Acta Medica et Biologica
Vol. 35, No. 1, 1-25, 1987

ULTRASTRUCTURAL ALTERATIONS OF NUCLEI OF EHRLICH ASCITES TUMOR CELLS AFTER MITOMYCIN C TREATMENT

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(Received December 26, 1986)

ABSTRACT

Ultrastructural alterations of Ehrlich Ascites Tumor (EAT) cell nuclei following Mitomycin C (MC) treatment were investigated.

The principal alterations in the nuclear ultrastructure were those of the chromatin and of the nucleolus. Unusual condensation or aggregation of chromatin was observed at 30 min as the initial change after MC administration. This pathologic condensation of chromatin developed to a maximum at 4 hr. Density of the nuclear matrix was gradually diminished thereafter to an extreme at 48-72 hr. The earliest morphologic alteration of the nucleolus was manifested at 4 hr after MC treatment as decreased density of the nucleolonema. Disintegration and decrease in density of the organelle became evident at 8-12 hr, and progressed to an extreme at 48-72 hr. Relationships between these ultrastructural alterations and the effects of MC on the nuclear metabolism, especially of DNA and of RNA, are discussed.

Mitomycin C (MC) is one of antitumor antibiotics isolated from the fermentation media of *Streptomyces caespitosus* by Wakaki et al[49]. The drug is cytotoxic and/or effective to various microorganisms[38,42], animal tumors[46] and human tumors[27,31,41], and has been used in clinical practice for patients with malignancies[3,32].

On the action mechanism of MC, previous biochemical and cytochemical studies revealed that MC binds itself directly to nuclear DNA molecule[16,26,51] and that it inhibits DNA synthesis rather than RNA and protein synthesis in the nucleus[22,43,55].

This molecular action of MC, which would correlate mainly with the cytotoxicity of

the drug, is presumed to induce morphologic alterations in the cell unclei. The present paper shows the ultrastructural alterations in interphase nuclei of Ehrlich Ascites Tumor (EAT) cells after MC treatment in vivo, and discusses the relationship between the morphologic changes and MC effects upon nuclear metabolism.

MATERIALS and METHODS

1. Animal

Male albino mice of ddk strain, weighing 20 to 22 gr, were used for successive cultures of EAT and experiments.

2. EAT cells

EAT cells, grown in the peritoneal cavity for 7 days after transplantation, were aspirated by percutaneous puncture. They were then prepared to make a cell suspension in normal saline with a final concentration of about $5x10^6$ cells/ml. Immediately after the preparation, 1 ml of the suspension ($5x10^6$ EAT cells) was intraperitoneally innoculated into eath mouse. The mice with satisfactory intraperitoneal growth of EAT were employed for the following experiments.

3. Experiments

MC treatment: A dose of 40 μg of MC (1/3 of LD_{50} ; 5.2mg/kg[52]) in one ml normal saline was administered into the peritoneal cavity of each mouse 3 days after innoculation of the cell suspension.

Aspiration of ascites: Ascites was aspirated at serial intervals of 0.5, 1, 2, 4, 8, 12, 24, 48, and 72 hr after MC administration. Three or 4 mice were sacrificed at each interval.

Control: Instead of MC, 1 ml of normal saline was applied to each mouse, and ascites was aspirated at the same intervals as the cases of MC treatment. Two mice were submitted to sacrifice at each interval.

4. Electron microscopy

The aspirated ascites was fixed immediately in 2.5% glutaraldehyde in 1/15 M phosphate buffer solution at pH 7.4 for 2 hr. They were then centrifuged for 10 min at 1,500 rpm to obtain pellets of EAT cell samples. The pellets were divided into small pieces of about 1 mm^3, and post-fixed for 2 hr in 1% $0s0_4$ buffered with 0.2 N S-collidine solution at pH 7.4. All fixation processes were carried out at 4℃. The samples were dehydrated with graded ethanol and propylene oxide, and then embedded in Epon 812 resin mixture. Ultrathin sections were prepared, and were picked up on 150-mesh copper grids coated with collosion films. To enhance the contrast of the sections, they were treated with uranyl acetate for 60 min and lead citrate for 10 min. Ultrastructural observation was performed under a JEM100S electron microscope at 80 Kv with an objective aperture of 60μ.

OBSERVATIONS

1. Ultrastructure of interphase nuclei of control EAT cells (Figs. 1-4)

There are some reports available on the ultrastructure of interphase nuclei of native EAT cells[47,53,54] and other neoplastic[36,37] or normal tissue cells[1,44]. The present observations essentially agree with these reports except on a few points.

The interphase EAT cells were usually mononuclear, and the nucleus was often bean-shaped or ovoid with a considerably irregular outline. Intranuclear cytoplasmic invaginations or processes were frequently seen (Fig. 2). Intranuclear canaliculi were also frequently observed (Fig. 3) as reported by Yasuzumi and Sugihara[53].

Chromatin: Condensed chromatin was seen as electron-opaque dense fibrillar plaques arranged along the inner membrane of the nuclear envelope. The plaques were often interrupted at the site of nuclear pores. Small clumps of condensed chromatin were also distributed in the nuclear field. A majority of the interphase nuclei showed such condensed chromatin (Figs. 1, 2, & 3). However, in some instances, where a greater amount of chromatin substance may be distributed in the dispersed form, the condensed chromatin was scarcely observed. The morphologic differences of chromatin possibly correlated with the cell division cycle[14] and/or reflected different functional activities of DNA molecules in the nucleus[45].

A description of nucleolar chromatin is given in the following paragraphs.

Nucleolus: One or two nucleoli were usually observed in the center of the nuclear field or close to the nuclear envelope. Five principal components of nucleolus were differentiated[36,37]; granular and fibrillar components, fibrillar centers, nucleolar chromatin and amorphous matrix.

Numerous dense particles, 15 to 20 nm in diameter, were distributed compactly in the nucleolus forming granular component. Fibrillar component was seen as a dense fibrillar texture distributed in the nucleolus with an irregular but continuous arrangement which showed a tightly convoluted skein, and was contiguous to the granular component and/or the fibrillar center with an indistinct border. This component often formed a circular configuration around the fibrillar center as well (Fig. 2) . Both the granular and fibrillar components formed a characteristic filamentous network called nucleolonema.

Fibrillar center was seen as a round and relatively low electon-opaque field of loose fibrillar substance, generally encircled by a ring of the fibrillar component (Fig. 2).

Nucleolar chromatin consisted of two components: nucleolus-associated and intranucleolar chromatin; the former was distributed around the nucleolus, and the latter as ramifications of the former penetrated into the nucleolar field[12,44]. Both components were scarcely differentiated in a large unmber of nucleoli, while they were significantly prominent as electron-opaque fibrils or strips in some instances where they were distributed in condensed state (Fig. 3).

Amorphous matrix was seen as a small, electron-lucent spot generally associated

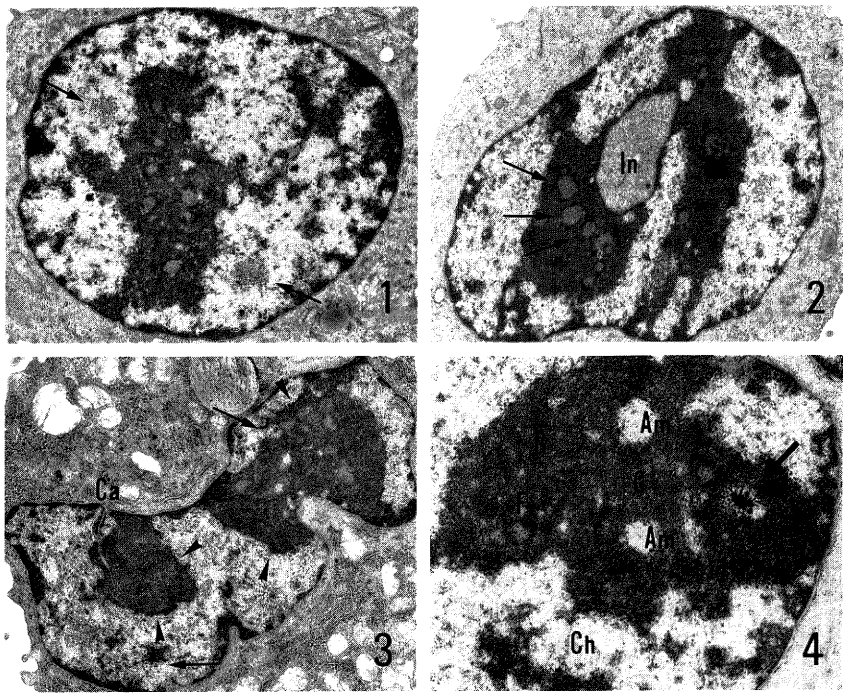

Fig. 1 Typical ultrastructure of an interphase nucleus of a control EAT cell. The nucleus is ovoid in shape. Electron-dense fibrillar plaques of condensed chromatin are well defferrentiated along the nuclear envelope. Small dots of condensed chromatin are also distributed in the nuclear field. They are interrupted at the sites of the nuclear pore. A nuleolus is identified in the center of the nuclear field. Two clusters of interchromatinic granules are observed (arrows). × 7,500

