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Comparative Pathology and Pathogenesis of Spontaneous and Experimentally Induced Fibropapillomas of Green Turtles (*Chelonia mydas*)

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Abstract. Tumor biopsy samples from 25 Floridian and 15 Hawaiian green turtles (*Chelonia mydas*) with spontaneous green turtle fibropapillomatosis (GTFP) and from 27 captive-reared green turtles with experimentally induced GTFP were examined microscopically to differentiate the histologic features that result from GTFP pathogenesis and those that result from incidental factors that may vary according to geographic region. Common histologic features for spontaneous and experimentally induced tumors included fibroblast proliferation in the superficial dermis, epidermal acanthosis and hyperkeratosis, epidermal basal cell degeneration with dermal–epidermal cleft formation, spinous layer degeneration with intraepidermal vesicle and pustule formation, and ulceration. Visceral tumors, found in eight of 10 (80%) free-ranging turtles with cutaneous disease that were examined after death, had extensive interstitial fibrous proliferation. The presence of spirorchid trematode eggs and associated foreign body granulomas, common secondary findings within spontaneous tumors, varied by geographic location, and these findings were not observed in experimentally induced tumors. Eosinophilic intranuclear inclusions and intranuclear herpesvirus-associated antigen immunoreactivity were found in 18 of 38 (47%) experimentally induced cutaneous tumors and nine of 119 (7.5%) spontaneous tumors from Floridian but not Hawaiian turtles. The possible involvement of GTFP-associated herpesvirus in the pathogenesis of epidermal degenerative changes and GTFP pathogenesis is discussed.

Key words: *Chelonia mydas;* dermatitis; fibropapilloma; green turtles; herpesvirus; immunohistochemistry; tumor.

Green turtle fibropapillomatosis (GTFP) is a disease of green turtles (*Chelonia mydas*) that is characterized by multiple cutaneous papillomas, fibromas, and fibropapillomas, as well as occasional visceral fibromas. Concern over recent increases in the prevalence of GTFP around the world has focused attention on identifying the cause and understanding the pathogenesis of this disease so that management strategies can be developed to minimize its impact on populations of endangered green turtles.[18]

A thorough description of the disease process should identify the relevant histologic features seen in spontaneous lesions and distinguish these from features that are incidental or result from secondary processes. In addition, any proposed etiologic hypothesis should account for the major histologic features of the disease, and the pathogenesis of these lesions should be consistent with what is known about the pathobiology of closely related pathogens. Therefore, careful systematic evaluation of lesions from different populations can sometimes yield the etiologic agent but more effectively can eliminate candidates not present in all groups.

The first histologic descriptions of GTFP were published about 60 years ago.[41,58,59] Subsequently, Jacobson et al.[31] provided a more detailed description of light and electron microscopic features of GTFP. These basic histologic findings have been confirmed by others in scattered case reports and case series.[1,4,17,18,29,46,65] None of these studies compared tumors among geographically disparate populations. Furthermore, small numbers of spontaneous tumors have been used to speculate on developmental features over time.[31] These can be misleading because the clinical history of the turtles and the age of the tumors studied are unknown. No study to date has compared spontaneous tumors in

free-ranging turtles with experimentally induced tumors, in which careful observations of developmental changes could be made.

This article presents histopathologic descriptions of spontaneous GTFP from a case series of turtles from Florida and Hawaii and an analysis of associations among various epidemiologic and histologic features of these tumors. Comparing spontaneous tumors in these wild populations with a series of experimentally induced tumors provided criteria for judging stages in the pathogenesis of GTFP. This study also evaluated pathogen loads, investigated possible pathogenic mechanisms, and provided evidence that a novel herpesvirus may be responsible for many features of this disease.

Materials and Methods

Animals and tissue processing

Case material was collected from 25 green turtles that stranded alive or were captured by net in Florida waters (Florida Bay, Florida Keys, or Indian River), 15 green turtles from the Hawaiian Islands, and 27 captive-reared turtles with experimentally induced GTFP.[22,24] Multiple tumor samples were obtained from each turtle under local or general anesthesia using a 6-mm Keyes biopsy punch (Hawaiian turtles) or by surgical excision (Floridian and experimental turtles). Biopsy samples were fixed by immersion in neutral buffered 10% formalin. After samples were embedded in paraffin, serial 6-μm sections were prepared and stained with hematoxylin and eosin (HE), Gomori's methenamine silver, and periodic acid–Schiff. Additional serial sections were mounted on silanized slides for immunohistochemical testing.

Tumor scoring

A minimum of three serial histologic sections were examined from each tumor. Sections were scored for the presence or absence of epithelial hyperplasia (acanthosis or orthokeratosis), epithelial integrity (ulceration, focal cellular degenerative changes, or vesicle formation), inflammation (granulocytes, mononuclear cell infiltrate, or foreign body granulomas), and potential pathogens (virus inclusions, spirorchid trematode ova, bacteria, fungi, and epibionts). Statistical associations of selected histologic features were determined by chi-square analysis using a P value of 0.05 as the criterion for significance.[66]

Immunohistochemical testing

All cutaneous tumors from free-ranging turtles and those from 11 captive-reared turtles were screened for the presence of herpesvirus-associated antigens by immunohistochemical testing following previously described methods.[21] The assay used an antiserum (green turtle plasma) that, when tested on GTFP sections proven by electron microscopy to contain herpesvirus, showed specific intranuclear reactivity within foci of ballooning epidermal degeneration corresponding to areas containing eosinophilic intranuclear inclusions and virus particles.[19,21] Briefly, tissue sections (6 μm) were incubated with antiserum (diluted 1/50 in phosphate-buffered saline [PBS]) and then washed and incubated with secondary antibody, a mixture of two biotinylated monoclonal antibodies specific for green turtle immunoglobulin (Ig) light chain and 7S IgY heavy chain, respectively.[23] After washing, the sections were incubated with horseradish peroxidase–conjugated streptavidin (Sigma Chemical Company, St. Louis, MO). Immunoreactivity was detected by color development following immersion in substrate (3,3′-diaminobenzidine, Sigma). Sections were counterstained with Harris' hematoxylin.

The presence of immune complex deposition in epidermal vesicles was determined by direct immunofluoresence staining. Frozen sections (6 μm) from two cutaneous tumors with epidermal cleftlike separations were cut, fixed in acetone for 10 minutes, and incubated for 30 minutes with one of the following monoclonal antibodies to green turtle immunoglobulins: HL857 (7S IgY specific), HL814 (5.7S IgY specific), or HL846 (IgM specific)[23] diluted 1 μg/ml in PBS with 1% bovine serum albumin. Slides were washed for 30 minutes in three changes of PBS and incubated with fluorescein isothiocyanate–conjugated sheep anti-mouse Fab′ (Sigma) diluted 1/1,000 in PBS for 30 minutes. After being washed, slides were mounted and examined using a fluorescence microscope.

