NUCLEAR CHANGES PRODUCED BY BLEOMYCIN IN THE
3-METHYLCHOLANThRENE-INDUCED MOUSE
EPIDERMAL CARCINOMA CELLS

(Plates LXXX-LXXXIV)

Katsuhiro Ogawa and Tamenori Onoe
(Department of Pathology, Sapporo Medical College)

Synopsis

Bleomycin has produced fairly specific ultrastructural alterations, including the reduction of chromatin in the nuclei, nucleolar segregation, the decrease of ribosomes, and other cytoplasmic degenerative changes in the 3-methylcholanthrene-induced mouse epidermal carcinoma cells. These alterations appeared to be related to each other and were assumed to be the morphological manifestations of the antineoplastic action of Bleomycin.

INTRODUCTION

Bleomycin is a new antibiotic isolated from Streptomyces verticillus in 1966 and separated into 11 or more effective components by Umezawa et al.\textsuperscript{33,34} Bleomycin has been in clinical use for its distinct antineoplastic action, especially on epidermal tumors.\textsuperscript{5,6,31,32}

However, the precise mode of action of this antibiotic is not well known and remains speculative. On the other hand, there have been few detailed descriptions about the morphological alterations of the cells treated with Bleomycin. In the present study, 3-methylcholanthrene-induced mouse epidermal carcinoma was examined by light and electron microscopy within a short period after the administration of a toxic dose of Bleomycin. In this condition, the action of Bleomycin was expected to be confirmed at the ultrastructural level and some morphological alterations induced there would give some clue as to the mode of biological action of Bleomycin.

MATERIALS AND METHODS

Mice of dd strain were painted 3\% solution of 3-methylcholanthrene in benzene on the back twice a week for 7 to 10 weeks. In the majority of these animals epidermal tumors were induced, which were histologically epidermal carcinoma. Two weeks after cessation of the treatment with 3-methylcholanthrene, 100 or 200 mg/kg of Bleomycin was administered intraperitoneally by one-shot injection, sacrificed after lag periods of 24 and 48 hr, and the epidermal tumors were prepared for light and electron microscopic examinations. For light microscopy, sections cut at 3 \( \mu \) thick were stained with Hematoxylin and Eosin or Methyl Green and Pyronine. For electron microscopy, materials were fixed in chilled 2\% OsO\textsubscript{4} buffered with Veronal acetate at pH 7.4 for 2 hr, dehydrated in graded ethanol, and embedded in Epon 812.\textsuperscript{31} Thin sections cut

* Minami-1-jo, Nishi-17-chome, Sapporo, Hokkaido 060 (小川勝洋，小野江為則)
with an LKB microtome were stained with uranyl acetate and lead citrate, and examined with a Hitachi HS-7D electron microscope.

Results

Histological findings of these tumors before and after the treatment with Bleomycin are shown in Photos 1 and 2. After the treatment with Bleomycin, in the basal cell layer and the lower layer of prickle cells, the carcinoma cells revealed the reduction of both cytoplasmic and nuclear basophilicity, and showed prominent nucleoli under the light microscope (Photo 2).

Electron microscopically, the most striking alterations in these cells after the treatment with Bleomycin were found in their nuclei. Before the treatment, the nuclei of these cells were round or ovoid, sometimes associated with invaginations of nuclear envelopes, and contained a moderate to abundant amount of chromatin consisting of numerous electron-dense chromatin granules (150–250 Å in diameter) mainly distributed both in marginal areas along the inner nuclear membrane (peripheral chromatin) and perinucleolar areas (nucleolus-associated chromatin). The interchromatin area consisted of amorphous matrix, finely dispersed chromatin granules, and interchromatin granules (Photo 3). In the majority of carcinoma cells in these layers, each nucleus contained one or two large nucleoli which were always pleomorphic in shape and mainly located in the nucleoplasm adjacent to the inner nuclear membrane (Photo 4). Each nucleolus was commonly composed of the following three components; particulate components which occupy the bulk of the nucleolar mass and consist of dense granules (1500–200 Å) embedded within a matrix; fibrillar components, a loosely meshed electron-dense network, which are scattered within particulate components and composed of fine fibrils, 80–100 Å in diameter, in a dense ground substance; amorphous components which are surrounded by cup-shaped cavities of the fibrillar components and are less electron dense and somewhat coarser in texture (Photo 3). The majority of nucleoli in these carcinoma cells revealed the characteristic pattern of “spotted nucleoli” composed of more of particulate components and less of fibrilar components.

