Effects of Bleomycin on Human Tongue Carcinoma Cells as Revealed by Electron Microscopy

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SUMMARY

Five patients with tongue carcinoma were treated for 20 days with bleomycin, 15 mg/day, given by one-shot infusion through the superficial temporal artery. Specimens were taken 7, 14, and 20 days after the initial administration for light and electron microscope observations.

The first alterations observed were the decrease of electron-dense chromatin clumps in nuclei and, simultaneously, the segregation of nucleolar components, i.e., amorphous protein component segregated from fibrillar and granular components. Subsequently, numerous nuclear bodies appeared in the nucleoplasm, which were observed for the first time in human tongue carcinoma cell nuclei in cases of bleomycin treatment. On the other hand, the cytoplasmic alterations were recognized by the decrease in number of mitochondria and by the appearance of numerous free ribosomes followed by the formation of tonofilaments. The cells enlarged because tonofilaments flocked together to make cancer cell nests, which were gradually transformed into a keratinic structure showing the so-called cancer pearl pattern. Finally, cancer cells were degenerated nearly to necrosis; there was no evidence of recurrence of cancer.

Although the precise mechanism of the effect of bleomycin on nuclear activities of human tongue cancer cells remains unsettled because of the lack of cytochemical analysis, it is believed that bleomycin inhibits DNA synthesis and might also activate ribosomal RNA synthesis.

INTRODUCTION

BLM,3 a water-soluble basic glycopeptide, is an antibiotic and antitumor agent that was isolated by Umezawa et al. (23, 24) from Streptomyces verticillus. It has been shown clinically to be effective on squamous cell carcinoma (7, 21, 22). In HeLa cells and Escherichia coli culture, BLM has a strong inhibitory action on growth and on DNA synthesis (19, 20). BLM may also interact with the sulfhydryl-combining sites of DNA molecules and make their double-helical structures labile, consequently facilitating depolymerization of these DNA molecules by DNase (12). Ultrastructural studies concerning the mechanism of BLM action on mammalian normal and tumor cells have been reported by several authors (4, 10, 14, 26), but no electron microscope study has been performed on human tongue carcinoma cells, except for our previous report (27).

The BLM-induced alterations in the appearance and texture of nucleoli of tumor cell nuclei have been described as segregation of the nucleolar components, reduction in the size of nucleoli (10, 14), and ultrastructural changes of fibrillar nucleolar elements and their loss from the nucleolus (4).

In the present study on human tongue squamous cell carcinoma we investigated certain aspects of the cells during the degenerating processes of the tumor cells treated with BLM. Therapeutic effects of BLM on human tongue carcinoma were brought about by pyknosis and keratinization, which were preceded by the segregation of nucleolar fibrillar and granular components from the amorphous component, occurrence of nuclear bodies, and production of abundant tonofilaments. This paper, then, is devoted to a subject that was an integral part of our research on the fine structure of nuclei as revealed by electron microscopy (29).

MATERIALS AND METHODS

Twenty Japanese patients with tongue carcinoma were admitted to Nara Medical University Hospital from 1970 to 1975. Five patients with no evidence of metastasis to other organ systems, except for the cervical lymph node, were chosen for treatment with BLM (Table 1). The drug, 15 mg/day, was administered to each patient by 1-shot infusion through the superficial artery as follows. A polyethylene or Teflon tube, 20 cm long and 1 mm in diameter, was retrogressively inserted into the arteria carotis externa. Since the total dosage of 1 course of treatment was 300 mg, a course was usually completed in 20 days. Specimens were taken by biopsy before treatment and 7, 14 and 20 days after treatment with BLM. The biopsy specimen, 5 cu mm including the normal tissue, was divided into 2 portions immediately after removal. One portion was fixed in 10% phosphate-buffered formaldehyde solution for paraffin embedding. Sections 5 μm thick were stained with hematoxylin and eosin. The other portion of the biopsy specimen was cut into tiny blocks that were fixed in 2.5% glutaraldehyde, postfixed in 1.0% osmium tetroxide, and embedded in epoxy Epon resin through a routine method. Each fixative...
Results of BLM therapy for human tongue carcinoma

Patients 1 to 4 returned to their usual activities. At the time of this report, there was no evidence of recurrence of either malignancy (February 29, 1976).

