times 4500$

of protein.

RESULTS

1) *Electron microscopic observations*

As previously reported (Michinomae 1974), electron microscopic observations revealed the specific structures in the degenerative cells, namely, simple vesicular figures (look like a necrotic foci), myelin-like figures and cell organelle fragments which were limited by a single membrane. These specific structures in the degenerating cells may be defined as lysosomes by histochemical detection of acid phosphatase. In the subsequent experiment the fine structural changes and relative frequencies of various lysosomes in the eye discs among the *Bar*, acetamide-treated *Bar* and wild type strains during the development compared by means of electron microscopical examination.

At the early larval stage, no degenerating cells in the eye discs were observed at 36 hours after hatching. The changes in specific structures in the degenerative discs first appeared as the simple vesicular figures in a few nuclei at 40-hour larval stage (Fig. 1). During 40 to 60 hours stage, the number of these specific structures gradually increased. As the development proceeded like in the nuclei at the 60-hour larval stage, the myelin-like figures appeared more frequently than before. Occasionally, some of 60-hour disc cells contained various fragmented cell organelles which were bounded by a single membrane. Among *Bar*, acetamide-treated *Bar* and wild type eye discs, no differences in the above-defined specific structures were observed at the 40-60 hour stage.

In *Bar* eye discs at the 70 to 85 hour stage, a number of myelin-like figures, lytic vesicles containing organelle fragments, and a few simple vesicular figures were detected. The nuclei of these cells in the degenerative process showed chromatin clumping and irregular nuclear envelope. In addition to these, the electron density of the cytoplasm greatly increased. After then, the nuclear envelope broke down, and the karyoplasm which was connected to the cytoplasm possessed myelin-like figure and dense chromatin (Fig. 2). During 85 to 95 hours after hatching, these events were followed by the stage of cytolysis, the characteristic pattern of degenerating cells, i.e., fragmentation of organelles, disappearance of the typical myelin-like figures and the membrane-bounded electron dense bodies. Eventually, these fragments were situated in numerous large phagocytic vesicles. The most active cytolysis seemed to occur during these period (Fig. 3). In fact, acid phosphatase which is one of the hydrolytic enzymes in the lysosomes was detected in the single membrane-bounded body or myelin-like figure

Figs. 2-4. Electron micrograph of an early stage of degenerating cells in 85-hour *Bar* eye disc. The nuclear envelope (Ne) breaks down, and the karyoplasma (Kp) is connected to cytoplasm. Note: the myelin-like figure (My) is detected in the nuclear region. $\times 16000$
 3. Electron micrograph of a later stage of a degenerating cell in 95-hour *Bar* eye disc. Myelin-like figure (My) and cell debris (Cd) are gradually resolved into the lipid droplet (*). $\times 23000$
 4. An acid phosphatase stained section through a 95-hour *Bar* eye disc. Reaction products (arrows) are localized on the peripheral region of single membrane-bounded structure. Note: the reaction products are not detected in the lipid droplet region (*). $\times 34200$

Table 1. The relative frequencies of the various lysosomes during the development of the eye discs

Time after hatching	Strain	Counted cell numbers	Pre-lysosomes	Lysosomes	Post-lysosomes	Total lysosome numbers	Frequency of lysosomes (%)
36 hours	W	33	0	0	0	0	0
	B	27	0	0	0	0	0
	BA*	—	—	—	—	—	—
48 hours	W	11	2	0	0	2	18.18
	B	49	5	0	0	5	10.20
	BA	134	15	0	0	15	11.19
60 hours	W	531	30	74	0	104	19.58
	B	357	34	35	0	69	19.32
	BA	—	—	—	—	—	—
70 hours	W	440	0	8	1	9	2.05
	B	257	0	23	2	25	9.73
	BA	842	4	13	0	17	2.02
85 hours	W	359	0	18	1	19	5.29
	B	1039	6	74	59	139	13.37
	BA	552	1	8	1	10	1.81
95 hours	W	751	0	8	2	10	1.33
	B	861	0	67	186	258	29.21
	AB	267	0	17	1	18	6.74
100 hours (pre-pupae)	W	327	4	27	5	36	11.01
	B	595	0	1	127	128	21.51
	BA	443	7	3	5	15	3.39

W: wild type (Oregon-R) eye disc, B: *Bar* eye disc, BA: acetamide treated *Bar* eye disc.

* The *Bar* larvae used for the acetamide treatment were reared in the normal medium from hatching to 42-hours, and then transferred to the medium containing 1.5% acetamide until they reached the specific stages according to our definition.

(Michinomae 1974). Then, these fragments (cellular debris) were gradually resolved into the lipid droplets. These lipid droplets appeared at the 95-hour stage (mature larvae). After puparium formation (100-hour stage), most of these structures—especially, the cell organelle fragments bounded by a single membrane—resolved into the lipid droplets and shrank. The enzyme activities that can be identified as acid phosphatase were also detected in the early resolving process (in the cell organelle fragments bounded by a single membrane), but not detected at the later stage (in the lipid droplets) of resolving process (Fig. 4).

