Multifocal Vascular Tumors in Fowl Induced by a Newly Isolated Retrovirus

Dov Soffer, Nitzan Resnick-Roguel, Amiram Eldor, and Moshe Kotler

ABSTRACT

The morphological features of a spontaneous, multifocal vascular neoplasm of chickens are described. Histologically, the tumor was characterized by areas consisting of freely anastomosing vascular channels with prominent papillary appearance and lined by bland-looking endothelial cells, which alternate with areas resembling cavernous hemangioma. Occasionally solid areas composed of plump, pleomorphic cells were also present. Although there was no clear evidence for metastatic spread, some tumors were obviously invasive. Electron microscopy and immunohistochemistry confirmed the endothelial nature of neoplastic cells, demonstrating in particular pinocytic vesicles, well developed junctional complexes, fragmented basal lamina, occasional Weibel-Palade bodies, and patchy factor VIII-related antigen immunoreactivity. The overall appearance of the tumor was that of a cavernous hemangioma with prominent papillary endothelial hyperplasia.

RESULTS

Retroviruses are able to induce a variety of malignant and benign diseases in vertebrates and are the causative agent of several forms of human cancer and other diseases including AIDS (1, 2). Therefore retroviruses are being used as an excellent tool for the study of human tumorigenesis and other diseases.

A new field strain of AHV was isolated by us from spontaneous tumors in layer hens (3). This virus belongs to the ALV group and induces similar lesions in birds infected as embryos or on the day of hatching (3). The AHV provirus was cloned by a DNA recombination technique. Viral progenies rescued from quail cells transfected with viral DNA were found to be similar to the field isolate in their molecular and biological characteristics.

Avian hemangiomata virus induces cytopathic effect in cultured avian and mammalian cells including bovine endothelial cells. Treatment of cultured endothelial cells with AHV leads to cell perturbation as defined by: (a) platelet aggregation near or on the treated cells; (b) decreased prostaglandin I₂ secretion; (c) elevated expression of tissue factors; and (d) increased expression of interleukin 1 mRNA (4). All of these effects can also be induced by a UV-inactivated virus and by the viral isolated envelope glycoproteins.

This report deals with the morphological features of the vascular tumors caused by AHV and includes light microscopic, ultrastructural, and immunohistochemical observations. Preliminary light microscopic data were reported previously (3). Morphologically the tumor most closely resembled a cavernous hemangioma but also displayed prominent papillary endothelial hyperplasia as seen in angiosarcoma and some features reminiscent of KS.

MATERIALS AND METHODS

Animals. Fifteen clinically affected hens with spontaneous tumors were selected for morphological study. The hens were killed by ether inhalation and underwent complete autopsy.

Light Microscopy. All grossly identified lesions were sampled for histology. In addition, random samples were taken from grossly unaffected organs including lungs, heart, liver, spleen, kidney, gastrointestinal tract, eyes, brain, and spinal cord. Tissues were fixed by immersion in 10% buffered neutral formalin and processed routinely. Sections were stained with hematoxylin and eosin and selected cases were also stained with Masson's trichrome, elastica-van Gieson, Masson's reticulin stain, and Gomori's iron stain.

Immunohistochemistry. The indirect immunoperoxidase method was used on formalin-fixed paraffin-embedded tissue. Selected lesions were stained with the following antibodies: cytokeratin [monoclonal DPC, Los Angeles, CA], prediluted; epithelial membrane antigen [Sera-Lab, Cranley Down, Sussex, United Kingdom] diluted 1:200; vimentin [monoclonal (V-9); DakoPatts], diluted 1:10; factor VIII-related antigen [DakoPatts] diluted 1:100, 1:200; and laminin [Biomakor, Rehovot, Israel] prediluted and Ulex europeaus I lectin. The same antibodies (except U. europeaus) were also applied to cryostat sections of a few tumors and examined by immunofluorescence.