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>Age</th>
<th>Sex</th>
<th>Histological examination</th>
<th>T.N.M. after treatment with 300 mg BLM</th>
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<tr>
<td>1</td>
<td>S. M.</td>
<td>60</td>
<td>Male</td>
<td>Poorly differentiated</td>
<td>T2NM, +</td>
</tr>
<tr>
<td>2</td>
<td>K. M.</td>
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<td>Male</td>
<td>Moderately differentiated</td>
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<tr>
<td>3</td>
<td>A. L.</td>
<td>69</td>
<td>Female</td>
<td>Well differentiated</td>
<td>T2NM, +</td>
</tr>
<tr>
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<td>71</td>
<td>Male</td>
<td>Well differentiated</td>
<td>T2NM, +</td>
</tr>
<tr>
<td>5</td>
<td>T. K.</td>
<td>65</td>
<td>Male</td>
<td>Well differentiated</td>
<td>T2NM, +</td>
</tr>
</tbody>
</table>

Tumor cell nest after treatment with 300 mg BLM.

Although the tumor cell nest was excised by operation.

Electron Microscopy

The human tongue carcinoma cell usually had a large nucleus that was irregularly outlined, giving a zigzag appearance. The inner layer of double-layered nuclear envelope was associated with dense chromatin clumps in a small amount. A few dense nucleolus-associated chromatin was occasionally encountered. The nucleus often contained 2 oval nucleoli, a large one, 3.7 x 2.7 μm, and a small one, 2.6 x 1.8 μm, consisting of the network of dense nucleolonemata. In addition, amorphous material of intermediate density was observed between the meshes of nucleolonemata. The fibrillar material, consisting of fine filaments, mitochondria, and ribosomes free and attached to the endoplasmic reticulum, were encountered in the cytoplasm (Fig. 9).

The fine structural changes occurred in the nucleolus 7 days after treatment with BLM in many nuclei; the nucleolar component was segregated into the dense and the less dense ones. The dense component was composed of granular and fibrillar elements, but the less dense component was composed of an amorphous material. Two nuclear bodies, consisting of an amorphous element of intermediate density, appeared in the vicinity of the separated, less dense component of the nucleolus. Occasionally, the less dense component of the nucleolus appeared in the nucleoplasm, which seemed to have been squeezed out from the nucleolus. The amounts of nucleolus-associated chromatin and nuclear envelope-attached chromatin were diminished. On the other hand, the cytoplasm was characterized by the increase of ribosomes in rosette form. A small number of mitochondria were encountered in the vicinity of the nucleus; these mitochondria were relatively large but were devoid of regularly arranged cristae (Figs. 10 and 11).

Remarkable cytoplasmic changes were observed after 2-week treatment with BLM. Bundles approximately 0.1 μm wide, consisting of tonofilaments 5 nm in diameter, appeared mainly parallel to the long axis of the cell, showing several stages of their development in different cells (Figs.
12 to 15). In the early stage of development of tonofilaments, the cytoplasm was characterized by the appearance of abundant free ribosomes. The nucleus had a relatively large nucleolus and several nuclear bodies consisting of amorphous and fibrillar components. The nucleolus consisted of dense nucleolonemata that were composed mainly of fibrillar elements and a few granular ones (Fig. 13). The development of tonofilaments was so remarkable in some cells that their bundles occupied almost all the cytoplasm where there were vacuoles of different sizes between the bundles of tonofilaments. The irregularly shaped nucleus decreased in size and contained a dense nucleolus and several nuclear bodies (Fig. 14). The process of keratinization was different in different cells. It occurred at the peripheral portion in some cells, being surrounded by an adjacent dense cell. Such a cell (round, 35 μm in diameter) probably corresponded to the cell showing so-called individual cell keratinization with light microscopy. The central portion was occupied by the irregularly shaped nucleus containing several nuclear bodies and invaginations, which was surrounded by numerous vacuoles containing several metabolic remnants, as well as by dense tonofilaments in a circular pattern (Fig. 12). In other cells the keratinization occurred in a similar manner simultaneously throughout the cell. The dense tonofilaments decreased their density, so that it was difficult to differentiate them from the matrix of cytoplasm. Vacuoles of different sizes and containing amorphous or granular components were observed between the keratinized material. The pyknotic nucleus contained several dense cell. Such a cell (round, 35 μm in diameter) probably corresponds to the cell showing so-called individual cell keratinization as already maintained by Reynolds et al. (1) and Zelickson (31).

