sphingoids (*9, 24*). The plasma membrane is a major site of sphingosine localization; it contains sphingomyelinase activity that could generate sphingosine from endogenous substrates (*25*), and it may be the primary site of inhibition of PKC. However, the solubility of sphingosine (>10 μM under intracellular conditions) permits its movement within the cytosol to internal membranes. We propose that at the ER membrane, sphingosine is converted most probably to sphingosine-1-phosphate and there mediates Ca^{2+} release. Analyses of sphingosine-1-kinase in platelets suggest that the enzyme is cytosolic, although with multiple forms (*22, 26*); a microsomal enzyme exists in *Tetrahymena pyriformis* (*27*), a location also suggested by our studies (*20*). Sphingosine-1-phosphate is curiously insoluble in either aqueous or nonaqueous solvents (*28, 29*); thus, if sphingosine-1-phosphate is formed in the ER membrane, it could stay trapped there. Sphingosine-1-phosphate lyase, an enzyme cleaving sphingosine-1-phosphate to palmitaldehyde and phosphoethanolamine, is a known microsomal enzyme (*28*). Thus, the ER appears to have the means to form, retain, and degrade sphingosine-1-phosphate. The regulation of either enzymic step could control Ca^{2+} permeability and hence Ca^{2+} signaling in cells.

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Mediation of Wound-Related Rous Sarcoma Virus Tumorigenesis by TGF-β

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In Rous sarcoma virus (RSV)–infected chickens, wounding leads to tumor formation with nearly 100% frequency in tissues that would otherwise remain tumor-free. Identifying molecular mediators of this phenomenon should yield important clues to the mechanisms involved in RSV tumorigenesis. Immunohistochemical staining showed that TGF-β is present locally shortly after wounding, but not in unwounded controls. In addition, subcutaneous administration of recombinant transforming growth factor–β1 (TGF-β1) could substitute completely for wounding in tumor induction. A treatment protocol of four doses of 800 nanograms of TGF-β resulted in v-*src*–expressing tumors with 100% frequency; four doses of only 10 nanograms still led to tumor formation in 80% of the animals. This effect was specific, as other growth factors with suggested roles in wound healing did not elicit the same response. Epidermal growth factor (EGF) or TGF-α had no effect, and platelet-derived growth factor (PDGF) or insulin-like growth factor–1 (IGF-1) yielded only occasional tumors after longer latency. TGF-β release during the wound-healing response may thus be a critical event that creates a conducive environment for RSV tumorigenesis and may act as a cofactor for transformation in this system.

ROUS SARCOMA VIRUS (RSV) WAS the first RNA tumor virus to be discovered (*1*). RSV rapidly transforms many cell types in culture (*2*), but its ability to induce tumors in vivo is highly dependent on the tissue environment (*3–5*). An example of this is seen in young chicken hatchlings, in which a sarcoma is rapidly formed only at the site of virus injection and at the site of experimentally induced wounds (*5, 6*). In spite of circulating infectious virus, other tissues stay generally free of tumors during the early stages of pathogenesis (*5, 6*). Thus the infliction of a wound and the subsequent healing process appear to confer a state conducive to RSV tumorigenesis. We have used this model system to identify factors that contribute to the generation of such a competent environment.

Growth factors are important in tissue reorganization during the wound-healing process (*7*) and have been implicated in the sustained growth of neoplasms. They may therefore be mediators in the creation of the competent environment. TGF-β has been shown to be one of the most potent effectors of the wound-healing reaction. It enhances

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collagen formation and tensile strength in experimental wound models (*8, 9*) and can by itself induce the formation of new tissue in normal skin that resembles granulation tissue in wound repair (*10*). We therefore analyzed the pattern and time course of TGF-β elaboration in our model at the site of experimentally induced wounds.

Wounds were inflicted by inserting surgical silk suture through the wing of 10-day-old chickens. This method was 100% effective in the induction of wound tumors (*11*). Leaving the suture in place to allow the precise localization of the wound, we immunohistochemically analyzed tissue sections from the wound site as a function of time after wounding (Fig. 1). The antibody used [anti-CC 1–30] (*12*) was raised against a conserved 30 amino acid sequence of TGF-β1 and detects predominantly extracellular TGF-β (*13*).

