

amino acid composition, amino acid ratios, and CD spectra. By these criteria, all phytochelatins were indistinguishable from those isolated from *R. serpentina*.

The induction of the peptides by Cd^{2+} was also observed in cell cultures of *Anethum graveolens*, *Berberis stolonifera*, *Catharanthus roseus*, *Fumaria parviflora*, *Galium mollugo*, *Malva sylvestris*, *Rhazya stricta*, *Solanum marginatum*, and *Thalictrum dipterocarpum*. In all cases, more than 90 percent of the Cd^{2+} taken up by the cells was complexed to the phytochelatin peptides. The traces of Cd^{2+} (3 percent) that were excluded from Sephadex G-50 (Fig. 1B) were associated with proteinaceous material with a relative molecular weight of more than 30,000. There was no indication of the formation of a 10-kD metallothionein as reported previously (5-9). However, in our system the metal ions were supplied at a 200 μM concentration, which is about 10 times higher than that used by others (5-9).

The phytochelatins may be viewed as linear polymers of the γ -Glu-Cys portion of glutathione and, indeed, may be formed from glutathione itself. Because of the repetitive γ -glutamic acid bonds, they cannot be regarded as primary gene products. Phytochelatins are apparently the simplest (composed of only three different amino acids) natural compounds so far reported that may be engaged in the detoxification and homeostasis of heavy metals through metalthiolate formation. They are completely different in structure from the metallothioneins reported earlier but may serve the same purpose of binding excess heavy metals through mercaptide complexes.

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Wounding and Its Role in RSV-Mediated Tumor Formation

Abstract. Tumors induced in chickens by Rous sarcoma virus remain localized at the site of injection even though the animals become viremic. Tumors have now been shown to be inducible at other sites if a wound is inflicted or if the tissue is injured by administration of tumor promoters. These findings indicate that local wounding plays a role in the spread of tumorigenicity of Rous sarcoma virus.

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In most studies of tumor formation mediated by Rous sarcoma virus (RSV), the virus is administered by subcutaneous or intramuscular injection, a process that creates some local wounding (1). Viral infection of newly hatched chicks results in the rapid growth of a localized sarcoma that becomes palpable within 1 to 2 weeks, in the production of circulating progeny virus, and in most cases, in the death of the host within a month (2). Tumors usually form only at the site of inoculation (3), whereas nonmalignant hemorrhagic lesions are often found in tissues throughout the animal (1). Occasionally, distal tumors have been reported in young chicks, but they appear with a much longer latency and only in addition to the rapidly forming local tumor (4, 5). If circulating virus is present and if, as has been proposed, RSV infection and concomitant *src* gene expression are sufficient for neoplastic transformation and sarcoma development in chickens (6), tumors should form elsewhere as well.

To determine whether progeny virus was indeed present throughout the animal, we assayed several types of tissues obtained from tumor-bearing chickens

for the presence of focus-forming units (ffu). Tissues were minced in buffer, serially diluted, and tested for their ability to transform cultured chicken embryo fibroblasts (CEF) in a focus assay (7). Progeny virus was present in all tissues assayed (Table 1). Although this does not constitute proof that the virus was present intracellularly, it does indicate that it was being circulated. Despite this circulating infectious virus, tumors formed preferentially at the site of inoculation; it was thus reasonable to suspect that either wounding associated with inoculation or the subsequent healing plays a part in the formation of tumors.

To test this hypothesis, we inoculated 10-day-old chicks intramuscularly in the right wing with 5×10^6 ffu of the Schmidt-Ruppin-D strain of RSV in a volume of 0.1 ml. The opposite wing was pierced with a small stainless steel clip that remained in place for the duration of the experiment (Fig. 1A). As expected, palpable tumors formed at the site of injection in all animals after 8 or 9 days. When a clip was inserted at the time of injection, tumors also formed at the site of the clip, with the same frequency as those inserted after injection, but with a 20 percent longer latency period (Fig. 1C). Tumors induced by injection were indistinguishable from those induced by wounding, as judged from histological examination of the tissue sections (Fig. 1B). The timing of the inflicted wound affected the latency of the resulting tumors. The longer the clip insertion was delayed after virus injection (up to 2 weeks), the shorter the latency in formation of the wound tumor (however, it was never less than 2 days) (Fig. 2). After 2 weeks, the animals either died or were killed. We speculate that the

Table 1. Seven-day-old chickens were given injections of SRD-RSV (5×10^6 ffu), and a clip was inserted into the opposite wing. Two weeks later, the animals were killed, and the tissues of interest were prepared for analysis. Focus-forming activity, expressed as focus-forming units per milligram of protein, and *src* kinase activity, expressed as the multiple of increase over corresponding normal tissue, were determined as described (7). The values are means \pm standard error of the mean for three separate experiments.