Results

Clinical features

Biopsy samples of 119 skin tumors were collected from 25 Floridian green turtles (Nos. 1–25). An additional 53 tumor samples were obtained from 15 Hawaiian green turtles (Nos. 26–40). Floridian green turtles ranged in size from 26 to >100 cm in straight carapace length (mean ± SD = 57.3 ± 20.9) and included four nesting adult females. Hawaiian turtles were all immature, ranging in size from 46.0 to 88.1 cm in straight carapace length (mean ± SD = 60.0 ± 13.2). One to 12 tumors from each Hawaiian turtle (mean ± SD = 3.7 ± 2.7) were sampled. Tumors collected from multiple sites included 11 eye (conjunctival) tumors. One to 20 tumors were sampled from each Floridian turtle (mean ± SD = 4.8 ± 4.5), and 12 eye tumors were included. On average, more sections were examined from each Floridian turtle than from each Hawaiian turtle, and samples from Floridian turtles were larger. Necropsy was performed on nine of 25 Floridian turtles and one of 15 Hawaiian turtles. Of these, seven turtles from Florida and one from Hawaii had firm white nodules affecting the lungs, kidney, gastrointestinal tract, or heart. In addition, for comparison, 75 biopsy samples (two to seven samples per turtle) of experimentally induced tumors from 27 captive-reared Floridian turtles (Nos. 41–67) were examined. Samples of experimentally induced tumors were obtained 1–28 weeks after first being detected grossly.[22,24] In addition, a kidney tumor found at necropsy approximately 3 years after inoculation was examined

Table 1. Relative frequencies of histologic features found in spontaneous and experimentally induced green turtle fibropapillomas.

Characteristic	Relative Frequency (%)		
	Spontaneous Tumors		Experimentally Induced Tumors
	Hawaii (n = 53)	Florida (n = 119)	(n = 75)
Histopathologic features			
Dermal proliferation	100	100	100
Acanthosis	96.2	93.3	100
Orthokeratosis	73.6	84.9	97.2
Basal cell degeneration	77.4	90.8*	73.3
Dermal–epidermal cleft	50.9	54.6	54.7
Spinous layer degeneration	15.1	25.2	78.7†
Intraepidermal pustule	17.0	18.5	30.1
Erosion or ulceration	35.8	38.7	44
Inflammation			
Granulocytes	32.1	47.9	53.3
Foreign body granuloma	50.9	40.3	0†
Perivascular lymphocyte	58.5	73.1	61.3
Potential pathogens			
Intranuclear inclusions	0	1.7	18.6†
Herpesviral antigens‡	0	7.5*	47.4†§
Spirorchid ova	50.9	30.3*	0†
Epibiota			
Bacteria and prokaryotes	66.0	46.2*	74.5
Fungi and algae	32.1	6.7*	
Metazoans	18.9	7.6*	1.3

* Tumors from Floridian turtles were significantly different from tumors from Hawaiian turtles (chi-square test).

† Experimentally induced tumors were significantly different from pooled tumors from Floridian and Hawaiian turtles (chi-square test).

‡ Findings of immunohistochemical tests (biotin–avidin–peroxidase complex method).

§ Thirty-eight experimentally induced tumors were tested.

from one turtle with experimentally induced disease (No. 50).

Histologic features of cutaneous tumors

Relative frequencies of histologic features of cutaneous tumors from the three populations are summarized in Table 1. Features that had statistically significant differences in relative frequencies between the two regions or between spontaneous and experimentally induced tumors are described below.

Cutaneous tumors were classified on the basis of gross appearance as either verrucous, with highly arborized papillary projections supported by an abundant fibrovascular matrix, or fibromatous, containing primarily fibrous connective tissue with a relatively smooth surface. However, even relatively smooth fibromas often had areas with some degree of epidermal folding detectable at the microscopic level. Tumors from the Floridian turtles had primarily highly arborized features (74.6%) compared to those from Hawaiian turtles (51.5%). This difference in relative frequency was statistically significant (chi-square = 8.24, $P < 0.005$).

Tumors were pigmented to various degrees, ranging from white or pink to gray-green and black. All pigmented tumors had dendritic cells with long, fine processes scattered throughout the dermis, concentrated in and around small arteries or in the stratum basale and spinosum of the epidermis. Heavily pigmented tumors had these dendritic cells in all layers and had free pigment granules scattered throughout the epidermis, whereas some tumors with unpigmented epidermis had pigment cells concentrated around deep dermal vessels. In most tumors, the pigment was always black. Normal, darkly pigmented skin from some areas also had these type of cells. Light green skin had aggregations of dendritic cells located in the superficial dermis that contained a pale gold-green pigment. These pigment cells were observed in few tumors.

Proliferation of fibroblasts in the papillary dermis was found in all cutaneous tumors. The dermal portion of tumors contained numerous well-differentiated fibroblasts haphazardly arranged in a dense ground substance composed of fine collagen bundles that could be easily differentiated from the underlying reticular dermis (Fig. 1). In some tumors, the ground substance was more myxomatous than fibrous, especially in the superficial layers. The superficial dermis was more densely populated with cells than deeper layers. Experimentally induced tumors were more highly cellular and had less extracellular matrix deposition. Small arteries and veins permeated the fibromatous portion of all tumors.

A number of epidermal changes were common features in the fibropapillomas. One or more of these features could be found to various degrees in any particular tumor regardless of site of origin. Compared with normal epidermis (Fig. 2), which was four to seven cells thick, tumors had varying amounts of epidermal proliferation, ranging from minimal (Fig. 1) to extensive (up to 30 cells thick) (Fig. 3). Acanthosis was observed in most (>93%) of the biopsy samples from each population. Orthokeratosis, in which the stratum corneum is thicker than surrounding normal skin without retention of nuclei, was also observed in the majority of tumors (Fig. 4), except for those arising from noncornified epidermis, such as the cloacal mucosa and conjunctiva (Fig. 3). When these cases were excluded from analysis, orthokeratosis was observed in 154 (89.7%) of the spontaneous tumors and 73 (97.2%) of the experimentally induced tumors. In several cases, hyperkeratosis was extreme and, when combined with papillary hyperplasia, led to retention of cornified inclusion cysts (Fig. 4).

