Electron Microscopic Studies on HeLa Cells Exposed to the Antibiotic Toyocamycin

Ursula Heine
Viral Biology Branch, National Cancer Institute, Bethesda, Maryland 20014

SUMMARY

The antibiotic toyocamycin (TCM) selectively inhibits the synthesis of ribosomal RNA in different mammalian cells by suppressing the cleavage of the 45 S RNA precursor to 18-28 S RNA (27). Present ultramorphologic studies on HeLa cells exposed to TCM reveal a highly specific response to the drug. When employed in low concentrations, TCM selectively induces an enlargement of the pars fibrosa in the nucleolus of the treated cells. High doses of TCM, however, provoke morphologic changes similar to those observed with the action of antibiotics interfering with the template activity of DNA.

INTRODUCTION

It was recently found that the antibiotic toyocamycin (TCM) selectively inhibits ribosomal RNA (rRNA) synthesis in HeLa cells, mouse fibroblasts, and Ehrlich ascites cells (27). At low concentrations, the drug exclusively suppresses the formation of 28 S and 18 S RNA in the cell systems studied, but at the same low concentration it does not affect the synthesis of 32-45 S RNA, 4 S RNA, DNA, or protein. According to the biochemical data published, it is preponderantly the 45 S RNA which accumulates in the nucleolus of the treated cells. It was shown that toyocamycin [4-amino-5-cyano-7-β-D-ribofuranosyl-pyrrolo (2,3-d)pyrimidine] (TCM) is at least partly incorporated into this 45 S RNA molecule, most probably replacing adenosine (27).

Previous investigations have emphasized the importance of the nucleolus in cellular metabolism, i.e., in the synthesis of rRNA, it is known that a nucleolar RNA with a sedimentation coefficient of 45 S is the precursor of rRNA (15, 16, 17, 18, 22). There is evidence that a distinct morphologic entity, a fibrillar component of the nucleolus, might represent the 45 S precursor of rRNA (see Refs. 2, 8). The high specificity of TCM, in selectively inhibiting the maturation of this precursor, provides an excellent tool for attempting to correlate the biochemical and morphologic parameters of this entity.

For these reasons, it is of much interest to study the highly selective influence of TCM on cellular fine structure, especially that of the nucleus and nucleolus, and to investigate further the 45 S component which presumably accumulates in the nucleolus in the presence of the drug.

This report demonstrates that changes in cellular ultrastructure are very specific when TCM is employed in low concentrations, whereas high concentrations of the same antibiotic produce nuclear changes comparable to those observed under the influence of actinomycin D.

MATERIALS AND METHODS

Cells and Media. In order to facilitate a comparison of biochemical data already compiled with the morphology to be studied, our experiments were designed to be as similar as possible to those already published (27). HeLa-Sq cells used in this study were obtained either from Flow Laboratories, Inc., Rockville, Maryland, or from the Tissue and Media Unit of the NIH, Bethesda, Maryland. The cells were grown in Erlenmeyer flasks as suspension cultures at a concentration of 5-6 X 10⁵ cells/ml. The medium used was Eagle's minimum essential medium (4), supplemented with 10% heat-inactivated calf serum, 3% glutamine, penicillin (100 units/ml), and streptomycin (100 µg/ml). Cultures were routinely checked for contamination with mycoplasma and were found to be negative.

Antibiotic. TCM was a generous gift from Dr. Suhadolnik, Albert Einstein Medical Center, Philadelphia, Pennsylvania. It was added to the tissue culture medium in concentrations of 0.02, 0.01, 1.0, or 20.0 µg/ml. The cells were exposed to the drug for different lengths of time, ranging from 1 hr to 2 days, depending on the concentration used in the different experiments.

Fixation and Embedding. Cells were fixed in the cold in suspension by diluting cultures with equal volumes of 3% glutaraldehyde buffered to pH 7.45 with sodium cacodylate (21). The fixation time was 15 min, after which the suspensions were pelleted at 2000 rpm in an International PR-2 refrigerated centrifuge. The pellets were postfixed with Dalton's chrome-osmium fixative (3), and embedded in Epon-Araldite (14). Ultrathin sections were cut on an LKB-Ultrotome, transferred to Formvar-coated grids, and stained with uranyl-acetate (28) and lead citrate (19). They were examined in a Siemens Elmiskop I with double condenser and 50 µ objective aperture. An accelerating voltage of 80 kv was used.

