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SCANNING AND TRANSMISSION ELECTRON MICROSCOPIC EVALUATION OF HUMAN MELANOMA CELLS TREATED WITH ADRIAMYCIN AND ACTINOMYCIN D

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Abstract

A tumorigenic human cell line was derived from a patient with metastatic melanoma. Cells were treated with adriamycin or actinomycin D in order to assess morphological alterations induced by these anticancer agents. Exposure to 0.01 μg/ml adriamycin for one hour caused no observable morphological abnormalities as determined by SEM, while 0.1, 1.0 and 10.0 μg/ml concentrations of adriamycin produced surface alterations in the form of blebs, filopodia, microvilli and cell rounding. These alterations may be drug-affected changes of the cell surface or may reflect phases of the cell cycle directed by adriamycin action on the nucleus. Cell morphology appeared normal by SEM for 0.01, 0.1, 1.0 and 10.0 μg/ml concentrations of adriamycin when the cells were allowed to recover in drug-free media for 24 hours after initial drug incubation. Concentrations of 0.1, 1.0, and 10.0 μg/ml actinomycin D for 24 hours created morphological alterations characterized by cell rounding and long, dendritic-like processes. TEM of colonies treated in soft agar for 24 hours with either 1.0 μg/ml adriamycin or 1.0 μg/ml actinomycin D showed major morphological effects identified by increased cytoplasmic vacuolization and nuclear disintegration.

KEY WORDS: Actinomycin D, Adriamycin, Anthracyclines, Scanning Electron Microscopy, Transmission Electron Microscopy, Soft Agar Stem Cell Assay, Melanoma, Human Cell Line, Tissue Culture, Tumor Biology

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Introduction

Adriamycin (Doxorubicin) is an anthracycline antibiotic with potent antitumor activity in animals and in patient care, and is among the most active anticancer drugs. In particular, it is employed in the treatment of small cell carcinoma of the lung and breast, soft tissue sarcoma, anaplastic carcinoma of the thyroid, Hodgkin's disease, non-Hodgkin's lymphoma, and melanoma.

Historically, DNA has been considered to be the primary target for cytotoxic action of the drug (Rusconi and DiMarco, 1969; Neidle, 1979). Adriamycin intercalates in DNA thus affecting DNA and RNA synthesis and/or damages DNA (Schwartz and Kanter, 1979). The mechanism of cytotoxicity, however, does not appear to be limited to either DNA damage or the blockage of DNA synthesis since adriamycin has recently been demonstrated to be actively cytotoxic without entering the cell (Tritton and Yee, 1982). Therefore, adriamycin initially described as having intracellular DNA as its major site of action can also exert its biological activity solely by interaction with the cell surface.

Adriamycin cytotoxicity is enhanced by the influence of local anesthetic on a human cell line (Chlebowski et al., 1982), thus supporting the view that local anesthetics may influence cell membrane structure and function. It is known to modulate membrane activities such as concanavalin A interaction (Murphree et al., 1976), glycoprotein synthesis (Kessel, 1979), phospholipid arrangement (Duarte-Karim et al., 1976; Tritton et al., 1978), membrane fluidity (Murphree et al., 1981), small molecule and ion transport (Solie and Yuncker, 1978; Dasdia et al., 1979), fusion properties (Murphree and Tritton, 1979), expression of hormone receptors (Zuckier et al., 1981), and cellular hypertrophy with development of axon-like processes (Raz, 1982). Certainly, the biological effect is complex.

Another pertinent drug in our study which has gained a great deal of recognition for its role as an anticancer agent is actinomycin D (AMD). This is an antibiotic with potent antitumor activity currently employed in the

treatment of metastatic melanoma, Wilms' tumor and gestational trophoblastic tumors. Actinomycin D is a transcription inhibitor (Jordan and McGovern, 1981) that has been shown to markedly affect the protein producing organelles and the nuclear components of neural crest cells (Kramer, 1980).

The increase in membrane fluidity after brief exposure to adriamycin combined with the devastating nuclear reaction subsequent to actinomycin D treatment prompted our study of the effects of these integral anticancer agents on human melanoma cells derived from a patient's metastatic tumor. Morphological alterations in drug affected cells and colonies grown in soft agar were examined topographically with scanning electron microscopy (SEM) and intracellularly with transmission electron microscopy (TEM). Pertinent information concerning the different modes of action of these two drugs is discussed.

Materials and Methods

Human Melanoma Clonogenic Cell Assay

A single cell suspension was obtained from a biopsy of a subcutaneous nodule from a patient with metastatic melanoma in accord with a protocol approved by the University of Arizona Committee on Human Subjects. The nodule was cut into one mm^2 pieces, placed in a 50 ml conical tube, and inverted several times. Tumor pieces were allowed to settle at unit gravity, and the supernatant containing the single cells was aspirated. The cells were then cultured in 30x10 mm dishes (Falcon) with a 1.0 ml underlayer containing 0.5% agar (Bacto), Hams F-10 medium, 10% heat inactivated fetal calf serum, penicillin (100 µg/ml), and streptomycin (100 units/µl). The plating layer contained the same media components in 0.3% agar with freshly added animal derived insulin (1.54 units/µl), glutamine (0.45 µg/ml), pyruvate (0.34 µg/ml) and mercaptoethanol (0.77 mM). One-half million cells were plated per dish. The dishes were incubated in a 5% CO_2, 95% air atmosphere at 37°C with constant humidity. After 14 days multiple colonies were plucked from the agar, placed in a conical test tube containing Hams F-10 medium, and mechanically dissociated. Cells were plated on plastic dishes with Hams F-10 media and 10% fetal calf serum and constantly replated upon confluency in order to develop a human cell line.

Drug Assay

For adriamycin treatment, passage 4 cells were seeded on 22 mm^2 coverslips in 35 mm dishes and grown for 3 days. The media were then removed from the growing cells (logarithmic phase) and replaced with fresh Hams F-10 media (4 dishes) or with adriamycin at 0.01, 0.1, 1.0, and 10.0 µg/ml concentrations in fresh Hams F-10 media (4 dishes each concentration), and incubated at 37°C. One hour later the media were removed and the cells were washed with fresh Hams F-10. Two dishes from each of the 5 groups were then immediately processed for SEM. The remaining 10 dishes were incubated an additional 24 hours in fresh Hams F-10 and then processed for SEM. The cytotoxicity and purity of our adriamycin was verified when checked against recent stem cell assay data and high pressure liquid chromatography respectively.

For actinomycin D treatment, passage 4 cells were similarly seeded in 35mm dishes on 22 mm^2 coverslips and grown for 3 days. The media were removed and replaced with Dulbecco's modified Eagle's media (DME) or with actinomycin D at 0.1, 1.0, and 10.0 µg/ml concentrations in DME. Twenty-four hours later the media were removed and the cells immediately processed for SEM.

Scanning Electron Microscopy

Melanoma cells were fixed by aspirating the media and then gently layering on several milliliters of modified Karnovsky fixative (Karnovsky, 1965) (1% paraformaldehyde; 1.25%

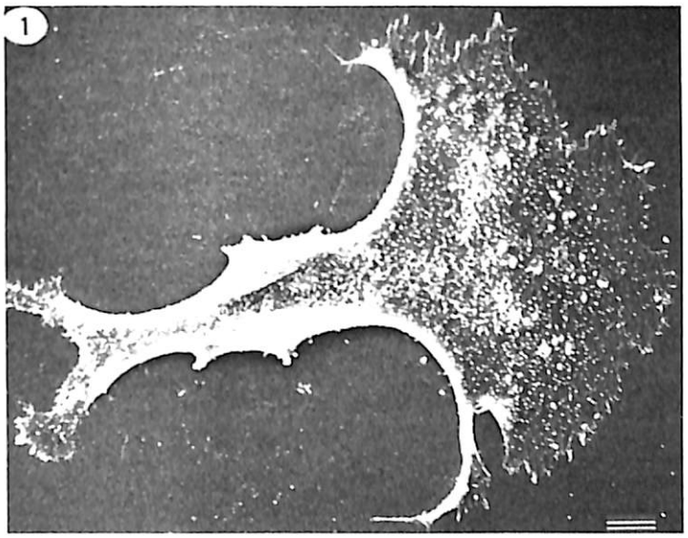
Figures 1-5 are of control and actinomycin D treated cells.

