# Studies on the Cytoplasmic Granules in the Tumor Cells of the MTK-Sarcoma, III. Observations on the Azur Granules[1)]

By
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*(With 6 Text-figures)*

The morphological features of cytoplasmic granules occurring in tumor cells is of significant importance in relation to their physiological phases. In the papers published by Okada 1954, Tonomura 1955, Okada & Nakahara 1956, Yuize 1956, remarkable evidence has been presented that in rat ascites tumors the morphological changes of cytoplasmic inclusions take place in close association with the physiological functions of tumor cells. These observations were carried out with living material following the supravital staining method with the aid of the phase contrast microscope. In the present study, the morphological features of the cytoplasmic granules were studied in both fixed and stained material, for comparison with the corresponding features in the living material.

Before proceeding further it is the author's pleasant duty to tender this sincere thanks to Professor Sajiro Makino for his direction and improvement of the manuscript for publication.

## Meterial and method

Pure bred Wistar albinos (*Rattus norvegicus*) were used to transmit rat ascites tumors used for study; they are Yoshida sarcoma, MTK-sarcoma II and Hirosaki sarcoma.

Morphological observations on the azur granules occurring in tumor cells of the Yoshida sarcoma and MTK-sarcoma III both being subdiploid tumors were made with daily material through the whole life span of certain tumor-bearing rats, using smear preparations stained by Giemsa's azuroeosin-methylenblue solution. Furthermore, the Hirosaki sarcoma (Usubuchi et al. 1955), a hypotetraploid tumor, was studied for contrast with the data obtained in the diploid tumor.

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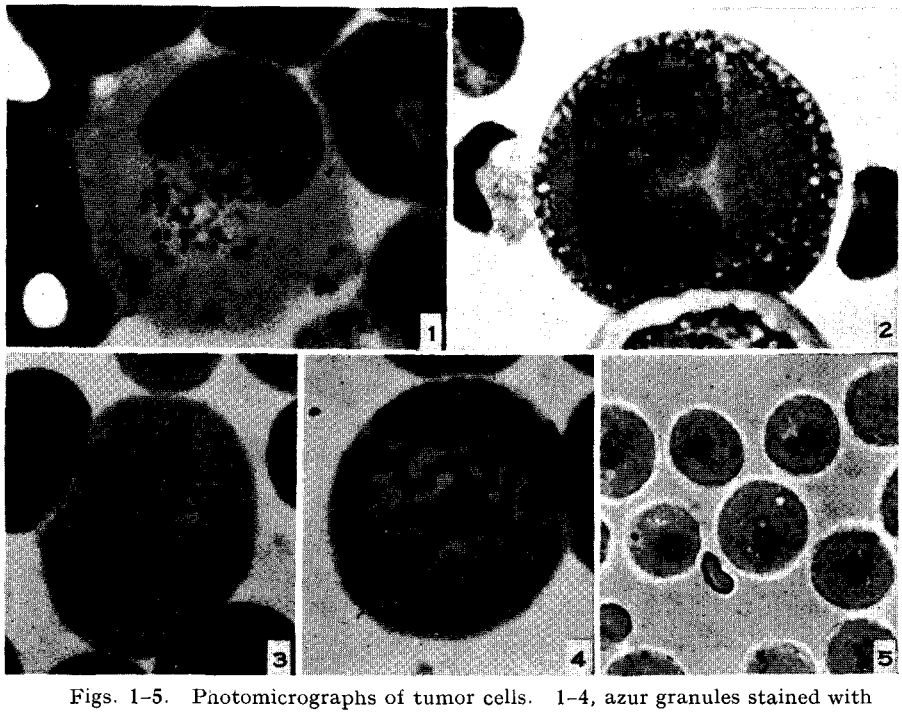
In order to learn the morphological relation of the azur granules in tumor cells to their metabolic activity irradiation experiments were undertaken. The experimental procedure is as follows : tumor-bearing rats were irradiated on the 3rd or 4th day after transplantation with X-rays at 200 r and 500 r in the total body exposure. At every one hour after irradiation, bits of the ascites were taken from the treated animals for the observation of the changes occurring in the azur granules in tumor cells.

