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19. Rat oxyntic mucosa was extracted in accordance with the method of Folch *et al.* (*20*), oxidized with OsO_4, and applied to a small column containing 0.8 g of neutral alumina. After 10 ml of chloroform and methanol (20:1) were added to the columns, the dipalmitoyl phosphatidylcholine fraction was eluted with 5 ml of chloroform, methanol, and 7*M* NH_4OH (70:30:2) and quantitated by phosphorus analysis (*17, 18*). The concentration of oxyntic dipalmitoyl phosphatidylcholine is expressed as micrograms per gram of tissue (wet weight).
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Polypeptide Transforming Growth Factors Isolated from Bovine Sources and Used for Wound Healing in vivo

Abstract. *Transforming growth factors, which are polypeptides that induce the transformed phenotype in nonneoplastic cells, have been isolated in bulk amounts from bovine salivary gland and kidney. In experiments in which wound healing chambers were implanted subcutaneously in the backs of rats, these bovine transforming growth factors accelerated the accumulation of total protein, collagen, and DNA in treated chambers. These studies thus show an effect of an isolated transforming growth factor in vivo.*

Although many new peptide growth factors have been isolated and characterized (*1*), there have been few studies on the activity of these materials in vivo. An important area for potential application of peptide growth factors is the enhancement of wound healing. Despite the need for rapid healing in the treatment of severe burns, trauma, diabetic and decubitus ulcers, and other conditions, there is no practical way at present to accelerate wound healing with pharmacological agents. Although it has been suggested that epidermal growth factor (EGF) might be of benefit (*2*), it has not yet been extensively used in a practical way for wound healing. The ability of a related and newly discovered set of polypeptides, the transforming growth factors (TGF's), to promote growth of cells under highly restrictive conditions in vitro suggests that TGF's might have useful applications in vivo for wound healing. We now report a large-scale isolation of TGF's from readily available bovine sources and a demonstration of the in vivo activity of an isolated TGF in an experimental wound healing system.

Transforming growth factors are a heterogeneous set of low molecular weight polypeptides defined by their ability to induce the transformed phenotype—particularly anchorage-independent growth in soft agar—in untransformed indicator cells that ordinarily do not grow in soft agar (*3, 4*). Transforming growth factors have been found in almost all tissues, both nonneoplastic and neoplastic, from many different species of animals, including man (*4, 5*). The finding of TGF's in blood platelets (*6*) suggests that TGF's may have a role in wound healing and tissue repair. Since TGF's have important functional interactions with EGF and its receptor, we have proposed a new classification of TGF's based on their relationships with EGF (*7*). Type α TGF's are those that compete with EGF for receptor binding and do not require EGF for induction of colony formation by indicator cells in soft agar, whereas type β TGF's do not compete with EGF for receptor binding, but do require the presence of EGF (or EGF-like polypeptides) for induction of colony formation in soft agar. Both types of TGF activity have been isolated from both neoplastic and nonneoplastic cells and tissues (*3–8*).

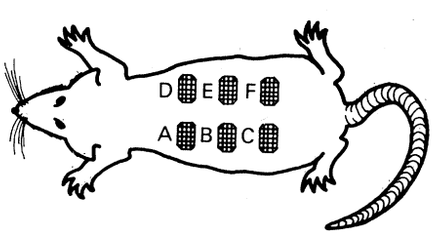

Fig. 1. Diagram of arrangement of wound chambers in the back of a rat. The chambers are made of stainless steel wire mesh and are 2 cm long and 1 cm in diameter. Male Buffalo rats, weighing 400 to 500 g and approximately 12 months old, were used in all experiments.

Full details of purification of bovine β-TGF's will be reported elsewhere. Briefly, salivary glands or kidneys, obtained fresh from the slaughterhouse and frozen immediately on dry ice, were extracted in 2-kg portions with acidified ethanol (*8*). Extracts from 6 to 8 kg of tissue were combined and chromatographed on Bio-Gel P-30 with 1*M* acetic acid on an 80-liter bed volume column (*9*). Most of the in vivo studies reported below were done with salivary gland or kidney TGF's purified to this stage; their activity in vitro was enhanced approximately 20-fold by the presence of 2 to 5 ng of EGF per milliliter in the assay. After chromatography on Bio-Gel P-30, the bovine β-TGF's were purified further by high-performance liquid chromatography (HPLC) on μBondapak C_{18} columns for which an acetonitrile gradient in 0.1 percent trifluoroacetic acid was used; this was followed by a second HPLC step on μBondapak CN columns with a gradient of *n*-propanol in 0.1 percent trifluoroacetic acid (*10*).

