ABSTRACT

When newly hatched chicks are given injections of Rous sarcoma virus, a tumor develops at the site of injection. In spite of the presence of the virus in the blood, no other tumors are found distant from the site of inoculation during the life span of the animal (4–6 weeks). However, if a wound is made away from the primary tumor, a tumor develops at the site of wounding. Work in our laboratory showed previously that these wound tumors do not develop as a result of metastasis, therefore, factors released upon wounding must contribute to the development of the wound tumors. In particular, we showed that transforming growth factor (TGF)-β, a growth factor implicated in wound healing, can replace wounding in tumor development. However, we also showed that epidermal growth factor and TGF-α, growth factors that also have roles in wound healing, do not induce tumors. To identify the critical event(s) and to determine the mechanism involved in wound tumor development, we have continued these studies. Here we show that: (a) wound tumor development correlates with the presence of circulating virus and inflammation; (b) the virus is present in serum and in heterophils of the peripheral blood; (c) cell division at the site of wounding precedes the expression of viral proteins; (d) in addition to TGF-β, acidic and basic fibroblast growth factors can also replace wounding in tumor development; (e) these three factors (TGF-β, acidic fibroblast growth factor, basic fibroblast growth factor) which promote tumors also induce inflammation, whereas epidermal growth factor and TGF-α do not; and (f) during the inflammatory response, blood vessel leakage occurs as tested by the release of fibrinogen into the tissues. To test the possibility that inflammation is the key element in the development of these wound tumors, we used β-methylprednisolone, an antinflammatory drug that inhibits inflammation (including blood vessel leakage), to determine if wound tumor development could be prevented. We found that when inflammation was inhibited, tumors were also inhibited; when inflammation could not be stopped, tumors developed as before. These results indicate that the effect of wounding on the development of wound tumors in Rous sarcoma virus-infected chicks is accomplished through the cytokines released by the inflammatory cells at the site of wounding. These inflammatory mediators play a critical role in providing the conducive environment for oncogene integration and activation, and subsequent development of tumors. Our results also suggest that in humans and other mammals, the tumors that develop at the sites of chronic irritation may be the result of the persistent action of inflammatory cytokines on cellular genes that are important in the maintenance of homeostasis. Overexpression or underexpression of these genes could lead to deregulation of cell function culminating in transformation.

INTRODUCTION

There are numerous lines of evidence that show that tumors develop as a result of a series of insults inflicted upon cells. Clinical and experimental studies have shown that there is an exponential relationship between cancer and age, supporting the idea that cancer is the result of a multistep event. Molecular studies have shown that these cancers are a result of a multitude of genetic changes. However, studies both in culture (1) and in vivo (2–4) show that the environment in which the cell finds itself can influence the outcome of these genetic alterations, promoting or suppressing the transformed phenotype.

For decades, scientists have observed that irritation, inflammation, and/or wounding are promoters of tumor development (5–7). Early in this century, Deel (8) observed that tumors develop at the margin of accidental wounds made in the skin of mice that had been treated with tar. Subsequent studies in several different species and tissues (see Ref. 7 for review) have shown that this is also true if skin that has been initiated with the chemical carcinogen 7,12-dimethylbenzanthracene is treated with the promoter 12-O-tetradecanoylphorbol-13-acetate. There are also many clinical reports that support these observations (5, 8–12).

More recently, it has been shown that even oncogenes require the cooperation of other oncogenes (13–15) and/or other factors (4, 16–19) to induce the transforming phenotype in cells. In at least four cases of tumors induced by viruses or oncogenes, wounding appears to play an important role: (a) v-src-infected chickens develop tumors at the sites of wounds 10–15 days after wounding (18); (b) bovine papilloma virus elicits skin tumors in transgenic mice 8–9 months after birth and in areas prone to wounding (2); (c) the tat gene of human T-cell lymphotrophic retrovirus type I induces mesenchymal tumors in transgenic mice at approximately 3 months of age, also in areas prone to scratching (14); and (d) v-juin transgenic mice develop tumors at 2–3 months of age only in areas where wounds have been made (19). It has also been observed that postvaccination sarcomas develop in cats that have been immunized against rabies; some cats develop vaccine-induced inflammatory nodules and a small percentage of those go on to develop sarcomas (20, 21). The correlation of wounding with carcinogenesis in humans, cats, mice, and chicks supports the notion that involvement of wounding or wound repair in carcinogenesis may be a general phenomenon.