Vacuolation of basal cell cytoplasm with individual

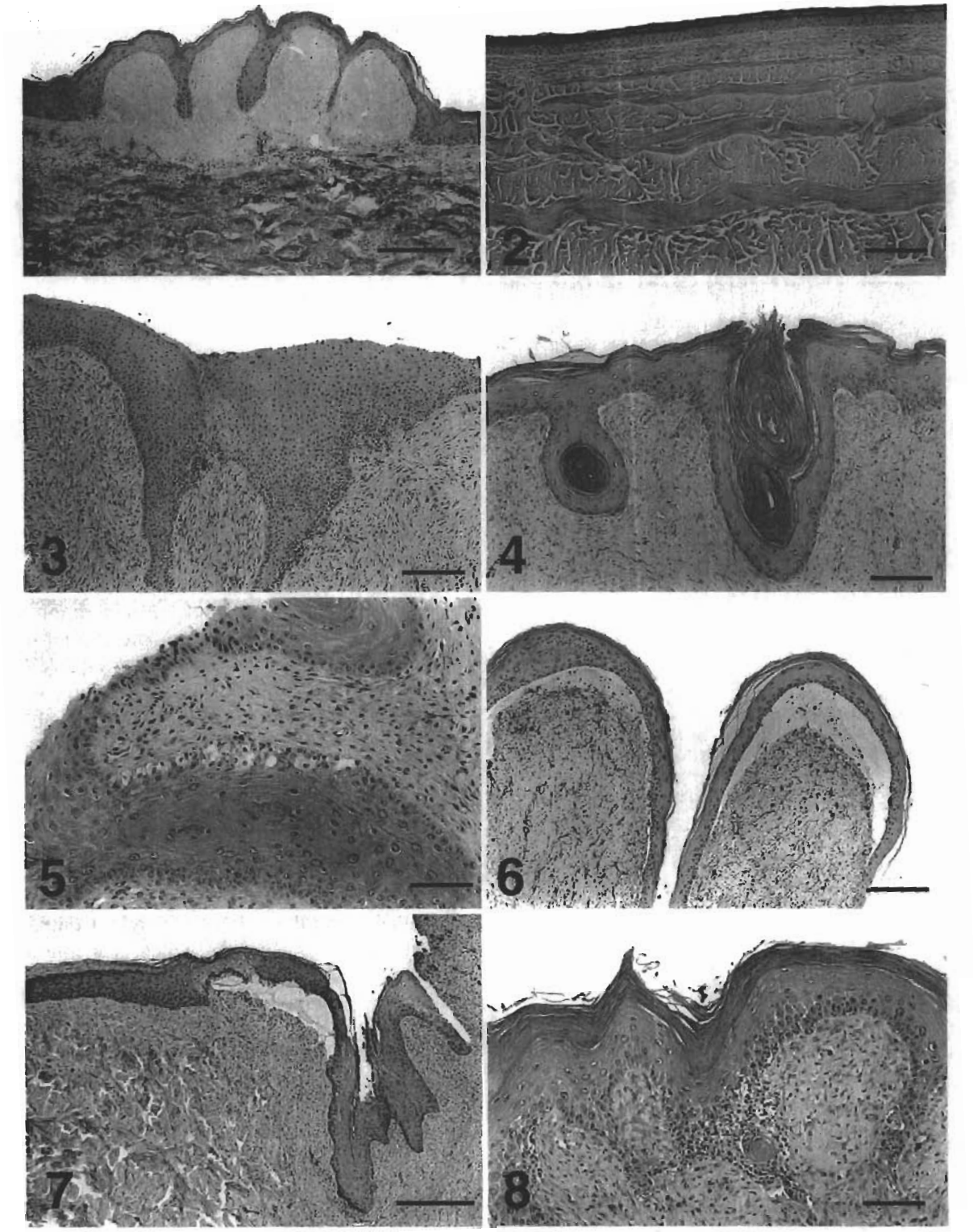

Fig. 1. Cutaneous fibropapilloma; green turtle No. 14. Papillary epidermal hyperplasia and fibrous proliferation in the papillary dermis with a sharp transition zone to underlying reticular dermis, which is infiltrated with mononuclear cells. HE. Bar = 300 μm.

Fig. 2. Skin; green turtle No. 46. Normal epidermis is four to seven cells thick, and normal dermis has a very thin papillary zone. HE. Bar = 200 μm.

Fig. 3. Conjunctival fibropapilloma; green turtle No. 6. Marked epidermal hyperplasia with absence of orthokeratotic hyperkeratosis. HE. Bar = 200 μm.

cell necrosis (Fig. 5) was a common feature found in 149 (86.6%) biopsy sections from free-ranging turtles and 55 (73.3%) sections from captive turtles, although significantly more Floridian samples (90.8%) had this feature than Hawaiian samples (77.4%) (chi-square = 5.63, $P < 0.025$). Sometimes, spongiotic changes in the stratum spinosum above locally extensive areas of basal cell degeneration were observed.

The degenerative changes along the basement membrane resulted in a separation between the dermis and epidermis (Figs. 6, 7). When this cleft became large, the epidermis above it underwent necrosis and ulceration. Cleft formation was observed in more than half of all biopsy samples. In both spontaneous and experimentally induced tumors, there was a statistically significant association of cleft formation with basal cell disruption (chi-square = 25.7, $P < 0.001$; and chi-square = 26.0, $P < 0.001$, respectively), but clefts also appeared to form at or below the basement membrane without adjacent basal cell degeneration. In some cases, separation of basement membrane from edematous papillary dermis (subepidermal cleft) was noted (Fig. 6). This was confirmed with periodic acid–Schiff staining of basement membrane. Clefts also resulted from disruption of the dermal–epidermal junction by lymphocytic infiltrates (Fig. 8).

Immunofluorescence labeling, with monoclonal antibodies to green turtle IgM, 7S IgY, or 5.7S IgY, did not detect antibody deposition in the stratum basale, basement membrane, or superficial dermis of frozen sections from two tumors containing clefts from two turtles (Nos. 17 and 40).

Cells in the stratum spinosum above locally extensive areas of basal cell degeneration contained solitary large, clear vacuoles that pushed the nucleus to the edge of the cell, giving it a crescent shape or perinuclear cytoplasmic clearing (edema) (Fig. 7). Degenerative changes in the upper layers of the stratum spinosum, however, were not always directly associated with basal layer changes or cleft formation (Figs. 9–11). These changes ranged from single-cell vacuolar changes described above (Fig. 9) to more extensive foci of reticular or ballooning degeneration, acantholysis, and intraepidermal vesicle formation (Fig. 10). In more advanced foci of degeneration and acantholysis, an extensive granulocytic infiltrate led to epidermal pustule formation (Fig. 11), which could progress to ulceration (Fig. 12). Erosions and ulcers were heavily infiltrated with granulocytes and were covered by cellular debris and desiccated proteinaceous material (Fig. 12).

