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by

Rani Karina Spudich

1997

ABSTRACT

EFFECT OF PHENOBARBITAL ON EXTRACELLULAR MATRIX PRODUCTION,

CELL REPLICATION, AND TRANSFORMING GROWTH FACTOR-β1 RECEPTOR

PROFILE IN RAT DERMAL FIBROBLAST CULTURES.

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Transforming growth factor-β1 (TGF-β1) enhances cell replication and extracellular

matrix synthesis, and associates with distinct cell-surface binding sites in human fibroblast

cultures. Phenobarbital therapy for seizure disorders has been shown to result in a 10%

increase in incidence of connective tissue disorders in patients taking the drug, an effect

that may be mediated in part by changes in local TGF-β1 actions in dermal tissue. The

purpose of our study was to evaluate whether phenobarbital stimulates extracellular matrix

production via TGF-β1-mediated effects in fibroblasts, thus directly playing a role in

connective tissue disorder pathogenesis. DNA and collagen synthesis were measured by

metabolic labeling techniques, while TGF-β receptors were examined by polyacrylamide

gel electrophoresis coupled either with ^{125}I-ligand binding, or specific anti-receptor

antibody probing. Preexposure of rat dermal fibroblast cultures derived from fetal rat

scalp to 30 µM phenobarbital did not alter basal DNA or collagen synthesis, nor did it

affect the stimulatory effects of TGF-β1 on these parameters. Binding studies revealed

that phenobarbital caused decreased TGF-β1 binding within M_r 250,000 (betaglycan), M_r

85,000 (type II), and M_r 65,000 (type I) TGF-β-binding complexes. In addition to decreased binding, total protein amounts of TGF-β receptor types I and II also decreased after exposure to 30 μM phenobarbital. In order for phenobarbital to cause a pathologic increase in matrix deposition, we had expected to see an increase in TGF-β1 binding to the cell-signaling receptors (types I and II), and perhaps a concurrent decrease in betaglycan which functions as a storage site for the TGF-β1 molecule. Our present data therefore suggests that phenobarbital does *not* directly modulate TGF-β1-stimulation of extracellular matrix protein synthesis or cell proliferation in rat dermal fibroblasts.

ACKNOWLEDGEMENTS

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INTRODUCTION

Transforming growth factor-β (TGF-β) is one of a family of molecules which regulates tissue development, cell physiology and many aspects of the immunological response. The continued investigation of the action of this growth factor and others contributes to our understanding of both normal cell function, and dysregulation of function in disease states.

Initially characterized by Roberts and Sporn in 1981, TGF-β was so named because its addition enabled normal rat fibroblasts to proliferate in soft agar – a feat previously accomplished by only virus-infected ("transformed") cells. Since the time of those studies, TGF-β has been found to have varied, and interestingly often opposing effects on cell growth and differentiation, embryogenesis, immune regulation, inflammation, and tissue repair. Overviews of the recent literature about TGF-β are presented in reviews by Border and Noble (1995), and by Okragly et al. The molecule's action appears to be both autocrine and paracrine, and some of its specific effects are to modulate bone formation, angiogenesis, hematopoesis, cell cycle progression, cell migration, and extracellular matrix production. The molecule has been isolated from many different cell types, including platelets, placenta, kidney (Roberts et al., 1983), and bone (Centrella & Canalis, 1985), suggesting its fundamental importance in cell physiology.

This ability to modulate cell growth in many ways groups TGF-β with other described cytokines such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), tumor necrosis factor (TNF), interleukins and colony stimulating factors. All of these cytokines function to maintain homeostasis within the cells of the body's tissues and in the surrounding extracellular matrix. This ability is all the more remarkable since the same cytokine can have stimulatory or inhibitory effects upon a given cell type depending upon such factors as the stage of the cell cycle, and the presence or absence of other cytokines. In general however, TGF-β appears to be stimulatory for mesenchymal cells and inhibitory for epithelial cells.

TGF-β is a regulatory protein belonging to a "superfamily" of structurally related polypeptide regulatory factors. This superfamily includes five TGF-β isoforms, TGF-β1 to TGF-β5, along with the activins, inhibins, and bone morphogenetic proteins (BMPs). Of the TGF-β isoforms, only TGF-β1, 2, and 3 are mammalian (TGF-β4 has been described in birds and TGF-β5 in amphibians). TGF-β1 is the best characterized and most abundant isoform in mammals. Its ubiquitous nature is evidenced by the fact that its amino acid structure is conserved across species, being identical in chickens, cows, pigs, monkeys and humans, and differing in the mouse by only one residue.

