Silver Staining of Nucleolar Granules in Tumor Cells¹

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ABSTRACT

With the aid of a simple silver-staining procedure, large numbers and unusual arrays of nucleolar argyrophilic granules were found in Novikoff hepatoma, KB, and HeLa cells. Some of these arrays consisted of linearly arranged discrete granules, and others were in two to three rows each containing three to five granules. Corresponding formations were not found in either the normal or regenerating liver nucleoli which contained an argyrophilic network in which the dark granules were apparently associated with the less dark argyrophilic fibrils of a reticulum. The nucleolar argyrophilic granules were readily identifiable in the separated daughter nuclei of the tumor cells in telophase, suggesting that the increased nucleolar activity of the G1 phase begins in these cells even before cell division has been completed.

INTRODUCTION

In addition to their aberrations in size and shape, and unusual antigens (2, 3), tumor nucleoli have a high rate of synthesis of pre-rRNA (1). In the search for approaches to light microscopic visualization of initiation and transcription complexes of nucleolar pre-rRNA synthesis, attention was directed to the possible usefulness of silver staining reported in the studies of Howell (9-11), Hubbell and Hsu (12), Goodpasture and Bloom (8), Schwarzacher (20), and Giminez-Martin et al. (7).

Silver staining of the nucleolus was developed first as an offshoot of the elegant methods for silver staining of tissues (19) during the last century and then was used for the demonstration of "nucleolonemas" (5, 13, 18), curled fibrils in the nucleolus. Its recent use has been the demonstration of NOR² in the specific "nucleolar" chromosomes which contain rDNA (4, 6-12). Preliminary cytochemical studies on these silver-staining NOR elements indicated that they were nonhistone proteins and not DNA, RNA, or histones (9, 10, 20). Particularly interesting questions from these results concern why the NOR regions of chromosomes carry with them nonhistone protein(s) and the nature of these nonhistone proteins.

Among the problems in the use of silver staining for biochemical studies on nucleolar proteins was the length of time and complexity of the methods used for silver staining and the degree of background (6, 8, 11, 13, 18). The present report indicates that silver staining could be simplified and shortened with a high reproducibility by increasing the concentration of silver nitrate in the staining solutions and developer. With this simplified method, large numbers and interesting formations of silver granules were detected in nucleoli of tumor cells including Novikoff hepatoma, HeLa, and KB cells; different types of silver-staining structures were found in normal and regenerating liver nucleoli. During these studies, analysis of the mitotic stages indicated that at metaphase the few granules present, usually in doublets, were associated with NOR regions of chromosomes (7-9, 11, 20). However, even before the completion of telophase, a large number and unusual formations of granules were present in the tumor cells. If these arrays of granules reflect the increased nucleolar activity in the G1 phase, the Novikoff hepatoma may be in the G1 phase even before termination of mitosis.

MATERIALS AND METHODS

Tumors and Other Tissues. Novikoff hepatoma ascites cells were transplanted i.p. into adult male albino Holtzman rats (The Holtzman Co., Madison, Wis.) weighing 200 g. Samples of these cells were taken 6 days after implantation of the tumor. HeLa and KB cells were grown in tissue culture in modified Eagle's medium. The original samples of the KB cells were obtained from Flow Laboratories (Rockville, Md.). Normal livers and regenerating livers were obtained from normal adult male albino Holtzman rats weighing 200 g. Hepatectomy was done 6, 12, or 18 hr prior to sacrifice of the animals. Smears of cells, cell fractions, and tissues were spread on microscope slides and air dried.

Silver Staining. The silver impregnation procedure (4, 8-12, 18, 20) was modified for tissues and cell smears to provide standard results in a relatively short period of time. Air-dried smears of the various types of cells were fixed in Carnoy’s mixture (methanol:glacial acetic acid, 3:1) for 10 min and then washed with running water and dried. The smears were covered with concentrated AgNO₃ solution (1 g/ml) for 5 to 7 min, after which the solution was drained off. The smears were next covered with a mixture prepared by adding 37% formaldehyde containing 12% methanol (Fischer, Houston, Texas) to an equal volume of AgNO₃ (1 g/ml) and then were incubated for 3 to 5 min at 40-50° on a warm plate. The silver nitrate solution should not be kept more than 2 days, and the formaldehyde-silver nitrate solution was prepared just prior to use.

To prevent the precipitation of silver salts, a thorough running-water wash was made. The sample was stained with May-Grünwald (1.6 g of methylene blue eosinate per liter of methyl alcohol solution) (Curtin Matheson Scientific Instruments, Houston, Texas) and diluted with 1:1 distilled or tap water for 1 min. After a thorough wash with the running water, the smears were air dried in the vertical position to prevent the formation of precipitates of silver salts.

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² The abbreviation used is: NOR, nucleolar organizer region.

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salts and then were examined on a light microscope under oil immersion.