Electron Microscopy. Four cutaneous lesions (from 4 different hens) were sampled for ultrastructural study. The tumors were excised under deep ether anesthesia and immersed in a phosphate-buffered mixture of 4% formaldehyde and 1% glutaraldehyde. Following 24 h fixation, the tumors were cut into small tissue blocks and fixed in the same fixative for an additional 24 h. They were then washed in phosphate buffer, postfixed in osmium tetroxide, and embedded in Araldite. Sections 1 μm thick were stained with toluidine blue, and thin sections were cut from selected areas, stained with uranyl acetate and lead citrate, and viewed with Philips 300 and JOEL 100 XC electron microscopes.

Virus Morphology. CEF were prepared from SFAFAS eggs (R. F. D., No. 3, Norwich, CT) and grown in Dulbecco's modified Eagle's medium enriched with 10% tryptose phosphate and 10% heat-inactivated fetal calf serum. AHV was isolated originally from spontaneously lesions in layer hens, as described before (3). Cultures of chicken embryo fibroblasts infected with AHV were processed for electron microscopy. Cultured cells were fixed overnight in a phosphate-buffered mixture of 4% formaldehyde-1% glutaraldehyde, postfixed in 1% buffered osmium tetroxide, washed, and dehydrated. The pellet was embedded in Araldite, and thin sections stained with uranyl acetate and lead citrate were viewed with a JOEL electron microscope.

Virus Isolation. Virus was isolated by cocultivation of the tumors with CEF and was further cloned by end point dilution on QT-6 cells, as described before (3).

RESULTS

Clinical Data. During the years 1988-1989 outbreaks of vascular tumors occurred among layer hens in several farms in...
the Jerusalem area. The clinical features of the disease were practically identical to those reported in earlier outbreaks (3).

In brief, the hens became emaciated and anemic, had dull plumage, and displayed grossly visible hemorrhagic tumors over the skin. They ceased to lay eggs and usually died 1 to 4 weeks after clinical presentation.

**Tumor Site and Gross Appearance.** The number of lesions in each hen and their distribution are summarized in Table 1. The disease was usually multifocal, and most hens had more than one tumor. The skin was by far the most frequently involved organ (85% of the hens), followed by the muscles of the trunk and extremities (61%), liver (31%), and other organs. It should be emphasized, however, that visible skin lesions constituted a major criterion for selecting birds into the study; therefore skin lesions might have been overrepresented. There was no particular site of predilection in the skin, inasmuch as the tumors were evenly distributed all over the body.

Grossly, the typical tumor presented as a small, well-circumscribed red hemorrhagic nodule, varying in diameter from 2–3 mm to 1.5 cm (Fig. 1). Skin lesions appeared as bluish or violaceous plaques or nodules, often ulcerated and hemorrhagic. Occasionally, larger lesions measuring up to 4–6 cm in diameter were noted, which on cut sections were shown to consist of a large blood clot enshrouded by a thin fibrous pseudocapsule. In addition to vascular tumors, fresh and old hemorrhages were frequently noted in the skin, muscles, and viscera, and emaciation was sometimes extreme, resulting in complete disappearance of subcutaneous and mesenteric fat.

**Histological Appearance.** This histological features of the various tumors were quite similar. A typical lesion was nodular and well demarcated (Fig. 2). It was surrounded by an incomplete collagenous pseudocapsule which formed extensions that partitioned the neoplasm into several coarse nodules. The tumor architecture varied from area to area within the same neoplasm. Large areas were composed of vascular spaces lined by neoplastic cells. Some spaces were large, dilated, filled with blood, and lined by flattened, spindle cells with the general configuration of endothelial cells. The overall appearance of these regions resembled cavernous hemangioma (Fig. 3). Other regions displayed a prominent papillary appearance with freely anastomosing vascular channels, as seen in angiosarcoma or Masson’s “vegetant intravascular hemangioendothelioma” (intravascular papillary endothelial hyperplasia) (5, 6) (Fig. 4). Typically, the papillary projections consisted of a connective tissue core, with occasional hemosiderin-laden macrophages, lined by a single layer of bland-looking endothelial cells. A few tumors also contained foci of endothelial proliferation with little stromal support. Here, slit-like vascular spaces were lined by plump, slightly pleomorphic cells forming sponge-like meshwork (Fig. 5) or solid cell clusters (Fig. 6). The neoplastic cells had scant cytoplasm with indistinct cell borders and rather large, oval nuclei and inconspicuous nucleoli. Very rarely, mitotic figures were present (Fig. 7).

