The Cytological Effect of Chemicals on Ascites Sarcomas

IV. A phase microscopy study of the damage to the tumor cells by podophyllin¹⁾

Sajiro Makino and Yuh H. Nakanishi

Zoological Institute, Faculty of Science, Hokkaido University, Sapporo

Received October 4, 1954

It has been well shown through the observations both *in vivo* (King and Sullivan 1947, Belkin 1948, Greenspan, Leiter and Shear 1950, Leiter, Downing, Hartwell and Shear 1950, MacCardle 1951, Makino and Tanaka 1953, etc.) and *in vitro* (Ormsbee, Cornman and Berger 1949, Makino and Cornman 1953) that podophyllin and its components do exert a powerful mitotic poisoning effect and do damage both malignant cells and normal tissues. The mechanism of their destructive effects as mitotic poisons, however, has remained unexplored. Ormsbee, Cornman and Berger (1949) suggested that podophyllin contains not only mitotic poisons but also some other substance or substances which selectively damage tumor cells by a mechanism other than that of disrupting cell division. Cornman and Cornman (1951) are of opinion that the destructive effects of podophyllin appear to exceed that which is explainable solely as a result of mitotic effects.

Previous observations of damage by podophyllin and its derivatives to rat ascites tumors *in vivo* (Makino and Tanaka 1953, Tanaka et al. 1955) have shown that these chemicals extensively attack mitosis. In podophyllin-treated animals, the division of tumor cells is arrested at metaphase, the chromosomes agglutinate into several clumps, and nuclear disintegration follows (Tanaka and Makino 1953). To obtain supplementary data upon the role of mitotic effects of podophyllin, the changes of living tumor cells in response to exposure to podophyllin have been followed in this study by phase microscopy using the temporary hanging-drop preparation.

The authors are grateful to Mr. H. Nakahara for his kind help in securing the data for this paper. They are also indebted to Dr. I. Cornman of the George Washington University Cancer Clinic, Washington, D. C., U. S. A. for his kindness in supplying the drug used for this experiment.

Material and method

The hanging-drop method in combination with the application of fluid paraffin has been proved to be very advantageous for the study of living cells

¹ Contribution No. 320 from the Zoological Institute, Faculty of Science, Hokkaido University, Sapporo, Japan.

Supported by a grant from the Scientific Research Fund of the Ministry of Education.

(Makino and Nakahara 1953). Podophyllin to be used in the following experiments does not mix directly with fluid paraffin. Carbon tetrachloride was, therefore, used as an intermediate agent to mix podophyllin with fluid paraffin.

One-tenth gram of podophyllin, after being dipped rapidly in absolute alcohol, was dissolved in 10 cc of carbon tetrachloride, and 0.05 cc of the carbon tetrachloride-podophyllin solution thus prepared was melted with 1 cc of fluid paraffin. Thus, 1 cc of fluid paraffin contains 0.5 mg of podophyllin¹. The procedure to prepare the hanging-drop preparation was as follows:

The depression of the slide is sufficiently filled with fluid paraffin containing podophyllin. A small amount of the tumor sample is dropped on a clean cover-slip without applying any medium. Then the cover-slip thus mounted is inverted over the depression slide. A slight pressure is applied on the cover-slip, and the excess of fluid paraffin is removed with blotting paper. The tumor sample for study was obtained from the tumor-bearing rat 3 to 4 days after the transplantation of the tumor by abdominal puncture with a fine glass. By this time, the most active proliferation of the tumor cells had been attained in the peritoneal cavity of the animal. The tumor used was the MTK-sarcoma III, a new ascites tumor of rats experimentally induced in this laboratory.

For control observations, the tumor sample was mounted with carbon tetrachloride-fluid paraffin containing no podophyllin (0.05 cc of carbon tetrachloride melted with 1 cc of fluid paraffin).

