Histogenetic and Morphological in Vivo and in Vitro\textsuperscript{1} Data Concerning Androgen-Estrogen-induced Scent Gland Tumor in the Syrian Hamster\textsuperscript{2}

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SUMMARY

The scent gland tumor of the Syrian hamster is induced by exogenous androgen and estrogen. Microscopic nodules are induced normally in old males by endogenous androgen. The histogenesis of the scent gland tumor is complex and not completely understood. In this study microscopic preneoplastic nodules and macroscopic tumors were studied by light and electron microscopy, and the macroscopic tumors were grown in tissue culture on collagen-coated coverslips and on sponge foam matrices by the organ culture method. The cultures were fed with an unfiltered fetal calf serum-bovine serum ultrafiltrate medium, which contained endogenous androgen-estrogen, 110-100 pg and could maintain growth without additional androgen-estrogen. Exogenous androgen-estrogen was also added to some cultures. Scent gland tumors grown in organ culture contained cells of two shapes, spindle and ovoid arranged in cords. Cultures on coverslips showed radiating outgrowths of spindle cells suggesting either mesenchymal or Schwann cells. By electron microscopy, both in vivo and in vitro preneoplastic and tumor samples contained cells with segments of basal lamina, micropinocytotic vesicles, and junctional complexes. These features were similar to those of poorly differentiated experimental malignant rat schwannomas maintained in similar in vitro systems. Tumors grown in vivo and in vitro were associated with collagen fibrils with a periodicity ranging from 400 to 1075 Å. The evidence reported in this paper suggests that one component of the scent gland tumor is an androgen-estrogen-induced poorly differentiated schwannoma.

INTRODUCTION

The TPR\textsuperscript{+}-DES-induced scent gland tumor of the Syrian hamster was first reported by Kirkman (16) in 1952. Its androgen-estrogen induction was confirmed by Oberman and Rivière (30). It has subsequently been studied histologically, histochemically, electrophoretically, and in culture systems (2-5, 11, 12, 16-28), and the pertinent literature was reviewed by Homburger (14, 15). After hormone treatment for approximately 250 days, slightly opalescent lobulated tumors are found in both male and female hamsters; the tumors cease growing if hormone treatment is discontinued, but they diminish very little in size. They can be transferred subpannicularly in hormone-supported male or female hosts (20, 23, 26). In old males or in males or females treated only with androgen, microscopic nodules appear; these nodules will enlarge into palpable scent gland tumors if the hosts are treated with TPR-DES (23, 26).

In vitro studies of the scent gland tumor reported by Algard (2-4) suggested that the cell cultures of this tumor were not hormone dependent, whereas primary tissue explants were. When enzyme-dissociated tumor cells were seeded on the surface of a thin plasma clot, estrogen and androgen supplements had no effect on cell division, morphology, or migration. Primary explants of the tumor grown on plasma clot required hormonal supplementation when a medium of Morgan's 199 with 10% 11-day chick embryo extract was used. Both cell cultures and tissue explants required hormones when transplanted back to host animals of either sex.

Kirkman and Algard (22, 23) divided the life history of the scent gland tumor into 3 stages, which can briefly be summarized as follows. Stage 1 nodules are preneoplastic microscopic spherical or ovoid masses which appear in untreated old males, TPR-treated males and females, and TPR-DES-treated males and females. Stage 2 nodules (maximum size, 1 mm) are found after about 200 days of TPR or TPR-DES treatment in males and females. Stage 3 tumors are palpable after approximately 250 days of TPR-DES treatment. These authors suggested that the tumors be named chaetepitheliomas [Gr. hair, + upon, + nipple + tumor]. Kirkman and Dodge (26) recently discussed the histogenesis of the scent gland tumor and concluded that the Stage 3 tumor originates from more than 1 type of tissue. It was felt that the laminated Stage 1 corpuscles are androgen-induced and arise independently in the connective tissue around the scent gland hair follicles, possibly from perineural cells or from the Schwann cells of perifollicular nerves, and it was suggested that under TPR-DES influence.
other epithelial components are added later from the hair
matrix, with possible contributions from the external root
sheath.

