An electron microscope study of rhabdomyosarcoma

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An electron microscope study of ultrathin sections of two rhabdomyosarcomas revealed the presence of cytologic characteristics previously described in neoplasias: nuclear envelope invagination, reduction in number of organelles, reduced size of mitochondria, swollen mitochondria. In addition, one group of seven centrioles was observed and many cells revealed the presence of aggregates of small, dense particles. Groups of fibrous structures were occasionally encountered.

The electron microscopic study of neoplasms offers a great deal of promise for contributions to many still clouded areas in our understanding of these conditions. Some neoplasms cannot as yet be accurately categorized by heretofore available techniques of study including light microscopy and chemical analysis. In the group must be placed the orbital rhabdomyosarcomas of children, which are fairly well delineated clinically since they are the most common malignant orbital tumor in children. Yet they are still the subject of some controversy as to the valid microscopic characteristics. The present study throws light upon this controversy.

In the broader and considerably more significant area of the etiologic study of neoplasms, it seems reasonable to expect, as the recent reviews by Beard, Bernhard, and Dalton suggest, that electron microscopy will be of tremendous value. The tumors which are the subject of this study were formerly called rhabdomyosarcomas when cross striations could be seen in the cells. However, it is possible to make the diagnosis without the presence of cross striations. In fact, such striations are usually not seen and rhabdomyosarcomas have extremely wide morphologic and cytologic variations.

This tumor is the third most common tumor in children, following leukemia and neuroblastoma in prevalence. It was formerly thought that tumors containing rhabdomyoblastic elements occurred only at sites where striated muscle is found, but it is now known that these tumors appear at other sites over the body including the bladder, uterus, vagina, and male genitals. Willis felt that the majority of these tumors arose in embryonal tissue, either immature prospective muscle tissue, or indifferent mesenchymal tissue with the potency for aberrant differentiation of muscle fibers. He thought that only rarely is the adult skeletal muscle implicated. Ashton also believes this and feels that these tumors should be classified as embryonal sarcoma. The consensus is that this tumor demonstrates extreme pleomorphism, and the diagnosis can be made without the presence of...
cross striations. 12 Porterfield and Zimmerman13 found cross striations in all of their differentiated type, in 60 per cent of the embryonal, and in 30 per cent of the alveolar. Reese12 divides the rhabdomyosarcomas into three groups: pleomorphic (which includes the differentiated of Porterfield and Zimmerman14), embryonal, and alveolar.

The pleomorphic type shows longitudinal striations (myofibrils) and less often cross striations. This type occurs in middle age and arises in relation to striated muscle. The embryonal type is most common. In this latter, there may or may not be longitudinal and cross striations. The alveolar type shows layers of cells closely applied to the connective tissue trabeculae separating the alveoli. Both the alveolar and embryonal types occur in the younger age groups. In one series of orbital rhabdomyosarcomas 32 were embryonal, 4 were alveolar, and 8 were pleomorphic.

Two observations of particular interest should be mentioned. First, it has been reported in Reese's recent textbook that two brothers, aged 9 and 14, died of rhabdomyosarcoma of the orbit 19 months after proptosis had been noted. Further, the primary tumors which have cross striations may give rise to extremely anaplastic metastases difficult to recognize as rhabdomyosarcoma. These facts raise the interesting question of whether a virus might be the causative agent in susceptible individuals. The purpose of the present communication is to report observations of the structural features of rhabdomyosarcoma.

Materials and methods

The tumors studied were obtained from a 4½-year-old white girl and a 7-year-old white boy. The diagnosis made with the light microscope at the pathology department at the Institute of Ophthalmology was rhabdomyosarcoma, embryonal type. Immediately following exenteration, portions of each tumor were excised and placed in a fixative consisting of 1 per cent osmium tetroxide buffered at pH 7.3 with acetate-veronal. Fixation was carried out at 2 to 5° C. for four hours. The blocks were dehydrated by passage through a graded ethanol series and were embedded in Epon according to the technique of Luft.6

The light micrographs were taken from 1 μ thick sections cut with a Porter-Blum ultramicrotome and stained according to the procedure of Richardson and associates.13 Ultrathin sections were cut with the same microtome and were picked up on collodion-coated 200 mesh copper grids. These sections were routinely stained with uranyl acetate according to the method of Watson4 for 1 to 3 hours. The sections were examined with an RCA EMU-2D electron microscope which had been equipped with a 0.015 inch platinum condenser aperture and a 50 μ copper aperture in the standard objective pole piece.