In all of these cases except for wound tumors in RSV-infected chicks, there is a significant time delay between initial infection and tumor development. Therefore, we have investigated the possibility that wounding is operating by a different mechanism in the rapid development of wound tumors in chicks. We have shown previously that wound-induced tumors (henceforth, called wound tumors) in newly hatched chicks infected with RSV do not develop as a result of metastasis and that cell proliferation alone is not sufficient for tumor promotion (22). Therefore, wounding must contribute to the rapid development of tumors via another mechanism(s). Growth factors released during wounding are important in the processes of healing during inflammation and granulation tissue formation (23), and they can potentially contribute to creating a conducive environment for wound tumor development. We have studied a variety of growth factors that are known to play roles in wound healing and showed that

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3The abbreviations used are: RSV, Rous sarcoma virus; CEF, cultured-chick embryo fibroblast; influenza virus; PBS, phosphate-buffered saline; RT, reverse transcriptase; BSA, bovine serum albumin; EGF, epidermal growth factor; TGF, transforming growth factor; aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; IL-1, interleukin-1; IL-2, interleukin-2; IL-6, interleukin-6; IL-8, interleukin-8; TGF-β, transforming growth factor-β.
TGF-β can replace wounding as a promoter of tumor development in chicks 100% of the time and platelet-derived growth factor can do so 25% of the time but that EGF and TGF-α do not promote tumors (24). This inability of TGF-α and EGF to induce tumors was not because these mammalian growth factors do not act upon chicken tissues; when tested on CEFs they were biologically active (24). Similar results were obtained when these growth factors were applied in vivo (24). As with wounding, proliferation at the site of application of the growth factor is not sufficient to promote tumor development, hence wounding and TGF-β must be acting via other mechanism(s) (22).

Here we show that in addition to TGF-β, aFGF and bFGF can replace wounding in the promotion of secondary RSV tumors and that the critical player in this process is inflammation.

MATERIALS AND METHODS

Experimental Induction of Wound Tumors. Five-day-old chicks were inoculated with 10⁴ ffu of the Prague C strain of RSV. A 30-gauge needle was used to inject the virus into the wing tissue. Five days later a clip or a suture was introduced in the opposite wing to cause a wound. The clip was introduced by piercing the wing between the ulna and radius and was left in place for the duration of the experiment. For suturing, we used sterile silk thread (3-0), and 10–12 loops were sewn in one area of the wing between the ulna and radius (22).

Detection of Infectious Virus Particles in Chick Blood. The blood was collected in heparinized tubes and centrifuged at 3000 rpm for 3 min. The serum was removed and added onto CEF cells that had been plated at 8 x 10⁵ cells/60-mm dish 3 h previously. These cultures were incubated overnight at 37°C, the medium was changed, and the cells were allowed to grow for 48 h. At this stage they were immunostained as described by Stoker and Bissell (25). Briefly, the cells were fixed in 2% paraformaldehyde for 20 min, treated with 0.1 mM glycerine, and rinsed and incubated with the primary antibody, anti-p19gag, overnight at 4°C. After a washing, cells were incubated with a biotinylated goat anti-mouse F(ab)_2 antibody (Amersham) and then with streptavidin alkaline phosphatase. The detection solution contained 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium in a detection buffer [100 mM Tris-HCl (pH 9.5)-1 mM EDTA].

