CALCIFYING EPITHELIOMA OF MALHERBE

HISTOCHEMICAL AND ELECTRON MICROSCOPIC STUDIES*

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Calcifying epithelioma is a rare, solitary tumor which often occurs in young individuals (1), most frequently on the face and the arms (1, 2). Since Malherbe and Chenantais (3) described "calcified epithelioma of sebaceous glands" in 1880, the histogenesis of this tumor has been attributed to various skin appendages such as sebaceous glands (3, 4) and hair (2, 5, 6, 7). Lever and Griesemer (7) regarded the basophilic cells in calcifying epithelioma as primary epithelial germ cells with a tendency to differentiate into keratotic hair cells. Forbis and Helwig (1), in their recent study on 240 specimens, agreed with the opinion of Lever (2) and Lever and Griesemer (7) that calcifying epithelioma arises from primitive cells of the hair matrix. They pointed out that the broad dense transitional zone from basophilic to shadow cells of this tumor, where sulfhydryl groups are abundant, is analogous to the keratogenous zone of the hair. They also demonstrated that hyaline shadow cells in some instances show a brilliant birefringence, as does the keratogenous zone of the hair.

In this study, an electron microscopic examination performed on a case of calcifying epithelioma proved that the tumor is composed mainly of hair cortex cells.

Materials and methods

I. HISTOCHEMICAL STUDIES

The specimen used in this study was excised from the lesion of a 70 year-old white man. The lesion was located near the medial aspect of the left elbow, was 5 x 8 cm in size, bony-hard in consistency and deep-seated. The covering skin, though unevenly elevated, appeared normal. The patient had been noticing a gradually growing subcutaneous tumor over 50 years, but had not obtained any treatment. A presumptive clinical diagnosis of calcifying epithelioma was made and the tumor was excised in toto under local procain anesthesia. A portion of the excised specimen was frozen and cut in a cryostat into sections approximately 8μ in thickness. These sections were used for the following histochemical stains: for demonstration of sulfhydryl (SH) and disulfide (SS) groups dihydroxy-dinaphthyl-disulfide (DDD) (8) and alkaline tetrazolium stains (8); for calcium chloride acid (9) and for carbonates and phosphates the method of von Kossa (8); for the study of the stroma metachromatic dyes such as methylene blue, toluidine blue and crystal violet (10), PAS stain (10) with and without diastase and collagenase digestion (8), Alcian blue (10) and Hale stains (10) with and without hyaluronidase digestion (8) and Congo red stain (10). As controls for each enzymatic stain a substrate-free incubation medium was used. Polarscopic examination was performed on each section stained for SH and SS groups. A part of the specimen was fixed in 10% formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin for routine histopathological studies. In addition, skin of the scalp from several adult volunteers as well as from human embryos (16 weeks to 22 weeks old) were used for comparative studies.

RESULTS

Light microscopic examination revealed that the tumor was composed of a parenchyma, which often formed solid masses, and a loosely connected stroma. In the solid mass of the parenchyma, punched out empty spaces lined by several layers of condensed parenchymal cells were often seen. These structures suggested hair follicles without hairs (Fig. 1). The specimen used in this study contained few areas of basophilic cells. The majority of tumor cells possessed characteristics of both basophilic and shadow cells (Fig. 1); in this report these cells will be referred to as transitional cells, which correspond to the "squamoid cells" described by Forbis and Helwig (1). They often retained nuclei, but showed a large amount of eosinophilic, hyaline cytoplasm rather than basophilic cytoplasm. Calcium deposits were seen as strongly basophilic, finely granular substances in large masses within the strands of shadow cells and transitional cells. There was a significant correlation between these basophilic deposits and the positive substance
FIG. 1. The Tumor Parenchyma is composed of solid masses of SH- and SS-positive cells. Some cells retain nuclei, while others show empty nuclear areas. There are three punched out spaces surrounded by rather condensed layers of the tumor cells. Although these spaces are empty, they resemble the hair follicles with extracted hairs. DDD stain. (× 430)

stained by chloranilic acid (Fig. 2) and von Kossa stains (Fig. 3). Since chloranilic acid stain has been regarded as specific for the calcium ion and von Kossa stain for carbonates and phosphates in general (8, 9), these deposits seemed to represent carbonate or phosphate salts of calcium.

