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Journal
The American journal of anatomy, 184(3)

ISSN
0002-9106

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Publication Date
1989-03-01

Peer reviewed

THE AMERICAN JOURNAL OF ANATOMY 184:212–224 (1989)

Immunohistochemical and Ultrastructural Study of Human Melanoma Colonies Grown in Soft Agar

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ABSTRACT **An immunohistochemical and ultrastructural study of human melanoma colonies grown in soft agar for up to 50 days was performed. Three morphological variants of developing tumor colonies are reported: 1) large light colonies, 2) small dark colonies, and 3) smooth-edged colonies. The large light colony variant is the most frequently observed in the soft agar assay (≈70%), followed by the dark colony variant (≈27%), and the smooth-edged colony variant (≈3%). Major morphological characteristics are associated with each variant, as shown with light microscopy (LM) and transmission electron microscopy (TEM). Both LM and TEM analyses demonstrated that the large light colony variant was hypomelanotic and contained a microfibrillar extracellular matrix (ECM). The small dark colony variant was found to be hypermelanotic and contained a less demonstrable ECM. The smooth-edged variant has an encapsulated periphery, no demonstrable ECM, and tightly packed cells with desmosome-like junctions. In order to characterize further the ECM in the most commonly observed variant, the large light colony, specific antibodies to fibronectin (FN) and collagen types IV and V (COLs IV and V) were applied and observed with immunofluorescence microscopy and immunoperoxidase. In paraffin sections of melanoma colonies, FN was observed associated with both the cell surface and the ECM. However, no specific staining was seen for COLs IV and V. In addition, ruthenium red was used to preserve and selectively bind to glycosaminoglycans (GAGs) and proteoglycans (PGs). TEM studies reveal GAG-like granules stained with ruthenium red in the fibrillar ECM and a dotted, punctate staining of the cell surface. Understanding the biological and architectural composition of developing melanoma tumor colonies in soft agar could contribute to the development of more efficient chemotherapeutic strategies.**

INTRODUCTION

The ability to predict the response of human tumors to various cytotoxic and noncytotoxic agents on the basis of in vitro tests in soft agar is currently being investigated by many laboratories. The soft agar assays of Hamburger and Salmon (1977) and of Courtney and Mills (1978) have been readily applied to study malignant melanomas, revealing chemotherapeutic sensitivity (Meyskens et al., 1981a; Salmon et al., 1978), karyology (Meyskens et al., 1981b; Trent and Salmon, 1980), self-renewal capacity (Buick and MacKillop, 1981; Thomson and Meyskens, 1982), and light and electron microscopic characteristics (Meyskens and Salmon, 1981; Meyskens et al., 1981b). To date, two distinct morphological variants of human melanoma colonies grown in the Hamburger and Salmon assay have been defined by light microscopy (Meyskens and Salmon, 1981) and analyzed by cell-kinetic studies (Meyskens et al., 1984a). These variants, called light and dark colonies (Meyskens, 1980), are worthy of more detailed studies.

In the process of colonization, multicellular growth units develop from stem cells. The critical events associated with the formation of miniature tumor colonies in an anchorage-independent environment involve cell-to-cell contact as well as cell-to-extracellular matrix (ECM) interactions. Much attention has been devoted to the analysis of the tumor cell microenvironment and its role in influencing cell behavior (Hay, 1981), specifically cell proliferation and cell shape (Gospodarowicz et al., 1978; Folkmann and Moscona, 1978); cell transformation (Liotta, 1986); and, most significantly, gene expression (Bissell et al., 1982; Spiegelman and Ginty, 1983). The importance of the ECM associated with transformed cells strongly suggests a complex interaction between the cell surface and its immediate environment. Specific ECM and cell-surface-associated macromolecules have been identified as potentially useful markers for metastatic transformation because they appear to be altered during this process. Among these are fibronectin, laminin, collagen types IV and V, and glycosaminoglycans or proteoglycans (for review, see Liotta et al., 1986).

Fibronectin (FN), a cell surface and ECM glycoprotein with a high molecular weight, has been shown to be involved in the adhesion of cells to each other and to a substratum (Wright et al., 1984; Terranova et al., 1984). The presence or absence of FN has even been used to distinguish carcinomas from sarcomas in vivo and in vitro (Stenman and Vaheri, 1981; Vlodavsky and Gospodarowicz, 1981). FN is found in a soluble form in the culture media of virally transformed rat kidney cells (Hayman et al., 1981), yet absent from the

Received June 9, 1988; accepted November 11, 1988.

surface of these same cells. Additionally, many transformed fibroblasts show a reduced level of cell-associated FN (Hogg, 1974; Hynes, 1973; Vaheri and Mosher, 1978). Much effort has been directed toward elucidating 1) whether cell-associated FN is reduced in tumor cells (Taylor-Papadimitriou et al., 1981) and neural-crest derivatives (Thiery and Duband, 1986), 2) whether FN can be used as a reliable marker for transformation (Chen et al., 1977; Marshall et al., 1977), 3) whether FN regulates cell attachment (Terranova et al., 1986), 4) whether there is a relationship between FN expression (cell-associated or cytoplasmic) and metastatic growth potential (Neri et al., 1981; Puricelli et al., 1985; Alessandri et al., 1986), 5) whether FN enhances tumor cell migration (McCarthy et al., 1985), and 6) whether a synthetic peptide from FN inhibits experimental metastasis (Humphries et al., 1986).

Successful metastasis of a tumor is dependent on several complex series of sequential events that involve both the tumor cell and host properties (Poste and Greig, 1986; Holmes et al., 1986). Two important events in the metastatic cascade are the ability of a cell to invade basement membranes in the process of dissemination and subsequently colonize near and distant host sites. Many metastatic tumors have shown a preferential digestion of basement membrane collagen, namely type IV collagen (Liotta et al., 1980, 1986). Since type V collagen has been found in fetal membranes and in vascular tissue, as well as in bone and cartilage, it too is undoubtedly affected during tumor metastasis (Liotta et al., 1986). It has been suggested that the specific digestion of ECM materials during the metastatic cascade of events promotes the absence of certain proteins from the tumor cell surface. The soft agar assay is a good system for testing the presence of important ECM proteins during the formation of metastatic tumor colonies in a three-dimensional microenvironment.

Our study of human melanoma colonies grown in soft agar details characterization of three morphological variants, the immunohistochemical profile of FN and collagens IV and V, and transmission electron microscopic evaluation of the cellular and extracellular components. This study of human melanoma colony-forming cells will allow us to understand more fully the cellular and ECM composition of developing tumors during the metastatic growth phase in an anchorage-independent environment.

MATERIALS AND METHODS

Human Melanoma Clonogenic Cell Assay

Our culture conditions have been described extensively (Meyskens et al., 1981b; Sipes et al., 1986). Briefly, single-cell suspensions were obtained from biopsies of subcutaneous nodules from patients with metastatic melanoma in accord with a protocol approved by the University of Arizona Committee on Human Subjects. Cells were cultured in 30 × 10 mm dishes (Falcon) with a 1.0 ml underlayer containing 0.5% agar (Bacto), Hams F-10 medium, 10% heat-inactivated fetal bovine serum (Gibco), penicillin (100 units/ml), and streptomycin (100 μg/μl). The plating layer was the same medium in 0.3% agar with freshly added animal-derived insulin (1.54 units/ml), glutamine (0.45 μg/ml), pyruvate (0.34 μg/ml), and mercaptoethanol (0.77 mM). Cells were plated at a concentration of 5×10^5 per dish and incubated for up to 50 days in a 5% CO_2: 95% air atomosphere at 37°C with constant high humidity in a specialized chamber.

