Correlation of Nucleolini with Fine Structural Nucleolar Constituents of Cultured Normal and Neoplastic Cells

Robert Love and Ramon Z. Soriano

Department of Pathology, Jefferson Medical College, Philadelphia, Pennsylvania 19107

SUMMARY

Alterations in the structure of nucleoli of normal diploid human embryo fibroblasts and of aneuploid HeLa cells produced by the conditions of in vitro culture and by treatment with thymidine have been studied by light and electron microscopy. During exponential growth of diploid cells, the dense fibrillar component of the nucleolus is mainly produced by the conditions of in vitro culture and by treatment with thymidine. These correspond to densely packed spherical nucleolini and fibrillar centers. These correspond to densely packed spherical nucleolini which are often too numerous to count or too small to resolve in the light microscope. The correlation between nucleolini and fibrillar centers becomes more striking in stationary cultures of human fibroblasts, where few large nucleolini in the light microscope correspond to few large light fibrillar centers in electron micrographs. Treatment of HeLa cells with 5 mM thymidine for 48 hr results in striking enlargement of nucleolini and fibrillar centers. It is thus clear that the nucleolini observed by light microscopy are the light fibrillar centers, with or without surrounding dense fibrillar material, observed by electron microscopy.

INTRODUCTION

Two types of RNP have been demonstrated in a number of different cells in vivo (2, 12) and in vitro (6–10). One type is stained metachromatically by TBM Method B and is frequently located in roughly spherical structures which correspond to some vacuoles in living cells (9). These spherical structures have frequently been observed by the classical cytologists, who called them nucleolini (13). Although these structures can be demonstrated by the TBM method, by silver (9) and lead (19) precipitation, and by an affinity for zinc (20), none of these methods are suitable for electron microscopic studies. Therefore, the relationship of the nucleolini to structures observed by electron microscopy depended upon indirect evidence. Studies in this laboratory of cells treated with antimetabolities or infected with viruses have been compared with electron microscopic observations of others (1), and the results suggest that collections of a fibrillar RNP component in electron micrographs may correspond to the nucleolini (8). Light and electron microscopic studies of HeLa cells exposed to hyperthermia also showed comparable changes in nucleolar RNP and in the fibrillar component of the nucleolus. In the heated cells, the nucleolar RNP diffused throughout the nucleolus; in electron micrographs, the nucleolus was almost entirely fibrillar (8).

Differences in the morphology of nucleolini have been observed between a wide variety of normal diploid cells and those of aneuploid, neoplastic, or transformed cells during exponential replication in vitro (6, 10). It was subsequently found that the morphology of nucleolini was influenced by conditions of culture as well as by karyotype (10). It has also been observed that treatment of cells with thymidine results in great enlargement of nucleolini. It becomes possible, therefore, to alter the structure by manipulating the conditions of culture. The present investigation utilizes this knowledge to produce in nucleoli changes which have been studied by light and electron microscopy.

MATERIALS AND METHODS

Cell Cultures. FF were obtained from Flow Laboratories, Inc., Rockville, Md., and were studied at early passage levels (13 to 17). HeLa JH cells (22) were used as a tumor cell model. All cells were cultivated in Blake bottles in Eagle's basal medium supplemented with 10% fetal calf serum and chlortetracycline (50 μg/ml). Bottles were seeded at 30,000 cells/ml and examined at intervals as described without any change of medium.

Light Microscopy. Regular microscope slides were halved longitudinally to make 2 slides, each 3 x ½ inches. These were inserted into the Blake bottles before sterilization. These slides were fixed and stained by TBM Method B after digestion with DNase (6, 9) at the same time that the rest of the cells in the bottle were being prepared for electron microscopy.

Electron Microscopy. The monolayers were fixed at 4° in situ with 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.2 for 30 min, followed by 1% osmic acid in the same buffer for 30 min. Cells were gently scraped off the glass and centrifuged at 1000 rpm for 5 min, and the resultant pellet was embedded in Epon. Sections were stained with 2% uranyl acetate in methanol for 30 min and/or with lead citrate (14, 16) and examined with a RCA EMU4 microscope.