In order to gain further insight in the histogenesis of the
scent gland tumor, we have undertaken an electron micro-
scopic study of its various stages of development in vivo
and an in vitro study of Stage 3 tumor explants grown in
sponge culture method on sponge foam matrices. The morpho-
logical features were compared with those, recently de-
scribed (9, 32), of malignant experimental rat schwannomas
maintained in similar culture systems. Levels of 17β-estro-
diol and testosterone were determined by radioimmunoas-
say (1, 7) in the non-hormone-supplemented nutrient me-
dium, in which hamster scent gland tumor explants could
be maintained for up to 5 weeks.

MATERIALS AND METHODS

Materials. Stage 1, 2, and 3 scent gland tumors from 34
Syrian hamsters (male and female) were induced by the
subpennicular implantation of TPR-DES pellets (30 mg
each). Stage 1 preneoplastic nodules were obtained from
scent glands treated for at least 150 days. Stage 2 and 3
tumors were obtained from scent glands treated for approx-
imately 250 days. Stage 3 tumors from 3 females and 1 male
were dissected aseptically and used for culture experi-
ments. All tumors were examined by light and electron
microscopy.

In Vitro Methods. Explants were grown on sponge foam
(Gelfoam; Upjohn Co., Kalamazoo, Mich.) matrices in an
organ culture system and on collagen-coated coverslips.1
Details of the culture techniques, condition of incubation,
monitoring, and tissue processing have been described pre-
viously (31, 32, 33). The nutrient medium used for explant-
ing and feeding the cultures contained: unfiltered fetal calf
serum, 40 ml (Reheis Chemical Co., Chicago, Ill.); Simms'
BSS, 35 ml; bovine serum ultrafiltrate, 25 ml (Microbiologi-
cal Associates, Inc., Rockville, Md.); dextrose, 3 ml (final
concentration, 6 mg/ml); insulin, 3 ml (final concentration,
0.09 unit/ml); low-zinc insulin, courtesy of Squibb Institute
for Medical Research, New Brunswick, N. J.); penicillin, 1
ml (final concentration, 50 units/ml); and Achromycin, 1 ml
(final concentration, 20 μg/ml). The hormone-treated cul-
tures were fed the above nutrient medium supplemented
with one of the following protocols: 0.1 μg TPR and 0.1 μg
DES per ml medium; 1.0 μg each of TPR-DES per ml me-
dium; or 5.0 μg each of TPR-DES per ml medium.

All cultures were fed biweekly. Those on collagen-coated
coverslips were maintained for 43 days, and viable cultures
on sponge foam were maintained for 36 days. Cultures from
both in vitro systems were sampled weekly and processed
for light and electron microscopy.

Light Microscopic Methods. Samples of the original tu-
mors were fixed in 10% neutral buffered formalin. Cultures
were first rinsed briefly in Simms' BSS and then fixed in
10% buffered formalin in alkaline Simms' BSS (sponge
foam cultures) or in Bouin's solution (collagen-coated
coverslips). Original tumors and sponge foam cultures were
embedded in paraffin; 6 μm sections were cut and stained
with hematoxylin and eosin, Bielschowsky's silver impreg-
nation for neurofibrils, Mallory's phosphotungstic acid-he-
matoxylin for glial fibers, Van Gieson's stain for collagen,
Wildor's silver impregnation for reticulin, Brown-Breen's
stain for keratin, and periodic acid-Schiff for glycogen. The
cultures on collagen-coated coverslips were stained with
hematoxylin and eosin.

Electron Microscopic Methods. The original tumors were
rapidly cut into 1-cu mm fragments fixed in a modification
of Ito-Karnovsky's fixative at 4° (10). Sponge foam cultures
were rinsed briefly in Simms' BSS at room temperature,
transferred to the chilled fixative for 30 min, and then
trimmed into 0.5- to 1-cu mm fragments for 3 to 4 hr. All
samples were postfixed in 0.2 M phosphate-buffered os-
mium tetroxide and dehydrated with progressive ethanol.
Some tissues were block-stained with 10% uranyl acetate in
methyl alcohol. All tissues were embedded in Epon 812.

RESULTS

Light Microscopic Observations

Original Tumors. The light microscopic description of the
appearance of the scent gland tumors in vivo at all stages
has been reported by Kirkman and Algard (23). They de-
scribed the cells as epithelial-like or spindle shaped, ar-
ranged in whorls or radial cords surrounded by connective
tissue stroma.