## Results

1. *Morphological features of azur granules in the tumor cells and their behavior in a transfer generation.* The observations with Giemsa's preparation showed that the azur granules appeared as spherical, purple-red minute bodies of varying sizes existing in the cytoplasm of the tumor cell (Fig. 1). The number




Figs. 1–5. Photomicrographs of tumor cells. 1–4, azur granules stained with Giemsa's azuroeosin-methylenblue. ×1300. 1, a tumor cell with azur granules arranging in a rosette form (MTK-sarcoma III). 2, azur granules in tumor cell with vacuoles in the peripheral part of the cytoplasm (Hirosaki sarcoma). 3, azur granules in a tumor cell with lobated nuclei (Hirosaki sarcoma). 4, azur granules in a prophase cells (MTK-sarcoma III). 5, cell showing neutral red granules demonstrated by supravital staining (MTK-sarcoma III). ×500.

of azur granules was inconstant. Generally, they were found surrounding the nucleus in a radial arrangement. Granules scattered near the nucleus were comparatively smaller than those distributed in the peripheral part of the cytoplasm. Sometimes considerably larger ones were observable in the peripheral part of the cytoplasm. In the tumor cells with lobated nuclei, the azur granules could be found in a rosette arrangement in the central nuclear area in a manner similar to the regular mononucleate cell (Fig. 3). They were variable in number and observable both in the resting and in the prophase stage. At early prophase, they showed a typical rosette arrangement, while at later prophase they mostly disappeared (Fig. 4). Through metaphase, anaphase and telophase, the azur granules were generally invisible though they occasionally occurred. Their orientation, number and some other features show nothing having connection with the cytoplasmic movement in cell division. The Hirosaki sarcoma (Fig. 2) was characterized by showing azur granules which were identical in arrangement and other morphological features with those occurring in the others to ascites tumors. Further, it was found by means of the supravital staining method that in the place where the azur granules occurred neutral red granules were also present (Fig. 5).

Through one transplant generation of the Yoshida sarcoma, MTK-sarcoma III and Hirosaki sarcoma respectively daily observations were made on the behavior of the azur garnules in tumor cells; special attention was directed to the increase of the cells with or without azur granules. The life span of the tumor-bearing rats, implanted with the Yoshida sarcoma, MTK-sarcoma III and Hirosaki sarcoma were 7, 9, 10 days, respectively. The daily frequency of the mitotic tumor cells was also observed for comparison with the acetic dahlia preparations sampled from the same tumor. The data are as shown in Table 1.

On the lst day after transplantation of the tumor, the tumor cells showing no azur granules appeared at a high frequency, while those containing azur granules

Table 1. The daily frequency of the tumor cells with azur granules through a transfer generation of the Yoshida sarcoma, MTK-sarcoma III and Hirosaki sarcoma.

| Days after transplantation | Yoshida sarcoma | | MTK-sarcoma III | | Hirosaki sarcoma | |
|---|---|---|---|---|---|---|
| | azur granule cells (%) | mitotic rate (%) | azur granule cells (%) | mitotic rate (%) | azur granule cells (%) | mitotic rate (%) |
| 1 | 16.3 | 3.2 | 23.6 | 2.3 | 11.9 | 2.3 |
| 2 | 40.5 | 5.3 | 34.3 | 3.6 | 40.4 | 5.3 |
| 3 | 22.8 | 3.6 | 36.4 | 4.2 | 38.6 | 4.0 |
| 4 | 16.4 | 2.1 | 51.4 | 4.4 | 36.6 | 2.7 |
| 5 | 2.0 | 1.2 | 28.2 | 3.6 | 28.7 | 2.9 |
| 6 | 1.3 | 0.6 | 10.0 | 3.5 | 25.8 | 2.0 |
| 7 | 3.0 | 1.0 | 11.7 | 3.0 | 13.1 | 1.1 |
| 8 | — | — | 6.5 | 2.2 | 16.0 | 0.9 |
| 9 | — | — | 0.9 | 1.4 | 6.3 | 0.7 |
| 10 | — | — | — | — | 4.1 | 0.3 |

were few in number. On the 2nd to 3rd day the cells without azur granules gradually decreased in number and were replaced by azur granule cells which showed a gradual increase in frequency towards the middle part of the life span of the tumor-bearing rat. On the 3rd to 4th day, the azur granule cells showed the highest frequency in occurrence. Toward the later part of a transplant generation, the cells without azur granules showed again a gradual increase. On the 7th to 10th day, nearing the death of the host, the cells with azur granules showed remarkable decrease representing only about 5 per cent of the observed cells, with considerable increase in frequency of the cells without azur granules at more than 95 per cent. On the other hand, the regularly mitotic cells, that is, the tumor stem-cells (Makino 1952, Makino & Kanô 1955, Makino 1956) showed the highest frequency on the 2nd to 4th day after transplantation. Thereafter, they showed gradual decrease towards the latter part of the transplant generation.