Analysis of Nucleolar Structure in the Cell Population. An attempt was made to measure the number of nucleolini in each cell in the FF cells in exponential growth and in the stationary phase of culture. A total of 100 cells was analyzed. During the

1 Supported by Grant GM813 from the NIH.
2 The abbreviations used are: RNP, ribonucleoprotein; TBM, toluidine blue molybdate; FF, human female embryo diploid fibroblasts.

Received January 26, 1971; accepted March 5, 1971.
log phase of growth, nucleolini could only be counted in 39% of the cells. In the remainder the nucleolini were either too numerous to count or not resolvable by light microscopy.

**Thymidine Treatment.** Thymidine was added to 3-day-old HeLa cultures in concentrations of 1, 2.5, and 10 mM. Nucleolini were examined at daily intervals, and it was found that treatment for 48 hr with 5 mM thymidine produced a striking increase in the size of nucleolini. The medium of control cultures was changed at the same time that the treatment for 48 hr with 5 mM thymidine produced a striking increase in the size of nucleolini. The medium of control cultures was changed at the same time that the thymidine was applied to the experimental cultures.

**RESULTS**

**FF Cells.** In light microscopic preparations, 24 hr after seeding, the nucleolini, where clearly distinguishable, were numerous and regular in size (isonucleolinosis) (Fig. 1). In order to give some idea of the morphology of the nucleolini in the cell population, an attempt was made to count them (Chart 1). It was impossible either to count or to resolve nucleolini in 61% of the exponentially growing cells, so that an exact mathematical analysis was not feasible. However, it is obvious at a glance that there is a striking difference between the 2 cell populations. Nucleolini, where countable in day-old cultures, were numerous, with a mode of 15 to 19, while in the stationary cultures the mode was less than 5, and nucleolini were resolvable in the entire population. Furthermore, the nucleolini of the stationary cells were uneven in size in all of the cells (anisonucleolinosis) (Fig. 2). The other type of nucleolar RNP, which stains orthochromatically by TBM Method B, was very scanty in the day-old cultures because the greater part of the nucleolus was occupied by the nucleolar RNP (Fig. 1). In the stationary cultures, on the other hand, the orthochromatically staining RNP was clearly visible between the nucleolini (Fig. 2). The ultrastructural basis for these differences between the 2 stages of culture is shown in Figs. 3 to 6. In the log phase cells the dense fibrillar component was very prominent and was organized in many places to give the appearance of a C or incomplete ring in thin sections (Figs. 3 and 4, arrows). In the center of the dense fibrillar C or ring, there is frequently a small amount of lighter fibrillar material. In some nucleolini, these centers of dense and light fibrillar material are not too well organized. Presumably, these nucleolini constitute the ones in which the nucleolini cannot be resolved by light microscopy. The correlation between light and electron microscopy becomes much clearer when we look at the stationary FF cells. Here the nucleolini were few, rather weakly stained, large, and readily resolvable by light microscopy (Fig. 2); in the electron micrographs, the light fibrillar centers were also prominent (Figs. 5 and 6). However, the associated dense fibrillar component was absent. The granular component of the nucleolus, like the orthochromatically staining RNP in the light microscopic preparations, was much more conspicuous than in the day-old cultures (compare Figs. 3 and 4 with Figs. 5 and 6). It is of interest too that heterochromatin was inconspicuous in the exponentially growing cells and was relatively abundant in the 9-day-old cells (Figs. 3 to 6). Free and membrane-bound ribosomes were relatively numerous in the day-old cultures and were scanty in the stationary cells (Figs. 3 to 6).

**HeLa Cells.** These were examined after 1 day and after 4 days in culture, since considerable differences in nucleolar morphology had been observed at these times (Figs. 7 and 8) (10). Nucleolini of 1-day-old cultures were relatively small and numerous and varied slightly in size (Fig. 7). In electron micrographs of the same cells, numerous light fibrillar centers were scattered throughout the nucleolus (Fig. 9). These were surrounded more or less completely by a dense fibrillar zone (arrows). The remainder of the nucleolus consisted of granules and the nucleolar-associated chromatin (Fig. 9). After 4 days in culture the nucleolini were fewer, larger, and more unequal in size (Fig. 8). In electron micrographs the light fibrillar centers and the surrounding dense fibrillar material were considerably larger and better defined than those of Day 1 cultures (Figs. 10 and 11).