Tissue	Infectious virus (ffu/mg)	Kinase activity (multiple increase)
Tumor at injection site	$1 \times 10^5 \pm 8.5$	25.4 ± 2.7
Spleen	$4 \times 10^4 \pm 3.1$	6.0 ± 2.9
Breast	$1 \times 10^3 \pm 0.4$	1.0 ± 0.1
Tumor at clip site	$2 \times 10^6 \pm 1.2$	14.3 ± 3.0

shorter latencies when clip insertion was delayed after injection may be due to the increasing titer of circulating virus resulting from the increasing size of the tumor. When the clipping wound was inflicted up to 1 week before virus injection, the latency of the wound tumor increased. Clips inserted more than 1 week before injection failed to cause tumors altogether (Fig. 2). We interpret this to mean that as the wound heals it loses its ability to complement the effect of RSV in tumor formation, and although the clip may act as a foreign body, it is not sufficient, in the absence of wounding, to cause induction of tumors. The latency of the tumors occurring after injections remained constant under all conditions.

Since wounding is believed to have tumor-promoting activities, we considered the possibility that other known tumor promoters such as phorbol esters might be able to substitute for the cocarcinogenic effect of wounding. To test this hypothesis, however, we needed to minimize the wounding that occurs in the delivery of the substances being tested. Our approach was to implant a 1-mm polyethylene tube, approximately 5 cm in length, into one wing of an 8-day-old chick. The wing was allowed to heal for 10 days with the catheter in place. Various tumor promoters were then administered through the catheter while RSV was injected into the opposite wing in the usual manner. Tumor promoters dissolved in a minimum amount of solvent (less than 10 percent methanol in water) caused tumors only at high concentrations (Table 2) and only when they produced necrosis and local inflammatory reactions. When the amount of methanol was increased, inflammation and necrosis were evident at the site of administration even with lower concentrations of the tumor promoters. Increasing the concentration of tumor promoters in the presence of high levels of solvent resulted in more prominent inflammation and necrosis, with subsequent appearance of tumors (Table 2). Methanol (100 percent) also caused tumors in half of the chickens, and these tumors were preceded by inflammation and edema. Thus, inflammation, as well as mechanical puncture or laceration, can act as a cocarcinogen in RSV-mediated tumor formation. Furthermore, at least in this system, irritation and local injury appear to be the mode by which tumor promoters allow the induction of tumors.

To ascertain that the wound-induced tumors were of viral origin, we assayed tissue from infected birds for the presence of viral kinase activity (Table 1).

Tissues from tumors induced by injection and those induced by wounding contained a 20- to 40-fold increase in src-specific kinase activity. However, no

other tissue tested showed any increase in this activity, with the exception of a small increase in the spleen. This suggests that wounding either allows target