In more than 90% of the tumor (parenchymal) cells, sulfhydryl and disulfide stains such as DDD (Fig. 1), performic acid-Schiff (Fig. 4) and alkaline tetrazolium were strongly reactive and the performic acid-Alcian blue reaction was moderately positive. With polarized light strong birefringence was observed in areas corresponding to those strands of tumor cells which were positively stained for SH and SS groups (Figs. 4 and 5). A similar birefringence was observed in adult and embryonic hair stained for SH and SS groups.

The stroma showed a weak to moderate metachromasia, a moderate to strong PAS reaction (which was diastase resistant but diminished

FIG. 2. Chloranilic Acid stain shows needle-shaped, reddish brown deposits of calcium chloranilate in islands of the transitional cells. (× 178)

FIG. 3. von Kossa stain shows dense, black deposits in islands of transitional cells. (× 178)

FIG. 4. Performic Acid-Schiff stain shows a strong reaction (k) in more than 90% of the tumor cells in various strands. There are several punched out holes (arrows) probably representing empty hair follicles similar to those shown in Fig. 1. c: collagen stroma. (× 178)

FIG. 5. Polariscopy of the same section as shown in Fig. 4 reveals a strong birefringence in areas corresponding to the performic acid-Schiff positive areas (k) and in collagen fibrils (c), indicating that both areas are composed of well-organized filaments. Arrows: empty hair follicles. (× 178)
Figs. 2 to 5.
slightly by collagenase), a moderate reaction to Alcian blue and Hale stains (hyaluronidase resistant) and was negative with Congo red.

II. ELECTRON MICROSCOPIC STUDIES

Materials and Methods

Part of the specimen used for histochemical studies was cut immediately after excision into small pieces and fixed in 1% osmic acid which had been adjusted to pH 7.2 with veronal buffer and to physiological osmolarity with 4.5% sucrose. After 1½ hours' fixation the pieces of the tissue were dehydrated through gradually increasing concentration of ethanol and propylene oxide and embedded in Araldite. Thin sections ranging from 400 Å to 800 Å were cut on the LKB Ultratome and stained with either 1% uranyl acetate in 30% ethanol or 1% phosphotungstic acid in 50% ethanol, then with Reynolds' lead citrate (11). Sections were examined with an RCA EMU-3G electron microscope.

RESULTS

1. Basophilic Cells.—The cells which corresponded to basophilic cells of light microscopy contained fewer electron dense fibrils than other types of tumor cells but more cellular organelles, notably mitochondria and ribosomes (Fig. 6). The individual filaments of the electron dense fibrils were 70—100 Å in width and comparable to α-keratin filaments of the hair cortex (12, 13). Some of these filaments were not yet cemented together and were, therefore, clearly visible individually (Fig. 6). The basophilic cells contained nuclei and were surrounded by a thin plasma membrane. These cells resembled the cortical cells of the embryonic as well as anagen adult hair at the lower level of the keratogenous zone (12—15) because of the abundance of mitochondria and ribosomes and relative scarcity of α-keratin filaments, which were not densely cemented together. Basophilic cells were few in number in our specimen.

2. Transitional Cells.—The cytoplasm of the transitional cells was filled with a number of concentric strands of α-keratin filaments surrounding nuclei or nuclear area (Fig. 7). In some transitional cells the electron density of these strands had increased, probably through further accretion of individual filaments and deposition of an interfilamentous cementing substance (γ-keratin), while in others they appeared homogenized (Fig. 8). The plasma membranes of the transitional cells were as thin as those of the basophilic cells (Fig. 7) or they disappeared entirely (Figs. 7, 8). Nuclei were represented as either a slightly dense area delimited by a nuclear membrane (Fig. 9a) or an electron dense nuclear membrane surrounding an empty space (where nucleoplasm was once present) in the center of the cell (Fig. 9b). In some transitional cells nuclei were not visible due either to the level of the section through the cells or to the fact that they had simply disappeared (Fig. 8). Calcium deposits occurred between strands of α-keratin, appearing as dense granular aggregations or thin thread-like crystals (Fig. 8, cf. Fig. 14). In some transitional cells endoplasmic reticulum were still present; in most of the cells various amounts of collagen fibrils were seen invading the strands of the electron dense fibrils (Fig. 8). Except for calcium deposition the transitional cells of this tumor resembled embryonic cortical cells at the middle to upper levels of the keratogenous zone (15) (Fig. 10) because of decreased subcellular organelles, thin plasma membranes and, in particular, an abundance of thickened bundles of α-keratin cemented with electron dense γ-keratin.