Unwounded controls generally did not show TGF-β staining in the muscular areas of the wing and only very rarely was staining detected in intermuscular connective tissue (Fig. 1a). At early time points after wounding (30 min, 1 hour, and 2 hours) there was no increase in specific staining for TGF-β. Beginning at 2 hours, the wound site and adjacent tissues became heavily infiltrated by inflammatory cells. TGF-β was detected extracellularly in association with these cells at 6 hours (Fig. 1c). A similar staining pattern but of increased intensity was seen at 11 and 20 hours (Fig. 1d). Inflammatory cells, identified by morphology, included thrombocytes, heterophils, monocytes, macrophages, and lymphocytes. Whereas thrombocytes together with released erythrocytes prevailed at early time points (30 min, 1 hour, and 2 hours), lymphocytes and heterophils were found predominantly at the time of increased TGF-β labeling (6, 11, and 20 hours). There were also monocytes present at 6 and 11 hours and macrophages at 20 and 42 hours. TGF-β was also detected at later stages of wound healing during the formation of granulation tissue (42 hours), when fibroblasts had begun to infiltrate the wound (Fig. 1e). It was still detected in fully developed granulation tissue (120 hours), notably in areas that appeared less organized, and at epithelial/mesenchymal interfaces of reepithelialization (Fig. 1, b and f).

The staining patterns reported above suggest that TGF-β is involved in several stages of the wound-healing process. The association of TGF-β with inflammatory cells is consistent with previously reported secretion of TGF-β in culture by stimulated neutrophils (which are the equivalent of chick heterophils) and macrophages (*14, 15*). Secretion of TGF-β by wound heterophils and macrophages may direct the further development of granulation tissue by its known effects on fibroblasts, including chemotaxis (*16*) and induction of increased deposition of extracellular matrix components (*16–17*). We have shown previously that wound fibroblasts express RSV proteins within 2 days after wounding and may be important in wound tumor formation (*11*). Thus both timing and location of TGF-β elaboration are consistent with a role for TGF-β as a molecular mediator in wound tumor induction.

If TGF-β elaboration were indeed causative rather than correlative in wound tumor formation, it should by itself be capable of induction of RSV tumors. To address this question, we applied TGF-β by careful subcutaneous injection to the wing dermis of RSV-infected chickens in four daily intervals (4×) with a tenfold excess of bovine serum albumin (BSA) in physiological saline. It is impossible to completely eliminate microinjury caused by the injection, but we minimized tissue damage by using thin needles and small volumes. Such minimal injury is not sufficient to induce wound tumors (*18*). Accordingly, control injections of BSA alone (4× 8 μg) did not result in any detectable change in phenotype over an observation period of 10 to 12 days (Table 1). By contrast, injection of 4× 800 ng of human recombinant TGF-β1 induced an appreciable response in less than a day, which resembled a granulomatous reaction [also reported in a mouse model (*10*)]. This reaction persisted during the period of growth factor application, but regressed after 7 to 8 days (all time periods mentioned