Foci of severe ballooning degeneration and acantholysis were found in eight (15.1%) biopsy samples from Hawaiian turtles and 30 (25.2%) samples from Floridian turtles. Experimentally induced tumors had a significantly higher frequency (59 [78.9%]) of spinous layer degenerative changes compared to the series of spontaneous tumors (chi-square = 68.9, $P < 0.001$). Intraepidermal pustules were observed in nine (17%) and 22 (18.5%) spontaneous tumor samples from Hawaii and Florida, respectively, and in 23 (30.1%) experimentally induced tumors. In addition, focal to locally extensive cutaneous erosions and ulcers secondary to cleft formation or degeneration in the stratum spinosum were observed in 36–44% of samples examined in all three populations.

Taken together, one or more of these epidermal degenerative changes, including basal-layer degeneration, spinous-layer degeneration, cleft formation, and ulceration, were found in 158 (92%) spontaneous and 71 (95%) experimentally induced tumor biopsy samples. The remaining samples appeared to represent tumors with healed epidermis.

Several types of inflammatory cell infiltrates were found in cutaneous fibropapillomas. Granulocytic infiltrates were found in 74 (43%) samples from free-ranging turtles and in 40 (53.3%) samples from captive turtles. These infiltrates were composed primarily of heterophils (large cells containing numerous oval eosinophilic granules) and fewer eosinophils (large cells with clear cytoplasm and seven to nine large, round eosinophilic granules). These cells were difficult to distinguish in sections stained with HE. In spontaneous tumors, granulocyte margination within dermal ves-

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Fig. 4. Cutaneous fibropapilloma; green turtle No. 13. Marked acanthosis and orthokeratotic hyperkeratosis with cornified inclusion. HE. Bar = 200 μm.

Fig. 5. Cutaneous fibropapilloma; green turtle No. 12. Focal ballooning degeneration of basal cells and formation of dermal–epidermal cleft. HE. Bar = 100 μm.

Fig. 6. Cutaneous fibropapilloma; green turtle No. 17. Separation of epidermis from underlying dermis along the basement membrane with eosinophilic material accumulating within the cleft. HE. Bar = 200 μm.

Fig. 7. Cutaneous fibropapilloma; green turtle No. 57. Experimentally induced lesion showing epidermal–dermal cleft with focal degeneration in the superficial layers of the epidermis and eosinophilic material accumulated within the cleft. HE. Bar = 300 μm.

Fig. 8. Cutaneous fibropapilloma; green turtle No. 13. Spongiotic changes along the basement membrane with diffuse lymphocytic infiltration. HE. Bar = 150 μm.

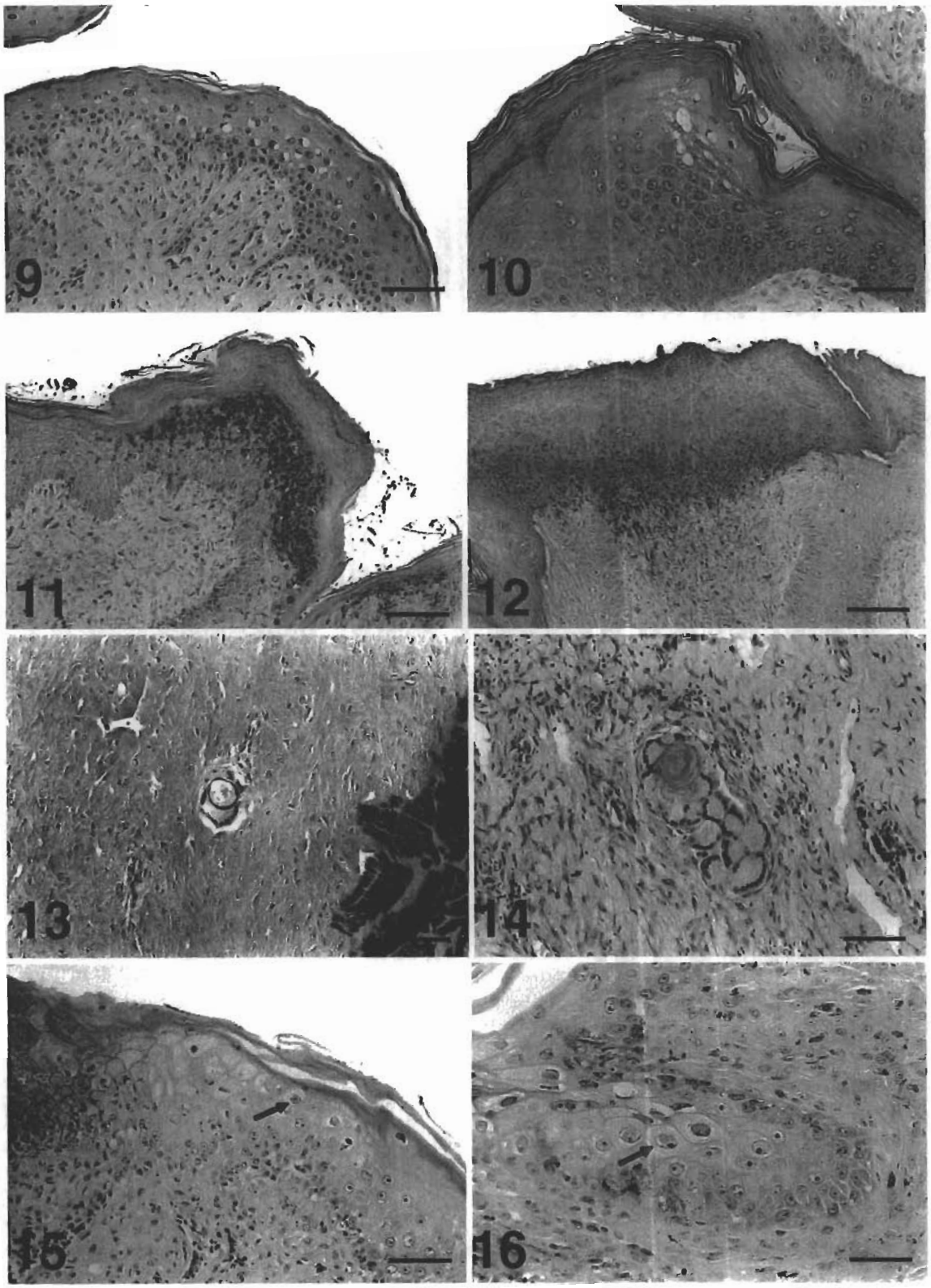

Fig. 9. Cutaneous fibropapilloma; green turtle No. 5. Vacuolar degeneration with displaced nuclei in spinous layer of the epidermis. HE. Bar = 100 μm.

