Studies on Canine Oral Papillomatosis

II. Oncogenicity of Canine Oral Papilloma Virus to Various Tissues of Dog with Special Reference to Eye Tumor

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Abstract. Canine oral papilloma virus (COPV) has been known to possess strict host and tissue specificities. The present study was undertaken to ascertain the oncogenic potential of COPV in a variety of tissues and organs of dogs. Inoculation with a phosphate buffered saline suspension of experimentally produced oral papillomas into the conjunctival epithelium of the eyelid induced tumors at the site of inoculation in 8 of 17 dogs. These tumors began to develop after an incubation period from 45 to 60 days, and attained maximum growth in 2 weeks. The general histological characteristics of the eye tumor were similar to those of oral papilloma. The lesions recovered spontaneously. With the filtrate of eye tumor emulsion, oral papillomas were induced in the oral mucosa of young dogs.

Papillomatous tumors were also induced experimentally in the eyelid and skin.

When administered with chemical carcinogen (MNNG), papilloma-bearing dogs showed a prolonged course of papilloma. Discussion was made on the progress of virus-induced papillomas.

In the course of the present experiment, a 5-year-old male mongrel dog was found to have spontaneous multiple growth of papillomatous tumors in the mucous membrane of the lips and right eyelid. Histologically, the tumors in these organs were papillomas and contained papilloma virus-like particles.

Infectious oral papillomatosis of dogs has been regarded as a self-limiting neoplastic disease caused by a canine oral papilloma virus (COPV), which is currently classified into the papovavirus group [18]. Naturally occurring growths are found usually on the inner surface of the lips of young dogs and rarely on the under surface of the tongue or the soft palate of the mouth. Experimentally, all the areas of the oral mucosa of young dogs have been known to be susceptible to the virus. In a few instances, papilloma developed in the skin and the muco-cutaneous junction of the eyelid [3]. The present study was undertaken to gain more comprehensive information about the oncogenic potential of COPV for a variety of tissues and organs of dogs.

Materials and Methods

Virus: The Miyazaki B strain of COPV was used.

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イヌ口腔内乳頭腫に関する研究 II. イヌ口腔粘膜以外の組織とくに眼における発病原性について: 時田尚志・小西信一郎（東京大学農学部家畜微生物学教室)
The details of the virus and the warts have been previously described [7]. The COPV inoculum was prepared from experimentally produced canine oral papillomas which had been stored in 50% glycerine-saline at 4°C or in frozen state at -70°C. Oral papillomas were ground in a mortar with phosphate-buffered saline solution (PBS, pH 7.2) containing penicillin and streptomycin. The final 5 or 10% emulsion of tumor tissue was clarified by centrifugation at 2,000 rpm for 10 minutes. Bacteria-free inocula were made by filtration of the resulting supernatant fluid through 220 nm Millipore filters. Both preparations, supernatant fluid and filtrate, were used as inocula in the experiments. They were frozen and stored at -70°C until use.

Dogs: The dogs used for most of the experiments were mongrels approximately 2-3 months of age. Some of them received canine distemper and infectious canine hepatitis vaccine 1 to 2 weeks before inoculation with papilloma virus.

Sites and methods of inoculation in the eye: The test animals were divided into 2 groups, 1 and 2.

In Group 1, 11 young dogs were inoculated with the supernate on the surface of the eyeball (the surface of the cornea and/or sclera) and the oral mucosa. They were anesthetized with pentobarbital and their eye surface was scarified with a surgical knife to such extent as to cause slight oozing of blood-tinged fluid. Inoculation was made at one eye, and the other eye was only scarified. For the purpose of confirming the infectivity of the supernate and susceptibility of dogs, these dogs were inoculated with 0.5 ml of the inoculum into the oral mucosa. Growth characteristics were compared between eye tumors and oral papillomas.

Group 2 consisted of 6 dogs. In it, 5 dogs were inoculated with the filtrate on the eye surface by the scarification method. The same material was titrated on the oral mucosa of these dogs. By the simultaneous-injection method, 0.1 ml of tenfold dilutions of stock virus was injected into multiple sites of the oral mucosa [7]. The endpoint of virus titration on the oral mucosa was determined as the highest tenfold dilution of virus capable of inducing a lesion at the site of inoculation.