Fig. 2 Interphase nucleus of a control EAT cell. An intra-nuclear cytoplasmic invagination (In) is observed in the center of the nuclear field. There are two nucleoli in this case. Several fibrillar centers each with a ring of the fibrillar component are seen in the left nucleolus (arrows). × 6,500

Fig. 3 Interphase nucleus of a control EAT cell. The nucleus is bean-shaped with an irregular outline. The nucleolus-associated chromatin is clearly seen around both nucleoli (arrow heads). Perichromatinic granules are indicated by arrows. An intranuclear canaliculus (Ca) is distinguished in the left side of the nuclear field. × 7,000

Fig. 4 Nucleolus in an interphase nucleus of a control EAT cell. Fibrillar component (F), granular compoent (G), and amorphous matrix (Am) are differentiated. Nucleolus-associated chromatin (Ch) is barely distinguishable. A typical dense nucleolar body is observed (arrow). × 12,000

with the fibrillar center or the nucleolonema. Some strips of fine filamentous fibrils were scarcely observed in the matrix.

Other nuclear and nucleolar constituents: Interchromatinic granules, somewhat polygonal particles of 10 to 25 nm in diameter, were occasionally observed. They often formed clusters in the interchromatin areas (Fig. 1).

A few to several perichromatinic granules were observed in some instances at the periphery of condensed chromatin (Fig. 3). They were characterized by a morphology consisting of round particles of 35 to 45 nm in diameter with a clear halo of 20 nm width.

Dense nucleolar body (Fig. 4) is a new term coined by the author. It designates an intensely electron-dense, irregular plaque periodically encountered in the peripheral region of the nucleolus. Some perichromatinic granules and an electron-lucent zone resembling the amorphous matrix of the nucleolus were usually present around this particular body. Nucleolar components, such as the nucleolonema and the fibrillar center also seemed to be intimately connected with this ultrastructure. Higher power view revealed that the dense nucleolar body consisted of coarse fibrillar matrix and dense round particles of varying size up to around 50 nm in diameter. Interestingly, the particles seemed to grow larger, ultimately developing into perichromatinic granules at the periphery of the body and separated from it by a clear halo.

Nuclear body was merely encountered on only a few occasions. Details on this specific nuclear constituent are mentioned in the following section.

2. Sequential alterations of EAT cell nuclei following MC treatment

Some remarkable alterations were distinguished in the ultrastructure of the interphase nuclei of EAT cells after MC treatment. Representative ultrastructural alterations observed at sequential intervals of the experiment are described, although progress of the alterations varied considerably in each indivisual.

At 0.5-2 hr: A majority of the interphase nuclei at the course of 0.5 to 1 hr showed apparently no remarkable alterations as compared to the controls. However, in some instances there were faint to modeately prominent aggregates of condensed chromatin distinguishable as relatively fine fibrillar, electron-opaque small dots scattered irregularly in the nuclear field (Fig.5). At 2 hr, the small dots of condensed chromatin became more prominent and denser concomitant with the appearance of slightly conspicuous nucleolar chromatin around and in the nucleolus.

At 4 hr: A large number of EAT cell samples aspirated at this interval predominantly showed an impressive nuclear ultrastructure with the distinct appearance of condensed chromatin forming a characteristic distribution pattern.

The chromatin aggregates coalesced and developed to from dense fibrillar nodules or bundles spreading throughout the nuclear field which came to show a peculiar mosaic-like appearance (Fig. 6). A rough polygonal outline of such nuclei was also noticeable.

As to the nucleolus, nucleolar chromatin in a condensed form was conspicuously distinguishable from other nucleolar constituents. A band of nucleolus-associated chromatin formed a prominent margination around the nucleolar body, and the intranucleolar chromatin stood out as dense electron-opaque plaques or strips in the nucleolus (Figs. 5 & 6). Also noted was a scarce distribution of particles composing the granular component, as well as a considerably diminished density of undeveloped fibrillar component (Fig. 6).

Intranucleolar stain, a new designation, was a relatively large (40 to 100 nm), round to ovoid dense particle-like element with high electron-opacity, rarely encountered in

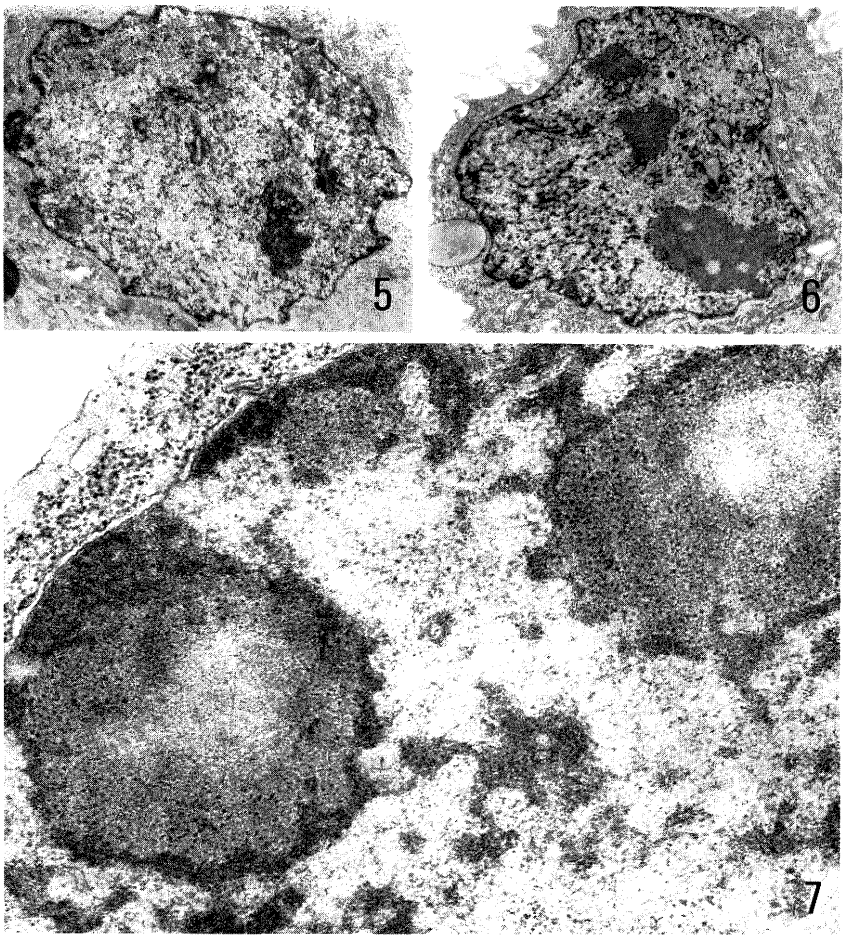

Fig. 5 Interphase nucleus of an EAT cell aspirated at one hr after MC treatment. Small dots of condensed chromatin are distributed throughout the nuclear field. × 7,500

Fig. 6 Interphase nucleus of an EAT cell aspirated at 4 hr after MC treatment. Aggregates of condensed chromatin are prominent in the nuclear field. The nucleolar chromatin is also well differentiated around and in the nucleolus. The lowest nucleolus shows prominent low density. The nuclear outline is polygonal and relatively rough. × 6,000

Fig. 7 Two nucleoli of an interphase EAT cell nucleus aspirated at 4 hr after MC treatment. Both nucleoli are ralatively round in shape. Nucleolus-associated chromatin clearly circumscribes the nucleolar field. Low density of the fibrillar component and sparse distribution of the granular component are evident. × 45,000

several nucleoli (Fig. 9). This element was observed by itself in the nucleolonema or the fibrillar center, and seemed to be an aggregation of the fibrillar or granular constituents of these nucleolar components.