Activity of isolated salivary gland and kidney β-TGF's in vivo was measured in a standard experimental wound healing model. Six empty wire mesh wound chambers (Schilling-Hunt) (*11*) were surgically inserted subcutaneously in the back of rats in a symmetrically paired fashion (pairs A and D, B and E, and C and F in Fig. 1). The animals respond to these chambers as if they were wounds, and eventually the chambers become filled with fibroblasts and collagen. By the fourth day after insertion, the chambers become encapsulated with connective tissue, but there are few cells within the chambers themselves. There is thus a defined, enclosed space within the chambers where a wound healing response can be quantitatively measured. At this time, daily injections of TGF (0.1 ml in sterile, phosphate-buffered saline) into

chambers A, B, and C were begun. Except where noted, a low level of murine EGF (*12*) was included in the TGF injections to potentiate TGF activity. Chambers D, E, and F were used as controls and were injected with an amount of bovine serum albumin (BSA), either alone or in combination with either TGF or EGF, to provide an amount of total protein equivalent to the amount of TGF injected into chambers A, B, and C. Injections were made once daily for either 5 days (Table 1) or 9 days (Table 2). All injected materials were sterile. The rats were killed 6 hours after the last TGF injection; the animals treated for 9 days (Table 2) were given intraperitoneal injections of [^{3}H]thymidine (0.5 mCi; specific activity, 6.7 Ci/mmole), with the last TGF injection. The chambers were removed from the rats, all connective tissue on the outside of the wire mesh was peeled away, and the contents of each chamber were determined.

Table 1 shows that 5 days of treatment with β-TGF from either bovine salivary gland or kidney caused a highly significant increase in total protein in the treated chambers, as compared to control chambers treated with an equivalent amount of BSA (experiments 1 and 3). The salivary gland TGF was still highly active after two steps of purification by HPLC (experiment 2). The effects observed are not the sole result of the minute amounts of EGF that had been used to potentiate the activity of β-TGF, since a highly significant difference between treated chambers A, B, and C, compared with control chambers D, E, and F was still observed when EGF was used as the control substance (experiment 4). Furthermore, when all chambers were treated with TGF, and only A, B, and C were treated with EGF, no significant difference was observed (experiment 5). At the end of experiments 1 to 4, we consistently observed that chambers A, B, and C were more firmly fixed in the surrounding connective tissue than the respective matched control chambers; this suggested that the effects of TGF were also manifested in the area immediately surrounding the chambers.

To measure the effects of bovine salivary TGF on DNA and collagen content of the chambers, we found it necessary to treat the animals for longer than 5 days. In an experiment in which 13 rats were treated for 9 days (Table 2), the increases in total protein, total DNA, thymidine incorporation into DNA, and total collagen were highly significant. Histological examination of the contents of chambers treated with TGF confirmed the occurrence of fibroblastic proliferation and formation of collagen. A sterile infiltrate of inflammatory cells, which are known to be involved in physiological wound healing (*13*), was also found within both treated and control chambers.

The results obtained in both experiments indicate that β-TGF's can significantly accelerate a wound healing response. Further studies in other wound healing models, such as measurement of effects on tensile strength of linear wounds and rate of healing of granulating wounds, are needed. The new growth factors that we have isolated are biologically active at the nanogram level in vitro and in vivo and can be obtained on a scale large enough for further investigation of their intrinsic physiological role or possible therapeutic application.

Table 1. Wound healing response to bovine salivary gland or kidney TGF after 5 days of treatment. Transforming growth factors were prepared and injected as described in the text. Each dose contained 25 times the amount of TGF found optimal for colony formation by normal rat kidney cells in a standard soft agar assay (*4*) and ranged from 18 to 42 colony-forming units (*9*) per dose. The amounts of protein injected per dose were: 7 μg in experiments 1, 4, and 5; 25 μg in experiment 3, and 0.7 μg in experiment 2. All doses of EGF were 20 ng. Total protein in wound chambers was measured by the method of Lowry *et al.* (*14*). Statistical analysis of the data was made by comparison of matched pairs of the chambers (A versus D, B versus E, and C versus F) shown in Fig. 1.