The other 3 dogs of Group 2 were inoculated with the filtrate on the eye surface and into the anterior chamber of the eye. The injection of the filtrate into the anterior chamber was accomplished by passing a needle through the conjunctiva laterally to the limbus of the eye, and then medially and anteriorly through the sclera, the outer border of the ciliary body and the iris into the anterior chamber. After a small amount of the inoculum was injected into the anterior chamber, the needle was withdrawn, while it leaked the virus in its path. No inoculation was done to the oral mucosa of these dogs.

In order to determine whether the eye tumors contained infectious COPV or not, and to recover, if possible, the virus from these tumors, a dog of Group 1 was killed on the 90th day after inoculation. Its tumor tissue was harvested, ground and filtrated. Some young dogs were inoculated with the filtrate on the eye surface or into oral mucosa. Three dogs were inoculated with the filtrate in the left eye by the scarification method. Two dogs served as controls with the right eye left uninoculated. The same filtrate was titrated on the left and right side of the lip in 2 dogs and on the left lip in one dog. One of the former 2 dogs was inoculated with COPV on the right side of the oral mucosa and in the right eye in order to determine its susceptibility to the virus.

Joint action of chemical carcinogen and COPV: An experiment was carried out with 4 dogs to test the joint action of chemical carcinogen and COPV on the oral mucosa and eye. The experimentally induced canine oral papilloma was benign and the oncogenic potential of the virus to various tissues was weak. From these results it was considered that an additional factor or condition was necessary for papilloma to progress into cancer and for the oncogenic potential of the virus to various tissues to increase.

As a chemical carcinogen, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) was used. Four young dogs were inoculated with the virus on the surface of the eye by scarification and into the oral mucosa. When papillomas appeared in them 28 days post-inoculation, all of them, but one (Dogs 1021, 1023, and 1025), were given 50 mg of MNNG in drinking water ad libitum daily for 40 days. After that, they were given 50 mg of MNNG every other day over a period of 24 days. On day 64, the administration of MNNG solution was discontinued and the solution replaced by tap water. The remaining dog (No. 1024) was given tap water only to serve as a control.

Sites of inoculation in other tissues and organs: Some dogs were inoculated with the virus on the skin by the scarification method. Four dogs were inoculated into the meninges through a trephine opening 5 to 10 mm in diameter of the frontal bone. The intact meninges were scarified and 0.5 ml of inoculum was injected into and onto them. In 2 dogs, the abdominal viscera were exposed surgically, and the stomach and bladder were inoculated with 0.5 ml of COPV on the inner wall. Inoculation was also done routinely to the oral mucosa in these dogs.

Observation: After inoculation, observation was
performed routinely at the sites of inoculation in the dogs every day. Specimens were collected from induced tumors, fixed in 10% formalin, embedded in paraffin and cut into sections, which were stained with hematoxylin and eosin for microscopic examination. The dogs were sacrificed for necropsy after the oral papillomas had regressed.

**Results**

**Eye tumor**

Findings in Group 1: The scarified areas of the eye healed soon and left nothing approximately 7 weeks post-inoculation. Tiny white, smooth elevations developed at the sites of inoculation in 6 of 11 animals after an incubation period of from 46 to 60 days. They increased slowly in size. Ordinarily, they were 1 to 3 mm in both height and diameter 3 weeks later, as shown in Figs. 1 and 2. All the lesions having appeared in 5 dogs were similar to one another in gross appearance. They were small, white, discrete nodules varying only slightly in size.

The inocula were placed in the oral mucosa, as well as on the surface of the eye, to study the relative susceptibility of dogs and tissues exposed. As a result, oral papillomas were produced in all the animals. Their incubation period ranged from 25 to 40 days, and their duration from 29 to 56 days. The incubation period for the appearance of oral papillomas was shorter in dogs which developed eye tumors than in those free from eye tumor formation.

In only one dog, eye tumors continued slowly to enlarge and fused with one another finally to form cauliflower-like tumor 5 weeks after their appearance. In this tumor, individual tumors reached a diameter of 6 mm and gave rise to a serious inflammatory reaction in the conjunctiva. Eyesight was lost on the tumor-affected side. Regression occurred to the mouth tumors up to the 56th day after appearance.

In 4 dogs, the lesions healed spontaneously, and regression occurred later to the eye tumors than to the oral papillomas.

Findings in Group 2: Of 3 dogs inoculated with COPV filtrate in the eye, one developed tumors in both eyes after an incubation period of 75 days. These tumors regressed spontaneously. The other 2 dogs failed to develop eye tumors. COPV was titrated in the oral mucosa in all the dogs. In dogs bearing eye tumor, oral papillomas appeared at the sites where 1:50, 1:500 and 1:5,000 dilution of the inocula had been inoculated. On the contrary, in 2 dogs which failed to develop papilloma at the eye, oral papillomas appeared only at the sites where 1:50 dilution of the inoculum had been inoculated.