Immunostaining of the Virus in Blood Smears of Infected Chicks. Blood was collected in heparin treated capillary tubes from 2-week-old chicks; blood smears were prepared, fixed in 100% methanol for 3 min, and allowed to dry. The cells were hydrated for 5 min in 1x PBS, blocked with 10% goat serum for 30 min at RT, and then incubated with an antibody against p19gag (a core protein of the virus) in PBS plus 1% BSA plus 1% goat serum overnight at 4°C. The smears were washed 3 times for 20 min each at RT in 1x PBS plus 1% BSA, incubated with fluoresceinated goat anti-rabbit IgG (Zymed Laboratories, Inc.) in 1x PBS plus 1% BSA for 1 h at RT, and then washed again as above. The blood smears were drained of excess wash buffer and mounted in 70% glycerol in 0.1 mM sodium bicarbonate containing 2% N-propylgallate and 0.1% sodium azide to reduce fading of the fluorochrome (26). Controls consisted of smears incubated with normal rabbit IgG in place of the primary antibody and at the same concentration.

Preparation of Tissues for Histology. The wings were removed and fixed for 8–10 h in 4% paraformaldehyde in 1x PBS and then decaclified as described previously (22). After decalcification, the wings were cut in half and processed for paraffin sections as described previously (27). Sections 4 μm thick were cut and either not stained to observe [³H]thymidine incorporation or stained with hematoxylin and eosin for histological observations.

[³H]Thymidine Incorporation at the Wound Site. Tissues were collected from uninfected birds at specific times (8, 18, 30, and 48 h) after wounding of the wings. Two h prior to removal of the tissues, the birds were given i.v. injections with 50 μCi of 5 mCi/mmol [³H]thymidine. Tissues were processed for histology as described above; 4-μm-thick sections were cut, covered with NBT-2 liquid emulsion (Eastman Kodak Co., Rochester, NY), exposed for 2 weeks, and developed in D-19: H₂O (1:1).

Induction of Tumor by aFGF and bFGF. Five-day-old chicks were given injections of Prague C in one wing as described above, and 5 days later the growth factors were deposited with a 30- gauge needle in the opposite wing under the skin. Either a single treatment of growth factor was applied or three separate treatments of growth factors were applied on 3 consecutive days. Each treatment contained 800 ng of growth factor alone or 800 ng of growth factor plus 100 units of heparin. The birds were left in the cages and observed for about 10 min and then stopped by 2 washes in a stop buffer [10 mM Tris-HCl (pH 8.0)-1 mM EDTA].

Fig. 1. Development of RSV-induced primary and wound tumors: (a) Injection of 10⁴ ffu of RSV into one wing of 5-day-old chickens causes primary tumors to develop at the site of injection (arrowhead), with 100% frequency, 8–10 days after inoculation (age, 13–15 days). When these chicks are wounded in the opposite wing 5 days after inoculation (age, 10 days, double arrowhead), a secondary tumor develops at the site of wounding, again with 100% frequency, 8–12 days after wounding (age, 18–22 days). (b) Micrograph of a primary tumor 3 weeks after RSV inoculation; these tumors result from the coalescence of multiple small nodules that result from individual infections. (c) Micrograph of a wound tumor (arrowhead) 2 weeks after insertion of a metal clip (star) between the ulna and radius.
frequently for the development of secondary tumors. Controls were given injections with 1× PBS plus 1% BSA and heparin. All growth factors used in this study were tested previously for their biological activity and showed no evidence of endotoxin contamination (24).

Inhibition of Development of Secondary Tumors Induced by Growth Factors. Chicks were treated as described above except that immediately prior to the application of the growth factor, 600 µg of β-methylprednisolone (dissolved first in ethanol at 10 mg/ml and then diluted in sterile 1× PBS and used immediately) were deposited under the skin. Six applications of 10 µl of the drug (each of which contained 100 µg) were done in various spots along the wing. The β-methylprednisolone was allowed to soak into the tissue for 3 min, and then the growth factor was applied in one area only at a concentration of 800 ng for TGF-β and 800 ng plus 1(K) units heparin for aFGF.