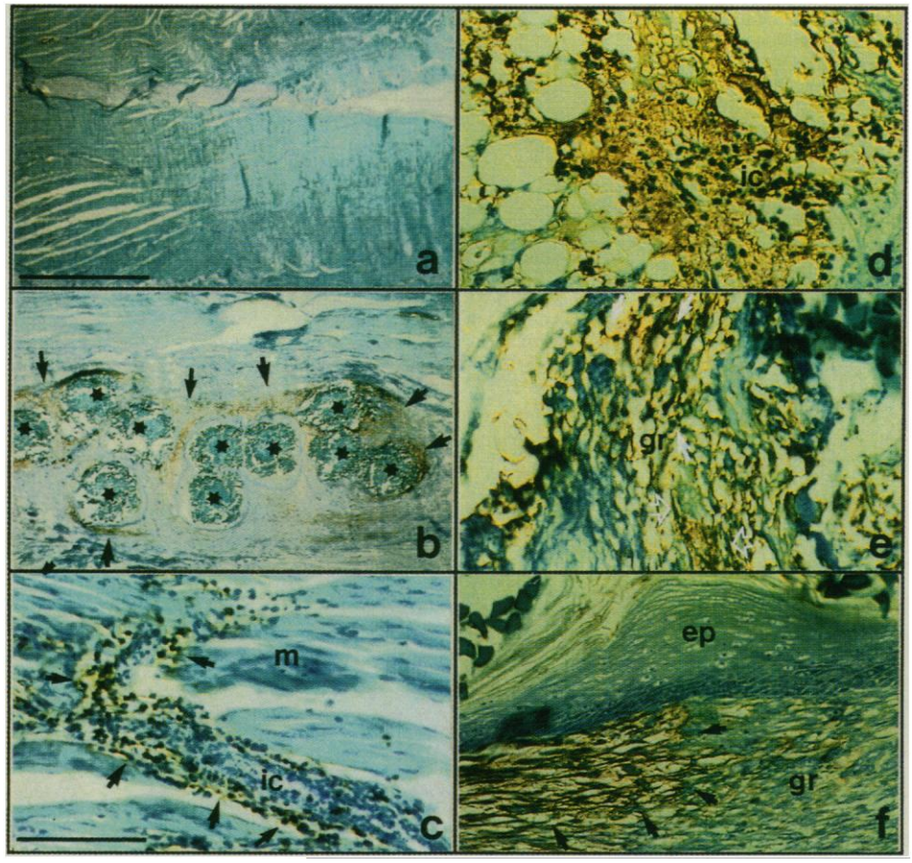

Fig. 1. Localization of TGF-β at the site of wounding. Ten-day-old chickens were wounded by inserting 15 loops of surgical silk suture through the central muscle blocks in the radius/ulna region of the right wings. Tissue samples with the suture in place were excised at the indicated times after wounding and processed for paraffin sectioning by standard techniques. Sections (5 μm) were stained by the Avidin-biotin-peroxidase technique with polyclonal rabbit anti-CC 1–30 antibody against the NH_2-terminal 30 amino acids of TGF-β1 (*12*), which are conserved between human, pig, mouse, and chicken. Positive staining is indicated by a brown precipitate. The counterstain was Giemsa/May-Gruenwald. (**a**) Unwounded control, (**b**) corresponding area 5 days after wounding showing TGF-β staining (arrows), stars indicate silk suture. Scale bar, 1 mm. (**c** to **f**) Time course after wounding. Scale bar, 100 μm. (**c**) At 6 hours, inflammatory cells (ic) infiltrating the wound site through disrupted muscle fibers (m) with associated TGF-β staining (arrows). (**d**) At 20 hours, inflammatory cells (ic) at the wound site with increased TGF-β staining. (**e**) At 42 hours, formation of fibrotic granulation tissue (gr), including inflammatory cells (solid white arrows) and fibroblasts (open white arrows) with associated TGF-β staining. (**f**) At 120 hours, TGF-β staining (arrows) at the interface of new epithelium (ep) and granulation tissue (gr).

refer to time after the first growth factor application). At the same time tumors formed at the TGF-β application site with 100% frequency (Table 1) and continued to grow until the birds were moribund and were killed (10 to 12 days). Tumors were frequently multinodular and sometimes showed very discrete hemorrhagic lesions (Fig. 2a). Porcine TGF-β1, purified from platelets, caused a similar granulomatous reaction, but there was a lower frequency of tumor formation (63%) (Table 1). This difference may reflect variation in purity and potency of the preparations. Control applications of TGF-β to uninfected chickens resulted in the same granulomatous reaction, but did not yield any tumors (Table 1). On day 10 the wings were morphologically indistinguishable from normal controls. Histologically the only apparent effect was a slight hyperplastic fibrotic thickening of the dermis (*18*).