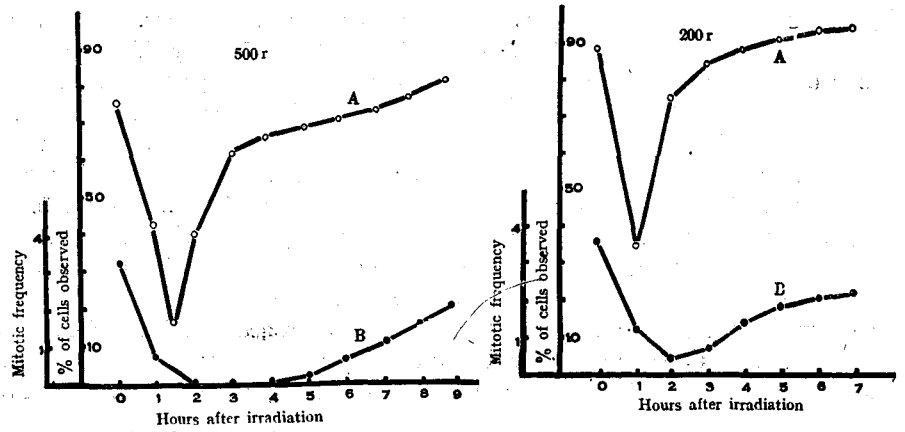



Fig. 6. Graphical representations showing the frequency of tumor cells after X-irradiation in the MTK-sarcoma III. A, tumor cells with azur granules, and B, mitotic frequency.

The results of the foregoing observations have revealed that the data derived from the observations of the azur granule cells and those from the tumor stem-cells run parallel; that is, both showed an increase in frequency from the early part of the transplant generation through the middle part and decreased towards the end of the generation. This evidence seems to imply that the azur granule cells are to be regarded as the tumor stem-cells showing a high metabolic activity in a resting condition. This is an item of evidence just similar to that obtained in the observations of the neutral red granules (Okada 1954), and indicates that the azur granules of tumor cells occur in close association with the metabolic activity of the cells.

2. *Behavior of the azur granules as observed after irradiaation.* In order to

obtain further data on the relation of tumor cells to the metabolic activity, the behavior of the azur granules in response to irradiation was investigated next. X-rays inhibit cell division but do not damage the cytoplasm. The whole body irradiation at 200 r and 500 r was made in tumor-bearing animals on the 4th day after transplantation of the MTK-sarcoma III. Samples of the ascites were taken from the treated animals every 1 hour after exposure and examined. The results of these experiments are illustrated in Figure 6.

Before irradiation, the tumor ascites contained azur granule cells in 70 to 80 per cent of the cells observed. At 1 hour after X-irradiation, mitotic tumor cells were nearly invisible in the ascites. This condition continued for 4 hours after treatment at which time the azur granule cells in the irradiated rats corresponded to 20 per cent of those occurring in untreated rats. At about 9 hours after irradiation, a few mitotic tumor cells were visible in the ascites. Thereafter, a gradual increase of mitotic cells occurred. At the same time, the tumor ascites showed many cells with azur granules in the cytoplasm in a rosette arrangement. Their frequency increased again in 70 to 80 per cent of cells observed. The evidence presented seems to show that the azur granules of the tumor cell occur in close association with the cellular functions.

## Concluding remarks

Based on the results from this study, the following inference can be drawn : the azur granules in the tumor cells of the Yoshida sarcoma, MTK-sarcoma II and Hirosaki sarcoma show a close similarity in form and distribution to the neutral red granules. Further, the azur granules seem to behave in close association with the metabolic cycle of tumor cells. This was shown, for instance, by the data involving the frequency distribution of tumor cells with and without azur granules through a transplant generation.

Okada (1954) and Tonomura (1955) have investigated the corresponding relationship between the cytoplasmic granules of tumor cells and the cellular functions in the MTK-sarcoma II, and III by means of supravital staining methods. They found that the granules stained with neutral red and toluidine blue appeared most markedly in the tumor cells sampled 2 to 3 days after transplantation, while as the end of life of the tumor-bearing rat approached, they were replaced by tumor cells bearing vacuoles. A similar relationship was established in the present study, showing that the metabolic activity of tumor cells is closely associated with the occurrence of the azur granules. It seems probable that these granules occurring in tumor cells are of the same nature as the neutral red granules or toluidine blue granules. The chemical nature of these cytoplasmic granules remains unknown at present, though the neutral red or toluidine blue granules are regarded as a polysaccharide ester sulfate of unknown composition.