RESULTS

Development of Wound Tumors and Correlation with the Presence of Virus Circulating in the Blood. Injection of 10⁴ ffu of RSV into one wing of 5-day-old chickens caused primary tumors to develop at the site of injection with 100% frequency 8–10 days after inoculation. When these chicks were wounded in the opposite wing 5 days after inoculation by insertion of a metal clip or by receiving 10–12 localized sutures, a secondary tumor developed 8–12 days later at the site of wounding, again with 100% frequency (Fig. 1a). These wounds also caused an inflammatory response (minor wounds that did not induce inflammation did not induce tumors). Micrographs of a primary tumor and of a wound tumor are shown in Fig. 1, b and c, respectively. Primary tumors are invariably composed of a number of small growths that result from individual ffu, whereas wound tumors are more localized to the area of the clip or sutures.

The frequency with which wound tumors developed depended strongly on the time of wounding (Fig. 2). If wounding was performed on chicks on the day of inoculation (age, 5 days), wound tumors did not develop. Wounding 2 days after inoculation (age, 7 days) yielded 50% wound tumors, and wounding at 4 or 5 days after inoculation (age, 9 or 10 days) resulted in 100% of the chicks developing wound tumors. However, the frequency of these tumors dropped to 40% if wounding was performed 7 days after inoculation of the virus at the primary site (age, 12 days). Wounding at 9 days or more after inoculation (age, 14 days or more) produced no tumors.

Previous studies in our laboratory found the virus circulating in the blood after the chicks were inoculated locally with RSV (18). However, the kinetics of viral circulation was not studied at that time. To determine if there could be a correlation between the presence of the circulating virus and the development of wound tumors, we gave chicks injections 5 days after hatching and collected blood from these chicks 0, 4, 5, 7, 9, 12 days after inoculation. The blood was centrifuged, and the serum was assayed on monolayer cultures of CEFs. Viral foci were detected as described in “Materials and Methods” by immunocytochemistry using an antibody against p19ppE. A typical focus is shown in Fig. 3, inset. Fig. 3 also shows that the number of foci on CEF cultures increased for blood collected up to 5 days after inoculation (age, 10 days) but that it dropped rapidly thereafter; no foci formed from blood collected 9 days or more (age, 14 days or more) after inoculation of the virus. These results correlate very well with the number of chicks that developed wound tumors.

We further investigated the presence of virus in the blood by staining blood smears with an antibody against the core protein of the virus p19ppE. We found that large leukocytes stain for this protein (Fig. 4a). In Wright-Giemsa-stained smears, we identified these cells as heterophils by the morphological appearance of the multilobular nucleus and the slightly granular cytoplasm (Fig. 4b).

Pattern of Cell Division upon Wounding. Despite the fact that we had previously shown that cells in the wing are dividing even in the absence of wounding, the strong correlation between the kinetics of viral circulation in the blood and wound tumor development prompted us to determine the kinetics of DNA replication after wounding. Fig. 5 shows hematoxylin and eosin-stained sections taken from wings of uninfected wounded birds given i.v. injections of [³H]thymidine 2 h before collection of the tissues. The cells that incorporated [³H]thymidine contained black nuclei that appeared larger than the nuclei in cells that did not incorporate the radioactive nucleotide insert; Fig. 5b, inset, is an enlargement of an area in the vicinity of the sutures to show that most labeled cells are elongated, suggesting that they are fibroblasts, not inflammatory cells that have come to the site of the wound. We observed that there was a 1.5-fold increase over the normal number of cells incorporating [³H]thymidine at 8 h after wounding, a 7-fold increase after 16–18 h, and no increase over normal after 30–32 h. However, the number of cells dividing increased 18.5-fold by 48–50 h (Fig. 5d). Expression of p19ppE was first observed 36 h after wounding (not shown, but see Ref. 22).
Promotion of Secondary Tumors by aFGF and bFGF. In addition to TGF-β, other factors that are released upon wounding could potentially promote tumor development. Here we studied the capability of aFGF and bFGF to promote secondary tumors. EGF was used as a negative control because it was shown previously not to promote tumors in this system (24).