Both the initial granulomatous reaction and tumor induction by TGF-β were dose dependent. The granulomatous response was less pronounced with each further dilution and not observed at a concentration of 4× 10 ng. Nevertheless, the frequency of tumor induction was still very high (80%) even at this dose (Table 1). A further 5-fold and 20-fold dilution resulted in a markedly reduced but still appreciable tumor rate (33% and 25%, respectively) (Table 1). A dose effect was also apparent from tumor size and latency. At the lower doses tumors were significantly smaller, frequently consisting of only a single nodule that appeared with approximately 2 days longer latency (Table 1). When 800 ng of human recombinant TGF-β1 was administered in only one or two instead of four applications, the rate of tumor induction was still high (71% and 100% respectively) (Table 1).

Histologically the TGF-β1–induced tumors had the typical characteristics of RSV-mediated neoplasms. The tumor nodules were identified as fibrous tissue sarcomas. Tumor tissue was found in confined areas of the dermis and in intermuscular connective tissue. Occasionally invasion of muscle fibers was observed (Fig. 2b). The observed discrete hemorrhagic lesions (Fig. 2a) were histologically identified as abnormally enlarged blood-filled vessels (region b on Fig. 2b).

To confirm the viral origin of the tumors, we performed in situ hybridization with a probe of the v-*src* oncogene of RSV on cross sections of the tumor-bearing wing. The tumor areas were strongly labeled and hybridization was confined to the tumor tissues (Fig. 2, c and d). Viral protein expression was confirmed by immunofluorescence with an antibody against $p19^{gag}$ (*12, 18*).

To test whether the tumorigenic response was specific to TGF-β or whether it could also be generated by other growth factors potentially involved in wound healing, we tested TGF-α, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and insulin-like growth factor–1 (IGF-1) for their potential to induce similar tumors. We used human recombinant proteins in all cases to ascertain purity, and applied a relatively high dose of 4× 800 ng (EGF and TGF-α) or 4× 200 ng (PDGF and IGF-1). In the case of EGF and TGF-α there were no observable effects for 10 to 12 days (Table 2). EGF isolated from mouse submaxillary gland showed some effect. However, since tumor frequency decreased and latency increased with increasing purity and no tumors resulted from the recombinant protein (Table 2), it appears that some impurity in the preparation was the cause of tumor induction (*19*). PDGF and IGF-1 application led to tumor formation in only one case out of six and seven (a frequency of 17% and 13%, respectively). Tumors were

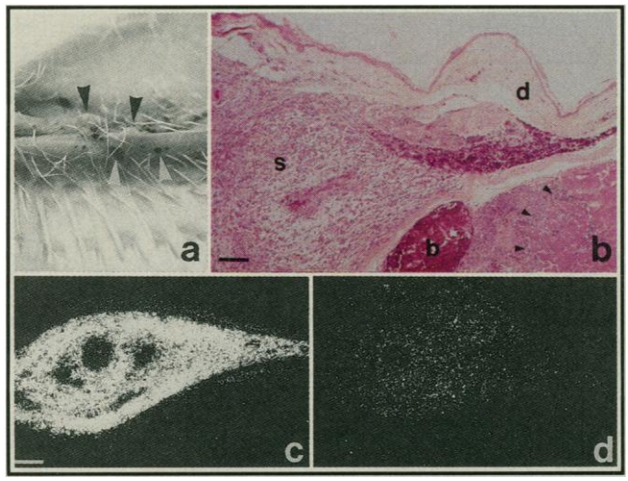

Fig. 2. Characterization of TGF-β–induced RSV-tumors. Analysis of tumors induced by 4× 800 ng TGF-β. Tumors induced by lower concentrations yielded equivalent results. Tumor-bearing wing segments were processed for paraffin sectioning by standard techniques and for frozen sectioning as described (*11*). Scale bars, 100 μm. (**a**) Photograph of tumor induced by 4× 800 ng of human recombinant TGF-β1 11days after the first application showing sarcomatous nodules (black arrowheads) and discrete hemorrhagic lesions (white arrowheads). (**b**) Hematoxylin and eosin staining of 5-μm paraffin section through the tumor area. Sarcomatous nodule (s) in the dermis (d) and abnormally enlarged blood-filled vessel (b); arrowheads, invasion of muscle block. (**c**) In situ hybridization with an α-^{35}S-labeled antisense riboprobe against the 812-bp Pst 1 fragment of the RSV-PC v-*src* oncogene on 5-μm frozen section of sarcomatous tumor area. The construction of the transcription vector and protocols for labeling and hybridization have been described (*11, 30*). The probe was added at 4×10^6 cpm/ml and exposure was for 2 weeks. Visualization was by dark-field optics. (**d**) Hybridization of same area on adjacent section with unrelated vector sequence (control).