Stage 3 Tumor Explants on Sponge Foam Matrices. At
first the explants retained the appearance of the original
Stage 3 tumors (Fig. 1). By the 3rd week they showed focci
of cell loss replaced by relatively acellular collagen (Fig. 2).
The cells of the culture explants were spindle shaped or
ovoid (Fig. 3). The intercellular connective tissue showed
a relative increase of collagen and a corresponding decrease
of reticulin fibers in the 42-day-old cultures. No neuritic
structures were demonstrated by specific silver impregna-

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tion in either the original tumors or the explants grown on sponge foam matrices.

**Stage 3 Tumor Explants on Collagen-coated Coverslips.** By 3 weeks an extensive outgrowth zone made up of parallel arrays of cells was usually present (Fig. 4a). The cells were elongated (Fig. 4b) or ovoid (Fig. 4c); some of the explants contained many macrophages. Occasional whorls of cells were seen in the outgrowth zone (Fig. 4c). Intracytoplasmic eosinophilic inclusions were noted after 3 weeks in culture. The inclusions stained negatively with the Brown-Breen stain for keratin. These inclusions stained positively for periodic acid-Schiff, and the reaction did not disappear after diastase treatment.

**Electron Microscopic Observations**

**Original Tumors.** There were fine structural characteristics common to all 3 stages of the scent gland tumor. The cells had an irregular ovoid or spindle shape; they were joined by junctional complexes and demonstrated isolated segments of basal laminae.

Stage 1 microscopic nodules demonstrated a central core of cells surrounded by collagen fibrils and elongated cells (Fig. 5). The cells contained granular endoplasmic reticulum, ribosomes, mitochondria, microcystotic vesicles, microfilaments, and lysosomes (Fig. 6). Stage 3 neoplasms were composed of ovoid and spindle-shaped cells similar to those observed in Stage 1 nodules (Fig. 7a). Long cytoplasmic processes extended from the cells and sometimes were surrounded by collagen fibrils (Fig. 7b). Basal laminae were observed along the margins of some cells and microcystotic vesicles were apparent (Fig. 7c); junctional complexes were observed (Fig. 7d). The periodicity of collagen fibrils in Stage 1 microscopic nodules and Stage 3 neoplasms ranged from 500 to 1075 Å (Fig. 8).

**Cultures on Sponge Foam Matrices.** Certain characteristics of the Stage 3 tumors persisted throughout the time of culture. The cells were irregularly shaped (ovoid or spindle) and contained granular endoplasmic reticulum, ribosomes, Golgi apparatus, mitochondria, lysosomes, and microfilaments that measured from 64 to 125 Å in diameter; microcystotic vesicles were present along the cell surface. The cytoplasmic processes often interdigitated and intercellular junctions were present. The periodicity of collagen fibrils ranged from 400 to 900 Å. Fewer segments of basal laminae were noted in the cultured explants than in the original Stage 3 tumors.

Eight-day-old cultures resembled the original tumor (Fig. 9) except for the appearance of areas of amorphous intercellular material (Fig. 9, a and b). The explants retained the cellular characteristics seen after 8 days up to 36 days *in vitro*. By 36 days in culture most of the cells observed had become spindle shaped and many produced collagen (Fig. 10a); there was a marked increase in collagen fibrils (as much as 50% of the area in Fig. 10b in the plane of section is collagen).

**Cultures on Collagen-coated Coverslips.** Explants demonstrated ovoid and spindle-shaped cells. Since the eosinophilic inclusions observed by light microscopy first appeared around the 3rd week *in vitro*, 29-, 37-, and 43-day samples were studied by electron microscopy in order to characterize this material both in the nuclei and in the cytoplasm. Dense bands of microfilaments were found in the cytoplasm of some cells, and crystal-like particles were seen scattered diffusely or packed around empty spaces (Fig. 12); the particles ranged in size from 150 to 250 Å. Round to oval bodies (0.35 to 0.8 μm in diameter) were observed in the nuclei; some appeared similar to the type 1 nuclear bodies described by Bouteille et al. (6, 13). They were usually surrounded by a less dense halo of chromatin material and some, when cut to show the center, had a center that was less dense than the periphery (Fig. 11).