Twenty days after treatment with BLM, nearly all the carcinoma cells had disappeared; only a few cells with heavy pyknosis and pronounced degenerating cytoplasmic alterations leading to keratinization were left (Fig. 16). Several nuclear bodies still appeared, each surrounded by an electron-lucent halo. The nuclear bodies, ranging in diameter from 0.5 to 1.6 μm, contained such different structures as amorphous, fibrillar, and granular elements. The tonofilaments increased in size until they reached a diameter of approximately 10 nm, showing the decrease of their density (Fig. 16). Such processes demonstrated the alteration of tonofilaments into keratinization, as already depicted by Brody (1) and Zelickson (31).

**DISCUSSION**

This paper is intended mainly to suggest that in the study of BLM action on human tongue carcinoma cells there is still much to learn about their degenerating processes. BLM is known to inhibit selectively the synthesis of DNA (21). In fact, the 1st and longest-lasting alteration in BLM-treated squamous epithelial cells is the apparent decrease of chromatin elements leading to nuclear hypochromasia, as already reported by Ogawa and Onoé (14), Krishan (10), Yamane (26), and Yasuzumi et al. (27).

Several reports are available on the effect of BLM on nucleoli (4, 10, 14, 19, 26). Suzuki et al. (19) showed that BLM does not inhibit RNA synthesis in culture experiments of bacterial and tumor cells. According to the report of Ogawa and Onoé (14), BLM decreases the ribosomes in 3-methylcholanthrene-induced mouse epidermal carcinoma cells. Krishan (10) has observed the initial nucleolar hypertrophy, followed by shrinking and segregation of the nucleolus, in cultured mouse fibroblasts and human lymphocytes of neoplastic origin. Recently, Yamane (26) has emphasized that nucleoli of cells affected by BLM produce many ribosomes in the oral mucous epithelium of normal rats. More recently, Daskal et al. (4) reported that BLM has inhibited rRNA synthesis in Novikoff hepatoma ascites cells in vivo and in vitro. Thus, it is clear that BLM can produce different effects on different cell lines.

BLM in a single dose causes a prominent, early nucleolar alteration in human tongue carcinoma cell nuclei, i.e., segregation of the amorphous component from granular and fibrillar components. The pattern therefore is similar to the form of nucleolar segregation that occurs in giant nuclei of human epidermis cells in precancerous diseases (29). Although Ogawa and Onoé (14) and Krishan (10) have noticed the segregation of nucleolus in BLM-treated cell nuclei, they have not described its structure in detail. The present segregation pattern differs significantly from other segregation patterns produced by such agents as actinomycin D (5), Mitomycin C (8, 9), 4-nitroquinoline N-oxide (15), and proflavine (18), since they have shown distinct separation between granular and fibrillar zones. Nucleolar segregation represents the morphological expression of specific biochemical reaction as already maintained by Reynolds et al. (15) and Simard and Bernhard (18). The segregation in the latter case is known to be induced by agents that inhibit the DNA-dependent RNA synthesis directly or indirectly.

Recently, it has been revealed from the ultrastructural and autoradiographic standpoints that nuclear bodies originate from the amorphous protein component of the nucleolus and develop to cooperate with the nucleolus in synthesis of precursor rRNA (28, 29).

Although the precise mechanism of BLM action on RNA and its synthesis is not known, it is suggested that BLM activates the synthesis of rRNA by its effects on nucleolar morphology, i.e., occurrence of nuclear bodies and formation of tonofilaments.

As BLM treatment proceeds, abundant free ribosomes appear in the cytoplasm, accompanying the formation of tonofilaments. However, the nucleolonemata constituting the nucleolus are composed mainly of fibrillar components. It is well known that the fibrillar component of the nucleolus is rapidly labeled with RNA precursor and can be a precursor of RNA in granular components of the nucleolus (3, 6, 25). Consequently, free ribosomes are considered to be the site of synthesis for protein-natured tonofilaments (17) in carcinoma cells treated with BLM; it has previously been suggested by many authors (2, 11, 13, 16, 26, 30) that this is true for the rodent or human epidermis as well as for the mucous epithelium under several physiological and pathological conditions.

Brody (1) and Zelickson (31) have reported that the tonofilaments undergo changes in their state of aggregation or in chemical composition through a stepwise transformation of tonofilaments to keratin filaments. The present study demonstrates that tonofilaments increase in size but decrease in density as treatment with BLM proceeds. In
tongue carcinoma cells a stepwise transformation of the tonofilaments to keratinization causes mainly the necrosis of the carcinoma cells, which indicates the value of the drug. It is therefore suggested that the occurrence of tonofilaments in human tongue carcinoma cells is important in assessing prognosis.