Table 1. Induction of RSV tumors by TGF-β1. Five-day-old chickens were injected with 10^5 focus-forming units (ffu) of RSV-PC in the left wing. After 5 days, saline solution (20 μl) containing the indicated amount of activated TGF-β1 (*29*) and a tenfold excess of BSA were administered at daily intervals to the dermis of the opposite wing by careful subcutaneous injections with a 25-gauge needle. Animals were scored daily for tumor formation until they were killed at 10 to 12 days after the first application.

Factor	Number of applications	Amount (ng) per application	Tumors/ treated chickens	Latency (days)
	RSV-infected chickens			
BSA	4	8000	0/18	
Porcine TGF-β1	4	800	5/8	7–8
Human recombinant TGF-β1	4	800	6/6	8–9
	4	400	3/4	8
	4	200	4/5	8
	4	50	4/5	8–9
	4	10	4/5	8–9
	4	2	2/6	7–10
	4	0.5	1/4	10
	1	800	5/7	7–8
	2	400	4/4	7
	Uninfected chickens			
Porcine TGF-β1	4	800	0/2	
Human recombinant TGF-β1	4	800	0/3	

smaller and had a longer latency period than those induced by TGF-β. The very low effectiveness of PDGF in tumor induction is especially noteworthy, since the action of PDGF and TGF-β appear to be connected in many instances. For example, it is thought that the mitogenic effect of TGF-β on fibroblasts is mediated by induction of PDGF/c-*sis* (*20*). On the other hand, it has been suggested that PDGF induces TGF-β mRNA in macrophages (*21*). Our results indicate that in this system TGF-β is the primary effector and that the response to PDGF is probably indirect, possibly via the induction of TGF-β in macrophages.

The absence of or limited effect of these growth factors on tumor induction was not due to poor interspecies cross reactivity. All peptides were biologically active as growth factors on chicken cells in culture. They induced increased DNA synthesis in density-inhibited chick embryo fibroblasts (CEF) as determined by [^{3}H]thymidine incorporation (Fig. 3A). To assure that the failure of EGF and TGF-α to induce tumors was not because of degradation and activity loss in vivo, we administered 800 ng of human recombinant EGF or TGF-α subcutaneously to the wing and assayed after 16 hours for DNA-synthesizing cells in frozen sections of the area by the use of 5-bromodeoxyuridine (BrdU) incorporation and immunofluorescence with an antibody to BrdU (*22*). Application of both factors resulted in a significant increase of proliferating cells in the dermis (Fig. 3B). Under the same conditions, TGF-β did not have a significant effect on proliferation. This demonstrates the retention of activity of recombinant EGF and TGF-α in vivo and also indicates that the induction of proliferation at the application site is not sufficient and a more specific event is required to permit RSV tumorigenesis (*11*).

In conclusion, the kinetics and localization of TGF-β in situ qualify it as a molecular mediator of wound tumor formation. TGF-β can induce RSV-mediated sarcomas in chicken hatchlings at 100% frequency and thus can substitute completely for wounding in tumor induction. Taking the discontinuous means of application into account, it is effective at very low concentrations that may approach physiological levels. The effect of TGF-β is specific. Other mitogens with suggested roles in wound healing fail to elicit the same response. TGF-β release during the wound-healing process may thus be a critical event that creates a conducive environment for RSV tumorigenesis. Ultimate proof for this would come from inhibition of wound tumor formation by

Table 2. Effect of other growth factors on RSV tumor induction. Chicken hatchlings were treated as described in Table 1, and the indicated amount of factor (*29*) was applied under the same conditions.

Factor	Amount* (ng)	Tumors/ treated chickens	Latency (days)
Mouse submaxillary gland EGF (80% pure)	800	5/10	8–9
Mouse submaxillary gland EGF (95% pure)	800	3/11	11–12
Human recombinant EGF	800	0/9	
Human recombinant TGF-α	800	0/9	
Human recombinant PDGF	200	1/6	10–11
Human recombinant IGF-1	200	1/7	10–11

*Each dose was applied four times in daily intervals.