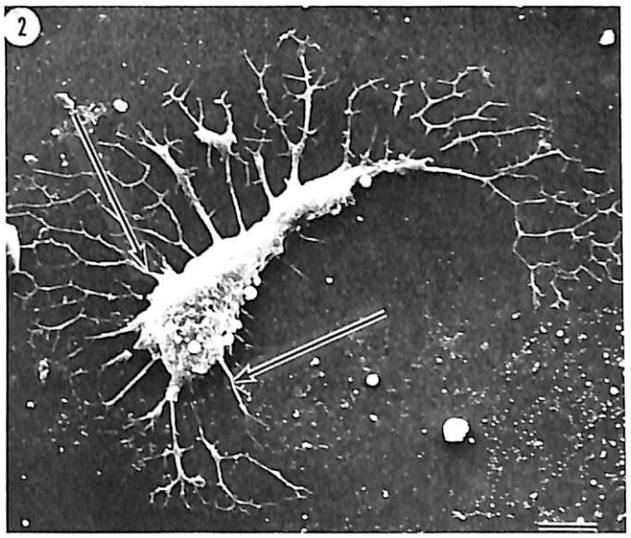
Fig. 1. Control melanoma cell which was incubated with fresh media for one hour and then fixed. The cell surface is relatively flat and covered with microvilli. An expansive pseudopodia extends from the bipolar cell body. Bar = 10 µm.

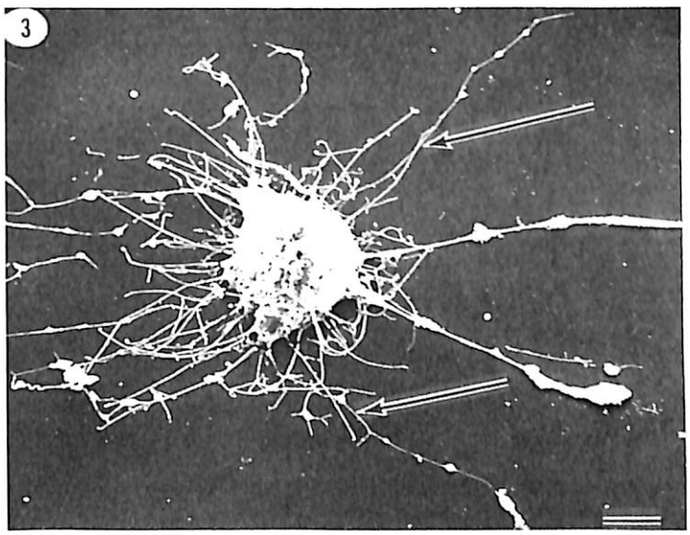
Figs. 2-4. Melanoma cells which were incubated with 0.1 (Fig. 2), 1.0 (Fig. 3), and 10.0 (Fig. 4) µg/ml actinomycin D for 24 hours and then fixed. Fig. 2. Long dendritic-like processes (arrows) are associated with the rounding cell body. Bar = 10 µm. Fig. 3. Fine, extensive cytoskeletal filaments (arrows) are usually noted on 1.0 µg/ml actinomycin D-treated cells. Note that the cell body is round in shape. Bar = 10 µm. Fig. 4. Filamentous structures are observed surrounding the rounded cell body. The filamentous-like debris (arrows) are cellular artifacts probably due to cell shrinkage in the dehydration process for scanning electron microscopy. Bar = 10 µm.

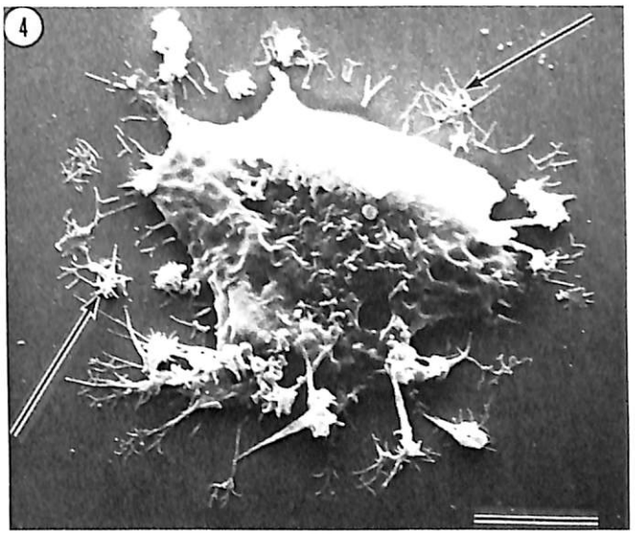
Fig. 5a. Transmission electron micrograph of a melanoma colony cultured in soft agar, incubated with 1.0 µg/ml actinomycin D for 24 hours and then immediately fixed. The cells are severely affected. The nuclear envelope of the nucleus (N) is discontinuous and often unrecognizable. The cytoplasm contains numerous vesicles, some of which are more electron dense than others (asterisks). Bar = 1 µm.

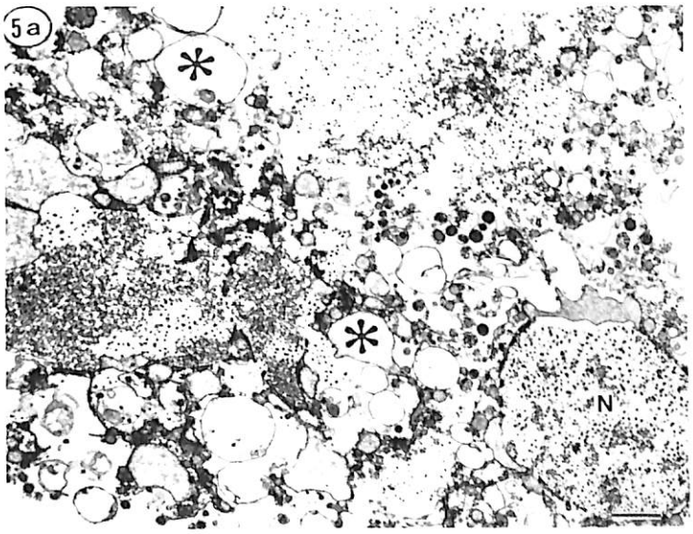
Fig. 5b. Transmission electron micrograph of a control melanoma colony cultured in soft agar. This cell contains a nucleus with a prominent nucleolus (N), mitochondria (M), secondary lysosomes (L), and rough endoplasmic reticulum. The nuclear envelope is continuous. Bar = 10 µm.