Experiment	Treatment		Number of matched pairs of chambers	Average amount of protein per chamber (mg)*		Average ratio†	P‡
	Chambers A, B, C	Chambers D, E, F		A, B, C	D, E, F		
1	TGF (salivary, P-30) + EGF	BSA	36	10	3.9	3.8 ± 0.6	< .001
2	TGF (salivary, HPLC) + EGF	BSA	9	8.4	2.9	4.6 ± 1.0	< .02
3	TGF (kidney, P-30) + EGF	BSA	9	8.1	3.5	5.2 ± 1.5	< .005
4	TGF (salivary, P-30) + EGF	EGF	9	9.6	5.3	2.1 ± 0.3	< .02
5	TGF (salivary, P-30) + EGF	TGF	9	11.2	9.6	1.4 ± 0.3	.5

*For method of computation, see (*15*). †Average of matched-pair ratios, A/D, B/E, C/F, ± standard error of the mean (S.E.M.). ‡One-sided P values based on the sign test.

Table 2. Wound healing response to bovine salivary gland TGF after 9 days of treatment. Chambers A, B, and C were injected once daily with 7 μg of TGF (P-30) plus 20 ng of EGF. Chambers D, E, and F were provided an equal amount of BSA.

Measurement	Number of matched pairs of chambers	Average content per chamber		Average ratio†	P‡
		A, B, C	D, E, F		
Protein (mg)	30	24	15	1.6 ± 0.05	< .001
DNA (μg)	30	21	8.6	2.6 ± 0.16	< .001
[^{3}H]Thymidine (counts per minute per microgram of DNA)	30	45	30	1.7 ± 0.09	< .001
Collagen (mg)	9	5.2	3.2	1.8 ± 0.2	< .005

*Measurements were made as discussed in (*16*). †Average of matched-pair ratios, A/D, B/E, C/F, ± S.E.M. ‡One-sided P values based on the sign test.

MICHAEL B. SPORN
ANITA B. ROBERTS, JAMES H. SHULL
JOSEPH M. SMITH, JERROLD M. WARD
National Cancer Institute, Bethesda, Maryland 20205
JARO SODEK
National Institute of Dental Research, Bethesda, Maryland 20205

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9. TGF activity, assayed as described (*4*), eluted in a broad peak between ribonuclease (13,700) and

insulin (5700) markers. At this stage of purification, the TGF's had a specific activity approximately 10- to 25-fold higher than that of the acidified ethanol extracts, with a range of recovery of 150,000 to 200,000 colony-forming units per kilogram of tissue. A colony-forming unit is defined as the amount of TGF that will induce the formation of 1000 colonies of normal rat kidney cells $> 3100\ \mu m^2$ under standard assay conditions (*4*), in the presence of EGF (5 ng/ml).

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15. In all experiments, chambers were inserted in several untreated rats and then removed on the 4th day, at the time when injections of TGF's were begun. Total protein was determined for these chambers, and these "zero-time" values were subtracted from the respective values obtained from rats treated with TGF or control materials. Comparison of the ratios (A/D, B/E, C/F) for the zero-time values (a total set of 33 matched pairs of chambers) showed no significant difference between the left and right side of the rat.
16. Protein was determined as in (*14*). [^{3}H]Thymidine incorporation and total DNA were determined on portions of tissue that had been dissolved in 1*M* NaOH, precipitated with ice-cold 0.3*M* perchloric acid (PCA), washed with ice-cold 0.2*M* PCA, and finally extracted in 0.5*M* PCA at 70°C. Portions of the final extract were used for determination of radioactive counts in a liquid scintillation counter, and deoxyribose was determined by the method of K. Burton [*Biochem. J.* **62**, 315 (1956)]. Collagen was determined as hydroxyproline after hydrolysis with 6*M* HCl. Typing of the collagen by gel electrophoresis of pepsin digests showed no difference between treated and control chambers; Type I collagen was the predominant form.
17. We thank G. Martin and M. Anzano for helpful suggestions, L. Lamb for assistance with the determinations, C. Brown for advice on the statistical analysis, and R. Morsillo for manuscript preparation.