At 8-12 hr: The prominence of condensed chromatin was distinctly reduced both in the nuclear and nucleolar fields. The native fine reticular appearance of the nuclear field

was recovered in many instances. Consistency and electron-opacity of the nuclear matrix in such cases were apparently sufficient enough compared to the controls. The rough polygonal outline of nucleus became amoother once again (Fig. 8).

Recovery of density of the fibrillar component and particles of the granular component in the nucleolus was fairly apparent. However, disintegration of the structure of this nuclear organelle seemed to have taken place to some degree. The fibrillar component was distributed in a discrete arrangement around the fibrillar center where the characteristic circular configuration of this component as seen in the control cases was lost. In such a nucleolus, small spots of the amorphous matrix were often observed between the interrupted fibrillar component (Fig. 9).

The perichromatinic granules increased in number and were encountered more often than at earlier intervals or than in the controls. Also discerned more often was the dense nucleolar body. In some instances, many of the perichromatinic granules were aggregated close to the well-developed dense nucleolar body (Fig. 8). These nuclear and nucleolar constituents were observed persistently in the following intervals as often as in this interval.

The intranucleolar stains were distinguished more frequently than during the other

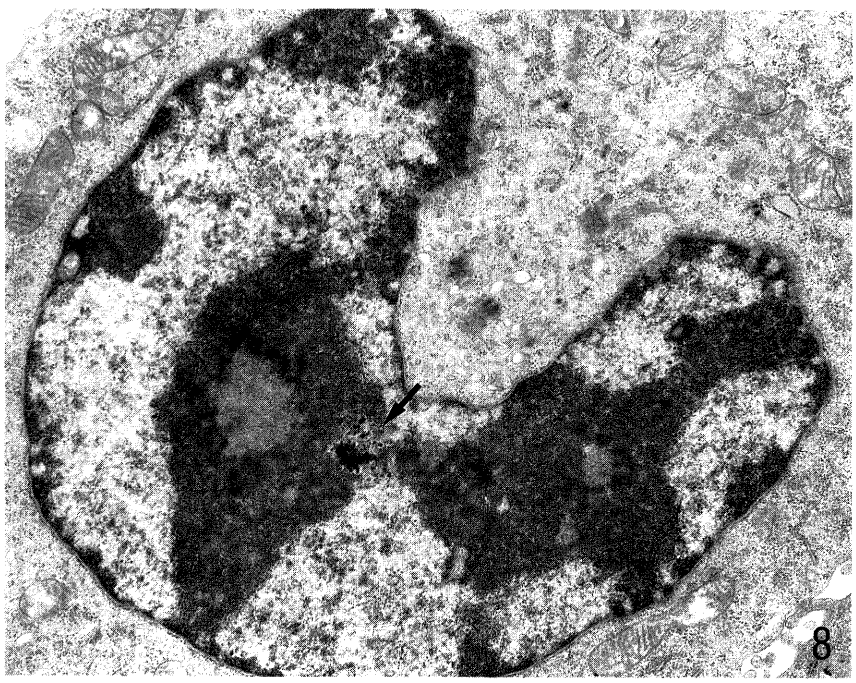

Fig. 8 Interphase nucleus of an EAT cell aspirated at 12 hr after MC treatment. The nucleus is bean-shaped with a smooth outline. The prominent aggregates of condensed chromatin are no longer seen. Density of the nucleolus is apparently sufficient. A well-developed dense nucleolar body and several perichromatinic granucles are recognized in the border between two nucleoli (arrow). × 15,000

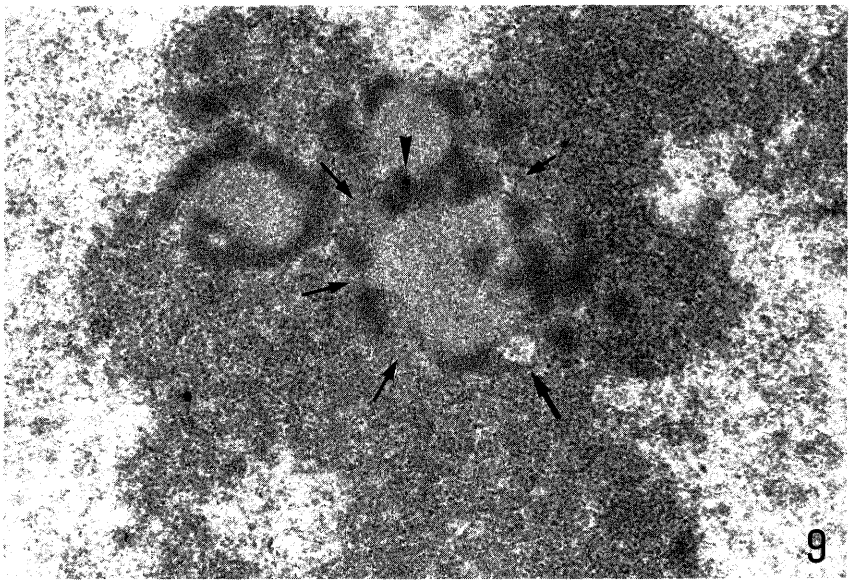

Fig. 9 Nucleolus of an interphase EAT cell nucleus aspirated at 12 hr after MC treatment. Note the inerruption of the fibrillar component around the fibrillar centers (thin arrows), and interposed small spots of the amorphous matrix (thick arrows). An intranucleolar stain is identified in this case (arrow head). × 35,000

intervals of the experiment, though they were considerably rare even at this interval (Fig. 9).

At 24 hr: The fine reticular texture of nuclear matrix became coarser in general. Consistency of the matrix seemed to be scarcer to some degree. Condensed chromatin was extremely decreased in quantity (Figs. 10 & 12).

Disintegration of the nucleolus was consecutively in progress in a large number of instances at this interval. The discrete arrangement of the fibrillar component around the fibrillar center became more prominent, usually accompanied by the enlargement of areas of the interposed amorphous matrix. A characteristic ultrastructure developed: the fibrillar center was alternately encircled by dense fibrillar plaques of disrupted fibrillar component and electron-lucent spots of amorphous matrix (Fig. 11). Plaques of the fibrillar component were sometimes seen even in the fibrillar center. In such instances, exhaustion of the fibrillar component was marked and only a few plaques of it were observed around the fibrillar center which was often significantly enlarged (Fig. 12). Several spots of the amorphous matrix also often developed within the nucleolonema as well as around the fibrillar center. All these alterations brought out a nucleolus which was somewhat sparser in consistency. Nucleolar chromatin was only faintly differentiated.