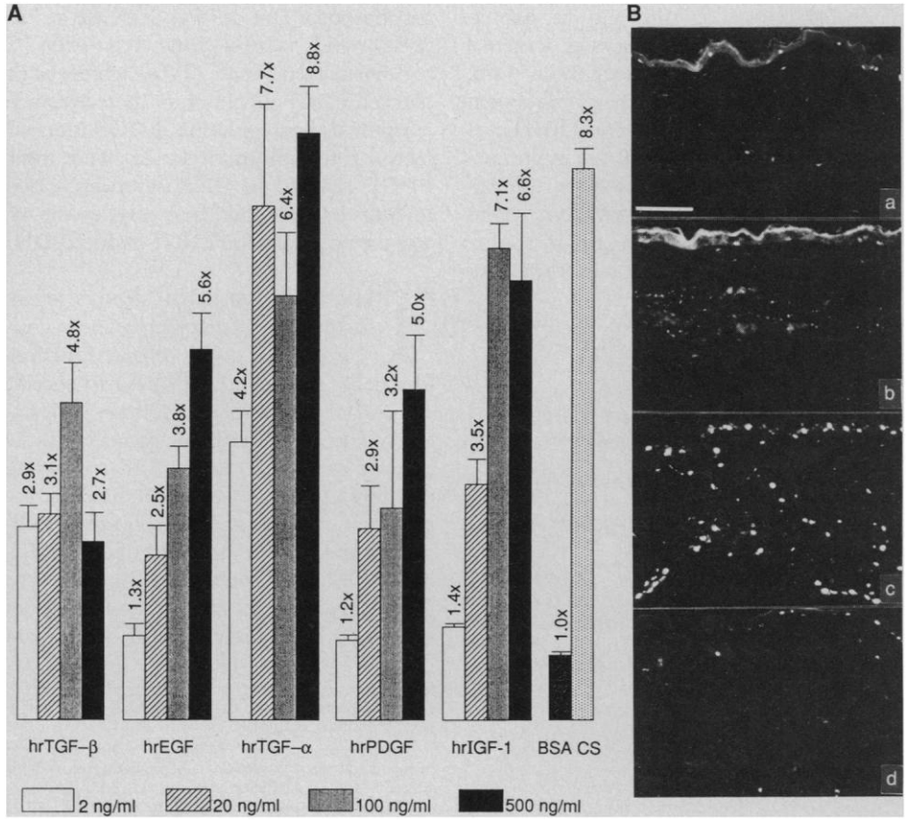

Fig. 3. Induction of proliferation by human recombinant (hr) growth factors. (**A**) In culture. Bar graph showing fold increase of [^{3}H]thymidine incorporation from mean of four separate samples over base level in density inhibited CEF; error bars indicate SEM. The factors were diluted into BSA solution (1 mg/ml) and added to the culture medium to give the indicated final concentrations. Control samples were mock stimulated with BSA (1 mg/ml) to give the base line for unstimulated proliferation (BSA) or stimulated with 10% chicken serum as a positive control (CS). Incubation was for 16 hours and [^{3}H]thymidine was added at 5 μCi/ml in nucleotide-free medium essential medium alpha (Gibco) for 1 hour. Cells were fixed in methanol/acetic acid (3:1 v/v) for 1 hour, washed twice in 80% methanol, and lysed with 1% SDS. Lysate was counted in Ecolite scintillation fluid (Westchem). (**B**) In vivo. Analysis of proliferative effect in vivo after growth factor application by BrdU incorporation and immunofluorescence. The indicated human recombinant factor (800 ng) was administered in 20 μl of saline containing a tenfold excess of BSA (8 μg) by careful subcutaneous injection with a 25-gauge needle to the radius/ulna wing region of a 10-day-old chicken. The tissue was excised after 16 hours, incubated for 2 hours in medium 199 (Gibco) containing 20 μM BrdU at 37°C and processed for frozen sectioning. Frozen sections (5 μm) were analyzed by immunofluorescence with an antibody to BrdU (*12*) as described previously (*11, 22*). (a) TGF-β, (b) EGF, (c) TGF-α, and (d) BSA (8 μg). EGF and TGF-α induce a significant increase of proliferation in the dermis compared to control injections of BSA alone. Scale bar, 100 μm.

neutralizing antibody to TGF-β. However, our initial experiments show that the effectiveness of currently available antibodies is too low to allow meaningful neutralizing experiments in vivo.