3. Shadow Cells.—By definition, each of the shadow cells contained an empty space in the center of the cells in lieu of a nucleus. Strands of α-keratin in these cells showed more advanced stages of accretion and cementing of the individual filaments: These strands were fused together and formed one large syncytial band surrounding an empty, often calcified nuclear area (Fig. 11). While most of these fused strands still revealed filamentous components, homogenization occurred in some portions (Fig. 11). The plasma membranes of these cells had completely disappeared. Except for the deposition of calcium the shadow cells resembled embryonic cortical cells at a very advanced stage of keratinization (15), since the latter show a homogenization of the keratin strands, disappearance of the nuclei and the plasma membranes.

4. Hair-like Structures.—In some sections there were structures composed of a large central core and several concentric layers (Fig. 12). They were regarded as mummified hair structures probably representing a more differentiated portion of the tumor than the portion which contained only individual tumor cells. These hair-like structures may correspond to
Fig. 6. A Basophilic Cell contains cellular organelles such as mitochondria (m) and ribosomes (r) in abundance. In immature small strands individual filaments of α-keratin are visible (arrows), while in more mature large strands (*) the individual filaments are only discernible at the periphery. Collagen fibrils (C) are invading the cytoplasm. N: nucleus. (× 33,750)
Fig. 7. Scanning View of the Tumor shows that the individual tumor cell is composed of a round mass of electron dense fibrils. Faintly stained plasma membranes (arrows) were more often absent than present. In the center of these cells empty spaces (N), where nuclei were present, are seen. These cells correspond to the transitional cells. C: collagen stroma. (× 4,234)
Fig. 8. A Transitional Cell contains strands of fibrils surrounding the empty nuclear area (N). Although individual strands show advanced stages of accretion of constituent filaments or homogenization (*), the filamentous nature of these strands are still discerned in most of them, especially at the periphery (arrows). Deposits of calcium salts (Ca) and collagen fibrils (c) are seen between these strands. (X 14,600)
the hair follicle-like, punched out structures described in the light microscopic findings and shown in Fig. 1.

The central core (the cortex) was usually a round area composed of moderately electron dense strands of α-keratin which were often homogenized as seen in some of the transitional cells and shadow cells. A heavy deposit of calcium salts was observed between these strands (Fig. 12). This central core, considering its position and configuration, was considered representative of the hair cortex.

Surrounding this core were a few layers of flat, imbricate cells delimited by thick, prominent plasma membranes (Figs. 12, 13, 14a). These plasma membranes were convoluted against each other. The adjoining cells were thus interlocked (Figs. 12, 13, 14a). These cells were regarded as cuticular cells of the cortex since, besides their flat configuration, topographical relationship to the cortex and thick plasma membranes, they contained uniformly small, round trichohyaline droplets of high electron density (Figs. 13, 14a), as seen in embryonic as well as adult cuticular cells (15, 16) (Fig. 10). These cells, as embryonic cuticular cells of the cortex, contained only small number of membrane-coating granules of Matoltsy and Parakkal (17) (Fig. 14a).