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RESULTS

Controls

In the controls a large nucleus is surrounded by a small rim of cytoplasm. The nuclear membrane has many invaginations. Mitochondria and free ribosomes, mono- and polysomes, are numerous, whereas lysosomes and rough endoplasmic reticulum are sparse. In the interphase nucleus the major part of the chromatin is distributed evenly (diffuse chromatin). Only small patches of condensed chromatin are arranged adjacent to the nuclear membrane and throughout the nucleolus. Perichromatinic granules are frequent. The nucleolus is large, with its granular component (pars granulosa) being outstanding (Fig. 1, A). Areas of fine fibrillar material are present in it (Fig. 1, C). These areas are surrounded by the fibrillar component (pars fibrosa) of the nucleolus (Fig. 1, B), which contributes very little to the whole structure.

Response to TCM in Low Concentration

0.01 µg TCM/ml. The influence of the drug on ultrastructural changes in the cells is minute when applied in this low concentration. Nucleoli appear to be slightly larger than those in controls, and a small increase in fibrillar material has been observed after exposure to the antibiotic for 2 days.

0.1 µg TCM/ml. First changes in fine structure are evident only in the nucleoli after exposure to the antibiotic for 1 hr. As shown in Fig. 2, they are expressed by a loss of many of the nucleolar granules. The remaining nucleoli are mainly composed of fibrillar material, arranged into irregularly intertwined cords (nucleolonema) with few granules in their immediate proximity. Further and striking changes in fine structure are seen after exposure to the antibiotic for 6 to 12 hr. The organelles are often round and exhibit a pronounced density due to a substantial increase of their fibrillar component, paralleled by a diminution of their granular part (Fig. 3). The overall size of these nucleoli is similar to that seen in the controls. This process, the accumulation of fibrils, accompanied by a loss of granules, continues during the following 12 hr and results finally in large, round nucleoli of high density.

1.0 µg TCM/ml. The ultracytologic response of the cells to this concentration is similar to that provoked by 0.1 µg TCM/ml. However, the spherulation of the nucleolus and the accumulation of the fibrillar component in it are already pronounced in less than 6 hr (Fig. 4).

Response to TCM in High Concentration

20 µg TCM/ml. Early changes, after 1 to 6 hr exposure, predominantly affect the nucleus. These changes are similar to those caused by other antibiotics and drugs, such as actinomycin D (9, 11, 13), mitomycin C (13), anthramycin (7), aflatoxin (1), and 4-nitroquinolin-N-oxide (20), and they are expressed by a "nucleolar segregation" (25), i.e., the separation and redistribution of amorphous, fibrillar, and granular components into distinct entities (Figs. 5, 6).

DISCUSSION

It is now well established that 45 S to 32 S rRNA precursors are formed in the nucleolus of the cells (15-18, 22). Kinetic studies give evidence that a substantial amount of the precursor can be detected in HeLa cells as early as 30 min after labeling with $^{32}$P, whereas a label applied for a shorter period of 5 min can only be recovered in an RNA of great heterogeneity of size (10-100 S), which is not of the ribosomal type (10). As shown, in the presence of 0.1 µg TCM/ml for 6 hr, the 45 S rRNA accumulation in mammalian cell nucleoli is about 4-fold. At the same time, its maturation to 28 S and 18 S RNA is completely interrupted. There is no effect on the 4 S RNA (27).

The changes in nucleolar fine structure presented here after exposure to the same doses of TCM (0.1 µg/ml) for a prolonged time, 12 to 24 hr, result in a profound increase of material resembling that of the pars fibrosa. In agreement with the biochemical data mentioned above, it might be assumed that this material contains the 45 S rRNA. The small amount of granules which remain recognizable between the electron dense fibrils might then represent the components containing 35-32 S RNA. According to the biochemical data the presence of 28 S RNA can be excluded (27).

A short exposure to 0.1 µg TCM/ml for 1 hr results in an obvious loss of nucleolar granules. The remaining nucleolus is represented by a twisted and skein-like structure which bears some resemblance to forms observed during “reconstruction” of hepatic nucleoli after treatment with ethionine that was followed by methionine. Those structures were designated so as to be fundamental units in the restoration of the nucleolus (24). The pronounced appearance of similar structures early in our experiments might be due to (a) the transfer of ribosomal components already present at the start of the experiment into other areas of the nucleus and into the cytoplasm, and (b) the relatively small amount of ribosomal precursors manufactured during the short exposure to TCM.

The results of this work find support in earlier publications discussing autoradiographic studies on nucleoli of different cells (5, 6). The Chironomus salivary gland cell, e.g., accumulates label-designating rRNA precursor in the nucleolus over
the pars fibrosa after exposure for 13 min. The label shifts later to the granular part of the same organelle and can be found there as late as 46 hr after application (6). In addition, recent investigations on cells under thermic shock led to the belief that the fibrillar component of the nucleolus might constitute the 45 S ribosomal precursor (26).