Colony Selection

Colonies were selected with the aid of an inverted light microscope and identified by making a circle on the petri dish below the colony. A characteristic pattern of circles for each petri dish was made so that each specific colony could be followed from the macroscopic level to the ultrastructural level.

Light Microscopy (LM)

The agar plates were generally fixed for 4 hr or longer at room temperature (25°C) in 10% neutral buffered formalin. Histochemical stains requiring a special fixative, such as Bouin's solution for glycogen, were used when appropriate. Fixation was followed by routine paraffin embedding of the entire agar plate in an Autotechnicon (Model 2A). Approximately 400 serial sections (6–7 μm) of the entire agar bilayer were cut in a cold room at 4°C, placed on cardboard trays, and stored at room temperature. Every tenth section was stained with hematoxylin and eosin (H&E) in order to locate colonies in the agar. Selected sections were stained with H&E, periodic acid-Schiff, Best's carmine, Masson's trichrome, and Snook's reticulum.

Sudan Black B staining of agar plates was performed on both unfixed and fixed frozen sections. Vital staining with acridine orange to identify the lysosomes of the melanoma cells within the agar was a modification of the method of Ohkuma and Poole (1978). Briefly, colonies were plucked from the agar, washed several times in phosphate-buffered saline (PBS), microcentrifuged, and incubated for 15 min in 1 μg/ml of acridine orange. Cells were microcentrifuged, washed two times in PBS, and examined with fluorescence microscopy.

Transmission Electron Microscopy (TEM)

Agar plates containing melanoma colonies were fixed for 90 min at room temperature by gentle immersion in modified Karnovsky fixative (1965), consisting of 1% paraformaldehyde and 1.25% glutaraldehyde, pH 7.4. The agar plates were rinsed in 0.2 M cacodylate buffer, postfixed for 1 hr in 2% OsO_4 buffered in 0.072 N cacodylate buffer at pH 7.4, washed in 0.144 N cacodylate buffer, dehydrated, immersed in 100% propylene oxide, and embedded overnight in a 50:50 mixture of propylene oxide/Spurr. Colonies in the agar plates previously selected before fixation were re-identified under the dissecting microscope, and small blocks of agar containing colonies were carefully dissected and placed in 100% Spurr for 24 hr. The small blocks were transferred to fresh 100% Spurr for final embedment. Sections were cut with glass knives on a Porter-Blum MT2 ultramicrotome. Thick sections were stained with toluidine blue. Thin sections were mounted on uncoated 200-mesh copper grids and stained with uranyl acetate and lead citrate. Grids were examined with a Philips 300 transmission electron microscope.

The ruthenium red technique was used to demonstrate the presence of glycosaminoglycans (GAGs) or proteoglycans (PGs) associated with the colonies and

followed a modification of the procedure of Luft (1971). Melanoma colonies and the surrounding agar microenvironment were carefully dissected from the culture plate and fixed (1% paraformaldehyde; 5% sucrose; 0.1M sodium phosphate buffer at pH 7.4) at room temperature for 1.5 hr, thoroughly rinsed in PBS 3 times (20 min each) and then left in PBS for 2 hr. The PBS rinse was followed by three rinses (20 min each) of sodium cacodylate buffer. The melanoma colonies were then postfixed (2.5% glutaraldehyde; 2.0% paraformaldehyde; 0.1M sodium cacodylate at pH 7.4; 0.2% ruthenium red) for 2 hr in a slow-shaking waterbath at 37°C and then in a postfix rinse for 45 min. Colonies were then further postfixed (2% OsO_4; 0.2M sodium cacodylate; 1% ruthenium red) for 1 hr on ice, rinsed in cacodylate buffer for 1 hr, dehydrated, and embedded in Spurr resin.

Immunohistochemistry

The in vitro/in situ localization of cell surface FN and collagen types (COLs) IV and V by human melanoma cells in colonies was determined initially by the indirect immunofluorescence technique as follows. Agar plates were fixed overnight at 4°C in absolute ethanol and glacial acetic acid (99:1), dehydrated, and embedded without infiltration in paraffin (56°C), according to the methods of Sainte-Marie (1962). The entire agar bilayer was serially sectioned (6–7 μm) and collected in a 4°C cold room; and every tenth section was stained with H&E in order to locate the colonies in the agar. Sections were then mounted on Chrom-Alum coated slides in a waterbath (40°C). Special care was taken to keep all temperatures below 40°C during processing so as not to denature the FN (Yamada, 1981) and collagen antigens (Olsen, 1981). The slides were deparaffinized at 4°C and rinsed overnight in 0.15 M Tris buffer, pH 7.4.

Rabbit antisera and absorbed antisera (control) to SDS-gel-purified FN were a generous gift from Dr. Richard O. Hynes, M.I.T., Cambridge, MA. The specificity and biochemical characterization of this antisera has been previously described (Mautner and Hynes, 1977). Antibodies to COLs IV and V were generous gifts from Dr. Charles Little, University of Virginia, Charlottesville, VA. The primary antisera and absorbed antisera, diluted 1:20 in PBS, were applied to the sections and left overnight at 4°C in a humidified chamber. After three (2-min) rinses in PBS, the secondary antibody (rhodamine conjugated goat antirabbit IgG; Cappel Labs, Inc., Downington, PA) was applied to the sections (1:30/PBS) for 1 hr at room temperature in the dark. The slides were then rinsed 3 times (for 2 min) in PBS, mounted with PBS and glycerol (10:90), and viewed with a Zeiss fluorescence standard-18 microscope. Photomicrographs were taken with Ilford HP-5 film, using a manual exposure time of 1.5 min for both control and experimental slides.

The appearance of cell surface FN and COLs IV and V on human melanoma cells in colonies was also demonstrated by the peroxidase-antiperoxidase (PAP) technique (Sternberger, 1986). Briefly, agar plates containing melanoma colonies were washed three times in PBS in a 37°C incubator for a total of 50 min prior to fixation. Subsequently, 1% paraformaldehyde/cacodylate buffer (w/v) was applied to the plates for 40 min at room temperature (24°C), followed by rinsing in cacodylate buffer (pH 7.4), dehydration in ethanol, and embedment in Spurr resin. Thick sections (1 μm) were cut with a Porter-Blum ultramicrotome. Selected sections known to contain colonies were placed on acid-cleaned slides; and the Spurr resin was etched away with a saturated solution of sodium hydroxide (NaOH) in 100% ethanol at room temperature, followed by hydration to water. Sections were incubated with the appropriate primary antibody [rabbit anti-FN (1:20 and 1:2000), or rabbit anti-COL IV (1:20 and 1:2000) or rabbit anti-COL V (1:20 and 1:2000)] overnight at 5°C, washed for 5–10 min in Tris-buffered saline (TBS), and treated with goat antirabbit IgG for 30 min at room temperature. Subsequently, slides were washed in TBS and treated with 1–2 drops of PAP (diluted 1:80) for 30 min. Slides were then washed in TBS and the H_2O_2-DAB-TB mixture applied for 7 min. The diaminobenzidene (DAB) was supplied by Sigma (St. Louis, MO). Slides were then rinsed in PBS and coverslipped with PBS/glycerol (10:90). Controls for PAP localization included substituting TBS or non-specific rabbit IgG (1:20 and 1:2000) for the primary antibody. Additional controls using DAB (no peroxidase-antiperoxidase) and PAP (no goat anti-rabbit) were performed to test the specificity of each sequential antibody reaction as well as to test for spontaneous polymerization of DAB.