When inoculated with virus on the eye surface and into the anterior chamber, 3 dogs had clear eyes presenting no evidence of bacterial infection 24 hours after inoculation. One of them which had received COPV developed tumor on the eye surface. No changes occurred to the anterior chamber in any dog. This tumor was formed after an incubation period of 68 days and persisted for 1 week. No tumor appeared on the oral mucosa in any dog. The results obtained from Group 1 and 2 are summarized in Table 1.

**Joint action of chemical carcinogen and COPV**

Chemical carcinogens were examined for effect on oral papilloma and eye inoculation. Oral papillomas appeared in 3 inoculated dogs during a period from the 22nd to 26th day after inoculation. After they developed, the 3 dogs were given MNNG solution. The oral papillomas of the treated dogs showed the usual growth characteristics and gross appearance during the first month. Then cauliflower-like
Table 1. Oncogenicity of canine oral papilloma virus to the eye of dogs

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Inoculum</th>
<th>Number of inoculated</th>
<th>Number of dogs positive for tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Eye surface</td>
</tr>
<tr>
<td>1</td>
<td>Supernate</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>Filtrate</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Filtrate</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2. Results in MNNG-administered dogs

<table>
<thead>
<tr>
<th>Dog No.</th>
<th>Incubation period of papilloma (days)</th>
<th>Existence period of papilloma (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNNG*</td>
<td>1021</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>1023</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>1025</td>
<td>22</td>
</tr>
<tr>
<td>Control</td>
<td>1024</td>
<td>26</td>
</tr>
</tbody>
</table>

Remarks.
*: N-methyl-N'-nitro-N-nitrosoguanidine.

Table 3. Virus recovery from eye tumor

<table>
<thead>
<tr>
<th>Dog No.</th>
<th>Oral mucosa</th>
<th>Eye surface $5 \times 10^{-1}$*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$5 \times 10^{-1}$</td>
<td>$5 \times 10^{-2}$</td>
</tr>
<tr>
<td>1 Right</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>—</td>
</tr>
<tr>
<td>2 Right</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>+</td>
</tr>
<tr>
<td>3 Right</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>+++</td>
</tr>
</tbody>
</table>

Remarks.
+++: Confluent papillomas.
++: Semiconfluent papillomas.
+ : 2–10 papillomas.
+: 1 papilloma.
— : Negative.
(—): Control.
*: Virus dilution.

Tumors developed one month after their appearance. In untreated control dogs, papillomas regressed and disappeared 45 days after their appearance.

The papillomas of the treated dogs, however, did not regress during the usual regression period. In dog No. 1021, tumors began to regress on the 82nd day, regressed slowly for about one month, and disappeared completely on the 117th day after their appearance. The papillomas of dog No. 1023 began to regress on the 211th day after their appearance. The regression period was 2 months in this dog. In dog No. 1025, regression occurred to papillomas on the 387th day after their appearance. These
results are shown in Table 2. No eye tumor appeared in these dogs. No tumor developed in the oral mucosa in the dogs administered with MNNG only. No remarkable changes were observed in any other organ or tissue in these dogs.

**Virus recovery test of eye tumor**

The filtrate of papilloma tissue collected from the eye induced tumors in the oral mucosa, but not in the eye, in 2 dogs. The endpoint of virus titration ranged from $5 \times 10^{-1}$ to $5 \times 10^{-2}$ in the 2 dogs. The filtrate of oral papilloma induced tumors in the oral mucosa and eye of dog No. 3. The results are indicated in Table 3.

**Eyelid tumor**

During the period of COPV inoculation on the surface of the eyeball by scarification, the eyelid was sometimes injured with the surgical knife and infected with the virus. Seven out of 17 dogs developed tumors at the mucocutaneous junction of the eyelid, where small white or grey nodules increased slowly in size to form rugose papillomas which resembled those developed on the oral mucosa. These eyelid tumors regressed in 2 to 4 weeks after their appearance. Several of them are shown in Figs. 1 and 3.

Other tissues and organs

When some puppies were inoculated with the virus into the scarified skin of the face, papillomas appeared in one of them (Fig. 4). These skin papillomas were white, firm, circumscribed dermal nodules approximately 1 to 2 mm in diameter. None of them, however, developed on the abdomen or back of any inoculated puppy.