After the treatment with Bleomycin, nuclei of the carcinoma cells in these layers became remarkably less electron dense and vesicular in appearance and, consequently, nucleoli became conspicuous (Photo 5). This reduction of electron density in the nuclei seemed to be attributable to the decrease in the amount of chromatin, including both peripheral and nucleolus-associated chromatin. Only a few of peripheral chromatin remained patchily in several places along the inner nuclear membrane and the nucleolus-associated chromatin made up cap-shaped satellites around the nucleoli (Photos 5, 7, 8, and 9). In these carcinoma cells with decreased chromatin, interchromatin areas were somewhat coarser in density than those in controls.

Along with the decrease of chromatin, nucleoli were variously altered in shape and texture. In slightly changed nucleoli of these cells, their fibrillar components were decreased and grouped into several aggregates located in their periphery (Photo 6). In further advanced stage, nucleoli were rounded up, had smooth contours, and decreased in size. In this stage, almost all the nucleoli were composed of particulate components, although only a small amount of fibrillar components was attached in cap-like form around the nucleoli (Photos 7 and 8).
NUCLEAR CHANGES PRODUCED BY BLEOMYCIN

Then, in more advanced stage, nucleoli were extremely diminished in size, in which not only fibrillar components but also particulate components were extremely depleted and amorphous substance occupied most of the nucleolar bulk (Photo 9). In addition to such nuclear changes, a decrease of ribosomes, which is likely responsible for the reduction of basophilicity of cytoplasm under the light microscope, was most strikingly observed in cytoplasm of these carcinoma cells after the treatment, while some of these cells showed additional cytoplasmic degenerations, such as swelling of mitochondria, appearance of secondary lysosomes, focal cytoplasmic degradations, widening of intercellular spaces, etc. (Photo 5).

The changes induced by the administration of 100 mg/kg of Bleomycin were similar in nature to those induced by a higher dose of 200 mg/kg, and the cellular alterations observed after lag periods of 24 and 48 hr were essentially similar in nature, although at a later period these changes became more pronounced in degree.

DISCUSSION

The result of the present study revealed that Bleomycin produced a reduction of chromatin, nucleolar alterations, and a reduction of ribosomes associated with other cytoplasmic degenerative changes in the 3-methylcholanthrene-induced mouse epidermal carcinoma cells. It had been demonstrated by Suzuki et al.28) that, in vitro, Bleomycin inhibited growth and DNA synthesis of HeLa cells and showed a moderate inhibitory effect on protein synthesis but no effect on RNA synthesis. Further, Nagai et al.14) have found that Bleomycin interacted with DNA in vitro and decreased Tm of DNA in the presence of sulfhydryl compounds such as glutathione, dithiothreitol, 2-mercaptoethanol, etc.

The latter authors considered that Bleomycin might interact with the SH-combining sites on DNA molecules and made their double helix structures labile, consequently making these DNA to be easily depolymerized by DNase. These biochemical data may offer an interpretation for the reduction of chromatin in carcinoma cell nuclei by the administration of Bleomycin as observed in the present study. Namely, it can be a morphological evidence caused by the denaturation of DNA which was induced by Bleomycin just as in in vitro.

On the other hand, various nucleolar alterations were also observed accompanying a decrease of chromatin, and some of them presented the so-called nucleolar segregation which was characterized by rearrangement of particulate and fibrillar components into two or more distinct zones. The nucleolus has been demonstrated to be a site for production of ribosomes1, 2, 10, 13, 14, 19, 23, 30, 35) and the nucleolar segregation was observed as an ultrastructural manifestation of its dysfunction. This nucleolar segregation is known to be induced by many kinds of agents which inhibit the DNA-dependent RNA synthesis directly or indirectly.3, 7-9, 12, 17, 18, 20-22, 25-27, 29) For example, this alteration is characteristically induced by Actinomycin-D3,22) which directly inhibits the DNA-dependent RNA synthesis,4,23) while the same change is induced by Mitomycin-C35) which inhibits the utilization of DNA precursors for DNA and accelerates the depolymerization of DNA.24) In regard to the inhibitory effect on DNA synthesis and inducing the nucleolar segregation, the action of Bleomycin seems to be closely similar to that of Mitomycin-C. The nucleolar segregation observed here is supposed to be due to
the inhibition of the DNA–dependent RNA synthesis secondarily derived from the denaturation of DNA by Bleomycin, although there still remains the possibility that Bleomycin may directly inhibit the RNA synthesis. Nevertheless, the reduction of chromatin prior to nucleolar alterations was observed in vitro a short time after the treatment with Bleomycin.\textsuperscript{15)} This may support the former possibility. Moreover, a decrease of ribosomes in the cytoplasm was noted in correspondence with these nuclear alterations. It is thought to be intimately related to the impairment of RNA synthesis within the altered nucleoli.