Observations and discussion

Light micrographs of the two tumors reported on in this study reveal the similarities and differences typical of representative fields of any single specimen of this material. One field (Fig. 1) reveals an area where there is an abundance of intercellular space. It will be shown subsequently that this space is occupied by collagen fibers and by cellular fragments. Prominent nuclei are visible in many of the cells and an appreciable variation in nuclear size is obvious. The most striking feature of Fig. 1 is the margination of chromatin, or chromatin capping, demonstrated by the nuclei designated N. A similar distribution of nuclear material has been noted in the case of cells of the human wart by Chap- man and co-workers,6 who have pointed out the interesting similarity between this phenomenon and the margination of chromatin demonstrated by bacterial cells following infection with bacteriophage, as illustrated so elegantly by Mudd and his associates.6

The appearance of the cells in the other field (Fig. 2) is quite different in that there is little intercellular space. On the contrary, the cells appear very closely packed together and it is rarely possible to distinguish the outlines of the individual cells. Variation in nuclear size is even more evident in this specimen than in the first one. The arrow near the center of the field designates a metaphase plate of one of the numerous mitotic figures observed in this material.

The variation in cytologic organization
demonstrated by these two tumors is strikingly similar in degree to that variation between the two retinoblastomas described by Allen, Latta, and Straatsma, one revealing a conspicuous rosette formation, the other not. (A rosette consisted of a group of tumor cells arranged radially around a central lumen.) The tumor cells, when studied in the electron microscope, reveal a considerable variation in state of preservation. Some (Figs. 3 to 7) appear very well preserved, with membranes and organelles in good order. Adjacent cells may differ markedly in the degree of development of one or another of their organelles. The cell containing the upper nucleus in Fig. 3, for example, contains an extensive granular endoplasmic reticulum with enlarged intracisternal spaces (ER). The cell immediately below it reveals only a few widely scattered elements of endoplasmic reticulum. The fact that only one mitochondrion (M) may be seen in these two cells is in agreement with Bernhard's general impression that there are fewer mitochondria in tumor cells. The lumen (L) of a blood vessel may readily be seen and pinocytotic vesicles are visible in an endothelial cell bordering that lumen; several areas of collagen fibrils are designated C (Fig. 3).

Invaginations of the nuclear envelope, similar to those noted by Bernhard, are quite obvious (Fig. 4). The uppermost nucleus is of particular interest in this regard for an invagination has been cut longitudinally at I and transversely at L. The lower right nucleus in this figure reveals blebs (arrows) or separations of the outer from the inner element of the nuclear envelope which closely resemble those shown by Allen and colleagues in the cells of retinoblastoma. Mitochondria (M) appear more numerous in the cells of this field, but are quite small in size. No significance can be attributed to this as Bernhard has reported that the mitochondria of tumor cells may become larger, smaller, or remain the same size as normal. However, it should be noted that Dalton reported small mitochondria in hepatoma cells. Mitochondrial cristae may be clearly seen. A nucleolus (NU) may be seen in this nucleus.

The blebs, which appeared rather small in Fig. 4, may become much larger (Fig. 5, arrows). A prominent nucleolus (NU) and quite numerous small mitochondria (M) may also be seen in this figure.

The other extreme in the range of mitochondrial variation may be seen in this study (Fig. 6). Here, the mitochondria (M) appear appreciably swollen and their cristae are sparse. This figure also reveals a curious fimbriation of portions of the nuclei (arrows). Other figures (Fig. 7) reveal a similar fimbriaion. This latter figure also reveals several tufts of intracellular fibrillar material (F). Several possible identities of this material may be suggested. Keratin filaments are a possibility. However, the mesodermal derivation of this tumor tends to rule out this possibility, keratin filaments normally being associated with ectodermal cells. One might also suggest the possibility that this fibrous material represents primitive myofilaments. Then, finally, there is the possibility that this fibrous material represents a degenerative process of the ground substance, as suggested by Bernhard. The true nature of this material remains to be determined. An area of collagen (C) and several swollen mitochondria (M) are also visible in this figure.

Areas of rather extensive cellular disorganization may also be found in this

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Fig. 1. Microscopic appearance of tumor. Nuclei, N, demonstrate chromatin margination or capping. The large amount of intercellular space may be seen. (Methylene blue, azure II. x1,100.)