Table 1 summarizes the results obtained when either aFGF or bFGF was deposited underneath the skin of the wing opposite to the primary tumor. These growth factors by themselves produced 80% and 60% secondary tumors, respectively. However, when the same factors were mixed with heparin [known to protect FGFs from degradation (28)], aFGF promoted wound tumors 100% of the time and bFGF did 80% of the time. These tumors are visible 8–10 days after application of growth factors (Fig. 6). Heparin alone did not cause inflammation or tumors.

All three growth factors that promoted secondary tumors (TGF-β, aFGF, and bFGF) also caused a large inflammatory response with edema, whereas EGF, which did not promote secondary tumors, did not cause an inflammatory response. EGF caused neither an influx of inflammatory cells nor any other significant morphological alteration of the skin (compare Fig. 7, a and b), whereas the other growth factors caused edema and a large influx of inflammatory cells to the site of application of the growth factor. Basic FGF induced only moderate inflammatory response and edema (Fig. 7c), but aFGF caused a marked inflammatory response, edema, and disruption of the skin structure (Fig. 7d), and TGF-β had an even more pronounced effect (Fig. 7e). Both bFGF and aFGF were chemotactic for heterophils, monocyte/macrophage cells and small lymphocytes (cells with densely stained nuclei), whereas TGF-β attracted mostly cells of the first two types. To demonstrate blood vessel leakage, we immunostained tissues with an antibody against fibrinogen and showed that tissues treated with aFGF (Fig. 7f) or TGF-β (Fig. 7g) contained large amounts of fibrinogen in the areas of inflammation, whereas no fibrinogen was detected in EGF-treated tissues (not shown).

Assessment of a Potential Mechanism for Secondary Tumor Promotion by TGF-β and aFGF. The marked correlation between the titer of virus in the blood, inflammation induced by growth factors and the development of secondary tumors led us to suspect that the critical step leading to tumor formation might be release of virus from the blood during the inflammatory response. Such release would naturally lead to infection and transformation of the growth factor-induced rapid division of resident cells. To test this hypothesis, we used a glucocorticoid (ß-methylprednisolone) because this class of drugs is known to inhibit a broad spectrum of processes involved in the inflammatory response (29). During inflammation, vaso-occlusion occurs to increase blood flow to the area and is accompanied by blood vessel leakage. Glucocorticoids inhibit the inflammatory response by decreasing vascular leakage and the formation of the inflammatory fluid and cellular exudates in a variety of ways: they stabilize mast cell granules and therefore reduce the release of histamines (the principal early mediators of vasodilation); they also stabilize lysosomal membranes, resulting in reduction of the release of enzymes that are important in the production of kinins, prostaglandins, and other inflammatory mediators that also induce vascular leakage; and they block the inactivation of catecholamines which are potent vasoconstrictors (29).

ß-Methylprednisolone was applied locally to the wing at the site of growth factor application. When applied alone, it caused no noticeable effect on the chicks. However, application of ß-methylprednisolone followed by application of aFGF or TGF-β was effective in inhibiting the inflammatory response elicited by aFGF but only slightly reduced that elicited by TGF-β. Under these conditions, chicks treated with aFGF did not develop tumors, whereas those treated with TGF-β developed tumors as in the absence of the drug (Table 2).