Fig. 10. Cutaneous fibropapilloma; green turtle No. 46. Experimentally induced lesion showing spinous-layer degeneration and intraepidermal vesicle formation. HE. Bar = 100 μm.

Fig. 11. Cutaneous fibropapilloma; green turtle No. 46. Experimentally induced lesion with subcorneal granulocytic infiltration forming an intraepidermal pustule. HE. Bar = 100 μm.

sels, migration through the tumor, and aggregation were strongly associated (chi-square = 12.88, $P < 0.001$) with disruption of the integrity of the epidermis (i.e., erosions, ulcers, or intraepidermal pustules) (Figs. 11, 12). In total, 60 (81%) sections with granulocytic infiltrates also had erosions, ulcers, or pustules. Similarly, 56 (86%) sections with erosions or ulcers had obvious granulocytic infiltrates. In contrast, granulocytic infiltration was not associated with cleft formation in otherwise intact epithelium (chi-square = 3.73, $P > 0.05$).

Small foreign body granulomas were found in 75 (43.6%) biopsy samples from free-ranging turtles but not in any of those from captive-reared turtles. These consisted of multinucleated giant cells, histiocytes, and lymphocytes associated with the presence of either trematode eggs (Fig. 13) or cornified inclusion cysts (Fig. 14) in various stages of degeneration. One or the other of these two foreign bodies accounted for all granulomas observed. Spirorchid ova were found more commonly than cornified inclusions, in 64 (85%) sections that contained foreign body granulomas.

Extensive lymphocyte infiltration at the junction of normal reticular dermis and proliferating papillary dermis of the tumor was a common feature in sections where this area was represented (Fig. 1). Because many biopsy samples did not include the deep margins of the tumor, the relative frequency of this type of infiltrate could not be determined. There were various degrees of inflammation around dermal blood vessels, which were graded as follows: 0 = no inflammation, 1 = a few scattered lymphocytes but not enough to surround the vessels in the section, 2 = numerous lymphocytes completely surrounding vessels, and 3 = lymphocytes surrounding vessels and extending into surrounding connective tissue. Perivasculitis within the fibromatous portion of tumors tended to be mild (grade 1 or 2) and was observed in 31 (58.5%) Hawaiian, 87 (73.1%) Floridian, and 46 (61.3%) captive turtle samples. Perivascular lymphocytic infiltration was not associated statistically with severe degenerative changes in the stratum spinosum, ulceration, or the presence of spirorchid ova but was most strongly associated with cleft formation in both spontaneous (chi-square = 5.87, $P < 0.025$) and experimentally induced tumors (chi-square = 5.96, $P < 0.025$).

Eosinophilic intranuclear inclusions (Figs. 15, 16) were found in HE-stained sections of single biopsy samples from two wild Floridian turtles (Nos. 5 and 17), yielding a prevalence estimate of 8%. Inclusions were found within a focus of early spinous-layer degeneration containing swollen keratinocytes with perinuclear edema (No. 17) and within an area of ballooning degeneration adjacent to an ulcer (No. 5). Similar inclusions were not observed in samples from Hawaiian turtles. However, eosinophilic intranuclear inclusions were found in 14 (18.6%) experimentally induced tumors from 11 turtles (42% prevalence).

Immunohistochemical screening for herpesvirus antigens revealed that formation of eosinophilic inclusions is a relatively transient and late event in cellular infection, thus limiting the effectiveness of detecting viral inclusions through routine HE staining. Immunoperoxidase testing of 38 experimentally induced tumors from 11 turtles detected antigen in four additional tumors that did not contain inclusion bodies on HE staining. Intranuclear immunoreactivity was detected focally in the spinous layer, in keratinocytes undergoing early to advanced ballooning degeneration in nine (7.5%) spontaneous tumors from free-ranging green turtles from Florida, increasing the prevalence estimate for this population to 24%. In contrast, none of the 53 biopsy samples from 15 Hawaiian turtles were positive.

Spirorchid trematode ova (Fig. 13) were detected in 27 (50.9%) histologic sections of tumors from Hawaiian turtles and in 36 (30.3%) sections of tumors from Floridian turtles. These relative frequencies were significantly different (chi-square = 6.8, $P < 0.01$). The prevalence of spirorchidiasis, diagnosed microscopically, among Hawaiian turtles was 73.3%, whereas the prevalence among Floridian turtles was only 52%. These prevalences, however, were not significantly dif-

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Fig. 12. Cutaneous fibropapilloma; green turtle No. 46. Experimentally induced lesion with focal ulceration and heavy granulocytic infiltrate. HE. Bar = 100 μm.

Fig. 13. Cutaneous fibropapilloma; green turtle No. 4. Multinucleate foreign body giant cell containing a spirorchid trematode egg. HE. Bar = 100 μm.

Fig. 14. Cutaneous fibropapilloma; green turtle No. 31. Foreign body granuloma surrounding a cornified inclusion. HE. Bar = 100 μm.

Fig. 15. Cutaneous fibropapilloma; green turtle No. 5. Spontaneous lesion with eosinophilic intranuclear inclusions (arrow) within a focus of ballooning epidermal degeneration adjacent to an ulcer. HE. Bar = 75 μm.

Fig. 16. Cutaneous fibropapilloma; green turtle No. 51. Experimentally induced lesion with eosinophilic intranuclear inclusions (arrow) within swollen degenerating keratinocytes. HE. Bar = 50 μm.

ferent. Spirorchid ova were not present in experimentally induced tumors.

Bacteria were often found in the desquamating layers of the stratum corneum. Small colonies of cocci, as well large bacilli that formed long chains, were frequent between epidermal papillae. Ulcerated areas were frequently infected. These organisms were found significantly more frequently in Hawaiian (66%) than in Floridian (46.2%) samples (chi-square = 5.8, $P < 0.025$). Fungi or algae in the desquamating layers of the stratum corneum also were found in a higher percentage of Hawaiian (32.1%) than Floridian (6.7%) samples, and this difference was statistically significant (chi-square = 18.9, $P < 0.001$).