The preparation thus prepared was studied exclusively with the aid of an Olympus phase-contrast microscope, the optics used being a positive-medium contrast 100 $\times$ objective in conjunction with a Periplan 10 $\times$ ocular. The microscope was set in a chamber equipped with an electric warmer. The experiments were run at a temperature of 35°C.

Test for the possible effect of carbon tetrachloride mixed with fluid paraffin

Prior to the experiment of podophyllin, the possible effect of carbon tetrachloride mixed with fluid paraffin was tested for comparison. The tumor sample was studied with the hanging-drop preparation prepared with the application of 0.05 cc of carbon tetrachloride mixed with 1 cc of fluid paraffin.

Figures 1 to 5 are photomicrographs taken with the aid of the phase-contrast microscope, showing the successive series of a division process followed through a single tumor cell, beginning with metaphase and ending with telophase. The division proceeded apparently regularly, showing no visible evidence of injurious effect of carbon tetrachloride.

¹ This is an appropriate concentration to make the mixture of podophyllin and fluid paraffin. At higher concentrations, podophyllin is difficult to mix well with fluid paraffin. Higher doses of podophyllin induced stark necrosis of cells at once without preliminary distortion.

The duration of each mitotic phase measured as an example was 48 minutes for metaphase, 4 minutes for anaphase and 17.5 minutes for telophase. The time relation here obtained is essentially similar to that observed by Makino and Nakahara (1953) in the hanging-drop preparation of tumor cells mounted with fluid-paraffin only.

Figs. 1 to 5. Photomicrographs of the tumor cell taken by the olympus phase microscopy, showing the successive series of a division process followed through a single cell, observed with the hanging-drop preparation prepared with carbon tetrachloride-fluid paraffin. 1, metaphase, 48 minutes after preparation. 2, beginning of anaphase, 51.5 minutes after preparation. 3, anaphase, 53.5 minutes after preparation. 4, telophase, 66 minutes after preparation. 5, completion of cell division, 71 minutes after preparation. $\times 1000$.

Generally the tumor cells were viable with no observed change for a period of 5 to 6 hours in the hanging-drop preparation prepared with the application of fluid paraffin containing a minute quantity of carbon tetrachloride.

Response of tumor cells following exposure to podophyllin

The changes of tumor cells in response to exposure to podophyllin were successively followed in single cells in the hanging-drop preparation made with fluid paraffin containing the drug.

As early as 20 minutes after the preparation of the hanging-drop slide, changes in response to podophyllin were observed under microscope in every one of the tumor cells. General pattern of response is a severe blebbing of the cell surface. A single cell produces many finger-like cytoplasmic buds of varying sizes and shapes through blebbing. They are then constricted off from the main body of the cell forming cytoplasmic fragments. With a repeated budding off of cytoplasmic fragments, the cell body becomes smaller and narrower. In addition to the above change, there occurs also a progressive pycnosis of the nucleus. The cytoplasmic fragments also undergo disintegration. Damage to the cell is thus attained and its death results.

Figures 6 to 17 form a successive series showing the course of cell damage in response to podophyllin followed in three tumor cells. Figure 6 shows three tumor cells at the resting stage, taken 7 minutes after the exposure to podophyllin. The first step of response is irregular indentation of the cell

surface. Figure 7 indicates the cells observed at 22 minutes after exposure; they begin to produce blebbing in their surface. The blebbing of the cell surface becomes more intensive with time (Figs. 8-11). Later, the blebbing

Figs. 6 to 17. Photomicrographs of tumor cells taken by phase microscopy (Olympus), showing the successive series of a course of cell damage in response to podophyllin. 6, three tumor cells at resting stage, 7 minutes after exposure to the drug. 7, the cells showing signs of blebbing in their surface, 22 minutes' exposure. 8, the cells showing blebbing of the surface, 33 minutes exposure. 9, the same, 36 minutes' exposure. 10, the same, 37 minutes' exposure. 11-14, the same, with budding off of cytoplasmic fragments, 49, 63, 66 and 74 minutes' exposure, respectively. 15-17, fragmentation of the cytoplasm into irregular masses, with degenerative changes of the nucleus, 89, 133 and 295 minutes' exposure, respectively. $\times 670$.