DISCUSSION

The results presented here provide insight into the mechanisms involved in the development of wound-induced tumors in newly hatched chicks infected with RSV. We have shown that: development of wound tumors correlates with the titer of virus in the blood; in addition to free viral particles in the serum, a number of heterophils (first type of inflammatory cells at the site of wounding) in the blood also contain the virus; wounding causes inflammation and stimulates cells to undergo division, providing the opportunity for RSV integration into the cell genome; aFGF, bFGF, and TGF-β all induce effects similar to wounding including the induction of tumors (see Ref. 24 for TGF-β induction of tumors); EGF, like TGF-α (24), stimulates replication of fibroblasts, but does not stimulate inflammation, blood vessel leakage or tumors; and ß-methylprednisolone inhibits both inflammation and tumors caused by aFGF but inhibits neither by TGF-β, establishing a direct correlation between the inflammatory response and the development of wound tumors.
In our previous work on wound tumor development (22, 24), we found that proliferation alone was not sufficient to cause wound tumors; we proposed that these tumors might be the result of activation of a previously integrated but silent virus, because it has been shown to be the case in other systems (2, 18) or, alternatively, that wounding would allow viral infection, integration, and expression. In the results presented here we show that the peak of wound tumor formation occurs when the birds are injured 4–5 days after infection and that this correlates perfectly with the presence of the virus in the circulatory system. If activation of an integrated virus were the cause of wound tumor development, as the bird grows and cells continue to divide, more and more cells would contain the silent virus and wounding should promote tumor development at any time. Therefore, the wound tumors must arise by integration of newly released virus rather than by activation of an integrated silent virus. This is further illustrated by the patterns of cell division and expression of viral proteins during the 2.5 days immediately following wounding. Had there been activation of a silent virus by wounding, one would expect to see evidence of viral proteins during the first peak of synchronized DNA synthesis at 16–18 h after wounding. However, the viral protein p138gag is not detected until 36 h after wounding (22). These results are consistent with the scenario that the virus released upon wounding takes advantage of the first wave of dividing cells at 16–18 h to integrate and then express the oncogene, with the second wave of synchronized cell division providing the ideal environment for rapid tumor development. However, this does not explain why specific growth factors can replace wounding in the development of wound tumors.

The common event shared by wounding and those growth factors that induce tumors is an inflammatory response. All three growth factors that are released upon wounding and can replace wounding in the promotion of tumors (TGF-β, aFGF, and bFGF) also induce a marked inflammatory response, with strong correlation between the strength of that response and the frequency of tumors. TGF-β and aFGF cause strong inflammatory responses and tumors 100% of the time, whereas bFGF causes a moderate inflammatory response and tumors at lower frequency. On the other hand, EGF and TGF-α do not cause inflammation or tumors. These results show that the frequency of tumor formation correlates with the intensity of the inflammatory response. This correlation led us to attempt inhibition of tumors through inhibition of the inflammatory response caused by aFGF and TGF-β by treating the tissues with the antiinflammatory glucocorticoid β-methylprednisolone (29) prior to the application of each growth factor. We succeeded in inhibiting tumors when the drug inhibited inflammation (aFGF experiment), whereas when the drug did not inhibit inflammation, tumor development was not inhibited (TGF-β experiment). These latter experiments confirm that inflammation plays a fundamental role in the development of wound tumors.

The inability of β-methylprednisolone to inhibit the inflammatory response by TGF-β indicates that the nature of stimulation of inflammation by aFGF and TGF-β is probably different. It is known that TGF-β has multiple functions. It can have the same effect in different tissues (30), have different effects in different tissues (?), or behave differently in the same tissue depending on the microenvironment (?). It also has been shown that in wounds treated with glucocorticoids, TGF-β was not able to enhance wound healing to the same degree as in normal wounds (31), indicating that TGF-β is unable to reverse all of the adverse effects induced by glucocorticoids on wound healing.