Two types of metazoan were occasionally identified on the surface of tumors. One type, found in two Floridian samples and interpreted to be a barnacle, was attached to smooth epidermis and had a broad base that separated the stratum corneum and reduced the epidermal thickness. This organism was heavily mineralized; thus, detail was poor. A thick, light pink band separated its base from the epidermis. The other type of metazoan was free-living and always located within folds of epidermis. These organisms had multiple legs and gills and other structures suggesting that they were arthropods, possibly crustaceans. There was a statistically significant positive association between the presence of these organisms and verrucous epidermis at the subgross level (chi-square = 4.65, $P < 0.05$). Even though the collection of biopsy samples from Florida contained more grossly verrucous tumors, the Hawaiian samples had a higher frequency of these free-living arthropods than the Floridian samples (18.9% versus 7.6%), and this difference was statistically significant (chi-square = 4.65, $P < 0.05$). Free-living surface arthropods were found on only one experimentally induced tumor.

Early pathologic changes

Twenty experimentally induced tumors were sampled within 1–2 weeks of becoming grossly apparent as raised plaques or sessile masses measuring <5 mm in diameter. This series of biopsy samples allowed us to recognize a progression in early tumor development. Compared to normal skin (Fig. 2), the earliest changes identified in this developmental series were proliferation of fibroblasts in the papillary layer of the dermis and mild proliferation of keratinocytes in the stratum germinativum (Figs. 17, 18). The dermal portions of these tumors were highly cellular compared to larger, older tumors. Superficially, the proliferating dermis resembled granulation tissue, with linear blood vessels aligned perpendicular to the skin surface (Fig. 18). As tumors enlarged, some developed papillary projections (Figs. 19, 20), whereas others remained relatively smooth. Within this set of relatively young tumors, degenerative changes could be detected in all but the earliest in the series. Sporadic basal cell degeneration was noted in 16 samples (80%), and in 13 of these, clefts had already formed at the dermal–epidermal junction. Degeneration in the stratum spinosum was noted in 14 tumors (70%) and had progressed in seven tumors to include either intraepidermal pustule or ulcer formation. Lymphocyte aggregates were noted at the deep dermal margins of 14 (70%) early tumors (Fig. 19), and in 11 (55%) cases, lymphocytic infiltration into the tumor stroma itself was noted. Eosinophilic intranuclear inclusions were found in four (20%) of these early biopsy samples and were associated with degenerative epidermal changes.

Older (>3 weeks) and larger experimentally induced tumors exhibited most of the proliferative and degenerative changes previously described, with no obvious correlation between various feaures and tumor size or age.

Histologic features of visceral tumors

Twenty-two visceral tumors were examined from seven free-ranging Floridian turtles (Nos. 3, 5, 6, 8, 9, 12, and 18). These included 11 lung, six kidney, two heart, one liver, one mesenteric, and one stomach mass sample. A sample of a small intestinal mass from one Hawaiian turtle (No. 39) was not diagnostic. A renal tumor from one experimental turtle (No. 50) was also examined. Visceral tumors were mostly smooth, firm, and white, but in one case, some lung nodules were gelatinous and translucent. Tumors were embedded within normal tissue parenchyma and appeared on gross examination to be well demarcated from surrounding tissue.

The common histologic feature of visceral tumors was extensive fibroblast proliferation with few mitotic figures. Abundant secretion of myxomatous ground substance appeared to be an early development, followed by deposition of fine collagen fibers in more mature tumors. In the lungs, extensive fibrosis occurred in the bronchial interstitium, and islands of normal bronchial epithelium became entrapped within the expanding fibroma (Fig. 21). Similarly, in the kidney, tumors began as extensive interstitial fibrosis that eventually separated and entrapped renal tubules (Fig. 22). Similar patterns of interstitial fibroplasia were seen in liver, stomach, and heart. Epithelial components involved in visceral nodules were histologically normal except for occasional foci of bronchial epithelial hyperplasia in some lung tumors. Occasional separations between bronchial epithelium and underlying fibroma were seen and could be attributed to edematous changes below the basement membrane. Trematode ova with associated foreign body granulomas