A noteworthy feature of the neoplastic disease was the proliferation of miniature blood vessels distant to the main tumor.

Table 1 Distribution and number of vascular tumors in affected hens

<table>
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<th>Hen</th>
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<th>Liver</th>
<th>Other organs</th>
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* Lesions examined ultrastructurally.
* Virus isolated from lesions.

Fig. 1. Gross appearance of an ulcerated skin tumor.

Fig. 2. Overview of a typical tumor showing large vascular spaces alternating with papillary areas. H & E, x 23.

Fig. 3. Large cavernous spaces in a skin tumor, one with organizing thrombus (top), bordered by a vascular sponge. H & E, x 16.

Fig. 4. Overview of a typical tumor showing large vascular spaces alternating with papillary areas. H & E, x 23.
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A mitotic figure is seen right of center. H & E, × 440.

Fig. 4. High power view of a papillary region. Vascular papillae are lined by bland-looking endothelial cells. Semithin Araldite section stained with toluidine blue, × 300.

Fig. 5. Details of the sponge-like meshwork shown in Fig. 3. Also shown are scar-like fibrous areas. H & E, × 70.

Fig. 6. Less-differentiated cellular area in tumor consisting of plump cells with little stromal support. H & E, × 275.

Fig. 7. A solid area composed of plump cells with vesicular nuclei and small nucleoli. A mitotic figure is seen right of center. H & E, × 440.

Fig. 8. Proliferating miniature blood vessels in the upper dermis accompanied by a few spindle cells and sparse mononuclear inflammatory infiltrate. H & E, × 80.

Fig. 8. Proliferating miniature blood vessels in the upper dermis accompanied by a few spindle cells and sparse mononuclear inflammatory infiltrate. H & E, × 80.

Fig. 9. A lesion similar to, but presumably in a more advanced stage than, the one shown in Fig. 8. Closely packed proliferating blood vessels occupy most of the upper dermis. H & E, × 160.

In most cases, the tumor was well circumscribed and noninvasive. Skin neoplasms were located predominantly in the dermis. The overlying epidermis was frequently ulcerated, but not directly infiltrated by neoplastic cells. A few tumors, however, displayed an obvious invasive growth pattern. This was characterized by the ingrowth of neoplastic vascular channels into neighboring tissues, including subcutaneous fat and striated muscle (Fig. 10). Similar aggressive growth was noted with hepatic tumors. Necrosis was never encountered.

Thrombi were commonly detected within the lesions, sometimes occupying a large segment of the tumor (Fig. 3). Occa-
Fig. 10. Tumor invasion of striated muscle tissue. Neoplastic vascular channels dissect through and between individual muscle fibers. H & E, x 80.

Fig. 11. Patchy weak, factor VIII-associated antigen immunoreactivity in endothelial cells lining papillary structures in the tumor. Indirect immunoperoxidase, x 63.

Fig. 12. Ultrastructural appearance of a vascular papilla from the area shown in Fig. 4. Note junctional complexes between neighboring endothelial cells (encircled) and thin cytoplasmic folds on the luminal surface. x 4,300. Inset, high power view of the area shown at the top to better illustrate the junctional complexes. Also seen are pinocytic vesicles and an incomplete basal lamina adjacent to a cell process (arrows). x 26,840.

Finally, the whole lesion consisted of a large organized thrombus surrounded by a thick fibrous pseudocapsule which contained small nests of proliferating neoplastic cells, hemosiderin- and lipid-laden macrophages and multinucleated foreign body giant cells. Presumably, those lesions represented "burnt out," regressed tumors which have been destroyed by a massive hemorrhage. Scar-like fibrotic areas were noted in many tumors (Fig. 5) and were probably the result of organization of a previous hemorrhage.

Only grossly identifiable lesions were proved to be vascular tumors on microscopic examination. Randomly sampled tissues from grossly unaffected sites failed to show any lesions.