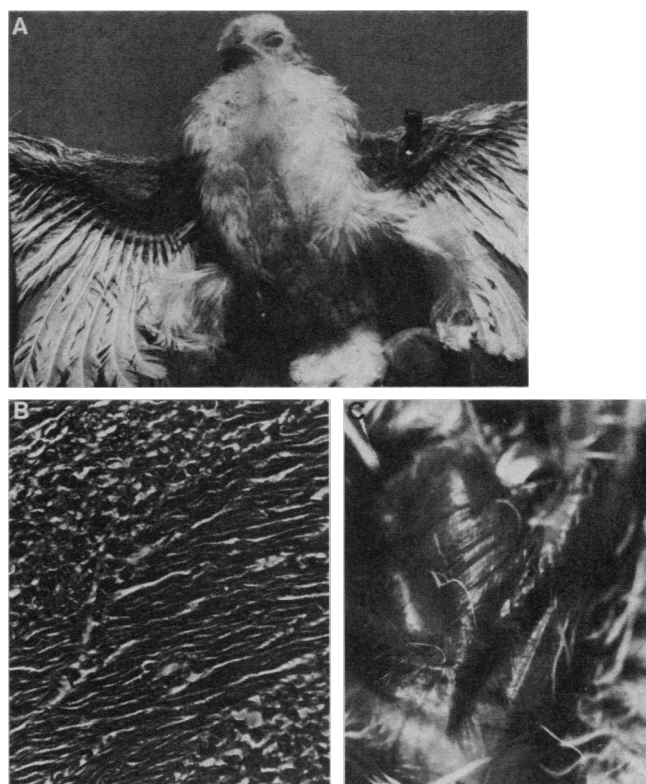


Fig. 1. Wound-induced, RSV-initiated tumors. (A) A 7-day-old chick was inoculated with 5×10^6 ffu of the SR-D strain of RSV. A 27-gauge needle was used to inject virus into the right wing web. At the same time a stainless steel clip was used to pierce the left wing web, and the clip was left in place for the duration of the experiment. The animal was photographed 2 weeks after injection, by which time tumors had formed in both wings. In the absence of virus, the clip caused only a minor transitory inflammation. (B) The tumor that formed at the site of the clip in (A) above was excised, fixed in Bouin's fixative, embedded in paraffin, and sectioned ($5 \mu\text{m}$). Sections were photo-

graphed (Nikon diaphot microscope) after being stained with hematoxylin and eosin ($\times 280$). (C) Detail of the wound-induced tumor shown in (A).

Table 2. Correlation of tumor induction with local irritation. Polyethylene catheters, 1 mm in diameter, were inserted into the wing webs of 7-day-old chicks. The catheters were attached with sutures and allowed to heal for 10 days. Solutions to be tested were then injected through the catheter in 0.1-ml volumes every other day for a total of three injections. Virus was injected in the opposite wing at the time of the first catheter injection. The extent of the local reaction was judged by the relative amounts of swelling, scabbing, and tissue necrosis and is shown as -, no reaction; \pm , minimal reaction; and +, ++, and +++, increasing necrosis; a slash indicates that some animals showed a reaction and others did not. Tumors are indicated by the percentage of animals with tumors in the wing containing the catheter, and n is the number of chickens tested. TPA is 12-*O*-tetradecanoylphorbol-13-acetate; PDD and 4 α PDD are phorbol and 4 α -phorbol 12,13-didecanoate, respectively.

Treatment	n	Extent of local reaction	Tumors (%)
Medium containing <10 percent methanol	5	-	0
Methanol*	6	++/-	50
TPA in medium containing <10 percent methanol (0.1 to 0.5 μg) [†]	4	-	0 \ddagger
TPA in methanol (<0.2 μg)	8	+	50
TPA in methanol (>0.2 μg)	4	+++	100
PDD and 4 α PDD in medium (<10 percent methanol) (0.1 to 2 μg)	0	-	0
PDD and 4 α PDD in medium (<10 percent methanol) (5 μg)	0	\pm /-	0
PDD in methanol (0.2 to 1 μg)	10	++	50
4 α PDD in methanol (0.2 to 1 μg)	24	++	50
Mezerin in medium (0.1 to 5 μg)	3	-	0
Mezerin in medium (10 μg)	3	+++	67
Mezerin in methanol (0.05 to 0.5 μg)	6	+++	83

*Tumors associated with methanol occurred almost exclusively when there was a local reaction. When there were no reactions, there were no tumors. [†]The amount refers to the total dose administered through the catheter in each application (total of three in the course of 6 days). [‡]In some cases, the catheters were pulled out by chickens in the middle of the experiment, and these chickens are not included.

tissues to be infected or enables a cryptically infected tissue to express the viral genes. If the latter is true, the presence of the virus should be evident from a restriction enzyme digest of the DNA from unwounded tissues.

Preliminary experiments indicate that nontumor tissues generally do not contain integrated provirus. However, newly acquired *v-src* sequences (Eco RI fragments) have been detected in some nontumor tissues, although at far lower concentrations than are found in tumor tissues (8). The significance of this finding is still unclear. Theoretically a single integration in a chick cell should be sufficient to cause tumors.