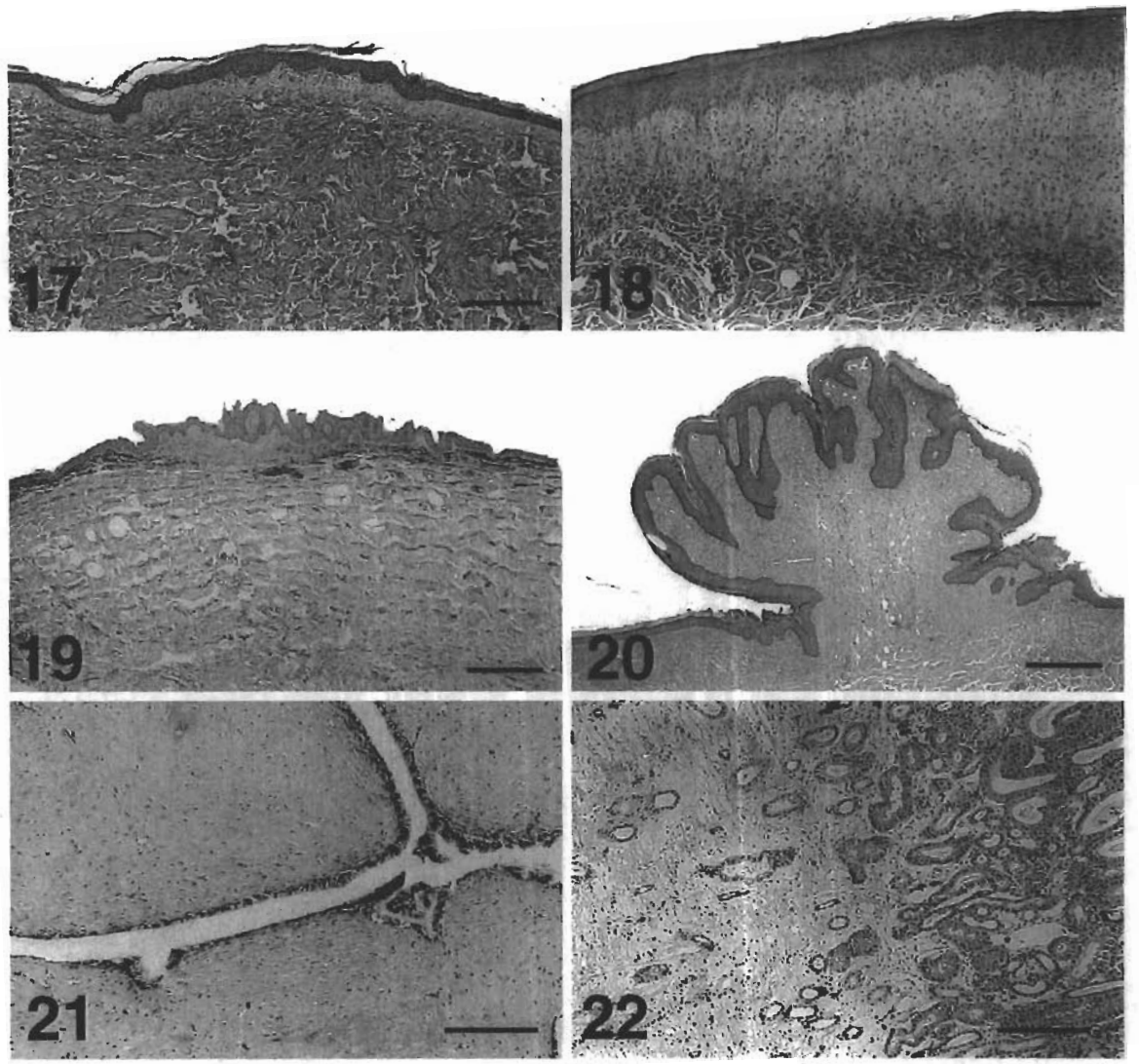

Fig. 17. Cutaneous fibropapilloma; green turtle No. 55. Early experimentally induced lesion (<1 week since detection) showing cell proliferation in the superficial dermis and mild epidermal hyperplasia. HE. Bar = 350 μm.

Fig. 18. Cutaneous fibropapilloma; green turtle No. 46. Early experimentally induced fibromatous lesion showing perpendicular orientation of dermal capillaries relative to the plane of tumor expansion, a feature shared with granulation tissue. Mild epidermal hyperplasia and perivascular and diffuse dermal lymphocytic infiltrate are also shown. HE. Bar = 200 μm.

Fig. 19. Cutaneous fibropapilloma; green turtle No. 53. Early experimentally induced lesion showing verrucous papillary epidermal hyperplasia and a heavy lymphocytic infiltrate at deep dermal borders of the tumor. HE. Bar = 700 μm.

Fig. 20. Cutaneous fibropapilloma; green turtle No. 51. Early experimentally induced lesion showing developmental progression to fibropapilloma; papillary epidermal hyperplasia supported on fibrovascular stalk. HE. Bar = 700 μm.

Fig. 21. Lung fibroma; green turtle No. 6. Bronchial epithelium entrapped within expanding fibrous tumor. HE. Bar = 200 μm.

Fig. 22. Kidney fibroma; green turtle No. 5. Transition from unaffected renal parenchyma to proliferating fibrous tissue showing entrapment of renal tubules within the expanding mass. HE. Bar = 200 μm.

were found both in visceral fibromas and adjacent normal tissue.

Discussion

The proliferative cutaneous lesions described here are consistent with those reported previously for GTFP.[1,4,17,18,29,41,46,58,59,65] This study is the first to compare experimentally induced and spontaneous tumors from turtles from two widely separated geographic regions to identify common features and possible pathogenic mechanisms. Experimental transmission studies provided a series of tumors of known age from indi-

viduals with known exposure history from which stages of progression could be described. Fibropapillomas developed in a manner similar to fibropapillomas in mammalian species.[34] Tumors begin as focal fibroblast proliferation in the superficial dermis, with some features consistent with granulation tissue. As these nodules enlarge, the epidermis also thickens and may become verrucous. Degenerative and lytic changes in the stratum basale and stratum spinosum develop later, although still early in the course of the disease, and lead to cleft and vesicle formation and ulceration, followed by local healing. Continued fibroblast proliferation may efface (stretch) the convoluted epidermis, resulting in a smooth, firm mass. Visceral tumors appeared to develop late (after 2 years) in the clinical course.

The series of spontaneous tumor biopsy samples shared all of the histologic features recognized in experimental tumors, including dermal fibroblast proliferation, epidermal proliferation with acanthosis and orthokeratosis, and epidermal degeneration. Statistical differences among biopsy series in the relative frequency of some of these histologic features can be attributed to differences in the age or stage of development of the tumors. Experimental tumors were sampled relatively early in their development and had the highest percentage of spinous layer degenerative changes. The lower percentage of tumor samples from Hawaii with epidermal folding and basal cell degenerative changes compared to Floridian samples may indicate that more samples from Hawaiian turtles were taken from older tumors with inactive or healing epidermal lesions, although this cannot be proven. Alternatively, some regional differences may result from sampling artifact. Sporadic lesions, such as focal basal layer changes, may have been underrepresented in Hawaiian samples, which were smaller (6-mm punch samples) than the samples from Florida (excisional samples).

The histologic features of GTFP described in this study may be caused by a single or multiple etiologic agents. The presence or absence of various potential pathogens in all three populations can define their role in the pathogenesis of GTFP. A potential etiologic agent should be found in all three populations to be implicated in the pathogenesis of GTFP. Trematodes are common in free-ranging turtles,[2,15,21] and the absence of spirorchid ova and associated foreign body granulomas in experimentally induced fibropapillomas suggests that these are incidental findings in spontaneous tumors. This conclusion is supported by the lack of association between antibodies to spriorchid trematodes and GTFP[21] and the successful transmission of this disease using homogenates from tumors lacking spirochid ova.[19]

Spirorchidiasis was diagnosed more frequently in Hawaiian samples than in Floridian samples, even though fewer and smaller biopsy samples were taken from Hawaiian turtles, suggesting that Hawaiian tumors had greater spirorchid ova burdens than Floridian tumors. In fact, egg counts from digests of Hawaiian tumors[10] do exceed those found for Floridian tumors (Greiner, personal communication). This regional difference may be due to the different species of spirorchids present,[9,16] higher fecundity among Hawaiian spirorchids, higher adult trematode burdens, or more persistent infections in Hawaiian green turtles.

Surface bacteria, fungi, and metazoans were recognized in all three population samples but were interpreted as incidental findings. Differences in the way turtles were handled may explain why biopsy samples from Florida had less evidence of surface organisms than Hawaiian samples. Most of the Hawaiian samples were collected in the field from free-ranging turtles that were captured, underwent biopsy, and were then released, whereas most of the Floridian samples came from stranded turtles that were brought to rehabilitation facilities for treatment. These turtles were often washed, and their tumors were scrubbed before surgery, thus reducing the number of surface organisms present.