However, as mentioned above, Suzuki \textit{et al.}\textsuperscript{28)} showed that Bleomycin did not inhibit RNA synthesis in vitro. This discrepancy is obscure, and further studies should be carried out on this problem by means of autoradiographic and biochemical methods.

\textit{(Received March 3, 1969)}

\textbf{REFERENCES}

15) Ogawa, K., unpublished data.
NUCLEAR CHANGES PRODUCED BY BLEOMYCIN


EXPLANATION OF PLATES LXXX-LXXXIV

Photo 1. The 3-methylcholanthrene-induced mouse epidermal carcinoma untreated with Bleomycin. Carcinoma cells in basal and deeper prickle-cell layers are relatively well differentiated, without any infiltration into the dermis, and show nuclear pleomorphism with a large nucleo-cytoplasmic ratio. A few mitotic figures are seen. Hematoxylin and Eosin. ×420.

Photo 2. After 48 hr following the administration of 200 mg/kg of Bleomycin. Basal cells and prickle cells in the lower layers become lighter because of the decrease in their cytoplasmic and nuclear basophilicity. Nucleoli are prominent in appearance. Hematoxylin and Eosin. ×420.

Photo 3. Nucleolus of a basal cell from untreated carcinoma. It reveals a pattern of 'spotted nucleolus' which is characteristic in abundance of particulate components (P) and in paucity of fibrillar components (F). Amorphous components (A) are surrounded by cup-shaped cavities made by fibrillar components. Chromatin (C), interchromatin granules (I). × 26,000.

Photo 4. Survey picture of 3-methylcholanthrene-induced mouse epidermal carcinoma in the basal and lower prickle-cell layers. Each nucleus contains moderate or abundant chromatin and one or two large nucleoli (N). Ribosomes are abundantly distributed through the cytoplasm. Basement membrane (B), desmosome (D), chromatin (C), tonofilament (T). × 6,000.

Photo 5. A portion of cancer nest, 48 hr after the treatment with 200 mg/kg of Bleomycin. Chromatin contents (C) are conspicuously decreased in these nuclei of the basal and lower prickle cells. In addition, they show a considerable decrease in the number of ribosomes, and some cytoplasmic degenerative changes, such as swelling of mitochondria (M), widening of intercellular spaces (arrow), and appearance of secondary lysosomes (S), can be seen. Nucleolus (N), basement membrane (B), desmosomes (D), tonofilaments (T). × 6,000.

Photo 6. Nucleolus from a carcinoma cell 48 hr after the treatment with 200 mg/kg of Bleomycin. Fibrillar components (F) are grouped into several aggregates and shifted to the periphery of the nucleolus. Chromatin (C). × 26,000.

Photo 7. Nucleolus is rounded up 48 hr after the treatment with 200 mg/kg of Bleomycin. Particulate components (P) are condensed and contain spherical amorphous components (A) within them. Fibrillar components (F), chromatin (C). × 26,000.

Photo 8. Nucleolus is decreased in size and almost composed of particulate components (P), 48 hr after the treatment with 200mg/kg of Bleomycin. Only a small amount of fibrillar components (F) are attached around the nucleolus. Chromatin (C). × 26,000.

Photo 9 Nucleolus (N) appearing as a round mass of amorphous substance, 48 hr after the treatment with 200 mg/kg of Bleomycin. In these nucleoli, with a disappearance of fibrillar components, particulate components are extremely decreased. ×26,000.