At 48-72 hr: In general, density of the nuclear matrix was further diminished. In some instances an extremely less electron-opaque nuclear field was seen, in which most

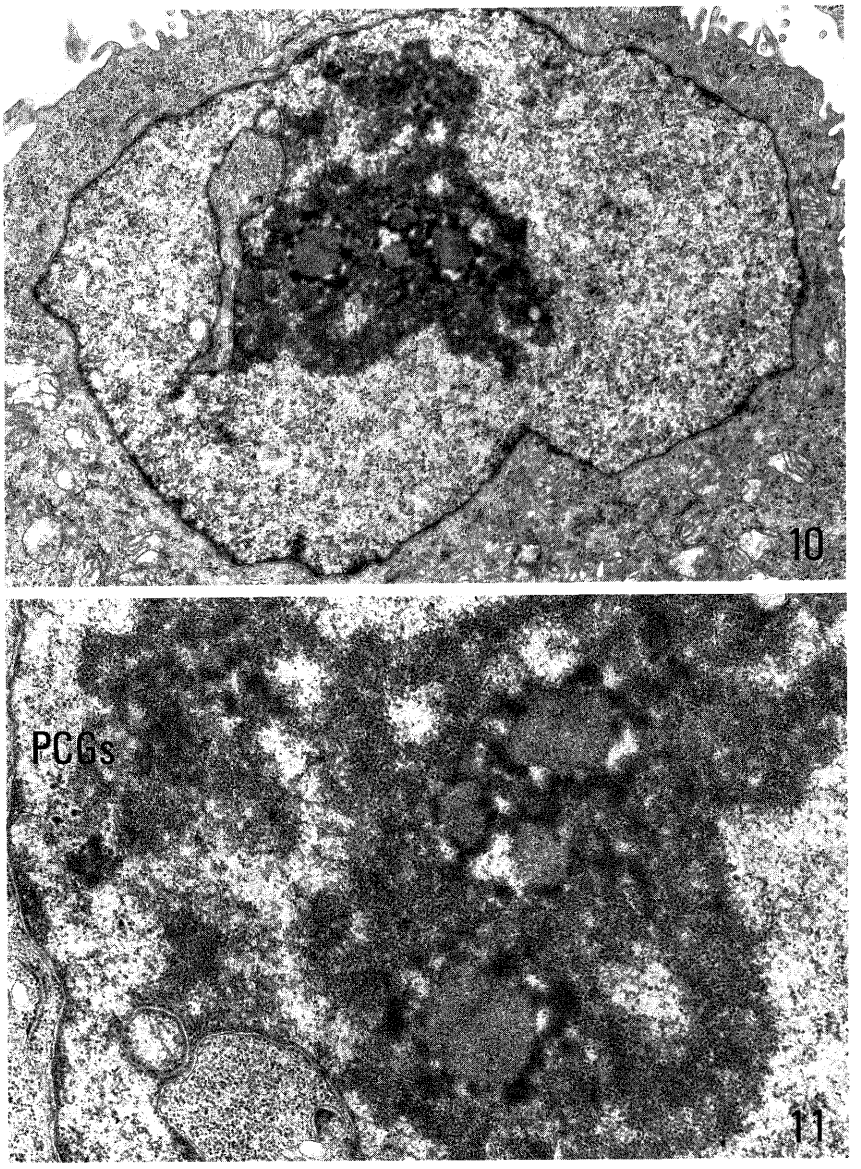

Fig. 10 Interphase nucleus of an EAT cell aspirated at 24 hr after MC treatment. Note the coarser consistency of the nuclear field with little condensed chromatin substance. × 11,500

Fig. 11 High power view of the same nucleolus shown in Fig. 10. Discrete arrangement of the fibrillar component and more developed interposed amophous matrix around the fibrillar centers is conspicuous, exhibiting alternate distribution of the fibrillar component and the amorphous matrix around the fibrillar center. Several spots of the amorphous matrix are also seen in the nucleolonema. Such a distribution of these nucleolar components are strong influences in causing the somewhat diminished density of the organelle. PCGs; perichromatinic granules. × 22,000

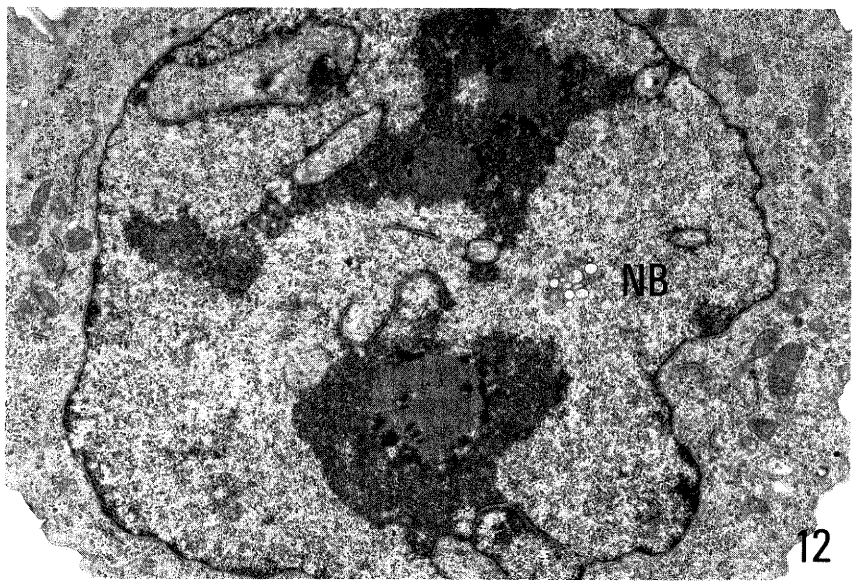

Fig. 12 Interphase nucleus of an EAT cell aspirated at 24 hr after MC treatment. The nuclear field shows sparse consistency. Exhaustion of the fibrillar component around the obviously enlarged fibrillar centers is distinct. Several small dots of the fibrillar component are left within the fibrillar center of the lower nucleolus. In this case, intranuclear cytoplasmic invaginations and intranuclear canaliculi are fairly developed. NB; type D nuclear body (ref. Fig. 21). × 12,000

of the matrix appeared to be emptied. Condensed chromatin remained in smaller amounts (Figs. 13 & 14).

Both a decrease in size and further diminution in density of the nucleolus with a round to ovoid configuration were apparent in many instances (Fig. 14). Alternate distribution of the discrete fibrillar component and the amorphous matrix around the fibrillar center still persisted in some cases. A relatively large amorphous matrix, which seemed to be derived from the fusion of the smaller spots of the matrix, sometimes developed around the fibrillar center (Fig. 15). A large fibrillar center often associated with an adjacent or neighbouring large, round amorphous matrix was occasionally seen (Fig. 13). In several nucleoli, there were peculiar aggregates of the fibrillar component evolved around the fibrillar center (Fig. 16) or sorted out in a round mass in the nucleolonema (Fig. 17). Cribrifrom appearance of the nucleolonema was remarkable in many instances (Figs. 13 & 15). This characteristic ultrastructure seemed to be mainly attributable to both the irregularly indented arrangement of the nucleolonema and the large number of small electron-lucent spots of the amorphous matrix distributed in this particular structure.

Dense satellite, one of the new designations presented in this article, was an electron-opaque dense body with an almost round shape of 120 to 220 nm in diameter noted in some instances (Figs. 14 & 16). It consisted of many particles of 15 to 25 nm in diameter

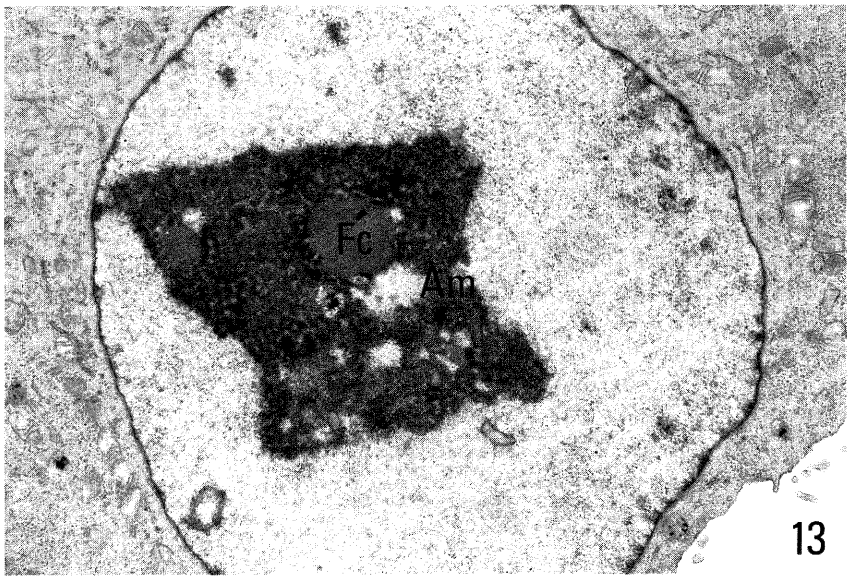

Fig. 13 Interphase nucleus of an EAT cell aspirated at 48 hr after MC treatment. The nuclear field is distinctly less electron-opaque. Few plaques of the condensed chromatin are distributed. The nucleolus is characterized by a large fibrillar center (Fc) and neighboring amorphous matrix (Am). A dense nucleolar body is observed in the center. Note a cribriform appearance of the nucleolonema (upper half of the nuclear field). × 9,500