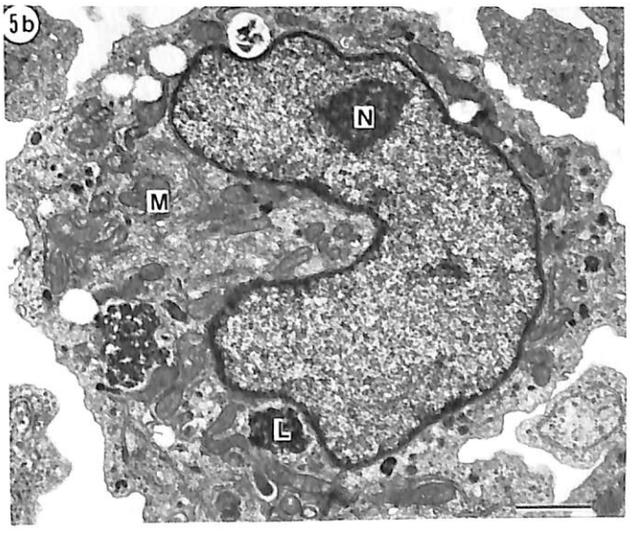












See page 984 for captions of Figs. 1-5.

glutaraldehyde; pH 7.4). The dishes remained at room temperature for 60-90 minutes. The coverslips were then rinsed twice in 0.2M cacodylate buffer and post-fixed in 2% OsO_4 buffered in 0.1M cacodylate buffer at pH 7.4 for 1 hour on ice in the dark. Subsequently, the coverslips were washed 3 times in 0.1M cacodylate buffer, dehydrated in ascending concentrations of ethanol, critically point dried with CO_2 in a Polaron E4850 unit or in a Tousimis samdri-790, coated with gold-palladium on a sputter coater (Polaron E5100), and observed in an ETEC Autoscan scanning electron microscope using 20 kV accelerating voltage.

Transmission Electron Microscopy

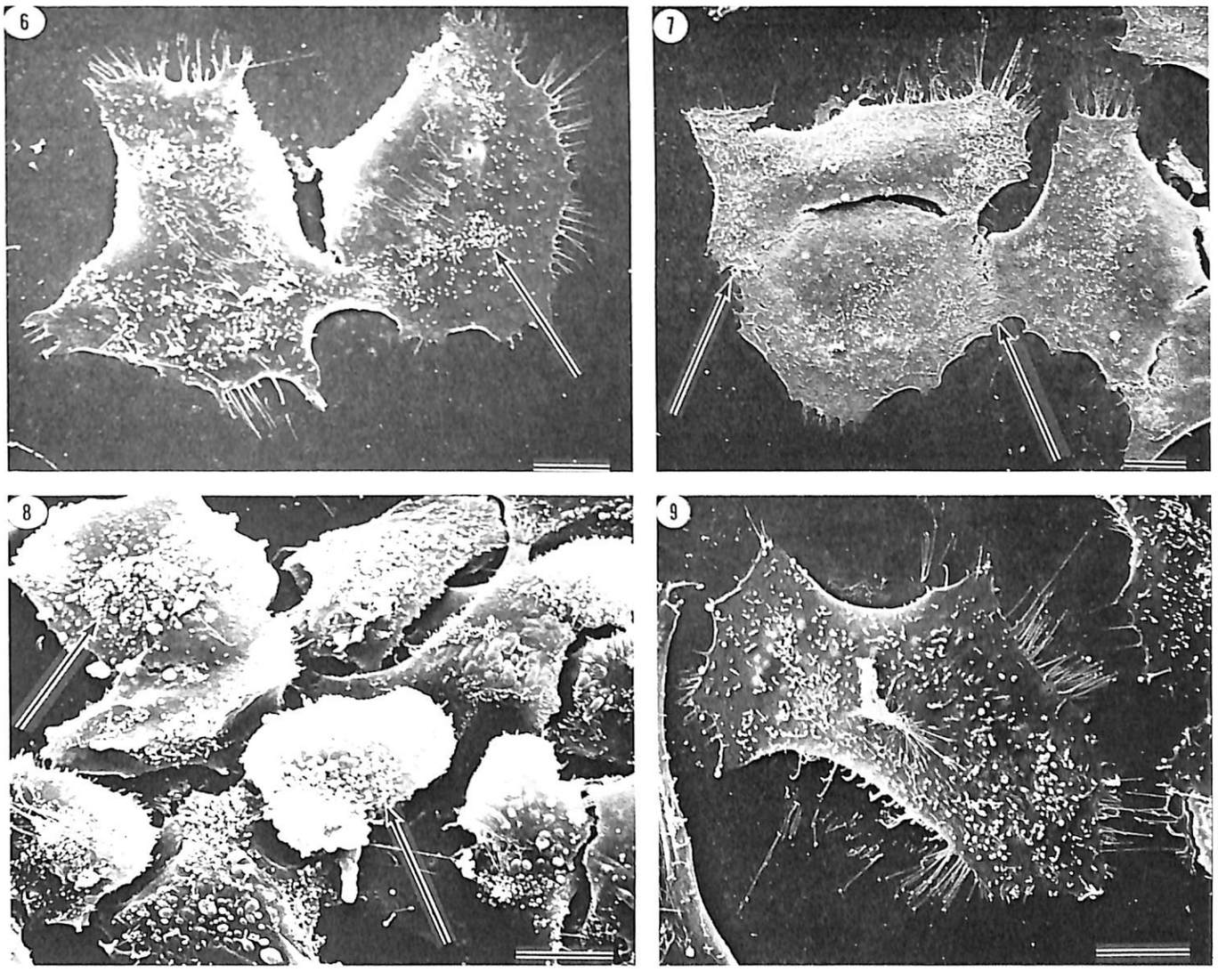
Melanoma cells cultured in agar for 12 days and 21 days were treated with 1.0 µg/ml adriamycin and 1.0 µg/ml actinomycin D respectively, and incubated for 24 hours. Agar plates were then fixed with modified Karnovsky fixative at room temperature, rinsed in 0.2M cacodylate buffer and post fixed in 2% OsO_4 buffered in 1.0M cacodylate buffer, at pH 7.4 for 70 min. on ice in the dark, and washed in 1.0M cacodylate buffer. Most of the agar plates were stained en bloc with uranyl acetate for 1 hour. Agar plates were dehydrated in ethanol, followed by 100% propylene oxide, and placed overnight in a 50:50 mixture of propylene oxide/Spurr followed by 100% Spurr for 24 hours, and finally embedded in fresh Spurr. Thin sections were mounted on uncoated 200 mesh copper grids, stained with uranyl acetate and lead citrate, and examined in a Philips 300 transmission electron microscope using 60 kV accelerating voltage.

Results

A human melanoma cell line grown on cover slips and in soft agar responded morphologically, as observed with scanning and transmission electron microscopy, to exposure with the anticancer drugs actinomycin D and adriamycin.

Control cells cultured on cover slips for the actinomycin D drug perturbation investigations are mostly flat, stretched out cells, some of which are fibroblastic in appearance in that they have a spindle-shaped bipolar appearance (Fig. 1). These untreated cells are generally covered with microvilli and have good cell-to-substratum adhesion as demonstrated by the anchorage sites associated with the well developed pseudopodia. Cell-to-cell contacts are quite prominent. In addition to the flat and bipolar cells, some cells (less than 5%) appear round. Cells incubated with actinomycin D for 24 hours at three concentrations (0.1 µg/ml, 1.0 µg/ml and 10 µg/ml), and then immediately fixed, demonstrate altered cell morphology (Figs. 2-4). Although cell morphology is heterogeneous, especially when observed by SEM, characteristic patterns are noted. At the lowest concentration, the majority of cell cytoplasm appears to have contracted leaving dendritic-like processes associated with the cell body (Fig. 2). With 1 µg/ml concentration, cells become more rounded and display more extensive and finer cytoskeletal filaments (Fig. 3). At 10 µg/ml concentration, less filamentous structures are observed in general. However, a few foci of fine filamentous structures are seen surrounding rounded cell bodies. An accumulation of filament-like debris is left in the vicinity of treated cells (Fig. 4). Transmission electron microscopy of colonies cultured in soft agar and treated with 1 µg/ml actinomycin D for 24 hours reveals severely deformed cells (Fig. 5a). The nuclear envelope is often discontinuous resulting in nuclear matrix-like material being located in the cell cytoplasm adjacent to the nucleus. Additionally, the cell cytoplasm is replete with vesicles of various diameters. In sharp contrast, transmission electron microscopy of control colonies cultured in soft agar depict normal appearing cells (Fig. 5b). Cells typically contain a nucleus with a prominent nucleolus, premelanosomes, mitochondria, secondary lysosomes, and other organelles. The nuclear envelope is continuous and the cytoplasm has very few vesicles.