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Demonstration of a Saturable Binding Site for Thyrotropin in *Yersinia enterocolitica*

Abstract. *Several lines of evidence suggest that there might be immunologic cross-reactivity between the thyroid plasma membrane in humans and antigenic determinants in the enteric pathogen* Yersinia enterocolitica. *Studies were therefore performed to determine whether* Y. enterocolitica, *like the thyroid membrane, contains a thyrotropin binding site. A saturable binding site for bovine thyrotropin was indeed demonstrable, particularly in preparations of the organism that have been treated with ethylenediaminetetraacetate and lysozyme. Hormonal specificity of the binding site, as judged from the inhibition of binding of ^{125}I-labeled bovine thyrotropin, was similar to that of the thyrotropin receptor in human thyroid tissue.*

In this report, we describe the presence of a specific, saturable binding site for the mammalian peptide hormone thyrotropin (TSH) in the pathogenic Gram-negative bacillus *Yersinia enterocolitica*. Several groups of observations prompted us to explore this question. First, patients infected with *Y. enterocolitica* commonly display several disorders thought to be autoimmune in nature, including arthritis, erythrema nodosum, Reiter's syndrome, and iritis (*1*). Further, the serum of such patients contains a variety of antibodies to epithelium (*2*), including some which by immunofluorescent techniques can be shown to bind to the cytoplasm and plasma membrane of human thyroid epithelium (*3*). The second group of observations relates to patients with the autoimmune thyroid diseases, Graves' disease and Hashimoto's disease; serum from these patients contains autoantibodies against a variety of thyroid antigens, including thyroid microsomal antigens and thyroglobulin (*4*). It is currently thought that among these antibodies in Graves' disease is an antibody against the TSH receptor which, like TSH, binds to the thyroid membrane, activates adenylate cyclase therein, and initiates the thyroid hyperfunction characteristic of this disorder (*4*). It seemed of particular interest, therefore, that patients with Graves' disease and Hashimoto's disease display, with inordinate frequency, both circulating agglutinins against *Y. enterocolitica* and evidence of cell-mediated immunity against this organism (*5*). Also of relevance is a report that indicates that rabbits immunized against human or rabbit thyroid tissue develop antibodies that are reactive against *Y. enterocolitica* (*6*).

Together these findings raise the possibility that one or more components of the thyroid membrane and of *Y. enterocolitica* share common or cross-reacting antigenic determinants. To explore this question, we looked for functional homology between *Y. enterocolitica* and the thyroid plasma membrane, reasoning that this might reflect structural similarities that could, in turn, account for immunologic cross-reactivity. The best characterized functional unit of the thyroid membrane is its receptor for TSH, the properties of which have been studied by well-established techniques (*7*). Therefore, we conducted experiments to determine whether *Y. enterocolitica* could be shown to contain any site functionally analogous to the TSH receptor in thyroid plasma membranes, and such indeed proved to be the case.

Highly purified bovine TSH (bTSH), labeled with ^{125}I by a stoichiometric chloramine-T method (*8*), was used as a probe. Cultures of *Y. enterocolitica*, serological type 0:3, preserved in the frozen state, were provided by one of us (S.W.). For each experiment, frozen organisms were thawed out and cultured on blood agar plates, and were then subcultured overnight at room temperature in brain-heart infusion broth.

Initial experiments were directed at determining whether saturable binding of ^{125}I-labeled bTSH to the *Yersinia* organism could be demonstrated. Organisms were harvested by centrifugation and washed once with 10 m*M* tris-HCl buffer, *p*H 7.4. Cells were either studied live, killed by treatment with heat or 0.4 percent Formalin, or treated with lysozyme or lysozyme-EDTA, according to techniques previously described (*9*). Various concentrations of cells were then suspended in 10 m*M* tris-HCl buffer, *p*H 7.4, containing ^{125}I-labeled bTSH (approximately 10^{-11}*M*) (*10*). Some tubes contained, in addition, 1.0 U of unlabeled TSH for measurement of nonsaturable or nonspecific binding. Suspensions were incubated for 60 minutes at room temperature, after which the bacterial residue was sedimented and counted. Under these conditions, nonspecific binding was regularly in the range of 1 to 2 percent of added radioactive bTSH. In all preparations, binding of ^{125}I-labeled bTSH in excess of nonsaturable binding was detected and remained constant for at least 6 hours. This specific binding varied in amount with the content of bacterial protein and the manner in which the bacteria had been treated. The least binding was evident in live and lysozyme-treated bacteria, and greatest binding in bacteria treated with a combination of lysozyme and EDTA, in which preparations more than 20 percent of added ^{125}I-labeled bTSH was bound (Fig. 1). We assume that the differences in tracer binding among bacteria prepared by the various methods were the result of differences in the ease of access of the tracer to the binding site. Because the greatest binding of ^{125}I-labeled bTSH was observed in lysozyme-EDTA preparations, this mode of treating the organisms was used in all subsequent studies.

Characteristics of the saturable binding were studied in comparable experi-