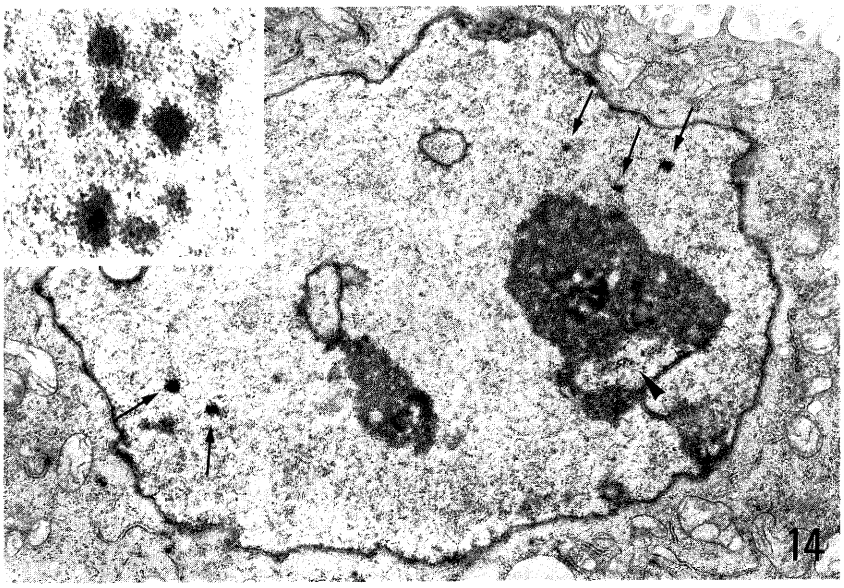

Fig. 14 Interphase nucleus of an EAT cell aspirated at 48 hr after MC treatment. Low density and coarse consistency of the nuclear field are apparent. There are two poorly developed nucleoli. An increase in the number of perichromatinic granules (arrow head) and several dense satellites are seen (arrows). × 12,000 Inlet: High power view of dense satellites. They consist of an aggregate of multiple dense particles in a fibrillar background. × 35,000

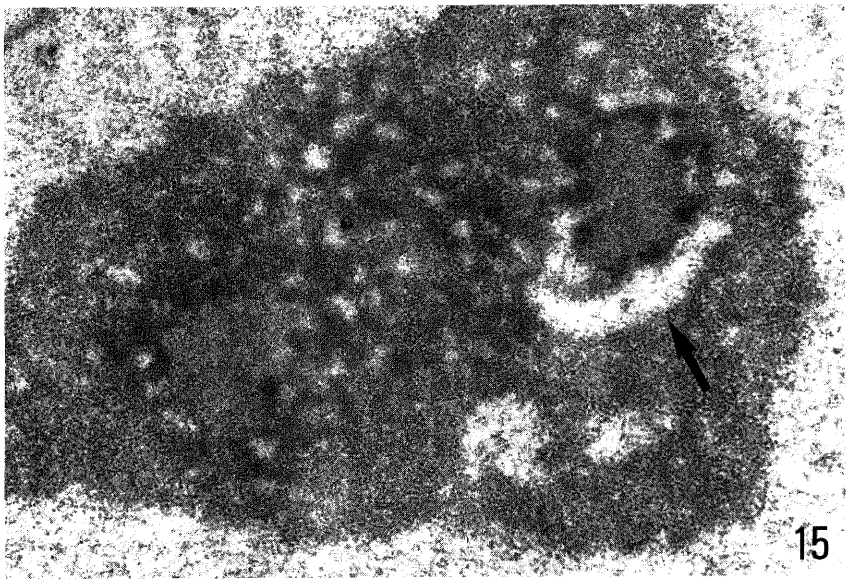

Fig. 15 Nucleolus of an interphase EAT cell nucleus aspirated at 72 hr after MC treatment. Characteristic alternate arrangement of the fibrillar component and the amorphous matrix is consecutively observed. Fusion of the smaller spots of amorphous matrix seem to form a larger matrix (arrow). The nucleolonema is arranged in a typical cribriform appearance in the center. × 28,000

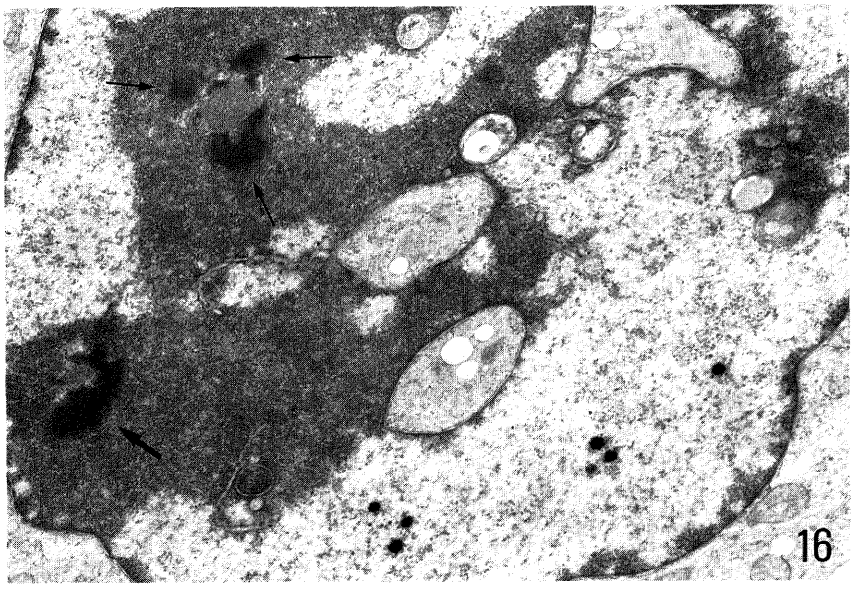

Fig. 16 Interphase nucleus of an EAT cell aspirated at 72 hr after MC treatment. There are peculiar aggregates of the fibrillar component around the fibrillar center (thin arrows) and in the nucleolonema (thick arrow) of the nucleolus. Several dense satellites are seen in the nuclear field. × 14,000

aggregated in a dense fibrillar matrix. Morphologically, both the particles and the matrix closely resembled granules and dense fibrils, respectively, in the nucleolonema. On occasion, several dense satellites were distributed in a scanty nuclear field, either near or some distance away from the nucleolus.

Dense nucleolar body sometimes appeared even in the nucleolonema distributed in the central part of nucleolar field (Fig. 17), as well as at the periphery of the nucleolus.

The interchromatinic granules were distributed in an essentially unaltered fashion through the course of experiment as compared with the control cases.

Nuclear body: Nuclear bodies were sometimes observed, especially in periods following MC treatment. They were basically round to ovoid structures with a diameter of 400 to 1000 nm, and might be classified into four types depending on the morphologic characteristics.

Type A nuclear body (Fig. 18) was a loose meshwork structure of intertwined fine fibrils, and stood out in electron-lucent relief against the nuclear matrix of a definite density.