Herpesvirus-like particles have been identified previously in spontaneous fibropapillomas from two Floridian green turtles[29] and in experimentally induced tumors.[22] In this study, eosinophilic intranuclear inclusions and herpesvirus antigens were found in experimentally induced tumors and in tumors from free-ranging Floridian turtles but not in tumors from Hawaiian turtles. This makes interpretation of the significance of herpesvirus, based on these data alone, difficult. Subsequent to this study, however, herpesvirus-like particles were found in other tumor specimens from Hawaiian turtles (T. Lipscomb, personal communication).[2] The quantitative differences in herpesvirus detection among sample populations may be explained by sampling errors or age and stage differences as discussed above. The negative cases are consistent with the pathogenesis of herpesvirus infection in vertebrate species, in which virions (and their antigens) are not present at all times during tumor development. Experimentally induced tumors were the youngest tumors sampled and had the highest frequency of intranuclear inclusions detected by HE and immunohistochemical testing. If virus production and shedding are early transient events in the progression of the disease, then evidence of active infection will decrease in older, larger tumors.

Successful experimental transmission of GTFP using cell-free tumor homogenates containing a filterable agent that is sensitive to organic solvents suggests that transmissible GTFP is caused by an enveloped virus.[24]

Coupled with evidence that turtles with GTFP develop antibodies to herpesvirus[21] and recent molecular biological evidence that novel alphaherpesvirus gene sequences can be detected by the polymerase chain reaction in a high percentage of fibropapillomas from both Florida and Hawaii but only rarely in unaffected tissues,[20,36,38,52] the histologic findings presented here support the hypothesis that the GTFP-associated herpesvirus is the etiologic agent of GTFP. However, the alternate hypothesis, that this herpesvirus is a secondary pathogen that preferentially infects and replicates in tumor tissue and as a result can be contransmitted with the true etiologic agent, cannot be completely ruled out.

To be the sole etiologic agent of GTFP, the herpesvirus must cause all of the key histologic features observed in this disease. The epidermal changes (acanthosis, degeneration, and necrosis in the stratum germinativum, followed by ulceration) found in all three sample populations are hallmarks of herpesvirus dermatitis[13,34,50] and have been observed in two other herpesvirus diseases of green turtles.[30,54]

Epidermal–dermal separation appeared to occur independently of degenerative changes observed in the stratum spinosum and is interpreted as a distinct process. The pathologic features of cleft formation are similar to those described for erythema multiforme in humans, which can occur as a sequela to herpesvirus infection.[8,27,56] These changes are nonspecific, however, having been observed in regressing papillomavirus-induced bovine fibropapillomas[40] and human warts.[62] Failure to identify the presence of immune complex formation, coupled with subsequent identification of a lytic herpesvirus infection, makes an autoimmune process unlikely. However, false-negative staining of clefts may occur,[61] and tests for other components, such as complement, which is possibly involved in immune-mediated cleft formation, were not conducted. Other processes leading to cleft formation, such as defective basement membrane attachment to the dermis, as seen in mechanobullous diseases, must also be ruled out.[8]

Epidermal keratinocyte and dermal fibroblast proliferation were consistent defining features of GTFP and were the earliest changes recognized in experimentally induced tumors. Epithelial proliferation has been associated with herpesvirus infections in other vertebrates, including several species of fish,[3] leopard frogs,[42] green wall lizards,[53] African elephants,[32] and immunosuppressed humans,[26,39,60,63] and is also a feature of the papular form of gray-patch disease, caused by chelonid herpesvirus 1, in green turtles after hatching.[54] Recently, herpesviruses have been implicated in the pathogenesis of mesenchymal tumors, Kaposi's sarcoma in humans,[6] and retroperitoneal fibromatosis in macaques.[55]

To be the sole etiologic agent of GTFP, herpesvirus would have to induce fibroblast proliferation, either via paracrine signals from infected epithelial cells[43] or via direct transformation of fibroblasts by either the presence of viral genes or by a hit-and-run mechanism.[11,12,14,57] In this study, herpesvirus antigens were not detected in visceral tumors or in cells below the basement membrane of skin tumors. Recently, however, detection by polymerase chain reaction of specific herpesvirus gene sequences in cultured fibroblasts derived from cutaneous fibropapillomas lends support to the hypothesis that fibroblasts are transformed by infection.[20] However, it is not known whether virus persistence is necessary to maintain the transformed phenotype.

On the basis of clinical observations of three captive-reared turtles (including No. 50), it appears that visceral fibrous tumors develop late in the course of disease. Visceral tumors could result from either metastasis from the primary cutaneous tumors (sarcomas) or multicentric infection during systemic virus dissemination, as seen in poxvirus infection in squirrels and rabbits[35,47] and papillomavirus infections in some cervids.[37,44] Molecular biological studies show that many visceral tumors are infected with herpesvirus.[20,36,38] The fact that tumor fibroblasts lack cytologic features suggestive of malignancy[25,49] favors the latter possibility.

Although a novel herpesvirus may alone account for most of the histologic features of GTFP described in this article, the evidence to date is insufficient to prove causation. Furthermore, GTFP may have a complex pathogenesis involving interactions among several viral agents. For example, the cellular proliferation characteristic of fibropapillomas may be caused by a different enveloped virus, such as a retrovirus like the one recently reported in Hawaiian green turtles.[5] Proliferating tumor tissue may provide favorable conditions for replication of a latent or coinfecting herpesvirus that causes the epidermal lesions described in this article. Another possibility is that a second transmissible agent may be needed to modulate herpesvirus gene expression or host responses, converting an otherwise inapparent or lytic herpesvirus infection into a tumorigenic one. Such a mechanism has been postulated for varicella-zoster–induced verrucous skin lesions in HIV-infected humans.[45,64] Related to this is the possibility is that a single transmissible agent, the GTFP-associated herpesvirus, may acquire and carry within its own genome portions of other virus genomes that have both *cis* and *trans* modulatory properties.[28,33] On the other hand, herpesvirus infection may be required to trigger or enhance the expression of other endogenous, latent, or coinfecting viruses (enveloped

and unenveloped), which themselves may carry the genes necessary for tumorigenesis or progression to malignancy.[7,12,48,51] Additional experimental work will be needed to resolve these questions. Only experimental induction of cutaneous fibropapillomas and visceral fibromas with purified infectious herpesvirus would fulfill Koch's postulates for this agent.

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