REFERENCES

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Figs. 1 to 4. Light micrographs of human tongue carcinoma in Case 1. H & E, × 270.

Fig. 1. Poorly differentiated squamous cell carcinoma adjacent to the normal squamous epithelium.
Fig. 2. Cancer cell nests are surrounded by the fibrous stroma, and a malignant squamous pearl is seen at the lower part of the field, after treatment with 105 mg BLM.
Fig. 3. At least 2 cell nests are clearly visible, after treatment with 210 mg BLM.
Fig. 4. After treatment with 300 mg BLM, the field is filled with necrotic masses, showing only a pearl-like pattern and a cellular feature.

Figs. 5 to 8. Light micrographs of the similar material in the 2nd case. H & E, × 270.

Fig. 5. Moderately differentiated squamous cell carcinoma.
Fig. 6. Several nests of polygonal cells are enmeshed within a fibrous stroma, and individual cell keratinization in high grade is seen after treatment with 105 mg BLM.
Fig. 7. After treatment with 210 mg BLM, keratinized cells have increased in size and many of them show nuclear hypochromasia.
Fig. 8. Large foci of necrosis after treatment with 300 mg BLM.

Figs. 9, 10, 13, 14, and 16. Electron micrographs of human tongue carcinoma from Case 1.

Fig. 9. Part of the carcinoma cell before treatment with BLM. The irregularly outlined nucleus includes 2 nucleoli consisting of the dense nucleolonemata and the less dense amorphous material. Dense chromatin clumps have appeared; they are attached to the nuclear envelope and the nucleoli. The fibrillar material (F) and ribosomes, free and attached to endoplasmic reticulum, are seen in the cytoplasm. × 18,000.

Figs. 10 and 11. One week after BLM treatment.

Fig. 10. The nucleolus is clearly separated into the dense and less dense components. A vacuole-like structure in the nucleolus may be due to the plane of section since the ground substance appears to be similar to the nucleoplasm. Two nuclear bodies (arrows) are seen near the amorphous component of nucleolus. A few mitochondria (M), many free ribosomes, granular endoplasmic reticulum, and fibrillar elements (F) are visible in the cytoplasm. × 23,000.

Fig. 11. Low-power electron micrograph showing the whole cell, its surface being provided with abundant processes. The irregularly shaped nucleus contains the constricted nucleolus in which the dense and less dense components were separated. A part of the less dense component appears in the nucleoplasm (arrow), being squeezed out from the nucleolus. Tonofilaments and ribosomes have increased in number in the cytoplasm. Enlarged mitochondria are devoid of cristae. × 6,000.

Figs. 12 to 15. Effects of 210 mg BLM.

Fig. 12. A peculiar individual cell keratinization appearing within a dense cell filled with tonofilaments. The pyknotic nucleus with nuclear bodies is surrounded by numerous vacuoles with granular elements and a large number of dense tonofilaments that are encircled by the less dense filaments. × 2,000.

Fig. 13. The nucleus has a net-like nucleolus and 2 nuclear bodies. The nucleolus is composed mainly of fibrillar components and a small number of granular components. Bundles of tonofilaments, abundant ribosomes, free and attached to endoplasmic reticulum, and 2 lipid droplets are seen in the cytoplasm. × 31,000.

Fig. 14. A small nucleus containing a relatively large nucleolus and several nuclear bodies in the nucleoplasm of intermediate density. Many bundles of tonofilaments are arranged mainly along the long axis of the cell, and between them are free ribosomes, mitochondria, agranular vesicles, and dense bodies. × 12,000.

Fig. 15. The pyknotic nucleus with a dense nucleolus and many nuclear bodies found in the cytoplasm of intermediate density where tonofilaments are scarcely visible. Mitochondria, ribosomes, and endoplasmic reticulum were no longer visible. × 7,000.

Fig. 16. Effects of 300 mg BLM. The nucleus contains a dense nucleolus and many nuclear bodies, each surrounded by an electron-lucent halo. The cytoplasm is filled with abundant bundles of the less dense filaments, rather than dense tonofilaments. Irregularly shaped agranular vesicles are found in the vicinity of the nucleus. × 15,000.
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