Control cells cultured on cover slips for adriamycin drug perturbation studies are typically flattened with extensive spreading of cytoplasmic veils and possess numerous microvillous processes protruding from the cell surface (Fig. 6). Keeping in mind the variations (heterogeneity) of cell morphology, these control cells are analogous to the control cells for actinomycin D (Fig. 1). Cells incubated with adriamycin for 1 hour at four concentrations (0.01 µg/ml, 0.1 µg/ml, 1.0 µg/ml and 10 µg/ml), and then immediately fixed, demonstrated altered cell morphology only at 0.1 µg/ml concentrations and higher. At 0.01 µg/ml concentration, the cells are very similar to control cells. There are no noticeable morphological differences (Fig. 7). However, at 0.1 µg/ml concentration, two basic morphological variants are common (Figs. 8 and 9). Cells display various degrees of cell rounding (contraction of cytoplasm) with a notable pattern of blebbing, especially over the nuclear region (Fig. 8). These surface vesicular structures measure approximately 0.2-1.5µm in diameter and project very prominently from the cell surface. Extensive blebbing over the entire surface is noted in completely rounded cells (Fig. 8). Additionally, long microtubulous projections are seen on some cell surfaces (Fig. 8). Not all cells demonstrate the aforementioned drug affected characteristics, however. As shown in Figure 9, various cells from the 0.1 µg/ml concentration of adriamycin appear similar to the control cells. With 1 µg/ml adriamycin, the cells characteristically have blebs and long microvillous projections on the cell surface and generally are rounder than the control cells (Figs. 10 and 11). The microtubulous projections, though not restricted to the nuclear region, are often observed over the nucleus and have a parallel arrangement (Fig.



Figures 6-9 are of control and adriamycin treated cells.

Fig. 6. Control melanoma cells which were incubated in fresh media for one hour and then fixed. The cells are flat with extensive peripheral cytoplasmic spreading. Microvilli (arrow) protrude from the cell surfaces. Bar = 10 μm.

Fig. 7. Human melanoma cells which were incubated with 0.01 μg/ml adriamycin for one hour and then immediately fixed. Cell-to-cell contacts (arrows) are prominent. Cell morphology appears similar to that of the control cells. Therefore surface features observed by SEM cannot be used to distinguish control melanoma cells from melanoma cells which were incubated with 0.01 μg/ml adriamycin for one hour. Bar = 10 μm.

Figs. 8-9. Scanning electron micrographs of human melanoma cells which were incubated with 0.1 μg/ml adriamycin for one hour and then fixed. In Fig. 8 cells display various stages of cell rounding with widespread blebbing (arrows) of the cell surface. The blebbing is particularly noticeable at the nuclear region where the blebs appear to be larger in size. The blebs range in size from 0.2 to 1.5 μm in diameter. Microvilli are observed primarily at the periphery of the cell surface. Bar = 10 μm. In Fig. 9, cells are flat with microvilli covering much of the cell surface. Long filamentous processes emerge from the peripheral margins of the cell and extend to the substrate. Bar = 10 μm.

11). At 10 µg/ml concentration, some cells are seen in close contact with each other and appear as a sheet, well adherent to the substratum (Fig. 12). Thus, at this highest dosage, certain cells appear similar to the control cells. However, other cells (Fig. 13) display similar morphology to the altered cells shown in Figures 10 and 11. These 10 µg/ml adriamycin-affected cells commonly have cell surface blebbing, predominantly over the nuclear region, and long microtubulous projections (Fig. 13). Transmission electron microscopy of colonies cultured in soft agar and treated with 1.0 µg/ml adriamycin for 24 hours reveals abnormal cells (Figs. 14 and 15). Cells appear to be lysing as the plasmalemma is discontinuous (Fig. 14). Cytoplasmic organelles appear disrupted, and the cytoplasm is full of vesicles (Figs. 14 and 15). The nucleus appears normal with a continuous nuclear membrane and heterochromatin (Fig. 15).

Cells incubated with adriamycin for 1 hour at four concentrations (0.01 µg/ml, 0.1 µg/ml, 1.0 µg/ml and 10 µg/ml), and then cultured in drug-free media for 24 hours prior to fixation, appear similar to their respective controls. Control cells have similar appearing morphology to all control cells (Fig. 16). Although Fig. 16 depicts bipolar cells with an abundance of microvillous projections, most cells are flat with various amounts of microvilli. The various concentrations of adriamycin at 0.01 µg/ml (Fig. 17), 0.1 µg/ml (Fig. 18), 1.0 µg/ml (Fig. 19), and 10 µg/ml (Fig. 20) produced similar morphology. The heterogeneity of flat smooth cells with few microvilli and the large bipolar cells covered with microvilli are discernible in Figure 19. Most often, cells are extremely flat such that intracellular organelles appear to protrude from inside the plasmalemma, especially around the cell periphery (Fig. 19). These 1-1.5µm diameter vesicular-like structures, which appear in the vicinity of the cell margin in many control and drug-treated cells, are not nearly as prominent as the smaller vesicular structures (blebs) observed in the nuclear region (see Figure 8). Varying degrees of cell elongation, cell-to-cell contact and cell-to-substratum contact, synonymous with healthy viable cells, are noted at all these experimental dosages (Figs. 17-20).

In summary, concentrations of 1.0 µg/ml of actinomycin D or adriamycin on colonies cultured in soft agar demonstrate morphological effects observed by TEM. All concentrations of actinomycin D demonstrate morphological alterations by SEM. Concentrations of 0.1 µg/ml and higher of adriamycin cause abnormal morphology, which is observed by SEM only when the cells are fixed immediately after drug perturbation. Cell morphology appears normal by SEM for all concentrations of adriamycin when the cells are allowed to recover in adriamycin-free media for 24 hours before fixation.