In acetamide-treated *Bar* eye discs, myelin-like figures and organelle fragments were also detected. However, the number of these figures decreased even more than before in larvae of the 70-95 hour. Lipid droplets were rarely observed in the eye disc cells at later stages of larval development.

Fig. 5. Specific activity of acid phosphatase in whole larvae or pupae at various developmental stages. Unit of specific activity is μ mol α -naphthol/mg protein. The arrow indicates the onset of puparium formation. Each point is the mean activity of 6-10 experiments. (*) the middle egg stage

The specific structural changes in degenerating cells in wild type eye discs at the 40-95 hour stage were almost the same as those of acetamide-treated *Bar* eye discs. In contrast, at the prepupal stage (100 hour stage) of wild type, the occurrence of myelin-like figures, cell fragments and lipid droplets were more frequently observed than at the earlier stage (36-95 hour stage) of the wild type. Most of these figures were detected in the presumptive hypodermis. The structural changes occurring in these eye discs during the developmental stages were related to functional changes in lysosomes (Michinomae 1974). Namely, simple vesicular figures are interpreted to be "pre-lysosomes", myelin-like figures and fragments of cell organelles are "lysosomes" and lipid droplets are "post-lysosomes". In the present observations, the frequencies of these various lysosomes observed are summarized in Table 1.

2) Enzyme assay

i) Enzyme activity in homogenates of whole larvae

The specific activity of acid phosphatase in whole homogenates of the *Bar* and wild type larvae at various developmental stages are illustrated in Fig. 5. The specific activity is indicated by μ mol α -naphthol/mg protein.

Fig. 6. Specific activity of acid phosphatase in the eye-antennal discs at various developmental stages. Unit of specific activity is μ mol alpha-naphthol/mg protein. The arrow indicates the onset of puparium formation. Each point is the mean activity of 6-10 experiments.

In the *Bar* strain, the most active reaction of acid phosphatase during the development [specific activity (S.A.) 1.6] was detected at the middle egg stage. In contrast to these, larvae immediately after hatching had the lowest value (S.A. 0.21) which was close to 1/9 activity at the egg stage. Thereafter, the activity continued to increase from S.A. 0.3 at the 40-hour stage to 0.55 at the 95-hour stage. At 95 to 100 hour stage (prepupa), the enzyme activity increased remarkably and reached about twofold increment of the activity of 95-hour larvae. The activity reached the maximum value (S.A. 1.0) at the early pupal stage (110 hours after hatching), and then, decreased slowly until the emergence of flies.

The enzyme activity of the wild type strain resembled to that of the *Bar* strain during the developmental process, except for a slight difference observed at the egg stage.

ii) Enzyme activity in homogenates of eye-antennal discs

The specific activity of acid phosphatase was examined in eye-antennal discs at various developmental stages of the *Bar*, the acetamide-treated *Bar* and wild type strains. The results of the experiments are summarized in Fig. 6.

At the 75-hour stage, the *Bar* eye discs had specific activity near 0.25 whereas both acetamide-treated *Bar* and wild type discs had slightly lower activity (near 0.2) than that of the *Bar* eye discs. The acid phosphatase activity continued to rise thereafter through the 85-hour stage; however, there was almost no difference in the increasing

tendency of the enzyme activity among these three strains during this period, except for the *Bar* eye discs. The *Bar* eye discs had the activity a little higher than that of acetamide-treated *Bar* and wild type eye discs. Subsequently, the enzyme activity of the *Bar* eye discs showed a remarkable increase and reached its maximum value at the 95-hour stage. In contrast, the acetamide-treated *Bar* eye discs showed only a slight increase in the enzyme activity, which was about 3/5 of that of the *Bar* eye discs at the same stage. The wild type showed a resembling pattern of the activity seen in the acetamide-treated *Bar* and had 0.42 of specific activity. There was no any striking increment of the enzyme activity of the acetamide-treated *Bar* and wild type strains at the same developmental stages.

Thereafter, the activity in all three strains decreased abruptly and continued to decline until the 100-hour stage (prepupal stage), when the specific activity reached only 0.3.

DISCUSSION

The cell death during the process of organization have been studied by a number of authors (Saunders *et al.* 1962; Robinson 1972; Hourdry 1974). We have also pointed out that the cell death occurred at a substantially higher rate in the *Bar* eye discs than in the wild type ones and the localization of these dead cells varied according to the developmental stages (Michinomae and Kaji 1973; Michinomae 1974). In the present paper, we indicated that the simple vesicular figure appeared at first in the nuclei at the early developmental stage (40-hour larval stage), but not in the cytoplasm. The marked changes such as the condensation of chromatin and the increase in electron density of the cytoplasm were observed in the *Bar* eye discs at the 70 to 95 hour stage. After that, the destruction of cell has progressed in the manner that the cell organelles (membranous structure and others) were disintegrated into many indistinct fragments. Then, these cells which contained many characteristic structural features i.e., typical myelin-like figure and membrane-bounded dense body, eventually were engulfed by neighbouring cells. In eye discs at later third instar larval stage (95-hour stage), these myelin-like figures or degenerated cells (cell debris) transformed into lipid droplets. These fine structural changes in lysosomes proceeded from "pre-lysosome" to "lysosome" and eventually to the "post-lysosome" type (Michinomae 1974). The "post-lysosome" type has been demonstrated to be enzymatically inactive by the histochemical method.