The brain, bladder, stomach and rectum were all refractory. No lesions were caused by COPV in the genital organ of any susceptible dog, even by injection or by rubbing into the scarified surface. In addition, no significant gross lesions were observed in any other organ. No lesions resulted either from experimental intravenous inoculation with the virus in any dog. The results obtained are summarized in Table 4.

**Eyelid and oral tumors spontaneously developed in a dog**

In the course of the present experiments, a 5-year-old male mongrel, which had been

### Table 4. Oncogenicity of canine oral papilloma virus to various tissues of dogs

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Total number of inoculated dogs</th>
<th>Number of positive dogs</th>
<th>Tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral mucosa</td>
<td>45</td>
<td>45</td>
<td>Papilloma</td>
</tr>
<tr>
<td>Skin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>abdominal</td>
<td>5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>back</td>
<td>5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>face</td>
<td>5</td>
<td>1</td>
<td>Papilloma</td>
</tr>
<tr>
<td>Eye</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>surface</td>
<td>17</td>
<td>8</td>
<td>Papilloma</td>
</tr>
<tr>
<td>eyelid</td>
<td>17*</td>
<td>7</td>
<td>Papilloma</td>
</tr>
<tr>
<td>anterior chamber</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Genital organ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vagina</td>
<td>4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>penis</td>
<td>4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Stomach</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>4</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

* Remarks:
  *: Injured with surface inoculation.
reared at a separate laboratory in the Tokyo area, was found to have spontaneous multiple papillomatous growths on the mucous membranes of lips and right eyelid (Fig. 5). These papillomatous growths gave neither apparent discomfort to the animal nor evidence of any generalized illness. The eye surface, genital mucous membranes and skin remained free of abnormal changes. These tumors disappeared without any treatment in one month.

When examined carefully, that male dog had 5 tumors on the oral mucosa. These papillomatous growths were 10–20 mm in diameter, white in color, bud in shape, and soft in consistency, with putrid smell (Fig. 6). Under the right eyelid, there was a white solid tumor. It was about 10 mm in diameter, well keratinized and had an irregular surface, being often split.

Portion of the eyelid and some oral tumors were excised for biopsy and used for histopathologic and electron microscopic examinations. Each oral tumor had a well-keratinized, hyperplastic epithelium and large swollen cells. It was similar to experimentally induced canine oral papilloma, and identified histopathologically as this tumor. The palpebral tumor consisted of a hyperplastic epithelium and a connective-tissue framework which supported this epithelium. The palpebral growth was also identified as papilloma.

Electron microscopy of oral and palpebral papilloma revealed large aggregates of particles in the nuclei of cells in the superficial keratogenic zone. The center-to-center distance of particles in well-formed crystals and the diameter of individual particles in such arrays were approximately 49 nm (Figs. 7 and 8).

Discussion

Papilloma developed on the eye surface in dogs which had been inoculated with a papilloma emulsion or filtrate. The appearance of this tumor at the site of inoculation can leave no doubt that the tumor was experimentally produced by COPV. The proliferative papillomatous lesion induced by COPV in the eye of the dog was classified into benign papilloma. These results indicated that the conjunctiva of the eyeball was susceptible to the virus, since eye tumor appeared in 8 of 17 dogs inoculated with the virus, even if a long incubation period was needed.

It is evident that the eye surface is less susceptible to COPV than the oral mucosa. It was presumed that the conditions responsible for the long growth period between the appearance and the beginning of regression of oral papilloma might favor the growth of eye tumor. Eye tumor was histologically regarded as benign tumor which regressed spontaneously, although it exerted a deleterious effect on the eye sight in animals. As to spontaneously developed eye tumor in dogs, its pathological and clinical findings have been reported, but it is yet unknown whether COPV is present in it or not. Therefore, the role of the virus in the etiology of such tumor must be speculative at this time.

The filtrate of tumor tissue of the eye surface induced papilloma on the oral mucosa, but not on the eye surface, in susceptible dogs. It was supposed that the eye tumor might have contained only a small amount of virus.

Anyway, in the spontaneous palpebral tumor, the characteristic cellular changes, the process of spontaneous regression, and the morphology and aggregation of particles contained in tumor cells were strikingly
similar to those described in the oral papilloma [3, 7]. On the basis of these findings, it was assumed that the particles described above might probably be those of papilloma virus and represent etiological agents of canine palpebral papilloma.