Immunohistochemical Findings. The tumors did not stain with antibodies to cytokeratin or epithelial membrane antigen, although these antibodies stained the normal epidermis intensely. No staining of neoplastic cells was obtained with antivimentin or with U. europaeus, presumably due to the species specificity of these antibodies. Antibody to factor VIII-associated antigen decorated tumor cells that lined vascular channels. Although staining was patchy and weak on paraffin sections (Fig. 11), the staining and the number of positively stained cells increased considerably when cryostat sections were used. Unequivocal factor VIII-associated antigen was rarely identified in tumor cells distant from vascular spaces. Intense immunoreactivity was noted around most tumor cells as well as around normal endothelium with laminin.

Ultrastructural Findings. The cells lining well formed vascular channels and papillary projections possessed many of the features of normal endothelial cells (Fig. 12). Typically, they had thin cytoplasmic projections on the luminal surface. The cytoplasm contained small amounts of smooth and rough endoplasmic reticulum, free ribosomes, mitochondria, and scattered dense bodies. Many cells had cytoplasmic collections of thin and intermediate filaments (Fig. 13). Pinocytic vesicles were frequent, commonly oriented along the plasma membrane. Occasionally, rod-shaped Weibel-Palade bodies (7) were also present (Fig. 14). The nuclei were oval, with a generally smooth outline, euchromatic dispersed chromatin, and occasional prominent nucleoli. Fragmented basal lamina was attached to the abluminal surface of some cells. Usually the cells formed elaborate interdigitations with frequent junctional complexes of the zonula adherens type (Fig. 12, inset).

Tumor cells not immediately lining vascular spaces possessed many ultrastructural features in common with the neoplastic endothelial cells. Some had continuous basal lamina and few organelles which may represent pericytes. Other cells, located in solid differentiated regions, had features of smooth muscle cells. They displayed marked pinocytic activity and had abundant thin filaments condensed into bundles and focal densities (Fig. 15). Cells with ultrastructural features of fibroblasts were intermingled among the latter cells.

Viral particles were frequently observed in the tumors (Fig. 16). They appeared singly or in clusters in the extracellular space adjacent to the tumor cell membrane. The virions were spherical, measuring approximately 80 nm in overall diameter. They had an electron-dense, centrally located nucleoid which was surrounded by an inner and outer membrane (Fig. 16). Rarely, viruses with crescent-shaped nucleoid, budding from cytoplasmic membranes, were also noted (Fig. 17). These morphological features are characteristic of type C retroviruses (8-
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Fig. 13. Details of an endothelial cell cytoplasm. There are abundant filaments, a Golgi complex, mitochondria, polyribosomes, and a few pinocytotic vesicles. × 32,000.

Fig. 14. Weibel-Palade bodies in an endothelial cell lining a papillary structure, × 32,000. Inset, higher magnification of a Weibel-Palade body demonstrating the parallel striation in the "tubulated body." × 67,600.

Fig. 15. Smooth muscle differentiation in a solid area of the tumor. The cell is surrounded by a basal lamina and contains bundles of cytoplasmic filaments, numerous pinocytotic vesicles, and focal densities. × 8,300.

Fig. 16. Extracellular viral particles adjacent to a neoplastic endothelial cell. The virions have centrally located condensed nucleoid and an inner and outer membrane, typical of mature type C retrovirus. × 70,950.

DISCUSSION

An outbreak of vascular neoplasms reappeared last year at several fowl farms in Israel. As in previous outbreaks (3), the tumor was induced by a newly isolated retrovirus of the ALV group, first isolated and characterized by us (3).4 This study describes the morphological features of the retrovirus-induced spontaneous, multifocal vascular tumor.

The tumor occurred in various tissues and organs, most commonly in the skin. Histologically it was characterized by areas consisting of freely anastomosing channels lined by rather bland-looking endothelial cells alternating with areas resembling cavernous hemangioma and, occasionally, less differentiated foci composed of solid masses of plump, pleomorphic cells. The electron microscopic and immunohistochemical findings confirmed the vascular origin of the neoplasm and the endothelial nature of the tumor cells. These included in particular the ultrastructural demonstration of pinocytotic vesicles, the well developed junctions to neighboring cells, the microvillous projections into the lumen, the (incomplete) basal lamina and the occasional occurrence of Weibel-Palade bodies, which are pathognomonic of endothelial differentiation (7, 11–14).