RESULTS

Three distinct morphological variants of human melanoma colonies grown in soft agar were identifiable. Certain morphological features were characteristic for each variant and are worthy of description. Also, the composition of certain cell surface/ECM macromolecules in the melanoma colonies grown in soft agar has been probed with specific antibodies.

A macroscopic view of typical melanoma colonies in soft agar is shown in Figure 1. In general, two distinct colony sizes are seen. These colonies measure 490 μm and 580 μm in diameter, which is very large in comparison to most colonies. Their size is due in part to having been grown in culture for 50 days.

At the LM level, three morphological variants are discernible, and each can be classified by a major morphological characteristic. These variants are large light colonies (Fig. 2), dark colonies (Fig. 3), and smooth-edged colonies (Fig. 4). The large light colony is the most common morphological variant seen in the agar system, and Figure 2 illustrates its growth at 50 days. Such colonies are hypomelanotic, hence, the light coloration (a light tannish color). As seen with inverted light microscopy, the size and shape of the cells in the center of this ellipsoid colony cannot be determined due to resolution problems associated with cell stacking. The periphery of a typical colony is composed of cells that appear circular and, due to their location, may be examined without difficulty. The second most common variant (Fig. 3) is brown-black in color due to its high concentration of melanin, and it, therefore, is hypermelanotic. A noteworthy characteristic of this variant is the halo surrounding the colony proper; this halo becomes more prominent with the increasing age of the colony. The composition of the halo is described below. The third and least observed variant (Fig. 4) appears to have a smooth-edged border and is encapsulated. The

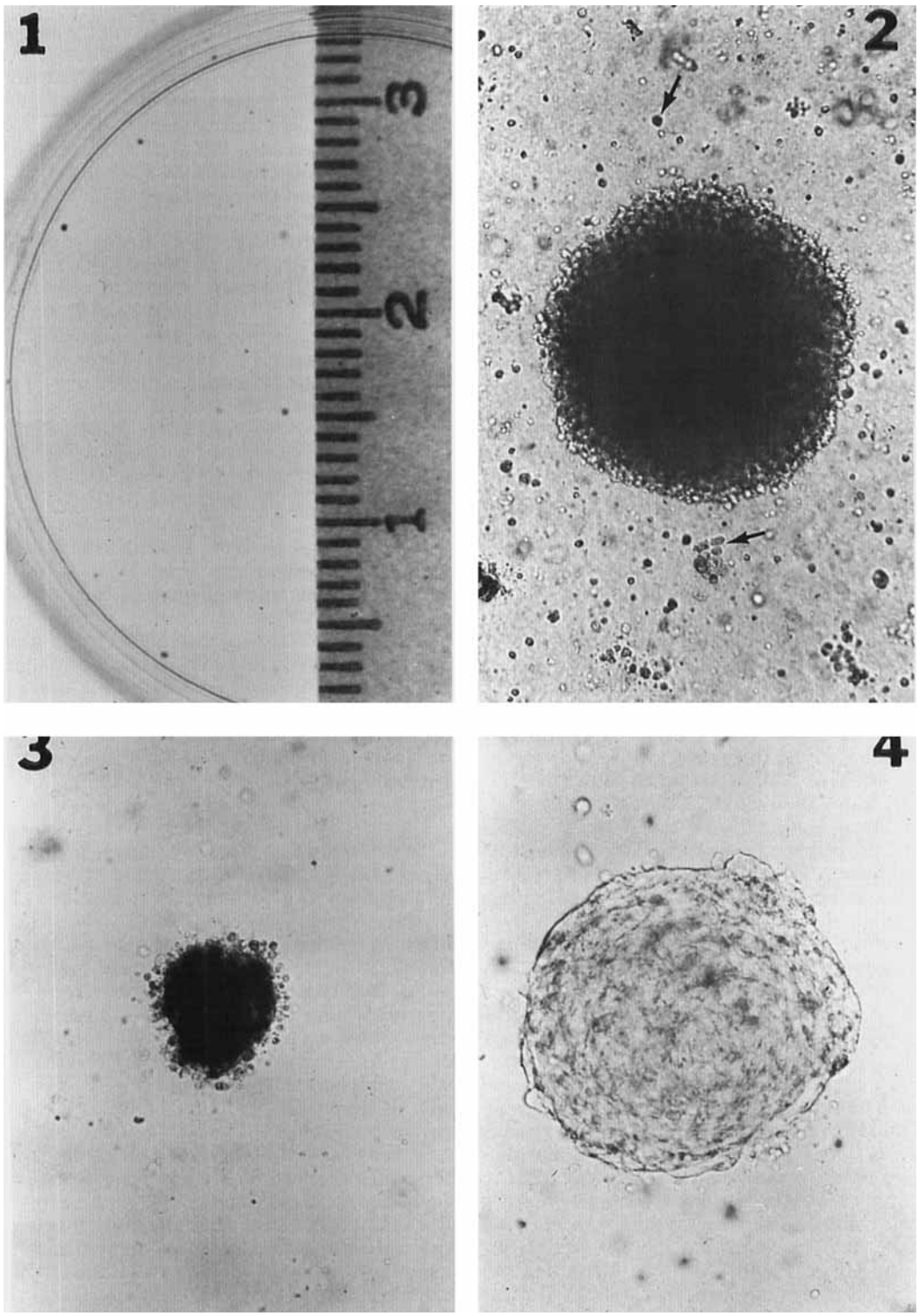


Fig. 1. Macroscopic view of a 35-mm tissue-culture dish containing large, light (hypomelanotic), human melanoma colonies, as observed after 50 days in soft agar. A 490-μm diameter colony and a 580-μm diameter colony are seen directly adjacent to the 1.5 and 2.4 markings, respectively, on the ruler. ×3.

Fig. 2. Higher magnification (with inverted LM) of the 580-μm diameter, large light colony shown in Figure 1. Nonclonogenic cells (arrows) from the original single-cell preparation are present throughout the agar layer surrounding the established tumor colony. ×80.

Fig. 3. Inverted LM view of a 140-μm-diameter, small dark (hypermelanotic) melanoma colony, as observed after 28 days in soft agar. A noteworthy characteristic of this variant is the halo surrounding the colony proper. ×160.

Fig. 4. A 220-μm diameter, smooth-edged colony variant demonstrated with inverted LM. The hypomelanotic coloration of this morphological variant is similar to that of the large light variant, shown in Figures 1 and 2. ×160.

coloration is similar to that observed for the large, light colonies. All three variants have been found to coexist in the same culture plate. In summary, the large light colony variant is the most frequently seen (≈70%), followed by the dark colony variant (≈27%), and the smooth-edged colony variant (≈3%).

Specific histochemical staining of the three colony variants demonstrated little, if any, accumulation of glycogen with either the periodic acid-Schiff reaction or Best's carmine method (not illustrated). Numerous lysosomes were identified by the fluorescent vital dye acridine orange (not illustrated). The lysosome concentrates the dye in situ so that the presence of collective lysosomes is easily detected by the characteristic bright white pattern when viewed under darkfield conditions.