Peripheral to this layer of flattened cells there were a few layers of relatively wide cells (Figs. 12, 13) which often contained homogeneously dense, non-filamentous trichohyaline granules (Fig. 14b), as seen in the inner root sheaths of the embryonic as well as adult hair (15, 18). These cells were regarded as representing the inner root sheath. Peripheral to these wide cells corrugated cells were occasionally seen,
Fig. 10. Cortical Cell (C), Cuticle of the Cortex (Cc) and Cuticle of the Inner Root Sheath (Ci) at the upper level of the keratogenous zone of an embryonic hair. The cortical cell (C) contains numerous dense strands of the α- and γ-keratin complex, abundant RNP particles, and a few mitochondria (m). Thin arrows indicate the filamentous component (α-keratin) at the periphery of these strands. The plasma membranes are very thin and inconspicuous (thick, short arrows). Insert also shows α-keratin filaments half-masked by dense γ-keratin, and RNP particles (r). The cuticular cells of the cortex (Cc) contain numerous small round droplets of trichohyalin (t) with few attached tonofilaments. This trichohyalin tends to align along the margin of the cell. There are an abundant amount of mitochondria (m) and RNP particles. The cuticular cell of the inner root sheath (Ci) contains a bundle of tonofilaments on which trichohyaline will deposit at a higher level. N: nucleus. (× 22,200) (Insert: × 33,300)
Fig. 11. A Shadow Cell shows fused strands of electron dense fibrils surrounding the empty nuclear area. While in most part of these strands filamentous patterns of $\alpha$-keratin are still recognizable, in some areas the strands are homogeneously dense (*). Ca: deposits of calcium salts. ($\times$ 14,600)
Fig. 12. Hair-like Structure. The central core (Cortex) is composed of homogenized strands of $\alpha$- and $\gamma$-keratin complex and heavy deposits of calcium salts (Ca) which occurs between these strands and also as a solid mass (Ca1). The plasma membranes are discernible in several places especially at the periphery (arrows). Cc: flat imbricate cells which correspond to the cuticle of the cortex. W: wide cells which correspond to the inner root sheath. C: collagen fibrils which are invading the outer layers of the hair-like structure. (X 4,234)
Fig. 13. **Trichohyaline Droplets** (Arrows) and **Membrane Coating Granules** (mc). The trichohyaline droplets (arrows) are of high electron density and found in the flat imbricate cells (Cc), which represent cuticle of the cortex. The membrane-coating granules (mc) are located in either intercellular spaces or in the cytoplasm near the plasma membrane. Note that in spite of the degeneration and disappearance of cytoplasmic organelles such as the nucleus (N), plasma membranes remain intact and prominent. W: wide cell which represent inner root sheath cell (×12,320)

filled with an electron dense amorphous keratin substance (γ-keratin) (Fig. 14c) which resembled the keratinized outer root sheath cells near the follicular orifice (Fig. 14d). Surrounding the outermost layer of the hair-like structure there was a collagen stroma which often invaded between outer layers of the hair-like structure (Fig. 12). A considerable amount
of membrane coating granules of Matoltsy and Parakkal (17), commonly found in the cytoplasm near the plasma membrane or in the extracellular spaces between apposing plasma membrane of the adjoining cells (Fig. 13), further characterized these two outer layers of hair-like structures as they did the inner and outer root sheaths of the embryonic hair (Fig. 14d). These granules were 1,000–6,000 Å in diameter, showed a matrix of moderate electron density and often contained parallel or spiral lamellae of high electron density. They tended to lie near the plasma membrane, fused with it and in some places emptied their contents, probably mucopolysaccharides (17, 19), into the intercellular spaces. According to Matoltsy and Parakkal (17), keratinizing epithelial cells, which invariably contain these granules, are thus coated from outside and increase the thickness of their plasma membranes.

5. Sebaceous Gland and Smooth Muscle.—Occasionally large masses of sebaceous cells (Fig. 15) and smooth muscle cells (Fig. 16) were found, very often in close proximity to each other and surrounded by tumor cells and connective tissue stroma (Fig. 16). Smooth muscle cells contained an abundance of myofilaments and a small amount of mitochondria and glycogen particles, and were interpreted as representing the arrectores pilorum muscle.

6. Stroma.—The stroma was composed of a very fine filamentous substance and premature and mature collagen fibrils. A moderate number of fibroblasts were found in the stroma (Fig. 16). The stroma invaginated between tumor cells, and, when the tumor cells were degenerated and disrupted, invaded their cytoplasm (Fig. 17). In such areas strands of α-keratin filaments of the tumor cells split up into individual filaments and became interwoven with mature and premature collagen (Fig. 17). There were a few straight filaments of the width of 60–100 Å which appeared similar to amyloid filaments. However, in view of the presence in the tumor of split up filaments of α-keratin and filaments of premature collagen, both of which are similar in size to amyloid filaments, the decision as to whether or not amyloid was really present in this tumor, as Peterson and Hult had recently reported (20) was extremely difficult. Such admixture of the epidermal and dermal fibrous components is unique and noteworthy. The fine filaments of premature collagen were apparently responsible for the diastase-resistant PAS and hyaluronidase-resistant Alcian blue stains (21, 22).