Discussion

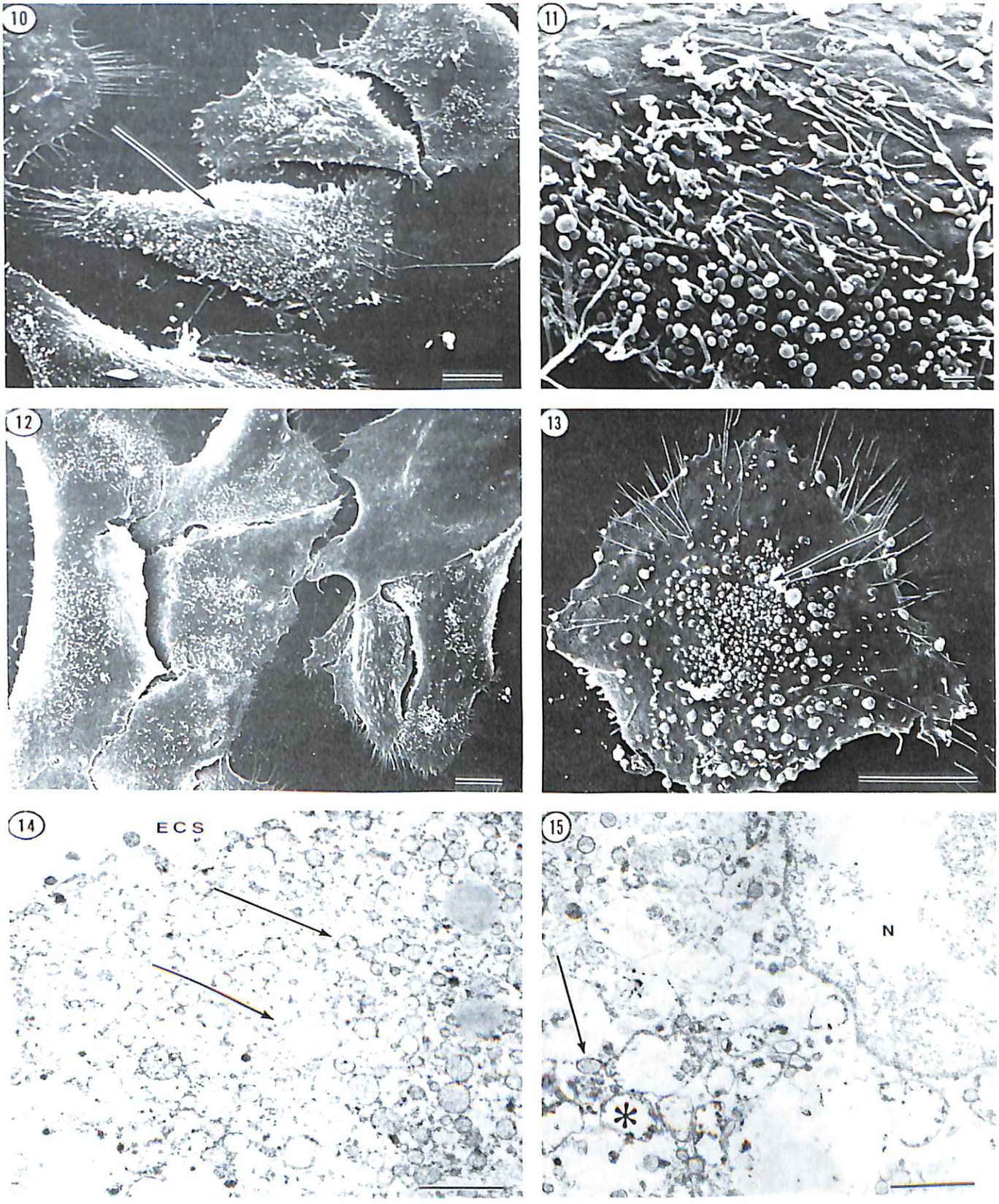
A tumorigenic human cell line derived from a patient with malignant melanoma was carefully chosen to study the morphological effects of adriamycin and actinomycin D. The aim of our research was to contribute to the basic understanding of this particular tumor presently under detailed investigation at the University of Arizona Cancer Center. Current *in vitro* chemosensitivity testing with the soft agar assay at the University of Arizona Cancer Center suggests that cytotoxicity for malignant melanoma, in general, is obtained in the range of 0.01 to 0.30 µg/ml for adriamycin and the range of 0.0001 to 0.1 µg/ml for actinomycin D. Previous experience with the assay shows these ranges to be pharmacologically achievable *in vivo* and correlated to clinical responses. Therefore the morphological surface and internal alterations observed in this study should be related or associated with cytotoxicity and hence facilitate interpretation of the results. Previous work with the actinomycin D analogue 87222 using dosages and exposure times comparable to this study resulted in a 55% - 59%

Figures 10-15 are of adriamycin treated cells.

Figs. 10-11. Cells incubated with 1.0 µg/ml adriamycin for one hour and then fixed. At this concentration, most cells are generally in the process of rounding, between being very flat and round. (Fig. 10, Bar = 10 µm). The long microvilli (arrow) and blebs that cover the cell surface in Fig. 10 are seen at higher magnification in Fig. 11. Bar = 1 µm.

Figs. 12-13. Melanoma cells incubated at 10.0 µg/ml adriamycin for one hour and then fixed. In Fig. 12, the cells appear flat and in close apposition to one another. Microvilli cover the cell surface. These cells are similar in appearance to control cells. Bar = 10 µm. Fig. 13, however, depicts a cell with prominent blebbing over the nuclear region (arrow). In these types of cells, long microtubulous projections extend from the peripheral cell surface to the adjacent substrate. Bar = 10 µm.

Figs. 14-15. Transmission electron micrographs of a melanoma colony cultured in soft agar and incubated with a 1.0 µg/ml adriamycin for 24 hours before fixation. In Fig. 14, the extracellular space (ECS) and the cell cytoplasm are separated by a disrupted plasmalemma. The cell cytoplasm is crowded with vesicles (arrows). Bar = 1 µm. In Fig. 15, the nucleus (N) contains both euchromatin and heterochromatin. The nuclear envelope appears continuous. Vesicles in the cytoplasm are translucent (asterisk) to electron dense (arrow) in nature. Bar = 1 µm.



See page 988 for captions of Figs. 10-15.

kill of the patient's malignant cells. One hour exposure to actinomycin D at 0.0005, 0.001, and 0.005 μg/ml resulted in 25%, 27% and 41% kill respectively. Continuous exposure to the drug at 0.005, 0.001, and 0.002 μg/ml yielded 27%, 54%, and 60% kill. In vitro chemosensitivity testing for adriamycin has not yet been done.

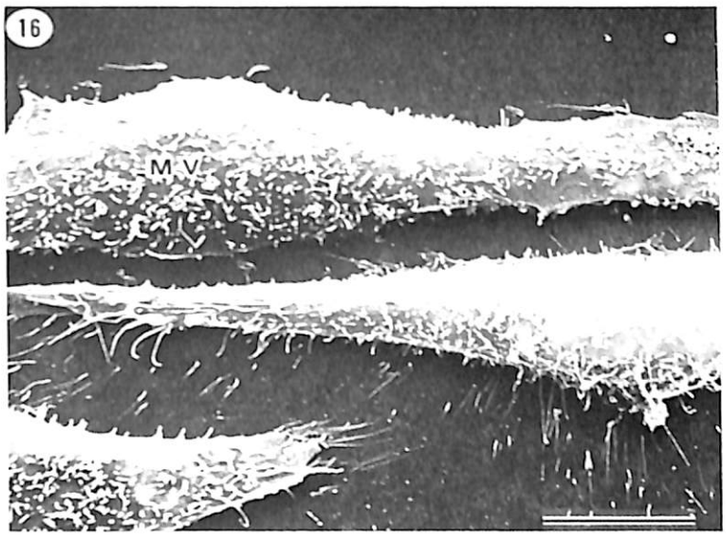
In addition to the drug-dosage response information received from this investigation, important morphological data rendered from this study permitted comparison with previous work dealing with the immunohistochemical and morphological characteristics of the human melanoma stem cell assay (Persky et al., 1982). The cytotoxicity of the drugs used in this study as well as the subsequent ultrastructural changes produced with specific concentrations and drug incubations are discussed below.

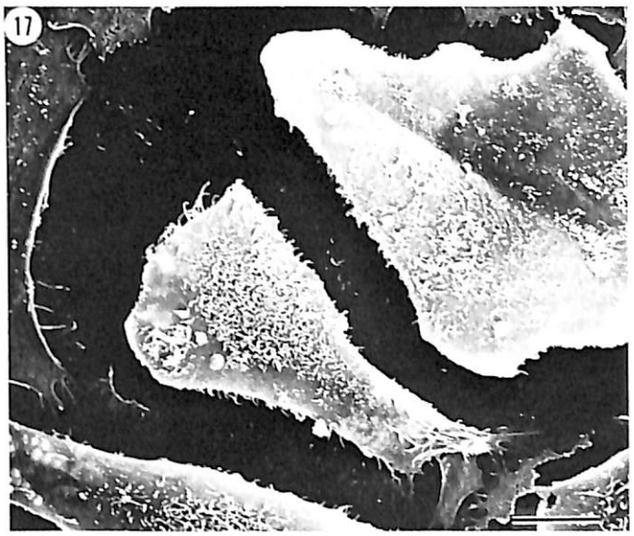
Previous reports indicate multiple sites of action of adriamycin and various mechanisms of this drug's action (Bhuyan et al., 1981; Myers, 1982; Neidle, 1979). It is not our intention to report on the many complex modes of adriamycin drug dynamics, but rather on morphological effects induced by adriamycin. Currently, there is a paucity of literature on this topic. It is worth noting, however, that biological effects are complex and that at different concentrations adriamycin can affect many DNA functions.