**Thymidine-treated Cells.** Since the medium of the control cultures had been changed 48 hr previously, the nucleolini and the fibrillar centers were relatively numerous and small (Figs. 12 and 15). On the other hand, the nucleolini of the thymidine-treated cells were very large (Figs. 13 and 14), and the electron micrographs of the same cells show that the light fibrillar centers with adjacent dense fibrillar material were very prominent (Figs. 16 and 17).

**Lead Staining.** In order to ensure that the fibrillar centers observed in the present experiments were similar to those
Robert Love and Ramon Z. Soriano

described by Recher, thin sections were stained with lead citrate without previous uranyl acetate stain (14). The dense fibrillar peripheral component was quite strongly stained, but the staining of the central light fibrillar component was weak (Figs. 18 and 19).

DISCUSSION

There seems little doubt that, although the lead staining of the light fibrillar centers was not as strong as in the studies of Recher et al. (14), the light fibrillar centers and associated dense fibrillar material described in the present experiments are the same as those they describe in 2 other types of carcinoma cells in culture. Fibrillar centers have been described under a variety of names in many different types of cells (1, 3, 4, 11, 14, 15, 17, 18, 21, 23—25). Schoell (17) described them in baboon kidney cells in vitro and called the light fibrillar component Zone c and the dense fibrillar material Zone b. Swift (21) and Yasuzumi and Sugihara (25) illustrate the fibrillar centers in Ehrlich ascites cells and Heine et al. (4) show them in cultured avian myeloblasts. Miller and Beatty (11) observed a fibrillar central core surrounded by a granular cortex in the extrachromosomal nucleolus of an amphibian oocyte. Electron-lucent fibrillar centers and associated dense fibrillar peripheral material have also been described in the nucleoli of human endometrial cells (23), in monkey nerve cells (3), and in phytohemagglutinin-stimulated lymphocytes (24). Smetana et al. (18) have described "ring-shaped" nucleoli, which consist of a light fibrillar center or centers associated with variable but rather sparse dense fibrillar material. These structures have been found in a wide variety of cells, e.g., endothelium, differentiated smooth muscle, mature lymphocytes, monocytes, plasma cells, and differentiated lymphosarcoma cells (18).

On the basis of enzyme digestion, it appears that the light (17, 25) and dense (1, 2, 15, 16, 18, 25) fibrillar components consist of RNP. Electron microscopic autoradiography (1) indicates that the dense fibrillar material is the most rapidly labeled component of the nucleolus and therefore probably contains ribosomal precursor RNA. This is consistent with the present observation that the dense fibrillar component is no longer demonstrable in the stationary diploid cells which are presumably not synthesizing any significant amount of RNA. The studies of Smetana et al. (18) also indicate that "ring-shaped" nucleoli, i.e., nucleoli with prominent light fibrillar centers, are characteristic of differentiated cells that are not likely to be active in RNA synthesis. On the other hand, they note that an organized fibrillar nucleolomena is characteristic of proliferating and immature cells of the lymphoblastic, plasmacytic, monocytic, and granulocytic series and of less differentiated lymphosarcoma cells. These observations are consistent with the present study of the diploid cells, where the dense fibrillar component prevails in exponential growth, while the light fibrillar centers are most conspicuous in the stationary cells. Similarly, the light fibrillar centers and nucleolini are few and large in the thymidine-treated cells, in which nuclear RNA synthesis has been shown to be inhibited (5). The observations of Smetana et al. (18) and the present results suggest that when the ribosomal cistrons are fully active the dense fibrillar component is abundant and what they term the "nucleolomena structure" predominates. In our interpretation, a 3-dimensional analysis of the C-like structures observed in thin sections suggests that they are incomplete spherical structures rather than threads. All transitions from the small C-like structures to well-developed fibrillar centers can be seen in electron micrographs. When there is some abnormality of rRNA synthesis or some disturbance in maturation or transport of ribosomal precursors, the light fibrillar centers predominate with or without the associated dense fibrillar peripheral material.