**Radioimmunoassay Studies.** Evidence from the microscopic studies of the cultured explants indicated that TPR-DES supplementation was not necessary for healthy growth of the explants in nonsupplemented medium. Radioimmunoassay of the unsupplemented medium showed that there were 110 pg of endogenous testosterone per ml of medium and slightly less than 100 pg of endogenous 17β-estradiol per ml of medium. These results suggested that the scent gland tumor could be maintained on minimal levels of TPR-DES under all growth conditions.

**DISCUSSION**

The electron microscopic and *in vitro* studies on the scent gland tumor of the Syrian hamster, described in this report, have given us a better understanding of the nature, uncertain until now, of the cell types of which this tumor is probably composed. By light microscopy, the evolution of the Stage 3 tumors maintained in organ culture systems provided no additional information, aside from the capacity of the explants to produce increasing amounts of connective tissue fibers and an apparent increasing conversion of reticulin fibers into collagen. The outgrowth of the explants cultured on collagen-coated coverslips showed a pattern of radiating spindle-shaped cells, which strongly argued against the epithelial nature of the tumor and favored either a mesenchymal or a Schwann cell origin. It is well known that, especially as regards the more anaplastic tumors, few or no morphological differences exist in tissue culture between poorly differentiated mesenchymal cells, such as malignant fibroblasts, and poorly differentiated Schwann cells.

By electron microscopy, the neoplastic cells, both in the original tumors and in the cultured explants, showed a number of fine structural features that most closely resembled those previously reported (9) in experimental poorly differentiated schwannomas induced in rats following transplacental tumor induction with ethylnitrosourea. Particularly noteworthy was the demonstration of frequent microcystotic vesicles along the plasma cell membranes, the presence of junctional complexes, and, especially, the presence of a patchy basal lamina. The increase of collagen fibrils in the late stages of cultures, noted in the present tumor, was similar to that described in the experimental rat schwannoma (9), both suggesting an increased interconvertibility *in vitro* of basement membrane material into mature banded collagen and confirming the now widely ac-
accepted concept that both normal and neoplastic Schwann cells are capable of manufacturing both reticulin and collagen (see discussion and references in Ref. 9). As previously discussed, the cells of the more differentiated of the nerves- or- induced neuroinomas approximate, in their electron microscopic features, normal Schwann cells, whereas the more anaplastic of the experimental nerve sheath tumors have features that recall some of those found in perineurial cells. It is apparent that, as far as the development of nerve sheath neoplasms is concerned, no morphological distinction can be made between Schwann cell and perineurial cell, which may represent purely functional variants of the same cell type. With respect to the experimental malignant rat schwannomas, however, observations on the early stages of tumor formation have established the origin of the tumors from the neoplastic transformation of adult Schwann cells. In the scent gland tumor, such an origin, determined by the presence of residual myelin sheaths or axons in the cytoplasm of the transformed cells, could not be determined in our studies.

Although the work reported above strongly supports the Schwann cell origin of the scent gland tumor, other evidence has been reported previously that argues for a possible epithelial contribution to the scent gland tumor. Kirkman and Algard (23) reported the close association of the developing Stage 3 tumors to the hair bulbs, and Kirkman and Dodge (26) reported that Stage 3 tumors did not develop from Stage 1 nodules when hair follicles were destroyed by $^{85}$Sr-β-ray irradiation. Native spaced collagen has been reported to have been formed by epithelial cells (8), and its presence in the scent gland tumor could be explained by its production by the epithelial cells in the tumor. The scent gland tumor appears to be a complex tumor of which one component is an endogenous androgen-induced nondifferentiated malignant schwannoma and another is an as yet unidentified epithelial component. Algard (4) and Kirkman and Algard (23) have discussed the implications of the fact that no hormones were needed for the growth of dispersed cell cultures of the scent gland tumor when grown on thin plasma clots, concluding that malignant cells might be able to grow in the living host because hormones rendered ineffective some control mechanism of the host. The present studies, in which we found that the tumors could be maintained and could multiply on the minimal amounts of endogenous androgen and estrogen found in the nutrient medium, lead us to suggest that the dispersed cell culture might have been maintained with the small quantities of hormones present in the plasma clot. It would appear then that the cells of the Stage 3 scent gland tumor are indeed androgen-estrogen dependent and have not reached the stage of hormone independence exhibited by other estrogen- and androgen-estrogen-induced hamster tumors (21).

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