However, the correlation of the granular component of the nucleolus with an RNA of specific sedimentation velocity is still under discussion. The autoradiographic studies mentioned above are comparable with the conclusion that 35 S and possibly 28 S rRNA, but little of the 45 S precursors are located in the granules. The effect of actinomycin D on cells of different types supports this assumption (18).

Although we could only detect traces of granular material in nucleoli under the influence of small doses of TCM, the accumulation and persistence of the nucleolar granules after exposure to high doses of the drug remains a puzzling problem. A possible explanation might be found by considering changes in the micro environment of the nucleolus, due to the conditions of the experiment, as a decisive factor in the formation of the granules. As shown in this report, high doses of TCM not only influence the morphology of the nucleolus, but are related to severe changes in the other parts of the nucleus including changes in the physical state of chromatin (redistribution and clumping). This holds true for the action of other drugs, such as actinomycin D (9, 11, 23), anthramycin (7), and aflatoxin (1), which were studied previously, and for the response to an infection with mycoplasma (12, see also Ref. 25). It might be feasible to speculate that these environmental changes influence much of the 45 S rRNA precursor which accumulates in the presence of TCM and promote its cleavage to the 32-35 S component, a process which would have its morphologic manifestation in the maturation of fibrils to granules.

The observed persistence of the granules, until cell death under the influence of TCM, is in good agreement with the biochemical findings (27) indicating that once the drug is incorporated into the precursor molecule, cleavage into 28 S and 18 S RNA-containing substructures is not possible.

Previous biochemical and present morphologic studies concerning the action of the antibiotic TCM on cellular metabolism open interesting aspects for further investigations in analyzing DNA-dependent RNA synthesis as a nucleolar function.

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REFERENCES


Figs. 1-7. All specimens were fixed with glutaraldehyde, postfixed with chrome-osmium, embedded in Epon-Araldite, and stained with uranyl acetate and lead citrate.

Fig. 1. HeLa cell, control. Nucleolus with abundant granules, pars granulosa (A), small areas of fibrils, pars fibrosa (B), and two areas containing material of very fine fibers (C). Chromatin (CH), nuclear membrane (NM). X 30,000.

Fig. 2. HeLa cell exposed to 0.1 μg toyocamycin/ml for 1 hr. The loss of nucleolar granules (A) is evident. The nucleolus consists mainly of cords of fibrillar material, nucleolonema (B), with few ribosome-like granules in their immediate proximity. Two structures (C) similar to those designated as C in Fig. 1 are recognizable. X 30,000.

Fig. 3. Nucleolus of a HeLa cell after treatment with 0.1 μg toyocamycin/ml for 12 hr. The nucleolus is very dense. The areas designated as C in the control are still recognizable (C). The distinction between fibrils and granules is difficult due to the extreme density of the structure. The nucleolus contains many fibrils (B), and few granules (A) are distributed at random throughout the structure. X 30,000.

Fig. 4. HeLa cell exposed to 1 μg toyocamycin/ml for 6 hr. The nucleolus forms a large, dense body. Its major component is fibrils (pars fibrosa). Few granules, remnants of the pars granulosa, with a diameter of approximately 150 Å can be recognized throughout the structure (see inset arrow). X 30,000. Inset, X 120,000.

Fig. 5. Nucleus of a HeLa cell after treatment with 20 μg toyocamycin/ml for 2 hr. The observed alterations affect only the nucleolus. The separation of its components is evident resulting in few accumulations of granules (A); areas of high density containing the fibrillar component (B) also result. Chromatin is distributed diffusely throughout the nucleus. X 18,000.

Fig. 6. Nucleolus of a HeLa cell treated in a similar manner as illustrated in Fig. 5, but at a higher magnification. The separation of the nucleolar components is very pronounced. The granular part of the nucleolus is large and contained in the center of the photograph (A). Fibrillar part of the nucleolus (B). X 32,000.

Fig. 7. Nucleolus of a HeLa cell exposed to 20 μg toyocamycin/ml for 24 hr. After prolonged exposure to TCM in high concentration, the visible alterations affect the nucleolus as well as other parts of the nucleus. A nucleolar segregation is evident. The granular part of the nucleolus is prominent (A), and a small area of fibrils is recognizable (B). Chromatin (CH) is clumped and accumulated adjacent to the nuclear membrane. Dense granules (arrows), about 200-300 μm in diameter, are found in clumps adjacent to the granular component of the nucleolus. Individual electron-dense granules are distributed throughout the remaining nucleoplasm. X 17,500.
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