The results clearly indicate that the nucleolini are the light fibrillar centers, with or without the peripheral dense fibrillar component. The nucleolini and the light and dense fibrillar material consist of RNP (7, 17). In all instances there is a close correlation between the variations in nucleolar structure observed by the light and electron microscopes. When the nucleolini are large and few, the fibrillar centers are also large and few. When the dense fibrillar component predominates, the metachromatically staining RNP in the nucleoli has the appearance of multiple small spherules or fine granules in the fibrillar centers (Fig. 1). They not uncommonly occur in rows like streptococci. This would suggest that each nucleolinus might be the site of ribosomal cistrons on euchromatin similar to those described by Miller and Beatty (11) in amphibian oocytes. Recher et al. (14) found that a fine reticulum persisted in the fibrillar centers after double digestion with pepsin and RNase. While it was felt that this remaining material might be euchromatin it was not possible to prove this by cytochemical or autoradiographic techniques (15).

REFERENCES


Fig. 1. Isonucleolar human lung fibroblasts after 1 day in culture. The nucleolini are very numerous, and all are approximately the same size. All light micrographs are of preparations stained by TBM Method B after digestion with DNase. X 2,200.

Fig. 2. Anisonucleolar human diploid fibroblasts after 9 days in culture without change of medium. The nucleolini are discreet, vary considerably in size, and are few in number. X 2,200.

Figs. 3 and 4. Cells from same culture as Fig. 1. The dense fibrillar material (nucleolonema) is organized into numerous C-shaped structures (arrows) in the center of which there is some light fibrillar material. The fibrillar component of the nucleolus predominates, and the granules are scanty. The ribosomes are fairly numerous, and heterochromatin is inconspicuous. All electron micrographs except Figs. 18 and 19 are of material stained with uranyl acetate and lead citrate. X 33,000.

Figs. 5 and 6. Cells from the same culture as Fig. 2. Light fibrillar centers correspond to the nucleolini in the light micrograph (Fig. 2). Note the paucity of ribosomes and the abundance of heterochromatin at the periphery of the nucleus and in association with the nucleolus (cf. Figs. 3 and 4). X 33,000.

Fig. 7. Relatively numerous, rather small nucleolini in a HeLa cell after 1 day in culture. X 2,200.

Fig. 8. Few, large, irregular nucleolini in a HeLa cell after 4 days in culture. X 2,200.

Fig. 9. Cell from the same culture as that shown in Fig. 7. The dense fibrillar material is arranged in C-shaped structures (arrows) in the center of which there is some light fibrillar material. X 33,000.

Fig. 10. Prominent light fibrillar centers with peripheral dense fibrillar material in a cell from the same culture as depicted in Fig. 8. X 33,000.

Fig. 11. Higher magnification of a cell from the 4-day culture depicted in Fig. 8. The granular component of the nucleolus is relatively abundant. X 44,000.

Fig. 12. Untreated HeLa cell culture after 6 days in culture. The medium had been changed 48 hr previously. Nucleolini are variable in size and not very large. X 2,200.

Figs. 13 and 14. Cells from a 6-day culture of HeLa cells that had been treated for 48 hr with 5 mM thymidine. The nucleolini are very large and less numerous than those of the control cells (Fig. 12). X 2,200.

Fig. 15. Cell from same control culture illustrated in Fig. 12. Light fibrillar centers with associated dense fibrillar component are of moderate size. X 33,000.

Figs. 16 and 17. Very large fibrillar centers in cells treated with thymidine (cf. Figs. 13 and 14). X 33,000.

Figs. 18 and 19. Four-day-old cultures of HeLa cells stained with lead citrate only. The dense fibrillar periphery of the fibrillar centers is strongly stained. X 33,000.
Correlation of Nucleolini with Fine Structural Nucleolar Constituents of Cultured Normal and Neoplastic Cells

Robert Love and Ramon Z. Soriano

_Cancer Res_ 1971;31:1030-1037.