In our previous work on wound tumor development (22, 24), we found that proliferation alone was not sufficient to cause wound tumors; we proposed that these tumors might be the result of activation of a previously integrated but silent virus, because it has been shown to be the case in other systems (2, 18) or, alternatively, that wounding would allow viral infection, integration, and expression. In the results presented here we show that the peak of wound tumor formation occurs when the birds are injured 4–5 days after infection and that this correlates perfectly with the presence of the virus in the circulatory system. If activation of an integrated virus were the cause of wound tumor development, as the bird grows and cells continue to divide, more and more cells would contain the silent virus and wounding should promote tumor development at any time. Therefore, the wound tumors must arise by integration of newly released virus rather than by activation of an integrated silent virus. This is further illustrated by the patterns of cell division and expression of viral proteins during the 2.5 days immediately following wounding. Had there been activation of a silent virus by wounding, one would expect to see evidence of viral proteins during the first peak of synchronized DNA synthesis at 16–18 h after wounding. However, the viral protein p138gag is not detected until 36 h after wounding (22). These results are consistent with the scenario that the virus released upon wounding takes advantage of the first wave of dividing cells at 16–18 h to integrate and then express the oncogene, with the second wave of synchronized cell division providing the ideal environment for rapid tumor development. However, this does not explain why specific growth factors can replace wounding in the development of wound tumors.

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**Table 1** Promotion of secondary tumors by aFGF, bFGF, and EGF

<table>
<thead>
<tr>
<th>Growth factor</th>
<th>Dose/injection</th>
<th>No. chicks used</th>
<th>% of chicks with primary tumors</th>
<th>% of chicks with secondary tumors</th>
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<tr>
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<td>100</td>
<td>?; 100</td>
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<tr>
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<td>100</td>
<td>80</td>
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<td>EGF + heparin</td>
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<td>100</td>
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</table>

*Applied on 3 consecutive days; tumors visible after 6–8 days. Results were similar with a single application, but tumors were visible only after 12–15 days.*

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**Fig. 5.** H & E-stained cross-sections of wings wounded by suturing. [3H]thymidine incorporation shows the wound-induced pattern of cell division. (a) Eight h after wounding there is a large influx of small round cells (inflammatory cells) (double arrowheads point to a few cells) but no [3H]thymidine incorporation by these cells or any others surrounding the suture (stars); (b) 18 h after wounding high numbers of inflammatory cells are still visible and now there are many cells around the suture (stars) that have incorporated [3H]thymidine. Inset quantifies the number of cells undergoing DNA synthesis normalized by a control in which [3H]thymidine incorporation was measured in an unwounded wing.

**Fig. 6.** Growth factor-induced secondary tumors. Tumors promoted by aFGF (a) and bFGF (b), 10 days after application of the growth factor. Arrowheads, small tumors, some of which are dark because they contain blood. This pattern is similar to the pattern observed at early stages of primary tumor development (compare with Fig. 1b).
Fig. 7. Cross-sections of the skin of wings treated with various growth factors and then stained with H & E. (a) Normal wing: note the thickness of the dermis (double arrow). (b) EGF-treated wing does not show an inflammatory response or any other significant morphological alteration (compare thickness of the dermis, double arrow). (c and d) hFGF and aFGF, respectively, cause an influx of inflammatory cells [heterophils, monocyte/macrophages (arrowheads), and small lymphocytes, i.e., small cells with dark nuclei (double arrowheads)] to the site of application of the growth factor, with aFGF causing a larger effect than bFGF; thickness of the dermis indicated by the double arrow. (e) TGF-β causes an influx of mostly heterophils and monocyte/macrophages (arrowheads); no small lymphocytes are seen but notice that the dermis is even more swollen than in the wing treated with aFGF. (f and g) Sections of wings treated with aFGF and TGF-β, respectively, and then immunostained with an antibody against fibrinogen showing fibrinogen on cells and matrix. This indicates that the fibrinogen must have come out of the blood vessels into the tissues.