results in the formation of many finger-like cytoplasmic buds of varying sizes and shapes constricted off from the cell body (Figs. 12-14). The cytoplasmic

fragments increase in number with time due to the repeated budding off, and consequent reduction in size of the cell body is induced. By this time, the cells develop cytoplasmic vacuoles of varying sizes, and progressive degenerative changes of the nucleus follow inducing its pycnotic degeneration (Figs. 15-17). There is also disintegration in the cytoplasmic fragments. Infrequently, the final damage takes place through a breaking down of the cell body with a following disintegration of the naked nucleus.

Figures 18 to 23 show the course of disintegration taking place in a mitotic cell. In figure 18 is shown a cell at metaphase observed 11 minutes after exposure to podophyllin. The cell exhibits irregular indentation of its surface at 17 minutes after exposure (Fig. 19). The cell surface becomes more distorted with

Figs. 18 to 23. Photomicrographs of tumor cells taken by phase microscopy, indicating a course of disintegration in a mitotic cell in response to podophyllin. 18, a cell at metaphase observed 11 minutes after exposure. Irregular indentation of the surface is seen. 19-21, the cell becomes more distorted, 17, 22 and 48 minutes' exposure, respectively. 22, the cell showing fragmentation of cytoplasm, 75 minutes' exposure. 23, the cell showing fragmentation of cytoplasm into many small masses. The chromosomes damage by pycnosis. 133 minutes' exposure. $\times 1000$.

time (Fig. 20-21). The division arrested at metaphase: the chromosomes are irregularly condensed. At much later time, the cell produces several cytoplasmic fragments through budding, and shows chromosome clumping (Fig. 22). The final damage is attained by fragmentation of the cytoplasm into many small masses and by pycnotic degeneration of chromosomes (Fig. 23).

Discussion

Podophyllin and its components are known as efficient mitotic poisons; they damage both malignant cells and normal tissue. Makino and Tanaka (1953) observed the effect of podophyllin on the division of tumor cells in rat ascites sarcomas *in vivo*. They showed that tumor cells were arrested at metaphase. The chromosomes become shorter and thicker with bizarre

outline. The cytoplasm showed irregular processes. Later the condensed chromosomes were agglutinated into irregular masses. They were then stuck together, and death of the cell was induced. MacCardle (1951) reported on the effect of podophyllin on tumor cells of Sarcoma 37 that in treated cells many clear vacuoles developed among the chromosomes arrested at metaphase. They coalesced into a single large sphere in the mid-plate region of the cell. A high dose of podophyllin induced stark necrosis at once, without preliminary distortion of mitotic figures. Ormsbee, Cornman and Berger (1949) indicated in tissue culture experiments with mouse sarcoma L 946 and lung tumor MA 387 that a sterile suspension of podophyllin exerts a toxic and regressive effect against tumor cells. Makino and Cornman (1954) studied cytological responses of malignant cells to podophyllotoxin *in vitro* and concluded that although podophyllotoxin was an efficient mitotic poison, it also damaged the cell as a whole, and appeared to exert a direct effect on the cytoplasm as evidenced by rounding, blebbing and vacuolization.