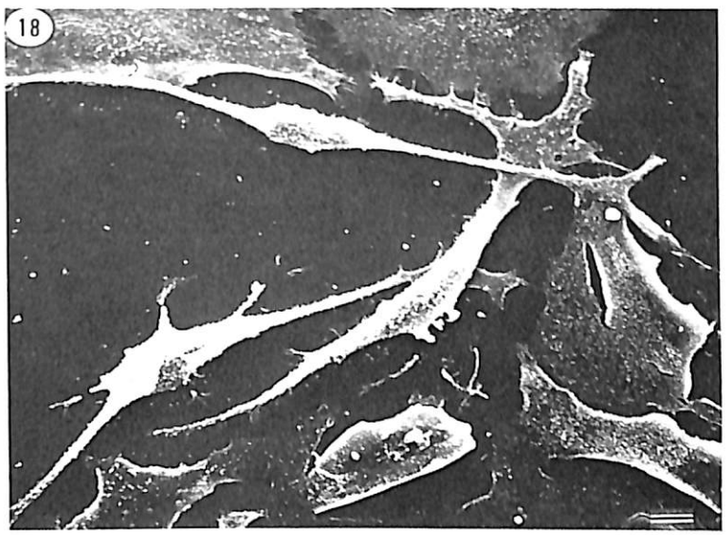
Adriamycin has been demonstrated to affect various components of the cell membrane. Treatment regimes consisting of low but cytotoxic levels of adriamycin show an increase in the rate of agglutination of sarcoma 180 cells by the lectin concanavalin A (Murphree et al., 1976; Murphree et al., 1981). These agglutination effects on the cell surface after 2-3 hours exposure to adriamycin suggest that the changes in the ceil surface may be partially involved in the antineoplastic action of the drug. Daunorubicin, an agent pharmacologically similar to adriamycin, has been shown to alter erythrocyte membranes (Schioppocassi and Schwartz, 1977). From their investigation, Schioppocassi and Schwartz suggested that the membrane effects induced by daunorubicin may result in protection or increased sensitivity of red blood cells to lysis by hypotonic salt solution. Changes in two cell surface properties, namely increased electronegativity and increased hydrophilic membrane glycoproteins, have been demonstrated after 30 minutes exposure to adriamycin (Kessel, 1979). In addition, exposure of B16 cell monolayers to adriamycin at noncytotoxic concentrations for 16 hours results in irreversible inhibition of proliferation of tumor cells and of their tumorigenic properties (Raz, 1982). Cellular and nuclear hypertrophy and the development of axon-like processes reportedly accompanied this cessation of cell proliferation. Our study confirms nuclear hypertrophy and the development of long cellular processes following treatment of human melanoma cells with adriamycin. However, the processes are more dendritic-like than axon-like, and generally appear concomitantly with cell rounding. These dendritic-like structures have been termed filopodia or microextensions (Porter et al., 1973). Our interpretation is that they occur when a cell undergoes "cell rounding" as in the M phase of the cell cycle rather than during the process of cell hypertrophy. Our ultrastructural observations agree in theory with recent findings that adriamycin increases cell membrane fluidity (Murphree and Tritton, 1979), and that it may exert at least part of its effect by interference with normal cellular growth controls through cell surface interactions (Zuckier et al., 1981). Additionally, when cells in G_1 are given lethal doses, but not excessive concentrations of adriamycin, they will proceed through S and then cease and die during G_2. Cells given increased doses of adriamycin will be blocked in S (Ritch et al., 1981). Our investigation notes the abundance of rounding cells which is known to occur in late G_2 and M phases of the cell cycle. Although there have been several detailed reports of changes in surface morphology and cell shape associated with the cell cycle, there is no definitive consensus concerning such surface morphology. Nonetheless, the majority of cells viewed after treatment with adriamycin appeared to be in G_1 and G_2. The visualization of flattened cells with many blebs, especially associated with the nuclear region, and some microvilli and ruffles have been determined to belong mainly to G_1, while rounding cells with increased microvilli and few ruffles and blebs are usually associated with G_2 (Porter et al., 1973). Thus, there seems to be the possibility that the majority of cells seen after adriamycin treatment may be correlated by SEM morphology to the observations of Ritch et al. (1981), which showed that adriamycin-treated cells stop in S and G_2 phases of the cell cycle.

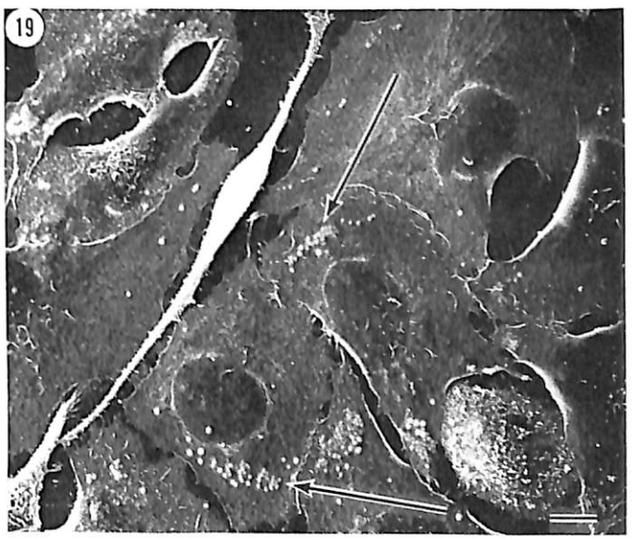
Human melanoma tumor cells exposed to adriamycin for one hour and then allowed to recover in adriamycin-free media for 24 hours prior to fixation are void of obvious (topographical) morphological variations. Also, they are observed in all phases of the cell cycle. A similar recovery phenomenon is noted in adriamycin-treated Hela cells in that the cells return from an increased number of epidermal growth factor binding sites to their normal status quo following a recovery period in drug-free media (Zuckier et al., 1981).

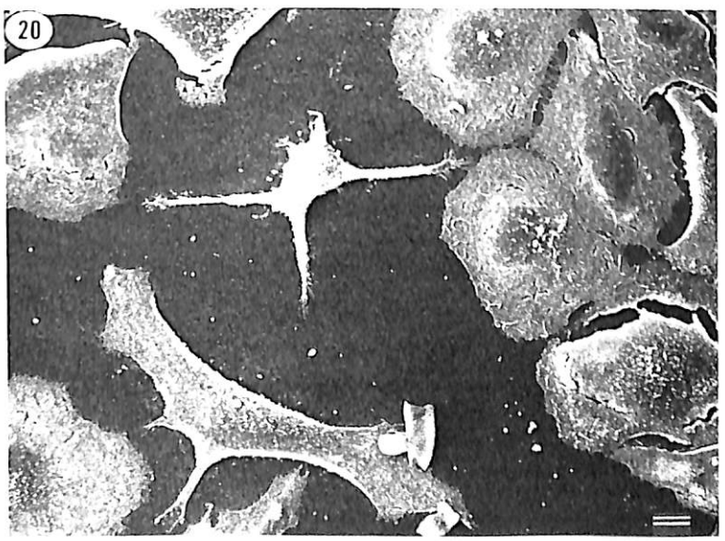
The appearance of a vacuolated cytoplasm after adriamycin or actinomycin D treatment of 1.0 μg/ml is similar to the cellular destruction seen with other cytotoxic drugs, such as hydrocortisone (Tew, 1982). Since anthracycline antibiotics like adriamycin, as well as nitrosoureas, nitrogen mustards, vinca alkaloids, and steroid hormones preferentially select macromolecular nucleophiles as their target, they will interact strongly with the nuclear membrane, the nuclear matrix and chromatin. It would be worthwhile to pursue, in greater detail, drug interactions with the nuclear membrane and matrix since such interactions may prove to be important mediators of drug-induced cytotoxicity, and thus have an important impact on the target dynamics of these integral anticancer drugs.