The immunohistochemical demonstration, in some neoplastic
cells, of factor VIII-related antigen provide additional evidence for their endothelial origin (15, 16).

Histologically, the tumor most closely resembled cavernous hemangioma but also had features of well differentiated hemangiosarcoma. The latter included: (a) the occurrence of multiple lesions; (b) the presence of freely anastomosing vascular channels lined by endothelial cells; (c) the sponge-like appearance of less differentiated areas; (d) the evidence of tissue invasion; and (e) the lack of epidermal involvement by skin tumors (14, 17, 18). Angiosarcoma can assume extremely well differentiated morphology, such that confusion with hemangioma may be a problem (14, 19). Still, certain microscopic features of the avian tumors were rather unusual for hemangiosarcoma. These included: (a) lack of obvious atypia in the cells lining the vascular channels; (b) the extreme rarity of mitotic figures; (c) the lack of necrosis; and (d) evidence of tumor regression.

Data from this study correlate with our previous studies, in which the etiological agent of the disease was defined (3). A member of the ALV group was isolated from spontaneous tumors and cloned in tissue culture and by a DNA recombination technique. The cloned virus induced vascular neoplasms in fowl upon inoculation and identical virus were isolated from the induced tumors (3). In the present study a retrovirus was isolated from several tumors and typical type C retrovirus particles (8-10) were observed in the tumors by electron microscopy. These particles were identical to the viruses seen in CEF cultures infected with AHV.

We have shown previously that AHV lacks an oncogene in its genome. Therefore, tumor induction by AHV could be explained by at least two different mechanisms: (a) the presence of the provirus is essential for the neoplastic transformation of the cell; or (b) proviral integration is not required for transformation. Enhancer, promotion insertion, or alternative splicing may be the mechanisms of neoplastic transformation used when provirus is integrated into the cell genome. Although such mechanisms could be implicated for all cell types, the preferential induction of vascular tumors can be explained by tissue tropism determined by the unique envelope of the AHV (1). Alternatively, tumor induction may be a result of the cytopathic effect of the virus. In order to overcome cell damage, affected cells increase their mitotic rate, and this leads to a high frequency of somatic mutations resulting in cell transformation.

The occurrence of retrovirus-induced vascular tumors in fowl brings to mind the analogy with KS, the most common malignancy in patients with AIDS (21, 22). However, both tumors share only few morphological features. Both are multifocal vascular tumors of endothelial cell origin (22-24) which occur most frequently in the skin but which may involve other organs. A hemangioma-like pattern, as seen in the AHV-induced tumors, is not uncommon in KS and is sometimes the predominant histological feature (18, 25, 26). Also, the collection of proliferating miniature blood vessels in the upper dermis of chickens, at some distance from a main dermal tumor, has some similarity to early (patch stage) KS (18, 27, 28). Occasional spontaneous regression of vascular tumor, as described in our previous study (3), has been suggested here by the presence of organized thrombi and scars in the present study. Tumor regression may also occur in KS (29, 30) and is sometimes related to discontinuation of immunosuppressive therapy (31-33).

Epidemiological, immunological, virological, and morphological data suggest that KS is a viral-associated if not viral-induced tumor, especially in AIDS patients (20, 34, 35). There is also some evidence suggesting a role for human immunodeficiency virus 1 in KS etiology. It has been recently shown that introduction of the tat gene of the human immunodeficiency virus 1 germ line of mice produces skin tumors in the transgenic mice which resemble KS (36).

We have shown recently that AHV is able to induce cytopathic effect on cultured endothelial cells (5). Shortly after infection and prior to any recognizable damage, the cells produce high levels of tissue factor, interleukin 1, and mitogens when treated with either the virus or its isolated glycoprotein. Similarly, cultivated KS cells were shown to produce various amounts of lymphokines and growth factors (37). While the relevance of the AHV-induced tumors to KS remains uncertain, this tumor model provides an opportunity to study virus-induced neoplasms in general and vascular tumorigenesis and transformed endothelia in particular.

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REFERENCES

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