The changes in acid phosphatase positive fine structures may follow the schematic diagram of deDuve and Wattiaux (1966) in which various forms of lysosomes and the related particles, along with the different types of interactions were illustrated. Accordingly, these characteristic figures in the degenerating cells with marked activity of acid phosphatase can be defined as the lysosome.

The same phenomenon was also described in the wing of chick embryo (Saunders *et al.* 1962). The regions of cell death appeared to be bluish particle-like structure in the Nile blue stain. We have found that organelle fragments which were bounded by a single membrane also appeared as blue particles in the Nile blue stain (Michinomae unpublished data). Nile blue has been known to stain acidic lipids into the blue color

(Cain 1947). These results, therefore, suggest that the acidic lipoids were formed by hydrolytic enzyme in lysosomes.

On the other hand, biochemical study of the *Bar* eye discs has revealed that the maximum value of acid phosphatase was observed at the 95-hour stage. The activity was two to three fold higher than that at the 75-hour stage. It appears that lysosomes and their lytic enzymes in the *Bar* eye discs may act effectively upon the breakdown of cell organelle in the degenerating area at the 95-hour stage. These results show that the peak of fine structural degeneration, biochemical activity as well as relative frequency of various lysosomes concurred at this stage. Such a correlation can be interpreted that the process of degeneration in the eye discs may be mediated by the lysosomal activity, either by the formation of lysosome or by the synthesis of hydrolytic enzyme.

However, the maximum activity of acid phosphatase in whole larva homogenates was observed in the prepupal to early pupal stage (100-110 hour stage). This suggests that the direct cause of larval organ degeneration may be due to an increase in hydrolytic enzyme. Essentially, the same pattern of degeneration has been found in other organisms during the metamorphosis, i.e., whole larvae of *Drosophila* (Mulherkar *et al.* 1972), larval organ in insect such as salivary gland (Price 1974; Lockshin and Williams 1965) and amphibian tadpole (Robinson 1972; Hourdry 1974). Modes of the acid phosphatase activity observed in whole larvae in the present work are consistent with those of these authors.

These investigations reveal an obvious difference in the maximum value of acid phosphatase activity between whole larvae and imaginal eye-antennal discs during the developmental stage. Such a difference may be resulted from the cell death accompanied by degeneration of the larval organs or in the specific degeneration of the mutant imaginal discs. The extent of degeneration which may be closely linked to these processes of cell death depends upon the activity of hydrolytic enzyme, such as acid phosphatase. It is not yet clear whether or not the increase in the activity of hydrolytic enzyme is directly controlled by the action of *Bar* gene. However, the changes in the activity of acid phosphatase can be caused by *de novo* synthesis of enzyme protein or by the results of an enzyme activation.

In the acetamide-treated *Bar* eye discs, the number of lysosomes and the activity of acid phosphatase were highly decreased (Michinomae 1974 and in the present experiment). In this respect, both the enzyme activity and the frequency of lysosomes in acetamide-treated *Bar* eye discs come close to those of wild type eye discs. These results suggest that the acetamide may act to inhibit the formation of lysosome or the synthesis of hydrolytic enzyme.

SUMMARY

The aspects of degeneration in the *Bar* eye discs at various developmental stages of *Drosophila melanogaster* was elucidated microscopically and biochemically. Acid phosphatase was identified histochemically in lysosomes or their derivatives in the

degenerating area of the 85-95 hour eye discs. The enzyme reaction appeared to localize selectively in the single membrane-bounded structure or myelin-like figure. The cell debris probably resulting from the resolving process, fail to demonstrate any enzyme activity at the prepupal stage.

Biochemical data indicated that the maximum activities of acid phosphatase were shown in eye discs at the 95-hour stage. This is in contrast to the weak acid phosphatase activity detected in eye discs of prepupal stage. However, whole larval homogenates showed maximum value of acid phosphatase activity at the same stage and the post embryonic development. This causal association of increased enzyme activity and histochemical observation in the resolving process of the eye discs suggests that the process of degeneration may be mediated by the lysosome activity, either by the formation of lysosomes or by the synthesis of hydrolytic enzymes. These lysosomes or activities of lysosomal lytic enzyme were reduced by treatment with acetamide. These data suggest that acetamide may act to inhibit the formation of lysosome or the synthesis of hydrolytic enzyme in the *Bar* eye discs.

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