Shear and Belkin (1937) performed a chemical test in living and non-living cells of mouse tumors with neutral red ; they said that the granules in the living

cells are different from those in the non-living cells. The data from the irradiation-experiment in the present study, indicates that the azur granules of the tumor stem-cell exist in close association with the cellular functions.

## Summary

The present paper describes the results of an investigation on the behavior of the azur granules occurring in the tumor cells of the following three rat ascites tumors, Yoshida sarcoma, MTK-sarcoma III and Hirosaki sarcoma, observed through the smear preparation technique with Giemsa's azuroeosin-methylenblue staining.

It was found that the general morphological features of the azur granules are nearly similar to those of the neutral red granules demonstrated by the supra-vital staining method.

From the results of the cell frequency observations made through one transplant generation of the tumors, and through an irradiation-experiment, it was revealed that the change in morphology of the azur granules is closely related to the variation in cellular functions of tumor cells, and that the azur granules occurred in a close association with the metabolic activity of the cells. The azur granule cells are to be regarded as the tumor stem-cells being in a resting condition.

## References

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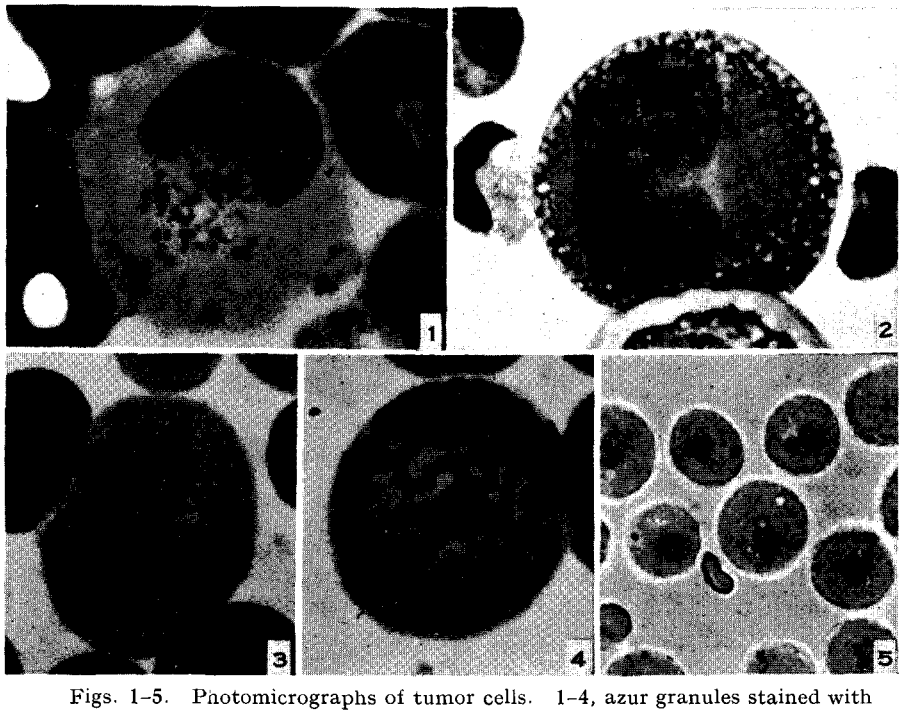


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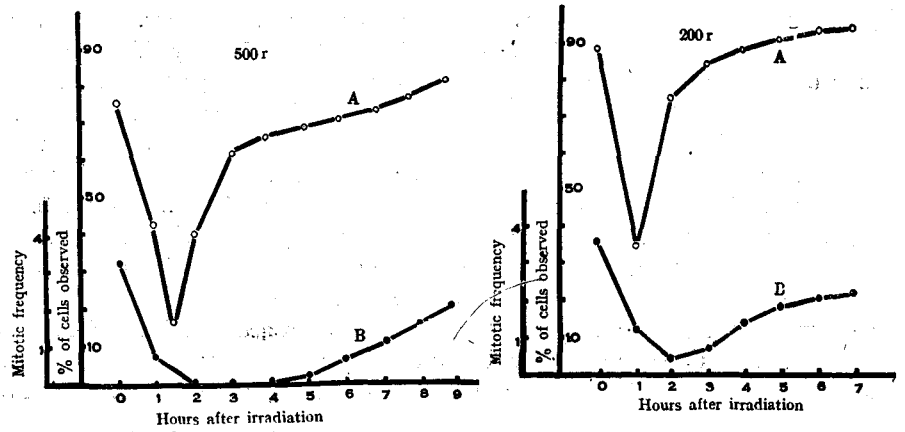


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