Figures 16-20 are of control and adriamycin treated colonies.

Fig. 16. Control melanoma cells which were incubated with fresh media for 24 hours prior to fixation. Cells are bipolar and covered with microvilli (MV). Cell processes extend from the cell surface to the substrate. Bar = 10 μm.

Figs. 17-20. Scanning electron micrographs of melanoma cells which were incubated with 0.01 (Fig. 17), 0.1 (Fig. 18), 1.0 (Fig. 19) and 10.0 (Fig. 20) μg/ml adriamycin for one hour and then incubated for an additional 24 hours in drug-free media before fixation. The heterogeneity of cell shapes is observed. Vesicular-like structures, 1-1.5 μm in diameter, appear in the cell margin of many cells (arrows, Fig. 19). These adriamycin-treated cells are similar in appearance to control cells. Bar = 10 μm in Figs. 17-20.

Current reports indicate actinomycin D to be a DNA-dependent RNA synthesis inhibitor. Changes to both the cell cytoplasm and nucleus have been described with most morphological alterations occurring in the nucleolus (Kramer, 1980; Nordback, 1981; Jordan and McGovern, 1981). Exposure to 10 µg/ml actinomycin D for 4.5 hours has caused intracellular alterations, such as decreases in rough endoplasmic reticulum, distended Golgi, increased number of membrane-bound vesicles, decreased melanosome production, and nucleolar segregation of fibrillar and granular areas (Kramer, 1980). Our observations of a 10 µg/ml actinomycin D dosage primarily noted: (1) large increases in membrane-bound vesicles in the cytoplasm, and (2) disintegration of the nucleus, including the nucleolus. Our 1.0 µg/ml dose for 24 hours had greater effect than a 2.0 µg/ml treatment for 4.5 hours seen by Kramer (1980), in which she reported clumping of the outer granular area of the nucleoli and an overall decrease in size of nuclear granular material. Because of the complete obliteration of nuclear material in our TEM study of actinomycin D-treated tumor cells, we are unable to adequately compare our results with those of Kramer (1980), who observed the disappearance of nucleolar granular material, and Jordan and McGovern (1981), who reported the absence of the fibrillar component of the nucleolus. The reduction in the number of ribosomes and rough endoplasmic reticulum by actinomycin D (reported in detail by Kramer, 1980 and Nordback, 1981) was greatly overshadowed in our study by the tremendous accumulation of vesicles.

Our observations of vesiculation in and around the Golgi complex and the increased accumulation of vacuoles after 24 hour exposure to actinomycin D treatment is in agreement with previous work on chick oviduct epithelial cells by Nordback (1981). In addition, Nordback demonstrated that a single intraperitoneal injection of actinomycin D caused invagination and swelling of the nucleus and dispersion of the heterochromatin, swelling of the Golgi complex, mitochondria and perinuclear cleft, in addition to the formation of vacuoles and smaller vesicles. Enlargement of the nucleus seen 2-7 hours after actinomycin D treatment and the concomitant increase in nuclear pores, polysomes and rough endoplasmic reticulum as reported by Nordback (1981) are likely signs of cell recovery, but were not observed in our investigation, as recovery was not permitted.

The primary morphological change associated with the majority of actinomycin D-treated cells is cell rounding, which has been discussed as being intimately associated with cell death by Pexieder (1975). The cell rounding seen in this study is assumed to correlate with cell death due to the abundance of nonviable cells as determined with trypan blue exclusion and a coulter counter in similar experiments. The long dendritic-like processes are probably cytoskeleton components. However, their true identity cannot be made until additional observations, especially utilizing TEM, are made on these and similar cells.

Future aims toward observing and understanding tumor cell dynamics associated with drug perturbation studies are worthwhile so as to gain an understanding of why many tumor cells become drug resistant. Our study reported a brief exposure of human melanoma tumor cells with either adriamycin or actinomycin D. Cells which were treated with actinomycin D expressed morphological alterations associated with cell death. Cells which were treated with adriamycin showed some morphological variations. In contrast, cells which were treated with adriamycin and then cultured in drug-free media for 24 hours before fixation demonstrated normal morphology. These observations suggest that it will be important to consider the cell surface as a visible target in the engineering of a new generation of anticancer agents.

Acknowledgements

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Discussion With Reviewers

T.R. Tritton: Since neither adriamycin or actinomycin D are considered to be important drugs in the treatment of malignant melanoma, why are these agents used in this study? Why not utilize either a different tumor or first line drugs used for melanoma? Is the tumor sensitive to either of these agents in an in vitro colony forming assay?
Authors: Actinomycin D is a standard drug used in chemotherapy for human malignant melanoma at the University of Arizona Cancer Center. Treatment of malignant melanoma with this drug at our University Hospital has now been expanded to developing an in vitro chemosensitivity test in soft agar. Patients who have received low doses of actinomycin D for long periods of time generally respond better than with other standard drugs such as DTIC, BCNU, melphalan, and vinblastine. Adriamycin is not a standard drug for human malignant melanoma. However, we are currently investigating adriamycin as a chemotherapeutic agent. This particular tumor line was chosen for investigation in order to augment other research studies already in progress. There are no adequate first line drugs for malignant melanoma at this time. The patient's biopsy cells tested in the soft agar assay are sensitive to actinomycin D. Inhibition to control ranges from 25% with 0.0005 µg/ml to 41% with 0.005 µg/ml. Adriamycin sensitivity was not evaluated for this patient or for the derived cell line.

T.R. Tritton: The adriamycin results (Figures 6-13) suggest a very heterogeneous response. This is somewhat surprising since the cells are clonal in origin. Would the authors care to speculate on the origin of these heterogeneous morphological changes elicited by adriamycin?
Authors: The cells are not clonal. They were obtained from multiple colonies which were grown in short term (early passage) tissue culture. The question of cell heterogeneity for this cell line has recently been demonstrated by sensitivity to retinoic acid and dexamethasone (Bregman, MD, Peters, EM, Sander, D, Meyskens, FL, Jr. (1983). Dexamethasone, prostaglandin A_1, and retinoic acid modulation of murine and human melanoma cells grown in soft agar. JNCI, in press). Bregman et al. showed that some cells are resistant to increasing concentrations of retinoic acid or dexamethasone by 15% and 40% respectively.

K.M. Tveit: In the SEM studies you incubated the melanoma cells with actinomycin D for 24

hours and with adriamycin for 1 hour. The rationale for using different incubation times for the two drugs in monolayer and the same incubation time (24 hours) for the drugs in agar is not apparent from the paper.
Authors: The cell line used in this investigation was known to have a doubling time of 51 hours. Actinomycin D is also known to be cell cycle specific for G_1 and S phases of the cell cycle. We therefore selected 24 hours for drug incubation to make sure that cells would be drug affected. Adriamycin is not cell cycle specific. Therefore a one hour incubation period was adequate for studying by SEM the monolayer, and a 24 hour incubation period was excellent to match for comparison the actinomycin D investigation. The investigation would be improved by having all drug exposure parameters the same.