The visible effect of podophyllin on tumor cells observed by phase microscopy appears to follow a pattern of response similar to that described for podophyllotoxin by Makino and Cornman (1954) in the tissue culture material. It was shown in the present study that podophyllin damaged the cells as a whole and exerted visible influence on the cytoplasm. The cells were damaged by blebbing, budding off and vacuolization of the cytoplasm, followed by a pycontic degeneration of the nucleus, or of the chromosomes. Further, phase microscopic observations demonstrate that podophyllin damages both resting and mitotic cells, effects that were hardly ascertained in the fixed and stained material with the *in vivo* experiment (Makino and Tanaka 1953). It appears that the way the drug affects the cells *in vivo* is different from that *in vitro*. Probably the damage to tumor cells as produced in this experiment is largely based upon the toxic effects of the drug. Apparently the effects seem to be governed by the dose: the severity of response may increase with increased concentration of the drug used and also by duration of exposure. Further, the type of response and its severity may vary with the type of tissue under study, as shown by Makino and Cornman (1954). Imagi (1953) reported different responses of tumor cells to different chemicals. To analyse the mechanism of the mitotic effects of podophyllin and its derivatives, the collection of further experimental data must be awaited.

Summary

This paper deals with a phase microscopy study on the effects of podophyllin on the tumor cells of the MTK-sarcoma III. The changes of tumor cells in response to exposure to the drug were followed in single cells with the utilization of the hanging-drop preparation made in combination of fluid paraffin and podophyllin.

It was shown that podophyllin damaged the cells as a whole and exerted

a visible influence on the cytoplasm. The cells were damaged by blebbing and vacuolization of the cytoplasm, followed by a pycnotic degeneration of the nucleus, or of the chromosomes.

References

- Belkin, M. 1948. Effect of podophyllin on transplanted mouse tumors. *J. Pharm. Exp. Ther.* 93: 18-25.
- Cornman, I. and Cornman, M. E. 1951. The action of podophyllin and its fractions on marine eggs. *Ann. N. Y. Acad. Sci.* 51: 1443-1487.
- Greenspan, E. M., Leiter, J. and Shear, M. J. 1950. Effect of alpha-peltatin, beta-peltatin, and podophyllotoxin on lymphomas and other transplanted tumors. *J. Nat. Cancer Inst.* 10: 1295-1333.
- Imagi, S. 1953. Some ecological observations on the Yoshida sarcoma cells with the aid of the phase microscope. II. On the influence of several chemicals upon the Yoshida sarcoma cells. (In Japanese with English résumé). *J. Kyoto Pref. Med. Univ.* 53: 187-206.
- King, L. S. and Sullivan, M. 1947. Effects of podophyllin and of colchicine on normal skin, on condyloma acuminatum and on verruca vulgaris. *Arch. Path.* 43: 347-386.
- Leiter, J., Downing, V., Hartwell, J. L. and Shear, M. J. 1950. Damage induced in sarcoma 37 with podophyllin, podophyllotoxin, alpha-peltatin, beta-peltatin, and quercetin. *J. Nat. Cancer Inst.* 10: 1273-1293.
- MacCardle, R. C. 1951. Effects of mitotic inhibitors on tumor cells. *Ann. New York Acad. Sci.* 51: 1489-1496.
- Makino, S. and Nakahara, H. 1953. Cytological studies of tumors, X. Further observations on the living tumor cells with a new hanging-drop method. *Cytologia* 18: 128-132.
- and Cornman, I. 1953. Different cytological responses of malignant cells to podophyllotoxin *in vitro*. *Cytologia* 18: 277-282.
- and Tanaka, T. 1953. The cytological effect of chemicals on ascites sarcomas, I. Partial damage in tumor cells by podophyllin followed by temporary regression and prolongation of life of tumor-bearing rats. *J. Nat. Cancer Inst.* 13: 1185-1199.
- Ormsbee, R. A., Cornman, I. and Berger, R. 1949. The effect of podophyllin on tumor cells *in vitro*. *Acta Unio internat. contra cancerum* 6: 657-664.
- Tanaka, T., Kanô, K., Tonomura, A., Okada, T. and Umetani, M. 1955. The cytological effect of chemicals on ascites sarcomas, III. Damage induced by podophyllotoxin, alpha-peltatin, beta-peltatin and quercetin. *Gann* 46:15-26.
-