Various histochemical stains were applied to paraffin sections of the three colony variants for identification of an ECM. A demonstrable ECM was observed in the large light colony variant and to a lesser extent in the small dark variant. Little, if any, ECM was noted in the smooth-edged variant. For the large light colonies, Masson's trichrome stain renders the connective tissue blue and is limited to the colony with no peripheral staining of the agar (not illustrated). A positive Snook's reticulum stain renders reticulum fibers in the ECM a gray-black color (not illustrated).

TEM analysis of large light colonies grown for 28 days (Fig. 5) and 50 days (Fig. 6) reveals premelanosomes interspersed throughout the cytoplasm of the cells. The nuclei have irregular borders with one or more prominent nucleoli. Sudan Black B staining of frozen sections confirmed the presence of lipids at the LM level (not shown). Lipids, vacuoles, and the fine, fibrillar extracellular material that surrounds the cells are evident at low magnification (Figs. 5, 6). At higher magnification, the fine, wispy, nonstriated nature of the extracellular material surrounding the cells within the colonies can be seen (Fig. 7). Periodic banding has not been observed in this fibrillar matrix.

The abundance of stage III and IV premelanosomes and melanosomes was characteristic of the dark colony variant (Figs. 8, 9), and the intense osmophilia of premelanosomes and secondary lysosomes is clearly distinguishable with TEM. Unlike the large light variant, many melanosomes are found in the extracellular space of the dark colonies, both as single organelles and in aggregates. The halo shows a cluster of premelanosomes, fully melanized melanosomes, and amorphous material (Fig. 9). These peripheral structures are generally encircled by a discontinuous membrane. Again, the only organelles observed in these structures are premelanosomes that are seen in all stages of melanosome development.

The third colony variant possesses melanization characteristics and ultrastructural morphology similar to the light colony variant (Fig. 10). Premelanosomes and melanosomes are sparsely scattered throughout the cytoplasm. The nuclei are irregular in shape and contain distinct nucleoli. Lipids and vacuoles are also present. The distinguishing characteristic at the ultrastructural level, compared with the other two variants, is the tightly packed nature of the cells. Colonies appear to lack a demonstrable ECM, except at their periphery. Therefore, the cells are found in close apposition to each other and possess desmosome-like cell junctions (Fig. 10), which were not observed in either the large light or small dark colonies.

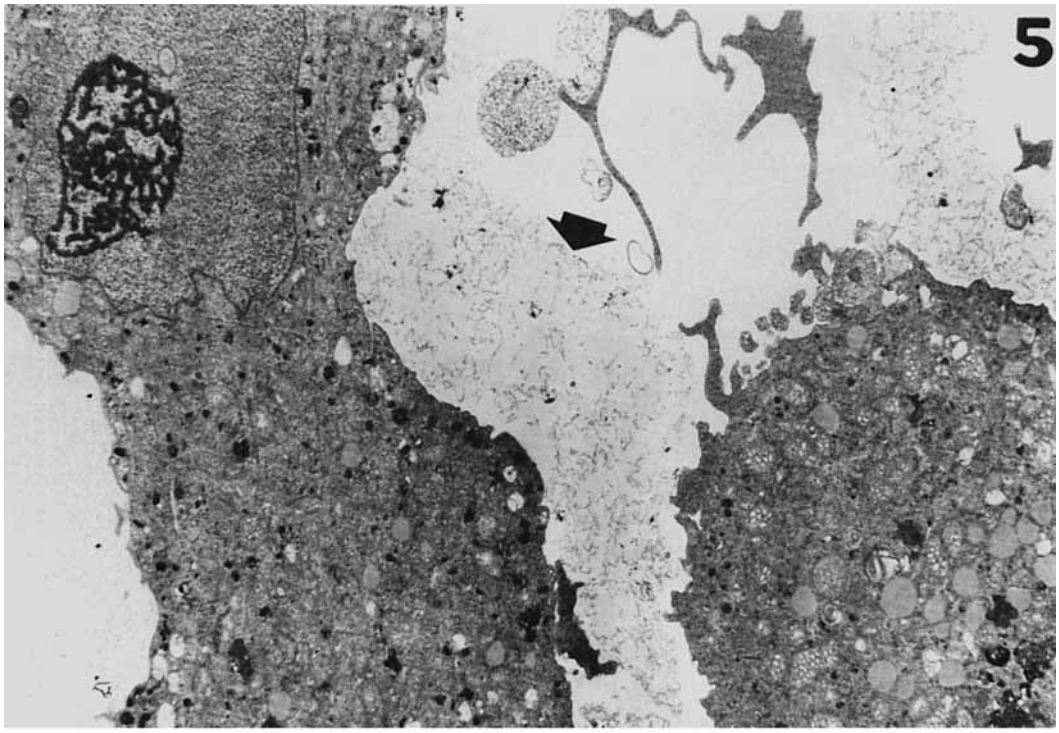
In order to characterize the ECM in the most commonly observed variant, the large light colony, specific antibodies to FN and COLs IV and V were applied. Immunofluorescence microscopy of colony sections reveals cell-surface associated staining for FN, and also suggests some staining of the associated ECM (Fig. 11). No staining was observed when absorbed anti-FN was used as a control (Fig. 12). Staining of paraffin sections of melanoma colonies with anti-COLs IV and V was inconclusive. Therefore, the more sensitive immunohistochemical assay of PAP was utilized to further resolve the localization of these antigens. A toluidine-blue-stained section through a large light colony (Fig. 13) provides the orientation for Figure 14 in which the colony, stained with anti-FN diluted 1:20, is faintly visible. Higher magnification (inset, Fig. 14) reveals the brown reaction product associated with the cell surface as well as in the ECM. Sections stained with antibodies to COL IV, diluted 1:20 (Fig. 15), and to COL V, diluted 1:20 (Fig. 16), showed no significant staining. Numerous controls testing the specificity of the primary and secondary antibodies, as well as the enzyme reactions, showed no staining pattern (data not shown).

Ruthenium red was used to preserve and to bind selectively to GAGs and PGs in order to elucidate further the composition of the ECM of the melanoma tumor colonies. Stained sections show GAG-like granules associated with the fibrillar ECM (Fig. 17), and a dotted, punctate staining of the cell surface, characteristic of a receptor pattern.

DISCUSSION

Development and use of the soft agar assay have significantly advanced the study of tumor cell biology (Hamburger and Salmon, 1977; Meyskens et al., 1981b; Thomson and Meyskens, 1982). The soft agar assay offers a tremendous potential for studying tumor colony forming ability as well as colony microenvironment. In this study, we have directed our efforts toward characterizing the variants of human melanoma colonies grown in soft agar morphologically, histochemically, and immunohistochemically. This investigation was necessary in order to provide background information of tumor development before initiating drug perturbation studies.