Fig. 2. Microscopic appearance of tumor. The arrow designates a metaphase plate. Great variation in nuclear size may be seen. (Methylene blue, azure II. x1,100.)
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Figs. 1 and 2. For legends see opposite page.
Fig. 3. Variation in appearance of cells of rhabdomyosarcoma. The upper cell reveals an extensive granular endoplasmic reticulum with rather enlarged cisternae, ER. The lower cell possesses very little endoplasmic reticulum. Several bundles of collagen, C, may be seen. L designates the lumen of a blood vessel, PV designates pinocytotic vesicles, and M designates a mitochondrion. (*11,500.)
Fig. 4. Invaginations, $I_1$, $I_2$, of envelope of uppermost nucleus, $N$. Blebs (arrows) represent areas where the two elements of the nuclear envelope have separated from one another. Small mitochondria, $M$, with cristae, and a prominent nucleolus, $NU$, may be seen. (x10,400.)
Fig. 5. The nuclear envelope blebs are more extensive in this field. There are no other signs of cellular degeneration. Mitochondria, M, and nucleolus, NU, are visible. (×10,400.)
Fig. 6. Swollen mitochondria, M, and fimbriated nuclear margins (arrows), together with the loss of integrity of the cell membrane, suggest that this is a degenerating cell. (*11,500.)
Fig. 7. An area of cellular degeneration, as indicated by the extracellular mitochondrion, M, just above the center of the field, and by the loss of plasma membrane integrity. A collection of collagen fibers, C, may be seen and the fibrillated surface (arrows) of the nucleus is prominent. Cluster of fibrils, F, may be seen at the lower left. (×8,000.)
Fig. 8. Region of cellular degeneration. Several aggregates of dense particles (arrows) may be seen. The one to the left is extracellular. Collagen, C, and a swollen mitochondrion, M, are also visible. (×11,500.)
Fig. 9. Adjacent serial section to Fig. 8, indicating that the aggregates of particles extend for some distance through the cytoplasm. Of particular interest is the group of seven centrioles (double arrow) seen at the lower left. (×11,500.)

Fig. 10. Area of cellular degeneration. The larger nucleus contains chromatin, nucleolar material, N, and the dense particles (arrow). The differences between these three intranuclear materials may thus be readily appreciated. The smaller nucleus contains a group of the particles (arrow). Several blebs B of the nuclear envelope are also visible. (×11,500.)

Fig. 11. Adjacent serial section to Fig. 10. The same features are seen. (×11,500.)
Figs. 10 and 11. For legends see opposite page.
Figs. 12 and 13. For legends see page 553.
Figs. 14 and 15. For legends see page 553.
Fig. 16. Nucleus containing two very large clusters of dense particles (arrow). It is interesting to note how each cluster has a dense central aggregate surrounded by more sparsely arrayed particles. (*15,600.)
Fig. 17. Adjacent serial section to Fig. 16. This section establishes the fact that this is a single nucleus (central connecting portion) and not a binucleate cell. Clusters of particles (arrows) are readily visible. (×15,600.)

Fig. 12. Area of cellular degeneration revealing blebs, B, of nuclear envelope and aggregates of particles (arrows). (×10,400.)

Fig. 13. Portion of a cell showing prominent chromatin capping or margination. Several somewhat swollen mitochondria, M, are also seen. (×15,600.)

Fig. 14. Binucleate cell with each nucleus showing chromatin capping. The nucleus at the right also contains a cluster of dense particles (arrow). (×11,500.)