Type B nuclear body (Fig. 19) consisted of several round particles and fine reticular matrix with an encircling faint fibrillar envelope around them. The particles, 60 to 120

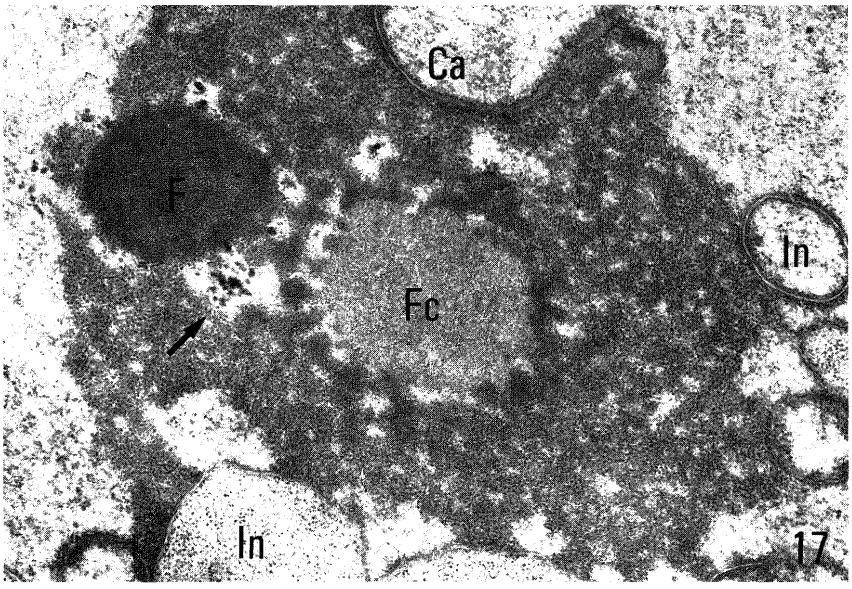

Fig. 17 Nucleolus of an interphase EAT cell nucleus aspirated at 48 hr after MC treatment. A round mass of the fibrillar component (F) sorted out in the nucleolonema. The nucleolonema is composed mainly of a granular component showing conspicuously sparse distribution. A large fibrillar center (Fc), surrouned by the alternately arranged fibrillar component and amorphous matrix, are also prominent. An atypical dense nucleolar body is seen in the central part of the nuclear field (arrow). In; intranuclear cytoplasmic invaginations. Ca; intranuclear canaliculus. × 23,000

nm in diameter, were made up of fine reticular matrix of various density and outer membrane-like fibrils.

Type C nuclear body (Fig. 20) was characterized by an inner limiting mambrane and an outermost thin fibrillar envelope. There was an electron-lucent zone of thin width (approximately 25 nm) between the membrane and the envelope. An electron-opaque core of moderate density and some fine filaments were observed in the center. The core consisted of several particulates (around 10 nm in diameter) with a narrow electron-lucent halo compactly distributed in the fine fibrillar matrix which seemed to consist of fine filaments.

Type D nuclear body (Fig. 21) referred to a structure consisting of several round vesicles with a clear limiting membrane. Diameter of these empty vesicles with occasional fine granular substance varied from 60 to 220 nm. Some amount of fibrillar matrix was discerned between the vesicles. There seemed to be an outer zone of relatively less electron-opacity which formed an obscure boundary between this body and the nuclear matrix.

Type A and B nuclear bodies were seen in both control and MC treated cells, although the chance of observation in the former cases was merely incidental. In MC treated cases, these nuclear bodies were occasionally observed at the intervals of 8, 12 and 24 hr during the experiment, still even this was not so often. Type C and D nuclear

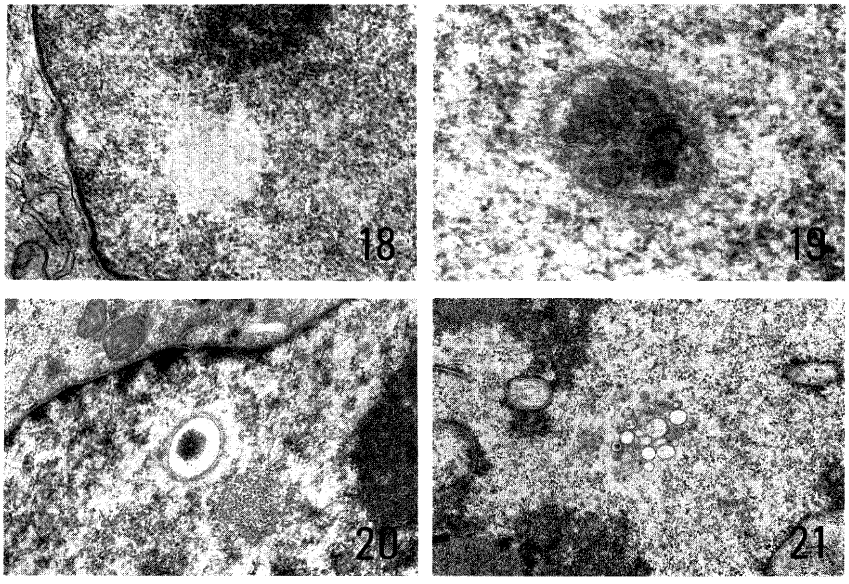

Fig. 18 Type A nuclear body, 8 hr after MC treatment. × 23,000
Fig. 19 Type B nuclear body, 12 hr after MC treatment. × 38,000
Fig. 20 Type C nuclear body, 12 hr after MC treatment. × 16,000
Fig. 21 Type D nuclear body, 24 hr after MC treatment. This typical type D nuclear body was observed in the case shown in Fig. 12. × 16,000

Table 1. Ultrastructural Alterations of EAT Cells After MC Treatment

Time (hr)	0.5-1——2	4	8-12	24	48-72
Nucleus					
Outline		—Polygonal—	Normalized		
Matrix			Essentially unaltered	Coarse	Depleted
Chromatin	Condensed, Prominent Condensation	Mosaic-like	Reducing	→	→
PCG			Increased	→	→
Dense Satellite					Present
NB			Specific NB: Type C & D, rarely		
Nucleolus					Decrease in Size
Chromatin NAC	Condensed	Margination	Reduced	→ Faintly (?)	→
INC		Plaques			
Granular C		Scarce	Normalized	Reduced	→
Fibrillar C		Lowered Density	Recovered Density	Reduced —— (Aggregates)	→
			Interrupted Distribution	Prominent Interruption ┐ Alternate Pattern	
Am				Enlarged	→
Fc				Enlarged	→
Nclnma					Cribriform Pattern, Reduced Density
INS		Present, rarely			
Dense NcB			Present more often than Control and well developed	→	→

PCG: Perichromatinic Granules — NB: Nuclear Body — NAC: Nucleolus Associated Cromatin
INC: Intranucleolar Chromatin — Granular C: Granular Component — Fibrillar C: Fibrillar Component
Am: Amorphous Matrix — Fc: Fibrillar Center — Nclma: Nucleolonema
INS: Intranucleolar Stain — Dense NcB: Dense nucleolar Body

bodies were considered specific to MC treated cells. They were rarely encountered, and then only in some instances with ascites aspirated at the intervals of 12 and 24 hr were either of these nuclear bodies demonstrated.

The overall observations in the present study are summarized in Table 1.

DISCUSSION

In the present study, predominant ultrastructural changes in the nuclear morphology of EAT cells following MC treatment might well be those of the chromatin and of the nucleolus. Because one of the main elements of chromatin is DNA[28] and the nucleolus is a machinery of ribosomal RNA (rRNA) synthesis[34,35,45], the relationship between morphologic alterations and effects on the metabolism of these nucleic acids produced by

MC should be discussed.

1. Ultrastructre of chromatin and DNA metabolism

It is now generally accepted that the principal action mechanism of MC is, as previously mentioned, to bind itself directly to DNA molecules leading to inhibition of DNA synthesis in the nucleus. On the fundamantal molecular interaction of MC with DNA, some hypothetic processes have been proposed.

One of the influential hypotheses is that MC acts as an alkylating agent when activated[4,40,48]. Some in-vitro studies have confirmend that the drug intercalates directly between DNA molecules to from firm interstrand cross-links causing alkylation of the nucleic acid[9,16,26,52]. From another angle, several in-vitro studies have proved DNA strand scission by MC and attached its importance to the cytotoxic effect of the drug[18,26,38]. On the other hand, Kaplan and Tomasz[17] insisted, in an in-vitro study with calf thymus DNA, that MC binds itself to minor grooves rather than between base pairs in DNA molecules leading to conformational changes of the nucleic acid.

Thus, some controversies still remain concerning the fashion of the molecular interaction between MC and DNA, while there seems to be little doubt that denaturalization of DNA does occur in the process of direct binding of MC to DNA molecules. Denaturalization of DNA, that is a change in the physicochemical property of DNA, may immediately correspond to loss of template activity of the nucleic acid in replication, resulting in inhibition of DNA synthesis. It would be expected that such biochemical effects of MC on DNA metabolism may produce morphologic alterations of chromatin in vivo.