Three types of melanoma cell colonies were characterized in this study. Two of the types were previously reported by Meyskens (1980) and were termed large light (LL) colonies or small dark (SD) colonies owing to their gross appearance in soft agar. These two morphologic variants were shown to contain 10–40 and 15–50 cells, respectively. We initially suggested that the dark colonies contained cells smaller in size than those composing the light colonies, and that the small dark cells contained more cellular pigmentation, i.e., fully melanized melanosomes. Light and electron microscopic analyses in this study and previous LM investigations (Meyskens and Salmon, 1981; Persky et al., 1981) confirmed that the small dark colonies are smaller in overall colony diameter than the typical large light colony. Although the initial LM study of small dark colonies



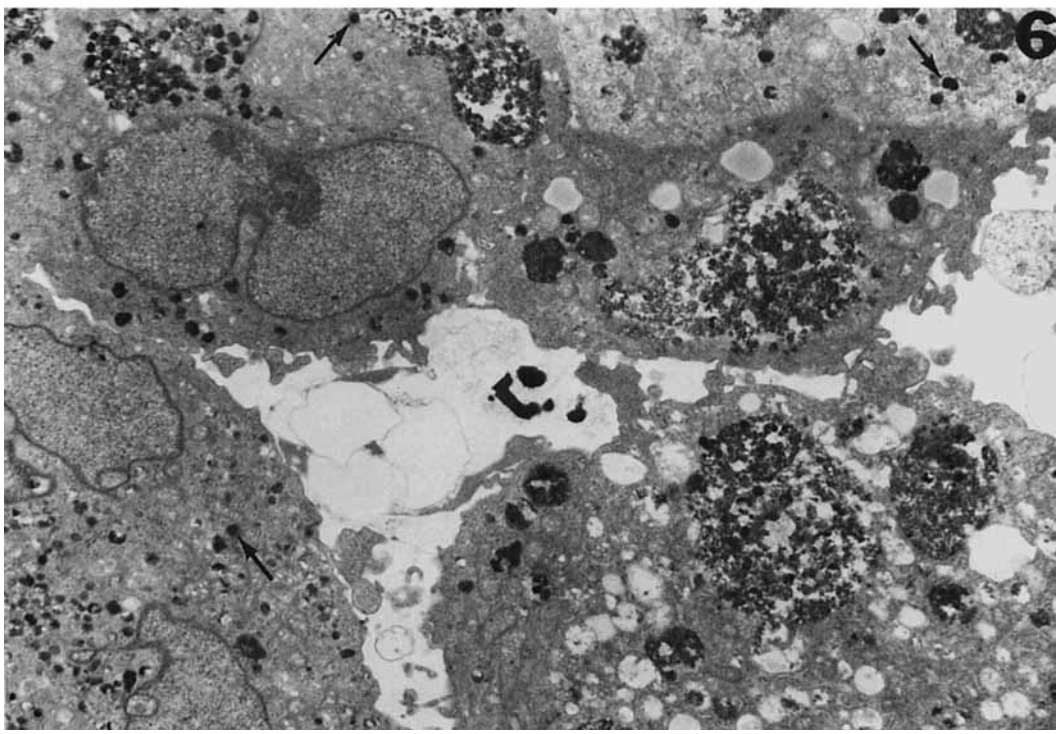
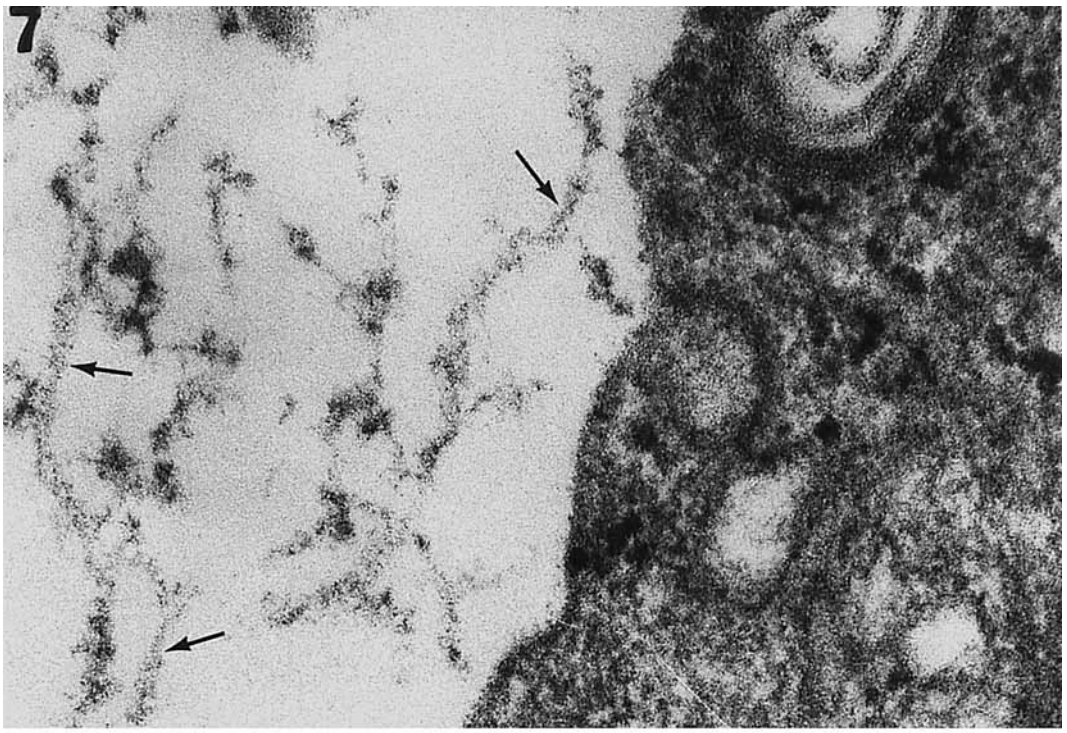


Fig. 5. TEM of two cells within a section of a large light colony grown for 28 days in soft agar. Note the fibrillar ECM (arrow) between these cells. ×6,200.

Fig. 6. TEM view of a portion of three melanoma cells within a large light colony grown for 50 days in soft agar. Melanosomes (arrows), lipids, vacuoles, and secondary lysosomes are prominent intracellularly; and a fine, fibrillar ECM is seen between cells. ×6,200.