K.M. Tveit: In all experiments you used Hams F-10 except when you treated the cells in monolayer with actinomycin D for the SEM studies. Why did you employ DMEM in this particular case? The change in type of medium alone could probably induce morphological alterations on the cell surface.
Authors: DMEM was employed for the actinomycin D SEM studies because total protein synthesis, especially collagen synthesis, was concurrently being evaluated for another investigation. DMEM is more conducive to measuring total protein synthesis than Hams F-10 medium. It is certainly true that different media can induce morphological alterations on the cell surface. To date, however, we have not noticed in SEM or TEM studies alterations in cell surface morphology between cells cultured in DMEM or Hams F-10 medium.

R.T. Chlebowski: Since vesicle formation is a relatively nonspecific morphological finding, do you have any information to suggest a primary relationship between vesicle membrane formation and cytoxic affects?
Authors: Many investigations have noted vesicle formation in cells after cytotoxic treatment. We, ourselves, have previously reported an increase in vesicles by cells of the choroid plexus after tertiary amine treatment (Persky, B, Friedenbach, DJ. Tertiary amine induced changes in the fine structure of the choroid plexus, published in: Scanning Electron. Microsc. 1979; III: 63-68). However, the demonstration of a primary relationship between vesicle formation and cytotoxic affects must involve dynamic investigative techniques. Light and electron microscopy are inherently limited to the static events at the time of fixation. The primary relationship for vesicle formation is thus suggested by this paper but is not proven.

K.M. Tveit: Did you employ other concentrations than 1 ug/ml in agar?
Authors: No. The concentration of the 1 ug/ml of adriamycin or actinomycin D in agar was selected to represent the upper limits of pharmacological achievable doses in the stem cell assay that could be correlated to patient responses.

K.M. Tveit: Why did you employ 12 day old colonies in the adriamycin study and 21 day old colonies in the actinomycin D study? Which size did the colonies have in these cases? Cells in the center of a large colony may be damaged by hypoxia and starvation and may exhibit morphological changes without the presence of a cytotoxic agent. In contrast, cells in the periphery probably take up more of the drug than cells in the center of the colony. Did you see any morphological heterogeneity in different cells in each control or treated colony? If so, which cells in the colonies do you report data on?
Authors: The selection of 12 and 21 day old colonies for adriamycin and actinomycin D treatment respectively is simply a matter of being able to visualize macroscopically several colonies (5 or more). Macroscopic recognition of a colony embedded in Spurr greatly facilitates TEM sectioning. Colonies varied in size with the largest colonies ranging from 600 μm to 800 μm in diameter. Hypoxia and starvation are unlikely factors for causing the morphological alterations because the colonies are small in diameter, especially since the colonies are more disc-like in shape than round. However, we have unpublished evidence that 22 day old colonies have distinct changes in cell-surface associated fibronectin, as determined by the peroxidase-antiperoxidase technique, for cells located at the periphery of the colony as compared to cells in the center of the colony. Morphological heterogeneity is noted for individual cells that comprise either control or drug-treated colonies. Heterogeneity is mainly recognized by the degree of vacuolization. Morphological heterogeneity for the cells at the center of the colony versus the cells at the periphery is not noted. The TEM micrographs illustrate those cells with the most severe vacuolization.

K.M. Tveit: Why did you perform the TEM studies on colonies in agar and not on cells in monolayer cultures as you did for SEM? It would probably be easier to compare the surface and the intracellular morphological changes if the cells were grown in the same culture system and treated with the same concentrations of drugs.
Authors: Our laboratory has focused for the last two years on melanoma cells cultured in soft agar. Because of the expertise in this technique and since severe morphological alterations are noted by TEM, SEM studies were initiated to augment the TEM studies. A SEM investigaton of cells cultured as a monolayer was the easiest way to acquire the SEM data. Comparison of monolayer cultures for both TEM and SEM would be ideal. Comparison studies in agar for both TEM and SEM would be possible, but difficult due to tissue preparation for SEM.

K.M. Tveit: How did you mechanically dissociate the colonies?
Authors: Colonies plucked from the agar are placed in a conical test tube containing Hams

F-10 medium. The test tube is agitated by roughly tapping the outside bottom of the tube with a pencil or finger. The test tube is inverted quickly several times and the tapping-inverting process continued until the colonies disappear from macroscopic view.

C.D. Bucana: Fig. 16 shows spindle shaped cells that are different from that seen in Fig. 6. What is the cycling time of the cell line before and after exposure to drugs?
Authors: The cell line has a doubling time of 51 hours before drug treatment. The cycling time of the melanoma cell line was not determined after exposure to drugs.

R.E. Moses: Could cell membranes play the primary role for adriamycin cytotoxicity?
Authors: The mechanism of adriamycin cytotoxicity is probably a complex phenomenon involving the plasmalemma, the nucleus, and other organelles. Changes in the cell membrane may represent a second mechanism of cytotoxicity in addition to an augmentive role for DNA interculation or the blockage of DNA synthesis.

C.D. Bucana: The filamentous structures described in actinomycin D-treated cells (Fig. 4) may be indicative of a cellular phenomenon called clasmatosis and not necessarily a reflection of the SEM preparative procedure.
Authors: Clasmatosis is the extension of pseudopodia-like processes in unicellular organisms and blood cells by plasmolysis rather than by a true pseudopodia formation. Since melanoma cells are not unicellular organisms or blood cells, the filamentous structures should not be due to clasmatosis. However, the morphological structures in Fig. 4 may certainly resemble structures caused by clasmotosis in other cells.

C.D. Bucana: In adriamycin-treated cells, what percent of cells show morphology similar to that shown in Fig. 9?
Authors: Approximately 20% of those cells incubated with 0.1 µg/ml adriamycin for one hour before fixation express morphology similar to that seen in Fig. 9. Most cells depict the morphology seen in Fig. 8.

C.D. Bucana: How is nuclear hypertrophy measured?
Authors: Nuclear hypertrophy was determined by comparing TEM micrographs of adriamycin-treated colonies to untreated colonies. More than 50 nuclei were evaluated from each group. Nuclear hypertrophy is expected since previous work using cytofluorescence demonstrated enlarged nuclei after adriamycin treatment (Raz, 1982).

K.M. Tveit: What is the relationship between the morphological changes seen after drug treatment at different concentrations and the inhibition of cellular proliferation in monolayer or inhibition of colony formation in agar after treatment with the same concentrations of drugs? Did you see morphological changes at drug concentrations too low to cause inhibition of proliferation?
Authors: The degree of inhibition of cellular proliferation in monolayer or of colony formation in agar after treatment was not calculated.

C.D. Bucana: Figures 6 and 10 appear to be similar and, in fact, the cells shown in Fig. 10 appear more bipolar than those shown in Fig. 6. Perhaps a definition of bipolarity would help (e.g. edge to edge measurement in one direction is twice as long as the measurement perpendicular to the first one).
Authors: The operational definition of bipolarity as stated above is excellent and certainly worthwhile to incorporate into experiments. An operational definition was not used in this investigation. Both scanning and transmission electron microscopy have potential analytical drawbacks if careful attention is not paid to many cells rather than a select few cells. Cells in Fig. 10 do appear more bipolar than those shown in Fig. 6. However, the overall statement that cells round up with increasing concentrations of adriamycin remains the key point to be acknowledged.

C.D. Bucana: The parallel arrangement of cytoplasmic projections shown in Fig. 11 are also discernible in Fig. 6.
Authors: This observation is correct. Cells incubated with 1.0 µg/ml adriamycin for one hour before fixation (Fig. 11) do have, however, more cytoplasmic projections than control cells (Fig. 6).

K.M. Tveit: Did you also study the effect of recovery on actinomycin D induced morphological changes?
Authors: No. This aspect was not investigated in the present study.

C.D. Bucana: What is the mitotic index of the melanoma cell line?
Authors: The mitotic index of the melanoma cell line was not determined in this experiment.