The abnormal condensation or aggregation of chromatin is the initial alteration in the nuclear morphology of EAT cells following MC administration. It appears as early as 30 min after MC treatment and reaches maximum at 4 hr with a mosaic-like distribution of chromatin aggregates in the nuclear field. Daskal and Crooke[6] also noticed, in their electron-microscopic study, an appearance of aggregates of condensed chromatin in rat hepatocyte nuclei at one hr after in-vivo MC treatment (a dose of 5 mg/kg MC, intraperitoneal administration). In a photomicroscopic observation of nuclei of malignant ascites tumor cells of mouse and rat following in-vivo MC treatment, Kobayashi[20] reported an unusual coagulation of chromatin and alterations in stainability of the nuclei to specific stainings for DNA, both suggesting certain changes in the nature of the nucleic acid. Nuclear DNA is dispributed in dispersed chromatin when it is in an active state, while it aggregates in condensed chromatin when in an inactive state[13,25]. Hence, the striking condensation of chromatin is considered to be a primary morphologic expression of the inactivation, i.e., the denaturalization of DNA caused by the direct action of MC against DNA molecules. However, in the in-vitro studies with cultivated KB cells[23] and Novikoff hepatoma cells[6], such chromatin condensation did not become prominent following treatment with the same drug. At present, the reason for such a discrepancy in the morphology of chromatin is obscure. It may owe to differences in the conditions

of the experiments, such as whether in vivo or in vitro, differences in the treated cells, and of actual doses or concentrations of MC employed. At any rate, a satisfactory elucidation relies on future investigation.

Similar unusual aggregation of chromatin has been confirmed in nuclei of cultivated cells following treatment with a relatively high concentration (50μg/ml) of actinomycin D[37] and platinum complex[7], both of which are known to bind themselves directly to DNA like MC. Simard[44] reported also in in-vitro studies that DNA intercalators, such as proflavin, ethidium bromide and daunorubicin, induced clumping of condensed chromatin, the morphology of which was characteristic of clumped chromatin distributed detouched from the nuclear envelope and apparently differing from that of the present case.

Thus, from these observations, along with the present study, it may be suggested that unusual chromatin condensation per se, regardless of whether it is in an in-vivo or in-vitro event, would be a plain morphology of denaturalized DNA brought about by direct binding of some chemical agents.

A lucid explanation is hardly proposed on the disappearance of inactivated condensed chromatin from the nucleus at 8 to 12 hr on. It may be transformed into some substance undetectable by the methodology employed in the present study through unknown biological mechanisms, or reactivated and distributed again in the form of dispersed chromatin if denaturalization of DNA by MC is reversible to some extent. On the other hand, existence of a certain, presumably more or less smaller than pretreatment, amount of active dispersed chromatin in the nuclear matrix could be assumed, at least st intervals of 8 and 12 hr, because of the ultrastructure of the nuclear field with a definite consistency. This assumption is partly supported by a previous study[22] which demonstrated that, although with less intensity as compared to the controls, new DNA synthesis was carried out in EAT cells in mice at 9 hr after intraperitoneal injection of 50 μg MC, using an experimental design similar to that of the present study. However, the source of the active chromatin is obscure. It may be derived from one or more of certain conceivable origins or processes, such as reactivation of denaturalized chromatin, native dispersed chromatin which survived the effect of MC, or newly synthesized DNA.

The progressive diminution in consistency of the nuclear matrix after 24 hr is thought to correspond to a decrease in the amound of chromatin resulting from the persistent inhibition of DNA synthesis by MC. This influence on DNA metabolism may be the secondary effect of the drug; it would be responsible mainly for impairment of replication under the denaturalized DNA which had already lost its template activity, although the possibility that impairment of DNA polymerase function by MC may participate partly in the inhibition of DNA synthesis cannot be excluded. In addition to the diminution of chromatin, the excessively emptied nuclear field observed at 48 and 72 hr would also reflect severe depletion of nuclear protein, a large amount of which was contained originally in the nucleus[28,44], at these latest intervals of the experiment. From this point of view, MC may either directly or indirectly affect the metabolism of nuclear

protein.

Thus, the ultrastructural alterations of chromatin substance are morphologically heterogenous between those observed at intervals of up to 4 hr and after 24 hr following MC treatment. The former, which are characterized by abnormal condensation of chromatin, may be morphologic representations of the primary denaturalization of DNA by MC, while the latter seem to reflect the secondary inhibitory effect of the drug against DNA replication or synthesis.

The significance and mechanism of the transformation of the nucleus into a rough polygonal outline is not clear. Although a close relation between such a nuclear morphology and abnormal condensation of chromatin can be speculated, because the altered nuclear outline appears and disappears with the movement of the condensed chromatin aggregates.

2. Ultrastructure of nucleolus and rRNA metabolism

The fibrillar and granular components, both main elemants of the nucleolus, have been confirmed to be precursors of rRNA, and the former is the original entity in the process of maturing to the latter in the organelle[1,13]. The rRNA is a ribosomal DNA (rDNA) dependent RNA[29] and, therefore, its biosynthesis is dependent on the active rDNA template. The template rDNA is distributed in the from of intranucleolar chromatin in ribosomal cistron of the fibrillar center, at the periphery of which rDNA transcription occurs to make a newly synthesized rRNA precursor appearing as fibrillar component around it[19,30].

The obvious morphologic alterations of nucleolus presented here most certainly show the MC effects on rRNA metabolism.

Condensation of the nucleolar chromatin observed at 2 hr after MC administration is the initial change in the ultrastructure of the nucleolus. This may be brought about by direct binding of the drug to DNA molecules in the organelle, as in the case of chromatin in the nuclear field, and may be assumed to indicate the inactivation of rDNA.

The considerably diminished density of the fibrillar component around the fibrillar center identified at 4 hr supposedly shows a drop in new rRNA synthesis. Decreased density of the nucleolonema at this interval is thought to reflect a decrease in the whole amount of nucleolar mass, which can be attributed mainly to the decline of rRNA synthesis. In considering the preceeding chromatin change, it may be most probable that the diminution of rRNA synthesis is a result of the secondary impairment of transcription due to the primary inactivation of rDNA by MC.

Reappearance of dense fibrillar component around the fibrillar center and restoration of density of the nucleolonema at intervals 8 and 12 hr signify the recovery of rRNA synthesis. The discrete arrangement of fibrillar component around the fibrillar center is a suggestive ultrastructure. This characteristic morphology may show that at least some active template rDNA has returned to be distributed partially around the fibrillar

center to which dots or plaques of the fibrillar component attach. In contrast, areas of the interposed amorphous matrix may indicate the intermittent absence of active rDNA at the peripheral portion of the fibrillar center. An ultrastructure such as the amorphous matrix is important when considering the morphogenesis and significance of this nucleolar component. The redistribution of active rDNA is attended by the disappearance of condensed nucleolar chromatin. The reactivation of rDNA and the disappearance of condensed nucleolar chromatin are noteworthy in relation to the redistribution of active dispersed chromatin in nuclear matrix at the same intervals of the experiment. The mechanism of reactivation of rDNA is not clear. However, it is possible that it is the same process(es) found in the nuclear chromatin.

Ultrastructural alterations of the nucleolus at an interval of 24 hr after MC treatment should be thought of as a complex of figures indicating the redecline of rRNA synthesis and progressive disintegration of the architecture. The more discrete distribution of the fibrillar component and the contrarily augmented area of the amorphous matrix around the fibrillar center may suggest reduction of the site with active rDNA and an accompanying decline of rRNA synthesis. Both of these may be directly connected with a decrease in the absolute amount of active rDNA in ribosomal cistron. Enlargement of the fibrillar center per se implies impairment or stasis of rRNA synthesis in the nucleolus[10,15,46]. The unusual morphology of the fibrillar component, such as fragmented plaques left in the fibrillar center and peculiar aggregates evoluted around the fibrillar center or soated out in the nucleolonema, may also suggest impaired metabolism of rRNA synthesis in the nucleolus. The cribrifrom appearance of the nucleolonema might develop in the process that occurs when the alternately arranged fibrillar component and amorphous matrix extend in a convoluted fashion away from the periphery of the fibrillar center towards the nucleolar field. Such a characteristic ultrastructure shows nothing but the reduced density of the nucleolonema. A decrease both in the density of the nucleolonema and in the size of the nucleolus represents a diminution of nucleolar mass, that is, a decrease in the amount of rRNA precursors. It should be significant that these ultrastructural alterations of the nucleolus, suggesting impeded metabolism of rRNA, appear alongside with the morphologic alterations of nuclear chromatin which seem to reflect disturbances in the metabolism of nuclear DNA. From this point of view, ultrastructural alterations of the devastated nucleolus are considered to be results of the primary disturbance in the metabolism of rDNA by MC.