For over 40 years it has been known that canine oral papilloma is caused by an infectious filtrable agent. Although it was proved that a virus had induced benign oral tumor in dogs, little attention has been paid to the oncogenic potential of the virus until recently. On the other hand, there are many papers on naturally occurring canine neoplasms, especially spontaneous papilloma which frequently occurs to the skin and mucous membrane. Nevertheless no etiological relationship has been made clear between these types of tumors and COPV.

Ajello and Gimbo [1], however, reported a cutaneous transmission of COPV in one of 3 experimentally inoculated dogs. Recently, Watrach [17] observed viral particles resembling those of papilloma virus in naturally occurring skin papilloma in 2 dogs. In the present experiments, COPV induced papilloma on the oral mucosa, eye surface, eyelid and skin. In addition, papilloma virus-like particles were observed in naturally occurring oral papilloma and palpebral tumor. On the basis of these results, it was indicated that COPV had oncogenicity to the skin and some other tissues of dogs.

Infectious papilloma has been found on the skin of many animals and on the oral mucosa of dogs and rabbits. Its etiology, papilloma virus, is known to possess a strict tissue and host specificity. For this reason, it has generally been known as a virus which induces only wart. At present, it is divided into 2 types, a cutaneous and a mucosal type, depending on the target tissue. In the cutaneous type, there are bovine, equine, human and Shope papilloma viruses, and in the mucosal type, canine and rabbit oral papilloma viruses. Shope papilloma and equine papilloma are confined to the skin, and oral papilloma of rabbits is to the oral mucosa. Bovine papilloma virus, however, evokes a prominent cutaneous fibroblastic proliferation in cattle. Besides, it can induce fibroblastic tumor of the brain, fibro-papilloma of the genital mucosa and polyoid tumor of the urinary bladder in the original host [6, 8–10]. In man, papilloma was reported to develop on the skin, oral mucosa, eyelid, vaginal mucosa and the mucous membrane of the penis. Little attention has been paid, however, to the oncogenic potential of the papilloma virus group to various tissues in the host.

The frequency of appearance of carcinomatous changes of Shope papilloma in the domestic rabbit has attracted attention of investigators. The incidence of papilloma-derived carcinoma has been reported to be approximately 75% in domestic rabbits and about 25% in wild rabbits [11]. Little attention has been paid to the occurrence of such malignant changes in any other viral papilloma, except human viral papillomatosus disease, in recent years. Canine oral papilloma was regarded as benign neoplastic disease, but Watrach found a malignant progression of naturally occurring papilloma in a Beagle dog in 1970 [17].

The carcinogenicity of MNNG as a potent mutagen was proved in 1966 by Druckrey et al. [4], Schoental [12] and Sugimura et al. [13]. Adenocarcinoma was produced in the glandular stomach of rats and hamsters by oral administration of MNNG solution in drinking water [5, 14]. In 1970, Sugimura et al. [15] reported the produc-
tion of carcinoma in the stomach of dogs. In the present experiment, no progression of papilloma into cancer occurred. The duration of papilloma in MNNG-administered dogs extended to 117 to 337 days, although that of oral papilloma experimentally induced in young dogs was usually 40 to 50 days [7].

The clinical case which Watrac found in a Beagle dog had been treated with papilloma vaccine. The effect of MNNG or the vaccine on the duration of papilloma is not known at this time. It seems reasonable to hypothesize that a break down might have occurred to the immune response of the dog and contributed to the malignant transformation of a usual benign neoplastic process. In other words, papilloma virus has an oncogenicity to some tissues other than oral mucosa, and may initiate malignant neoplasia in some tissues under conditions of immunologic collapse. The exact role of COPV in the etiologies of canine neoplasms is still to be determined.

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References
CANINE ORAL PAPILLOMATOSIS. II


Explanation of Figures

Fig. 1. Experimentally produced small tumors on the eye surface and mucocutaneous junction of the eyelid of young dog.
Fig. 2. Section of an experimentally produced papilloma on eye surface.
Fig. 3. Experimentally produced palpebral papilloma of young dog.
Fig. 4. Papillomas developed at the site of inoculation on the skin of nasal region.
Fig. 5. Naturally occurring papillomas on the oral mucosa and mucocutaneous junction of the eyelid of adult mongrel dog.
Fig. 6. Naturally occurring papillomas on the oral mucosa of adult dog.
Fig. 7. Numerous closely-packed aggregate of virus-like particles observed in naturally occurring palpebral papilloma.
Fig. 8. Aggregates of virus-like particles of naturally occurring oral papilloma.