Wounding acts as a tumor promotor in many other animal models of chemical and viral carcinogenesis [referenced in (*11*)]. It has been shown that 12-O-tetradecanoyl phorbol-13-acetate (TPA) induces expression of TGF-β in mouse epidermis (*23*) and that TGF-β can mimic the promoting effects of phorbol esters in vitro (*24*) and in the mouse skin model of multistage carcinogenesis in vivo (*25*). Together with our results, TGF-β elaboration may thus offer a more general explanation for the promoting effect of wounding in conjunction with activated oncogenes or initiating chemical carcinogens. The following mechanism may be envisioned: It has been suggested that tumors subvert the host's wound-healing response by inducing a supportive stroma that resembles closely the stroma in healing wounds and that is essential for further growth (*26*). The action of growth factors, in particular TGF-β, have been implicated in the generation of such stroma (*27*). Conversely, it may be argued that an already existing stroma such as that found in a healing wound or induced by TGF-β (*10*) could provide a conducive environment for tumorigenesis.

In apparent contrast to our findings, TGF-β is a potent growth inhibitor for most epithelial cells in culture and its role in carcinogenesis has been discussed in terms of escape from this regulatory mechanism, since many tumor cell lines have been shown to have lost their responsiveness to TGF-β (*28*). The actions of TGF-β are multifunctional and dependent on the tissue context. Therefore, its role in carcinogenesis has to be evaluated cautiously, taking both epithelial and stromal elements of tumor development into consideration.

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PDGF-Induced Activation of Phospholipase C Is Not Required for Induction of DNA Synthesis

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Platelet-derived growth factor (PDGF) induction of DNA synthesis is believed to involve activation of phospholipase C (PLC) and subsequent accumulation of inositol 1,4,5-trisphosphate [I(1,4,5)P_3], increase in intracellular Ca^{2+}, activation of protein kinase C (PKC), and receptor down regulation. Generation of these events is triggered by the tyrosine protein kinase (TPK) activity of the PDGF receptor. The TPK inhibitor genistein blocked PDGF induction of these events, including DNA synthesis, with the exception of receptor down regulation. PDGF-induced phosphotyrosine phosphorylations, including receptor autophosphorylation, were inhibited by genistein. Removal of genistein and PDGF resulted in DNA synthesis without the occurrence of PLC activation. These findings indicate that these early events, with the exception of receptor down regulation, are not necessary for PDGF-induced DNA synthesis.

THE PROCESSING OF INFORMATION across the plasma membrane is critical for successful cellular function. Mitogens, such as PDGF, bind to specific cell surface receptors that contain TPK activity. The early signals emanating from the binding of PDGF to its TPK-containing receptor are thought to be required for the initiation of DNA synthesis and cell proliferation and include increased phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis (*1–3*) resulting in the accumulation of I(1,4,5)P_3 and diacylglycerol (DAG), a transient I(1,4,5)P_3-induced increase of intracellular Ca^{2+} (*2, 3*), PKC-dependent phosphorylation of an 80-kD protein (*4, 5*), and receptor down regulation (*6*). With the use of receptor mutants that lack TPK activity, PDGF-receptor TPK activity has been shown to be required for most of these early signals as well as DNA synthesis (*2, 7, 8*). An additional PDGF receptor mutant lacking the TPK insert region, but still containing TPK activity, mediated the PDGF induction of these early signals but not DNA synthesis (*9*). However, from these and other studies (*10*) it cannot be concluded that the early signals resulting from PDGF binding to its receptor are essential for a DNA synthetic response. To answer this question, we have used a pharmacological agent, genistein, which offers the advantage over re-

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