As a whole, the ultrastructural alterations of the nucleolus following MC administration seem to represent the secondary impairment of rRNA metabolism due to the primary injury of rDNA metabolism by the agent. However, other effects of MC which either directly or indirectly take some part in the impaired rRNA synthesis cannot be entirely excluded. They are, for example, the direct inhibition of rDNA transcription, obstruction of rRNA polymerase activity, desolution of metabolism of the essential protein for rRNA synthesis[10,33], depolymerization or breakdown of once polymerized rRNA.

Morphologic proofs of the inhibitory effect of MC on rRNA metabolism were obtained by Lapis and Bernhard[23] and by Daskal and Crooke[6]. These anthors confirmed that MC brings a characteristic nucleolar lesion, nucleolar segregation, in cultured KB cells and Novikoff hepatoma cells, respectively, following in-vitro treatment with the drug. The nucleolar segregation is a ultrastructure with nucleolar components sorted out respectively to distinct areas in the nucleolus, and has been observed in various cells treated with actinomycin D[11,37,39], with 4-nitroquinoline-N-oxide[24,39], and with many other antimetabolites or chemicals[44]. This unique lesion is specific to agents that bind themselves directly to DNA molecules, and is considered to be a morphologic manifestation of either direct or indirect inhibition of nucleolar rRNA synthesis caused by the direct binding of some chemical agents to rDNA, leading to the loss of its template activity[11,44].

Absence of the nucleolar segregation in the present study is compatible with the observation made by Daskal and Crook[6] in studing the hepatocyte unclei of rats that had received in-vivo MC administration. It should be evident that there is a discrepancy in the nucleolar morphology between the cases influenced by MC in vivo and in vitro. The difference in the resulting morphologic alterations of nucleolus may be possibly due in part to difference in the cells employed. However, attention to a difference in the environment of the experiments, that is, whether they were maintained in vivo or in votro, is fundamental for making such a discrepancy. It is of particular interest that the inhibitory process of MC on rRNA metabolism may vary between cases which are in vivo or in vitro; a resolution of this problem remains to be clarified.

3. Relationship between other ultrastructures and effects of MC

The increase in the number of perichromatinic granules occurring after 8 to 12 hr following MC treatment is one of the noteworthy ultrastructures in the present study. The same phenomenon has also been recognized in various cell nuclei following in-vivo administration of the same drug[6], or of cycloheximide[5] and platinum compounds[7], and in in-vitro treatment with α-amanitin[8]. These nuclear particles contain both RNA and DNA[1], and are thought to increase in number under conditions where severe inhibition of chromatin transcription[7] or interruption of nucleolar RNA maturation[8] exists. The simultaneous occurrence of an increase in number of perichromatinic granules and remarkable morphologic alterations of the nucleoli in the present study may well be consistent with these hypotheses. In the case of MC, the increased granule content may be thought of as one of the results brought about by the secondary inhibitroy effect of the drug on RNA metabolism.

Just as in the case of perichromatinic granules, both an increase in the number and abnormality in the distribution of dense nucleolar bodies could result from the impediment of nucleolar RNA metabolism caused by MC. This explanation may be most plausible since the morphology of the dense nucleolar bodies strongly suggests their close participation in the process of development or metabolism of the perichtomatinic gran-

ules.

In addition, the metabolic disorder of nucleolus may be closely associated with the appearance of dense satellites at the terminal intervals of the experiment, since the morphologic resemblance of their components to those of the nucleolonema is undeniable. Some ultrastructural alterations similar in morphology to dense satellites have been reported in various culured cell nuclei following in-vitro treatment with MC[23], 4-nitroquionline-N-oxide[24] and actinomycin D[37]. The authors of these reports also proposed a correlation of impaired nucleolar metabolism with these ultrastructures, though with insufficient convincing proofs.

Intranucleolar stains seem to be morphologically similar to dense nucleolar particles which have been observed in various normal and pathologic cell nuclei with unclear significance[45]. In the present study, the appearance of the intranucleolar stains is possibly thought to reflect disturbances of DNA and/or RNA metabolism in the nucleolus. However, this hypothesis is precarious, and the singnificance of these two ultrastructures needs to be further evaluated in the future.

The appearance of nuclear bodies has been recognized in various normal, degenerative or neoplastic cells[2,21,50]. Among four types of nuclear bodies observed in the present study, type A and B seem to be native to EAT cells, since these two types are observed in both untreated and MC-treated cells. On the other hand, the remaining two types, C and D, both of which are specific to treated cases, are thought to be produced in the process of cell degeneration.

Summary and Conclusion

Ultrastructural alterations in interphase nuclei of EAT cells following MC treatment in vivo are demonstrated, and the relationships between these alterations and the effects of the drug on the nuclear metabolism are discussed.

The initial alteration is the appearance of aggregates of condensed chromatin in some nuclei at as early an interval as 30 min after MC treatment. This pathologic chromatin condensation develops to a maximum at 4 hr, when the prominent aggregates of chromatin substance are distributed with a characteristic mosaic-like appearance throughout the nuclear and nucleolar field. By uncertain mechanism(s) or process(es), these aggregates are distinctly diminished and a certain amount of dispersed chromatin becomes distributed in the nucleus at intervals of 8-12 hr. The dispersed chromatin is decreased in amount at 24 hr reaching a severe depletion at 48-72 hr, when the greatly diminished consistency of the nuclear matrix is conspicuously apparent morphologically. The appearance of condensed chromatin aggregates is regarded as a plain morphologic expression of the alteration of physicochemical properties of nuclear and nucleolar DNA molecules caused by the primary action of MC to the nucleic acid. On the other hand, the decrease in the amound of dispersed chromatin occuring by degrees after 24 hr may result from the secondary inhibition of DNA replication, which can be mainly attributed

to the primary denaturalization of the template DNA by MC.

The earliest morphologic alteration of the nucleolus is distinguished as an abnormal condensation of nucleolar chromatin at 2 hr after MC administration and is followed by a decrease in density of the fibrillar and granular component manifested at 4 hr. Variegated ultrastructures develop thereafter in the nucleoli; discrete arrangement of the fibrillar component, alternate distribution of the fibrillar component and the amorphous matrix around the fibrillar center, cribriform appearance of the nucleolonema, large fibillar center and amorphous matrix, and a decrease in nucleolar density and size. All these morphologic alterations are considered to reflect a process or a state of either exhaustion of the nucleolar constituents or disintegration of the organelle. The initial triggering of these morphologic alteratios seems to originate from around the fibrillar center, where the synthesis of rDNA dependent rRNA may be impaired considerably in MC treated cells. A hypothesis is proposed: alterations of the nucleolar ultrastructure are morphologic manifestations of the impaired rRNA synthesis secondary to the primary loss of template activity of rDNA cansed by MC.

Some other alterations in the nuclear ultrastructure following MC treatment, such as an increase in number of perichromatinic granules and dense nucleolar bodies, and appearance of intranucleolar stains, the dense satellites and specific nuclear bodies, may reflect the metabolic disorders in the nucleus, including those of DNA and RNA, caused either directly or indirectly by MC. However the significance of these morphologic alterations remain to be clarified in the future.

ACKNOWLEGEMENT

The author is indebted to Dr.Jun Soga, Professor of Surgery, College of Biomedical Technology, Niigata University, for his helpful suggestion and advice in the course of the present work and the preparation of this manuscript.

Thanks are also due to Prof. Terukazu Muto, Director of Department Surgery, Niigata University School of Medicine, for his encouragemant throughout this investigation.

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