DISCUSSION AND SUMMARY

Histogenesis.—Histochemical and electron microscopic studies of one case of calcifying epithelioma of Malherbe revealed that the tumor is no doubt an appendage tumor of the hair and its follicle. Several specific histochemical stains demonstrated an abundance of SH and SS groups in the tumor parenchyma. The simultaneous demonstration of a brilliant birefringence in the sections stained for SS and SH groups left little doubt that very well organized filaments are closely incorporated with SS- and SH-rich proteins, such as cystine and cysteine, and compose the main constituents of the tumor. Such a substance is most compatible with the hair keratin formed by the cortical cells, since it contains filamentous α-keratin and sulfur-rich γ-keratin (13, 14). Electron microscopic examination confirmed that a majority of tumor cells were indeed cortical cells at various stages of development; the basophilic cells represented the youngest stage and the shadow cells the most mature stage of one single cell line, that is, cortical cell. That the tumor was not only composed of the individual cells of the cortex but also contained hair-like structures, including sebaceous cells and smooth muscle cells, indicates that the tumor has attained a certain degree of differentiation towards mature hair structure.

Calcification.—Calcification of keratin is not unknown (23); it has been reported in horse tail hair and porcupine quill and in some other hard keratin such as claw of a bear, horns of cattle and baleen of a whale. Although many of the deposits of calcium salts so far observed seem to be associated with nuclei (23) and it was inferred that the nuclear remnants might play some inductive role for the mineral deposits (23), the deposits found in this investigation were not necessarily restricted to the nuclear area. Upon examination of the hair-like structure of the tumor, it was apparent that the area corresponding to the cortex showed an exceedingly heavy calcium deposit. In such cortical cells calcium deposits oc-
Fig. 14a. Central Core (Cortex) and Flat, Imbricate Cells (Cc) of the hair-like structure of the tumor. The plasma membranes of the cortical cells (large arrows) are but vaguely visible, while those of the flat, imbricate cells representing cuticular cells of the cortex (Cc) are convoluted and interlocked with each other. Only a few membrane-coating granules (small arrows) are present in the cuticular cells. A few small droplets of trichohyalin are seen (*). Individual crystals of the deposited calcium salts (Ca) are seen as an electron dense, needle-like or granular substance. (X 16,848)

Fig. 14b. Trichohyaline Granules found in wide cells (inner root sheath cells) of the hair-like structure of the tumor are homogeneously electron dense. C.: invading collagen fibrils. (X 12,672)

Fig. 14c. Keratinized Cell of the hair-like structure of the tumor shows amorphous, elec-
Fig. 15. A Sebaceous Gland found near the mass of smooth muscles shown in Fig. 16. Bm: basement membrane. C: collagen. G: germinative cells. g: glycogen particles. S: globules of sebum in mature sebaceous cells. (× 11,100)

Fig. 14—Continued.

tron dense keratin and a corrugated outline, whose configuration and contents appear similar to the outer root sheath cells near the follicular orifice. (cf Fig. 14d). *: membrane-coating granules. (× 16,848)

Fig. 14d. Outer Root Sheath Cells (O) of embryo hair (22 weeks old) at the level of the sebaceous gland (Se) keratinize by becoming homogeneously electron dense, flattened and shed (arrow) into the space (S) of the hair canal. The membrane-coating granules (*) are abundant. (×5,688)
Fig. 16. Smooth Muscle Cells (SM) are surrounded by a collagen stroma (C) and the basophilic (B) type of tumor cells. F: fibrocyte. (× 2,016)
Fig. 17. Admixture of the Strands of the Tumor Cells and Collagen Fibrils. Some strands are split up into component α-keratin filaments (arrows) and intermingled with mature and premature collagen fibrils (c). Ca: deposits of calcium salts. (× 22,800)
curred in the spaces between the strands of α-keratin filaments where RNP particles abound. The significance of this is unknown.

REFERENCES