This latter effect is similar to our observation that β-methylprednisolone can only partially inhibit the TGF-β-induced inflammatory response. It would appear, therefore, that in our system TGF-β induces inflammation through a pathway that β-methylprednisolone cannot inhibit, whereas aFGF functions via a pathway in which inhibition occurs. In that regard, it is interesting to note that, contrary to the FGFs, TGF-β does not attract small lymphocytes, immune cells that are important in the latter stages of healing, to the site of inflammation. Rather, TGF-β attracts mostly heterophils and monocyte/macrophages, immune cells that play an important role in the early stages of the inflammatory response and in chronic inflammation. In addition, TGF-β causes more edema and a more prolonged inflammatory response than does FGF. It has been shown that pm amounts of TGF-β stimulate monocytes to express a variety of cytokines, including tumor necrosis factor (32). It is therefore possible that the inability of β-methylprednisolone to inhibit the TGF-β inflammatory response is due to the autocrine production of TGF-β (therefore a persistent stimulation of the inflammatory response) and/or it is the induction by this growth factor of cytokines, such as tumor necrosis factor, that causes chronic inflammation (32, 33).

Table 2  Effect of β-methylprednisolone on promotion of tumors by aFGF and TGFβ

<table>
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<th>Growth factor</th>
<th>Dose of growth factor (ng)</th>
<th>Dose of β-methylprednisolone (μg)</th>
<th>Inflammatory response</th>
<th>% of secondary tumors</th>
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<td>aFGF</td>
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<tr>
<td>TGFβ</td>
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<td>600</td>
<td>Yes</td>
<td>100</td>
</tr>
</tbody>
</table>
In conclusion, our results demonstrate that inflammation is the critical factor in development of wound tumors in RSV-infected chicks. During the inflammatory response caused by wounding or by TGF-β, aFGF, and bFGF, a plethora of cytokines are released that cause blood vessel leakage and stimulate cell division which are key elements in the process leading to oncogene integration and activation and then to tumor development. In all cases, tumor promotion is indirect and is accomplished through the inflammatory response. Although some of the specific events in the inflammatory response differ for the two most effective tumor-promoting growth factors (aFGF and TGF-β), their effects on RSV tumor development are the same; they both stimulate a pronounced inflammatory response, and they both release fibrinogen from the blood, and in both cases tumors develop with the same kinetics.

The results presented here may have direct relevance to tumor development in humans. Even though the viral etiology of cancer in humans is obscure, we know that in some cases viruses are associated with inflammation and tumor development (34, 35). For example, epidemiological studies of patients infected with hepatitis B virus show a strong correlation between chronic inflammation of the liver and development of hepatocarcinomas (36). Inflammation could also be involved in the immunodeficiency disease caused by human immunodeficiency virus. In this case, the retrovirus is transmitted only through wounds or direct introduction into the blood stream. In the case of wounds it is possible that the local inflammatory response leads to the release of cytokines that are chemotactic and growth stimulatory for T-lymphocytes which are the host cells for this virus. Inflammation would not only provide the right host cells but would also provide them in the appropriate biological stage for integration and expression of the virus.

It also is known that, even in the absence of virus or active oncogenes, areas of chronic irritation are prone to development of tumors (7-12, 34). Using the liver again as a specific example, tumors frequently develop in patients with cirrhosis, a chronic inflammation of the liver that is caused by excessive alcohol consumption (37) or exposure to carcinogenic chemicals such as CCl₄ (12). Repeated exposure of tissues to the cytokines released by inflammatory cells appears to play a crucial role in the development of these tumors (albeit requiring a longer time in the absence of oncogenes). This could be the result of accumulated cellular alterations and stimulation or inhibition of expression of specific genes similar to the effect caused by viral oncogenes, leading to deregulation of cell function and culminating in transformation.

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Inflammation Is Responsible for the Development of Wound-induced Tumors in Chickens Infected with Rous Sarcoma Virus

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