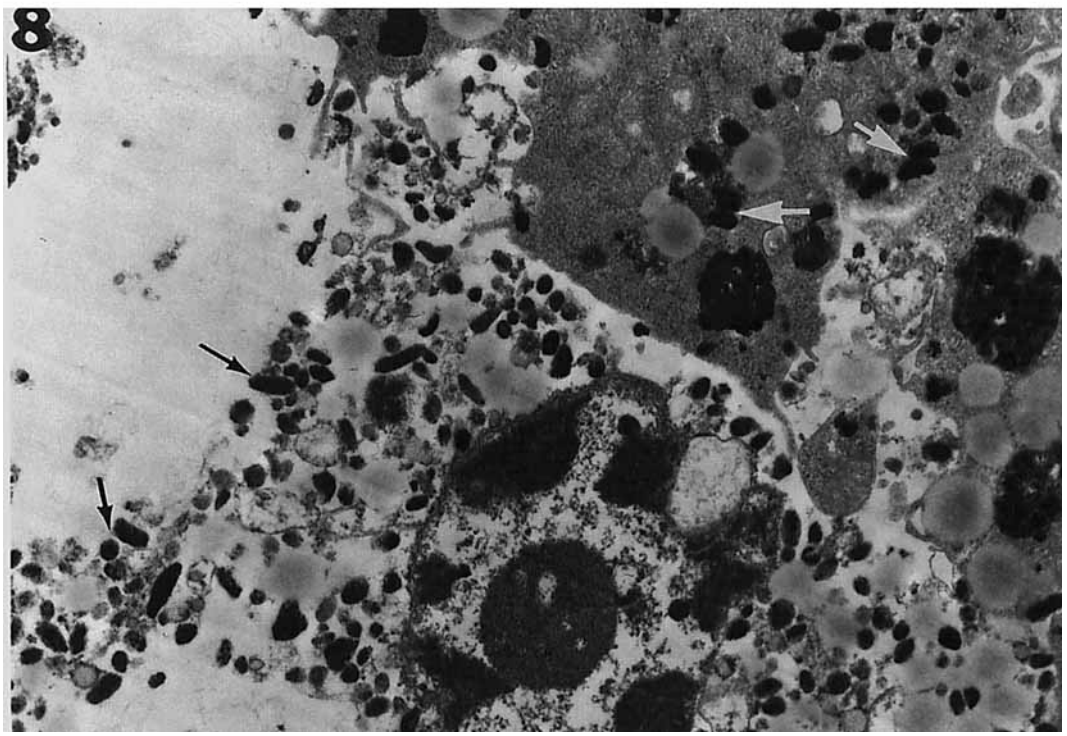
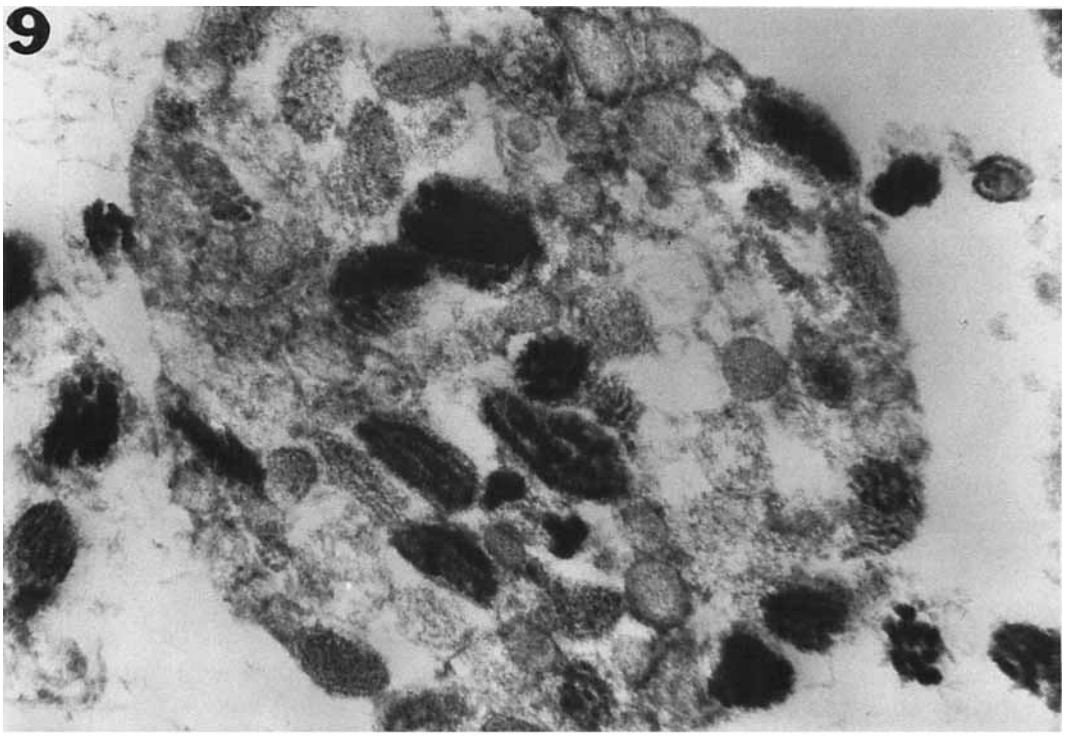


Fig. 7. Higher magnification TEM of the fine, wispy, nonstriated extracellular material (arrows), previously shown in Figure 6. ×170,000.

Fig. 8. TEM of a portion of a small dark colony. An abundance of intracellular (white arrows) and extracellular (black arrows) melanosomes are characteristic of this colony variant. ×10,500.



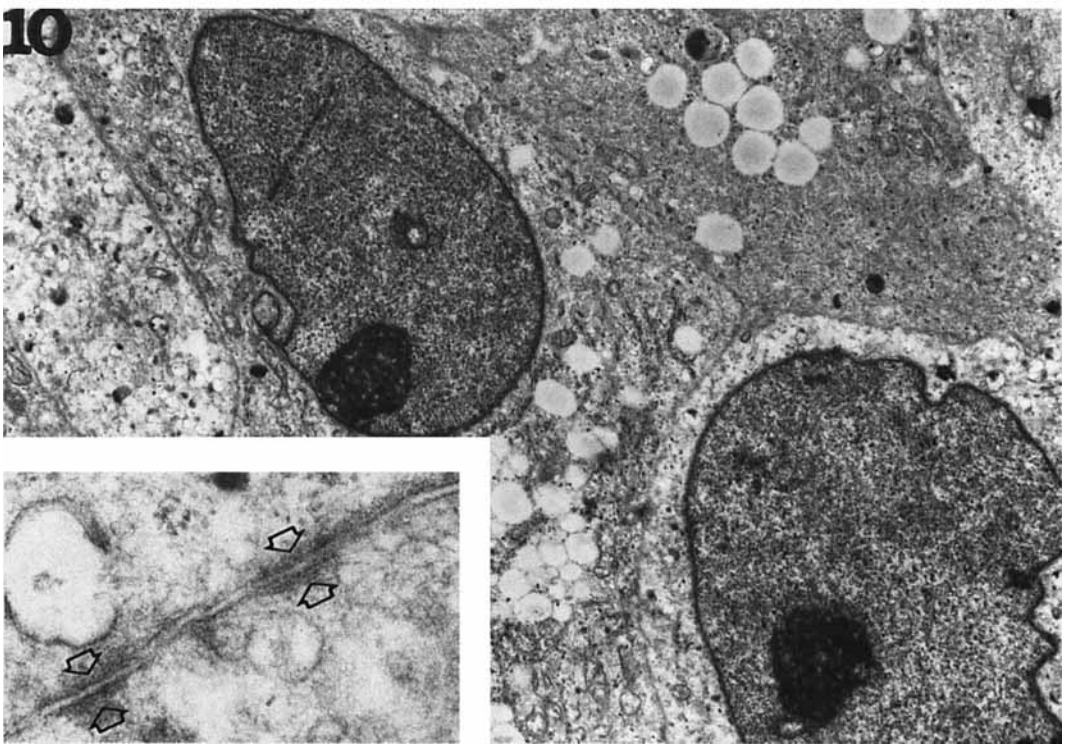


Fig. 9. Higher magnification TEM of the halo material surrounding the small dark colony variant (shown with LM in Fig. 3). This is a typical cluster of premelanosomes, fully melanized melanosomes, and amorphous material. ×55,000.

Fig. 10. TEM view of the central region of a smooth-edged colony-variant. The distinguishing characteristic of this variant is that the cells are tightly packed within the colony, leaving no discernible extracellular space. **Inset:** Typical desmosome-like junctions (arrows) found between cells. ×8,700; inset, ×32,000.