Fig. 15. Another nucleus containing a large cluster of dense particles (arrow). It may readily be seen that these particles are larger than the ribonucleoprotein particles which are abundant in this cytoplasm. (×15,600.)
Fig. 18. Mitotic figure in a tumor cell. These anaphase chromosomes reveal no peculiarities. (×10,400.)
Fig. 19. Portion of an anaphase mitotic figure. A lipid inclusion, L, and spindle fibers, S, may be seen. (×15,600.)
material (Fig. 8). This figure shows several aggregates (arrows) of very dense, somewhat irregular particles. These particles appear both within the nuclei and within the cytoplasm. It is tempting to suggest that these particles may be viruslike in nature. However, an extensive survey of the literature has disclosed no particles which are identical in appearance. These particles clearly resemble none of the three classes of particles described by Bernhard,\textsuperscript{5} being both smaller and less complex. Reluctantly, then, in the light of Beards'\textsuperscript{3} comments concerning the confusion introduced by preoccupation with theory as to the nature of viruslike particles, one faces the obligation to consider the possible origin and nature of these particles. Several possibilities seem worth mentioning. It is possible that these particles represent aberrant fragments of chromosomes or of nucleoli. However, the particle size is appreciably larger than that found in components of either chromosomes or nucleoli. This is particularly well demonstrated (Figs. 10 and 11) in figures which contain the particles, masses of nuclear chromatin, and prominent nucleoli all within the same nuclear envelope. There is no evidence that these particles multiply at the expense of nuclear chromatin as has been suggested for the wart virus.\textsuperscript{6} Nor can any relationship be seen between the particles and the nucleoli. It may then be reasonable to suggest that the particles possibly represent a primitive type of viruslike particle. This possibility does not, of course, imply that the particle is the etiological agent in this tumor. It remains for further extensive work by virologists and electron microscopists, working in collaboration, to elucidate the nature of this apparently new particle. This report, then, is presented in the hope of helping to contribute to the need indicated by Bernhard\textsuperscript{5} when he wrote: "A systematic morphological description of all experimental and human tumors, regardless of their etiology, is very desirable."\textsuperscript{*}

In the area under scrutiny (Fig. 8), it can be seen that cellular disintegration is occurring, a swollen mitochondrion (M) is visible, and a bundle of collagen fibers (C) may be seen.

An adjacent serial section is included to prove that the particles are not contaminating stain. It is clear that the aggregates of particles extend for some distance through the cytoplasm (arrows). This figure is of particular interest as it reveals an astonishing group of seven centrioles (double arrow). To our knowledge, no such concentration of centrioles has been previously described. This concentration may reflect the fact that this is a highly malignant tumor. This fact (high malignancy) also has significance in the light of Bernhard\textsuperscript{5} statement* that "Virus-infected tumor cells are probably malignant only if they are not overwhelmed by virus particles, which is a serious handicap for rapid growth." In this connection, it is notable that in no case did an extensive area of the particle-containing cell become occupied with the particles. This is in striking contrast to the situation occurring in the wart where entire nucleus may ultimately be occupied by viruslike particles.

In addition to demonstrating the particles, Figs. 10 to 12 show clearly the blebs (B) from the nuclear envelope which have been discussed previously. Figs. 10 and 11 are also adjacent serial sections.

The nuclear chromatin capping, or margination, discussed earlier (Fig. 1), is especially striking in some electron micrographs (Figs. 13 to 15). Fig. 15 presents the largest array of particles encountered in this study and clearly illustrates the fact that the particles are appreciably larger than the ribonucleoprotein particles which literally pack the cytoplasm of this cell.

Figs. 16 and 17 are two more adjacent serial sections. The dense particles are particularly clearly seen (arrows). The largest particles attain a diameter of about

\*Page 504.

\*Page 721.
50 nm and thus are slightly larger than the viruslike particles associated with cells of the wart but are smaller than the viruslike particles associated with a wide variety of tumors.

The appearance of mitotic chromosomes in these tumor cells has also been observed (Figs. 18 and 19). Careful study of the micrographs reveals no aberrations from normal chromosome structure as seen in the electron microscope. In the latter figure, spindle fibers (S) are visible and a lipid inclusion (L) has been indicated.

At the conclusion of this phase of this study, we must agree with Allen and associates10 that "there is no known ultrastructural feature or combination of features specific for cancer cells." We have found characteristics (small mitochondria, swollen mitochondria, nuclear envelope blebs, nuclear envelope invaginations, reduction in number of cell organelles) which other investigators have reported to occur in cancer cells. However, no characteristic can be conclusively said to occur in each cancer cell. This is partly due to the fact that any single section represents only a very small fraction of the total cell volume. It would, then, be quite easy to exclude from the section a mass of particles or a nuclear envelope bleb and the investigator would be unable to recognize his diagnostic feature. It may well be that, until some diligent workers analyze serial sections through several complete cells, we shall lack definitive information which will be of diagnostic value. The problem here is so tremendous that it is likely that information will be added rather haphazardly for some time to come as it has been in the past. The fact remains, however, that any information we can obtain in this area is worthwhile.

The limited amount of material sampled and the degree of variation encountered leave us unwilling, at this stage of our investigation of this tumor, to attempt to duplicate Allen's heroic effort to establish a sequence of events in the development of this tumor. We prefer, for the present, to indicate our belief that our most significant finding is the aggregation of dense intranuclear and intracytoplasmic particles. It is our hope that further work may be done to clarify the nature of these particles.

REFERENCES