RESULTS

Silver-stained Granules in Tumor Cells. Fig. 1 is a composite showing various formations of silver granules in a number of tumor cells. The number of granules varied considerably from nucleolus to nucleolus and cell to cell in these preparations. The mean number was 53 granules/nucleus and 21 granules/nucleus in the populations of Novikoff hepatoma ascites cells; 13 granules/nucleus and 20 granules/nucleus in KB cells, or 11 granules/nucleus and 39 granules/nucleus in HeLa cells (Table 1). Many nucleolar granules appeared to be part of a reticulum in these tumor cells as noted earlier by others (12, 17). However, quite frequently, the nucleolar granules appeared to be isolated free of reticulum. Frequently, they were arrayed in one or more rows containing several granules per row (Figs. 1, a to d, and 3a) in each of the types of tumors studied.

Silver-staining Nucleolar Elements in Normal and Regenerating Liver. In normal liver (Fig. 2a) and in the 6-hr (Fig. 2, b and c) and 18-hr regenerating liver (Fig. 2d), the nucleolus contained the characteristic reticulum described as a "nucleolonema" by Estable and Sotelo (5) and subsequent workers (1, 7, 13, 18). The nucleolar granules were thickenings of this network at a number of points; thus the structure resembles a reticulum. Unlike the tumors, the liver nucleoli did not contain individual granules or the types of rows noted above. Both the density of the staining and the size of the nucleoli were markedly increased in the regenerating liver nucleoli (Fig. 2, c and d). An analysis of the numbers of "granules" present per nucleolus indicated they increased from approximately 4/nucleolus and 13/nucleus in the normal liver to approximately 15/nucleolus and 33/nucleus in the 18-hr regenerating liver. These values correlate well with the increases reported in 45S RNA synthesis from 3 to 5 to 15 fg/min/nucleolus in the 18-hr regenerating liver (1).

Nucleolar Granules in Cell Division of Novikoff Hepatoma Cells. Fig. 3 shows that during the course of cell division there were marked changes in the number and appearance of the silver-staining nucleolar granules. During metaphase (Fig. 3), "doublets" of granules associated with chromosomes in the NOR (7-9, 11, 20) were present around the metaphase equatorial plates, and their total number was approximately 8 to 11. The size and density of these granules were approximately the same as that of the nucleolar granules, but it is not clear whether they contain the same silver-staining proteins.

During anaphase, the number of granules appeared to have increased but the granules were largely single; i.e., the doublets appear to have been separated (Fig. 4a). In telophase (Fig. 4, a and b), when the cytoplasm of the daughter cells had not separated, there was a marked increase in the density and the number of the silver granules in the newly formed nuclei. As noted in Fig. 4b (arrow), there was a reappearance of the linear arrays of the separated nucleolar granules. Apparently, even before the daughter cells had fully formed, the nuclei contained the large number of dense granules found in interphase cells.

DISCUSSION

The present studies represent an extension of attempts in this laboratory to investigate the elements of the nucleolus of the cancer cell, particularly with respect to the aberrations in nucleolar size, shape, and hyperactivity in human neoplasia (1). An important basis for such investigations has been the development of rapid methods for staining nucleoli and their substructures as an aid to biochemical analysis of nucleolar components. The present report of a rapid, simple, and reproducible method for silver staining will permit detailed studies on the constituents of the argyrophilic nucleolar elements, their roles in nucleolar function, and their changes during the cell cycle.

Staining with silver nitrate revealed several interesting properties of nucleoli of tumor cells including a large number of nucleolar argyrophilic granules and interesting formations of discrete granules in linear arrays, which were different from those observed in normal and regenerating liver cells. There appears to be a relationship between the number of granules and the rates of nucleolar synthesis of preribosomal 45S rRNA. In mature lymphocytes, where pre-rRNA synthesis is negligible (1), very few granules (0 to 4/nucleus) were found in rat (Fig. 1a) or human (20) lymphocytes. In normal liver (1), where the rates of synthesis of 45S pre-rRNA are low (3 to 5 fg/min/nucleolus), there were approximately 4 granules/nucleolus and 13 granules/nucleus. In the advanced G1 state of 18-hr regenerating liver, there were approximately 15 granules/nucleus and 33 granules/nucleus. In the Novikoff hepatoma, there were approximately 21 granules/nucleolus and 53 granules/nucleus (1).

These cytochemical findings must be extended and confirmed in a number of systems before conclusions can be drawn either with respect to common features of these granules in tumors and their potential diagnostic or functional significance. From the point of view of biology of cancer, it was interesting that even before termination of telophase, the nucleus and nucleoli contained large numbers of these granules in similar arrays to those found in interphase tumor cells.

The nature of the argyrophilic elements in the nucleolus is not known. Preliminary studies have suggested that

![Table 1: The number of argyrophilic granules in nucleoli of various cells](image)

- Cells: Rat hepatocytes, Regenerating liver hepatocytes, Rat Novikoff hepatoma cells, HeLa cells, KB cells
- Grains/nucleus: Mean ± S.D.
- No. of cells analyzed: 1500
- From 3 animals
- Obtained 18 hr after partial heptatectomy
nucleolar phosphoproteins such as C23 and B23 (13-16) stain positively in one-dimensional gels with the method used in this study. The relationship of these proteins to those in the NOR and those that accumulate during telophase remains to be biochemically analyzed. The silver-staining techniques in smears and polyacrylamide gels will be of value in such analyses.

REFERENCES

Silver-staining Nucleolar Granules

2a

2b

2c

2d
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