If wounding does not act directly on an already integrated virus, what are other possible mechanisms of its action in this system? The integration of RSV in cells in culture requires active cell division (9). It is possible that wounding may act as mitogen for otherwise quiescent cells. We measured the change in the rate of DNA synthesis by both thymidine incorporation into the DNA and by flow cytometry of isolated cells from the wing tissue of wounded and control 1-week-old chicks. Wing tissues were minced, dissociated, and incubated with [³H]thymidine for 1 hour. The amount of label in TCA-precipitable (TCA, trichloroacetic acid) material was determined for the wounded tissue and compared with that of similarly prepared unwounded tissue. Using this method of measurement, we were able to detect a slight, transient stimulation of DNA synthesis within the 24 to 48 hours immediately after wounding. However, flow cytometry of cells indicated that 4 to 5 percent of the cells were in S phase both before and 24 hours after wounding. While we cannot rule out the possibility that wounding may stimulate the proliferation of a significant target cell subpopulation that is lost upon isolation, wounding does not appear to be overtly mitogenic in growing young chicks.

If circulating virus cannot pass through the intact capillary endothelium into the interstitium, wounding could cause tumors by allowing access to the target tissues. Since this hypothesis is difficult to test directly, we investigated the possibility that the virus was able to cross other epithelial barriers. Rous sarcoma virus (5×10^6 ffu) was administered as an aerosol or by mouth to 5-day-old chicks, and a clip was inserted into their wings as described. Tumors formed in 7 of the 39 chicks, and only at the site of the clip. A blood-borne infection is probably established as a result of oral administration, but because of the ineffi-

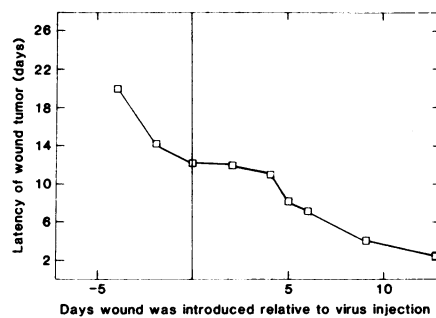


Fig. 2. Relation of tumor latency to time of wounding. RSV (5×10^6 ffu) was injected on day 0 as described in Fig. 1. The opposite wing was clipped either before (-) or after (+) virus injection. Latency represents the number of days elapsed between insertion of the clip and appearance of a nonregressing, wound-induced tumor (larger than 5 mm). Each point represents three or four chicks with latency periods differing by no more than 1 day.

ciency of establishing infection by this route (10) the percentage of tumors is low. Thus, while infection can be established in the absence of wounding, wounding may be required for the growth of solid tumors.

Transformed, non-virus-producing chick embryo fibroblasts infected with a replication-defective strain of RSV (Bryan strain) occasionally metastasize when introduced into the wing web of a chick (5). This probably explains the infrequent appearance of tumors in distal sites in the absence of wounding (4). We have considered whether wound tumors in our experiments could also arise from metastasis. However, in the aerosol experiments, the wound tumors arise de novo, and when a primary tumor has been induced, the kinetics of the appearance of wound tumors are too rapid to be accounted for by metastasis. Restriction digest of DNA from wound tumors is necessary to clarify this point unambiguously.

The aerosol experiment demonstrates further that RSV is capable of effectively crossing the epithelial barrier of the lung or gut (or both). Furthermore, the non-malignant hemorrhagic lesions that frequently occur in these animals show that the endothelial barrier of the capillaries can be breached without subsequent tumor formation (1, 4, 7). Even if injury were to alter the compartmentalization of the virus, wounding and wound healing would be good candidates for an active role in tumor formation. It is suspected, for instance, that wounding is a first-stage promoter in chemical carcinogenesis (11) and, in some animal models, tumors develop at wounding sites (12-14) regardless of the method of carcinogen application. Some human tumors are

also found at sites of wounding (15), and the process of wound healing involves factors that are believed to participate in tumorigenesis and tumor promotion. Wounding stimulates the release of several growth factors, one of which, the platelet-derived growth factor, is homologous to the protooncogene, *c-src* (16). Wounding also generates free radicals (17) and activates protein kinase C and the arachidonate metabolic pathway (18, 19).

Even with an overt, oncogenic virus such as RSV, tumor formation appears to be a more complicated process than the transformation of cells in culture would lead us to believe. The notion that a complete understanding of RSV-mediated tumor formation in birds could be achieved by measuring pp60 *src* levels alone may be an oversimplification of a complex relation between virus and host. Although RSV infection obviously initiates the tumor, the course of the disease is influenced by the cellular environment and the stage of development of the animal, as we (7) and others (20) have shown.

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