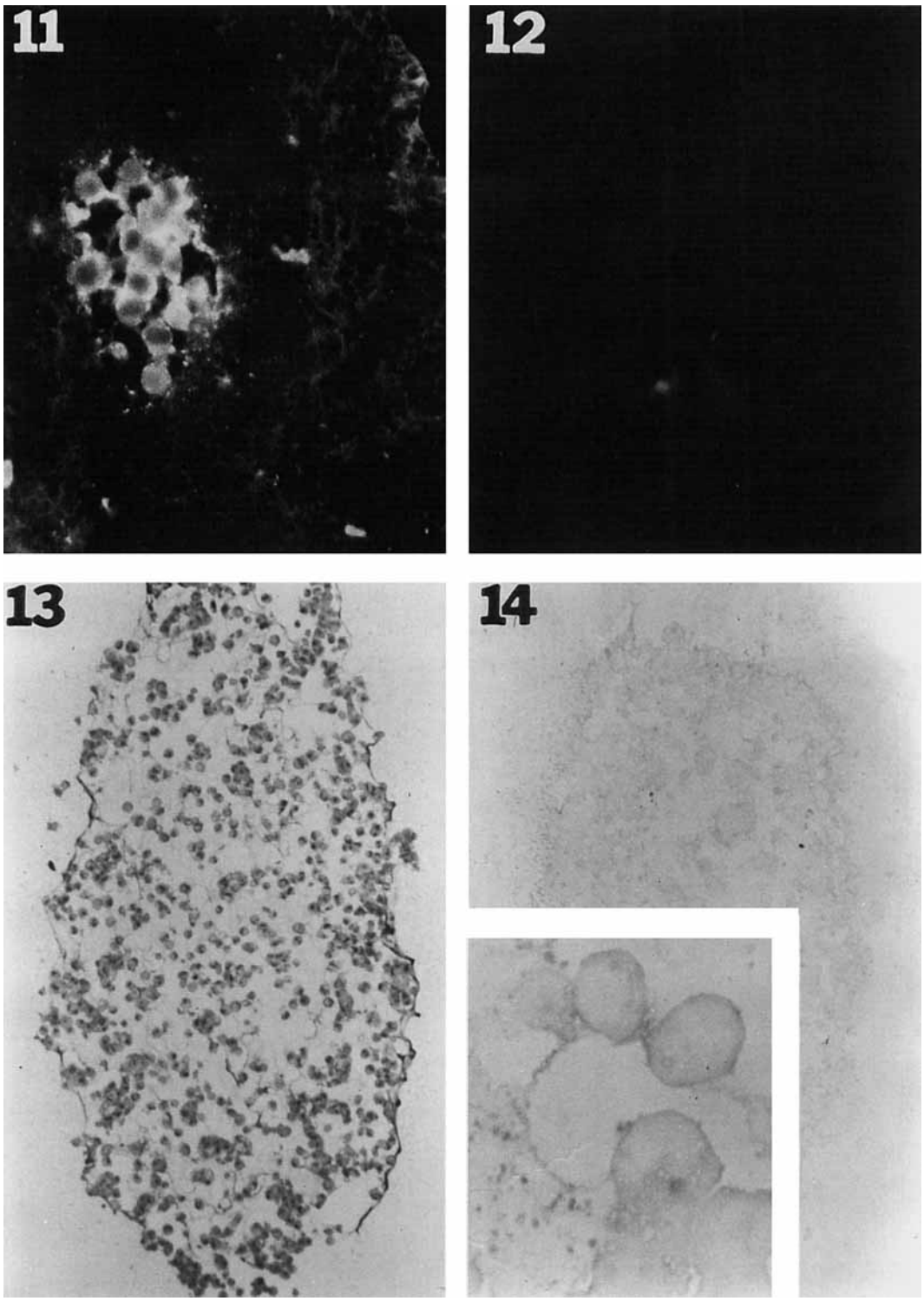


Fig. 11. Immunofluorescence microscopy of a section through a large light colony embedded in soft agar reveals cell-surface-associated staining for FN and suggests ECM staining as well. ×230.

Fig. 12. Staining for FN in a section similar to the one shown in Figure 11 is not observed when absorbed anti-FN is used as a control. ×230.

Fig. 13. LM view of a toluidine-blue-stained paraffin section of a large light colony embedded in soft agar provides orientation for Figure 14. ×100.

Fig. 14. The large light colony is faintly visible when stained with PAP for the localization of FN. **Inset:** A higher magnification LM view of both cell-surface-associated and limited ECM staining for FN. ×100; inset, ×1,100.

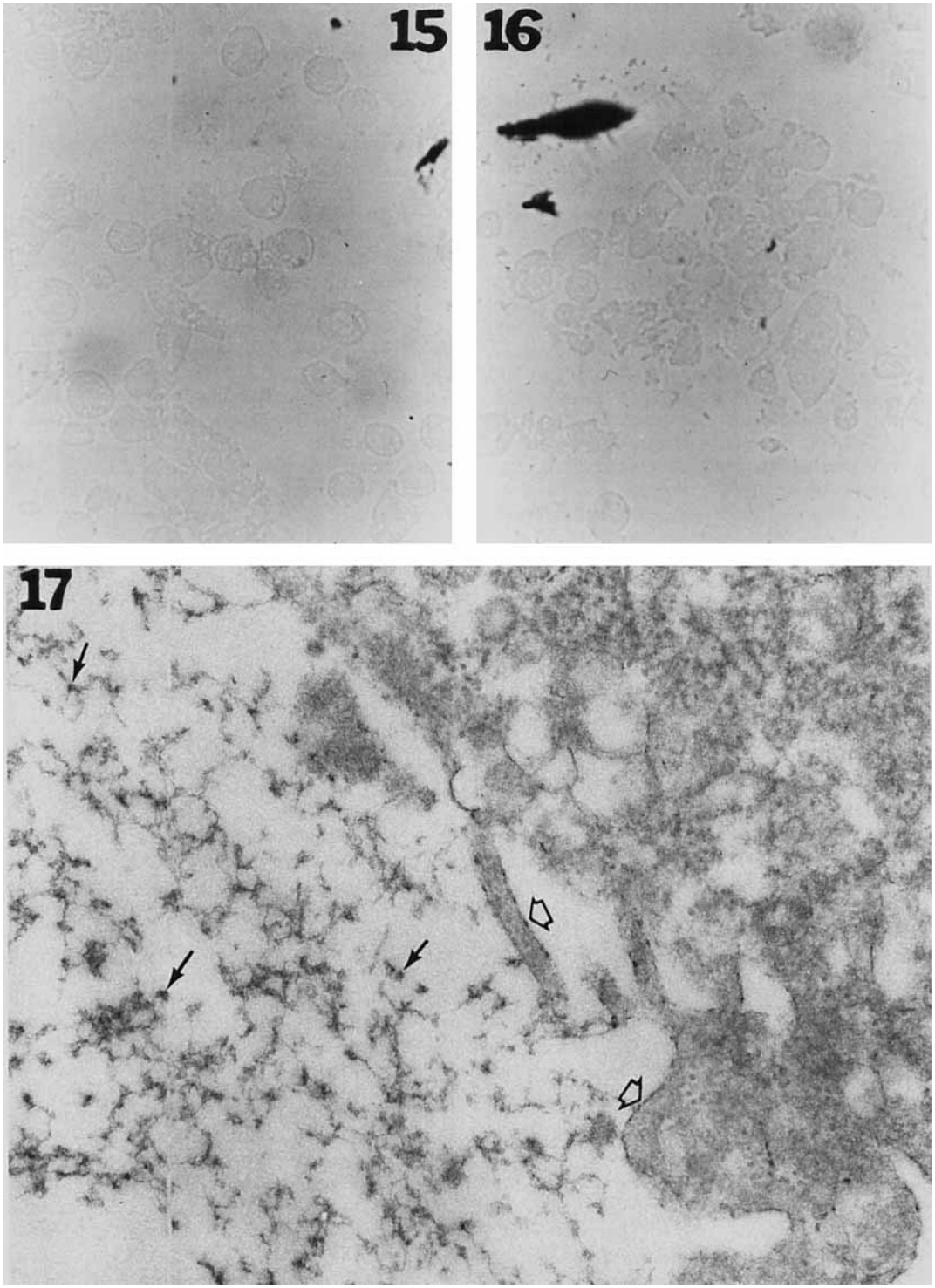


Fig. 15. There is no detectable staining for COL IV with PAP on sections of large light colonies. ×430.

Fig. 16. There is no detectable staining for COL V with PAP on sections of large light colonies. ×420.

Fig. 17. TEM of ruthenium-red staining for GAGs/PGs in a section of a large light colony. GAG-like granules are apparent in the ECM (arrows), and a dotted, punctate staining pattern is seen on the cell surface (arrowheads). ×78,000.

(Meyskens, 1980) interpreted the dark cells to be smaller in diameter than light cells, we have also demonstrated that there is wide variation in the diameter of human melanoma tumor colonies cultured in soft agar, and that comparisons of cells from small dark colonies may not relate directly to the large light colony cells described earlier (Meyskens et al., 1984b). Subsequent studies have shown that the individual cells of the small dark colony are approximately the same size as those comprising large light colonies (Persky et al., 1981).

From our ultrastructural analysis in this study, it is apparent that the darkness of the dark colony can be attributed to the hypermelanotic condition of the colony. There are more stage III premelanosomes and stage IV premelanosomes (i.e., mature melanin granules) in the dark cells compared with the other two variants. In addition, there were more premelanosomes and melanosomes released into the extracellular space of the agar by these cells. The smooth-edged variant demonstrates pigmentation characteristics similar to those of the cells of the large light colony; but it differs in outline from the large light colonies which have an acinar appearance. At the TEM level, the smooth-edged variant is distinct from the two previous types in that it contains cell junctions, which appear to be desmosome-like. Although the desmosome is the most common junction observed in cell-cell contacts in growing tumor spheroids in vitro (Sutherland, 1988), human melanomas contain vimentin, not keratin (Osborn et al., 1985); therefore, intermediate filament typing suggests that the cell junctions are desmosome-like only in appearance, not in composition.

Special attention was given to preserving the ECM produced by the melanoma cells composing the large light variant so that it could be analyzed by immunohistochemical methods and possibly related to metastatic growth potential in an anchorage-independent environment. To the best of our knowledge, our report is the first to demonstrate that melanoma cells in the soft agar assay produce an associated ECM. However, in the three-dimensional, multicell tumor spheroid model (which does not test clonogenic ability), extracellular volumes of 35–55% have been detected, which are similar values for tumors in vivo (Sutherland, 1988). In addition, it has been suggested that the production of ECM in the in vitro model stimulates differentiation within some cell types (Nederman et al., 1984).

The ECM in general is known to contain fibrillar components, glycoproteins, and GAGs/PGs (for review see Hay, 1981). Of particular interest for tumor biologists are laminin, FN, COLs IV and V, and GAGs. In this investigation, melanoma cells of the large light colonies were permitted to establish an ECM during a 28- to 50-day incubation period. Electron microscopic analysis suggested increased accumulation of ECM with longer incubation time. This observation supports the hypothesis that cells produce and constantly modify the surrounding milieu to accommodate growth and development (Schirrmacher and Barz, 1986). We would like to emphasize that the soft agar assay allows the study of tumor cell growth as cells form miniature tumor colonies; it also allows the direct observation of the production of a highly specialized ECM microenvironment which supports this growth.

The literature contains many diversified reports on the role of tumor cell surface molecules and important interactions between the cell surface and ECM during neoplastic events, as well as the production and absence of certain molecules during metastasis (for reviews, see Nicolson, 1984; Liotta et al., 1986; McCarthy et al., 1985). It is obvious that tumor cell surfaces are important in the metastatic process. During the dissemination of tumor cells and subsequent formation of metastases, the cell surface is intimately involved in adhesion, enzymatic secretion, migration, and general manipulation of the microenvironment (Liotta et al., 1986). An important glycoprotein involved in the adhesion of many cell types is FN. In our study, cell-surface-associated FN was demonstrated within the melanoma tumor colonies with both immunofluorescence and immunoperoxidase techniques. There have been a number of studies performed trying to elucidate the role of FN in neoplasia, and there is a strong suggestion that lack or loss of cell surface FN is an important determinant in tumorigenicity and/or metastatic potential (for review, see McCarthy et al., 1985). However, the definitive, unequivocal relationship between absence of cell surface FN and metastatic potential has not been shown (McCarthy et al., 1985). What has been demonstrated in previous studies (Hayman et al., 1981; Ruoslahti, 1984) is that malignant cells are incapable of depositing FN into a surrounding insoluble matrix. Our data may suggest that FN is important in the adhesion properties of tumor cells during colony formation, thus maintaining the three-dimensional architecture of the miniature tumor.

It was not surprising to discover, by immunoperoxidase and immunofluorescence techniques, an absence of COLs IV and V in growing melanoma colonies. Tumor cells have been shown to produce specific basement-membrane collagenases in culture, presumably for degradation of ECM barriers in vivo (Liotta et al., 1981, 1986), that could cleave these proteins from the cell surface/ECM interface and render them undetectable with immunological methods. In addition, some of the abnormalities noted in transformed cells have been related to a general reduction in ECM components (Ruoslahti, 1984). Similar observations have been made by Hendrix and coworkers (1986) regarding the absence of COLs IV and V in high and low metastatic variants of human melanoma cells as well as a difference in the amount of FN associated with the cells before and after invasion through a basement membrane.

Another interesting observation contributed by TEM and ruthenium-red staining was the discovery of GAGs/PGs in the ECM associated with the melanoma colonies, as well as the punctate staining of the plasma membrane. GAGs and PGs have been shown to have important biochemical interactions with collagens and FN (Gay and Miller, 1978; Geiger and Singer, 1979), as well as with interstitial structural components, as demonstrated by staining patterns observed with ruthenium red (Greenberg et al., 1980). These latter ultrastructural studies demonstrate that the PG complexes consist of a central granular core with fine radiating filaments (3–7 nm in diameter), which ap-

pear to interact with collagen fibrils and other extracellular fibrils, possibly FN, in the ECM. ^{35}S-methionine labeling of GAGs in the tumor spheroid system has shown the active production of these macromolecules in the tumor microenvironment (Nederman et al., 1984). In addition, the number of receptors for the GAG hyaluronate on the plasma membrane of nontransformed cells was shown to be substantially greater than those on the transformed counterparts, indicating alterations of these proteins associated with transformation (Underhill et al., 1987). In our study, TEM demonstrated a receptor-like staining pattern on melanoma colony-forming cells with ruthenium red.

Definition of the ECM may be of crucial importance in determining fundamental properties of metastatic tumor growth. The balance of the composition of the ECM may possibly serve as a marker for malignancy as well as provide a nutritive microenvironment for tumor development, for it is this complex interrelationship of cell-cell and cell-matrix interactions which determines the fate of tumor progression. Understanding the architectural arrangement of the ECM observed in the soft agar assay could contribute to the use of this milieu as a possible target for future chemotherapeutic intervention.

ACKNOWLEDGMENTS

This research was supported in part by Potts Estate grant 842-35 to BP, American Cancer Society grant PDT-205 and National Cancer Institute grants CA42475 to MJCH and CA27502, CA17094, and CA23074 to FLM. We thank Ms. Maria Felix and Ms. Donna Vandenberg for their skilled secretarial assistance.

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