TGF-β1 is secreted in a latent, or inactive form, and is modified for active receptor binding. The active form of TGF-β1 is a 25 kDa protein dimer composed of two identical 12.5 kDa subunits joined by disulfide linkages. The molecule is translated as a 390 amino

acid propeptide which becomes cleaved by proteases to create two fragments: a C-terminal peptide of 112 amino acids and a "latency-associated peptide," 278 amino acids in length. The active dimer mentioned above is formed by the disulfide bonding of two C-terminal peptides – however, the latency-associated peptide immediately noncovalently binds the dimer to form the 100 kDa "latent TGF-β1 complex." Yet another factor, the "latent TGF-β binding protein" may also attach. It is hypothesized that latent TGF-β1 is stored on the cell surface or in the neighboring extracellular matrix until it is needed. Activation of the cytokine occurs by removal of the latency-associated peptide. The mechanism by which this occurs *in vivo* is not well understood, but likely involves protease action.

The many effects of the TGF-β1 molecule are realized via a cell-signaling cascade initiated at the level of cell-surface receptors. Nine proteins have been recognized to bind TGF-β1 at or near the cell membrane (reviewed by Massague, 1992). Three of these are most abundant: two are true transmembrane receptors, known as receptor types I and II, while the third is an extracellular proteoglycan somewhat misleadingly known as receptor type III. The current evidence, however, suggests that the type III "receptor" is not itself involved in signal-transduction, therefore, it is perhaps more appropriately known as "betaglycan." The type I and II receptors have molecular weights of 53 kDa and 70 kDa respectively, while betaglycan is largest at 200-400 kDa.

Understanding the TGF-β1 signal transduction pathway is a current topic of research. To date, the data has suggested that this pathway may differ among cell types. One body of knowledge suggests that both receptor types I and II, shown to be serine-threonine kinases, are necessary for signal transduction (reviewed by Massague et al., 1994; Miyazono et al., 1994). This model supports the idea that the TGF-β molecule first binds the type II receptor, forming a complex that "recruits" the type I receptor. In this model, the type I receptor becomes phosphorylated by the constitutively active protein kinase domain of type II, thus initiating the signaling cascade (Wrana et al., 1992; Wrana et al., 1994). In the chemically mutated mink lung epithelial cells where the model was first formulated, cell responsiveness to TGF-β is lost when only one of the two receptors is in existence (Attisano et al., 1993). In other words, the evidence from these studies suggests a constant ratio between receptor types I and II is necessary for cell signaling to occur. In our laboratory, however, research by Michael Centrella and Thomas McCarthy has shown that in several cell culture models the type I receptor may bind TGF-β1 and propagate the signaling process independently of type II sites, depending upon the stage of differentiation of the cell. If the type I receptor truly functions "downstream" of type II in the signaling process (discussed further below), Centrella and McCarthy postulate that it may become activated via phosphorylation by *other* cellular kinases as yet undefined, and thus independently initiate the cell signal.

Because it is not required for signal transduction in some cell types, the role of betaglycan (type III receptor) remains undefined. Its core protein contains 853 amino

acids, and at least half of its mass derives from glycosyl side chains. The proteoglycan may be found in a soluble, extracellular form, or alternatively may be anchored in the cell membrane. The membrane-bound form has a transmembrane segment and short intracellular region, but is primarily made up of a large extracellular domain (Lopez-Casillas et al., 1991). It has been hypothesized that in its soluble form, betaglycan acts as a storage vehicle for TGF-β1, keeping the growth factor from interacting with the signaling receptors. It has also been proposed that a membrane-bound form of betaglycan is involved in presenting TGF-β1 to the receptor types I and II, thus assisting the signaling cascade (Lopez-Casillas et al., 1993).

The specifics of the ligand-receptor binding leading to physiologic response within the cell are only just becoming elucidated. Quite recently a new gene family named "Mad" has been identified as playing a role in the TGF-β signal transduction pathway (reviewed by Derynck & Zhang, 1996; Massague, 1996). "Mad" was described in Drosophila, where its function was found to be required for activity of DPP (Decapentaplegic), a TGF-β analogue. DPP initiates its signal via a similar interaction of a Type II receptor (named "Punt" in Drosophila) with a Type I receptor (two variations being "Thick veins" and "Saxophone") as previously described for TGF-β1. Such findings as that the overexpression of Mad partially rescues DPP deficiencies have led researchers to conclude that Mad functions *downstream* of the DPP Type I receptor. The products of the Mad genes are proteins of ~450 amino acids with highly conserved N-terminal and C-terminal domains, and a variable proline-rich intervening region. In response to receptors binding

ligands, it appears that Mads undergo serine phosphorylation, and accumulate in the cell nucleus. It is hypothesized that Mads may then interact with specific response elements in target genes, thereby activating their transcription. Mad homologues have been identified in other organisms, including the human (Smad1). It has therefore been suggested that, in humans, different TGF-β family members may signal through different Mad isoforms. Further, the same TGF-β molecule may effect different biological responses, mediated by different Mads, or different amounts of Mad transcript.

So, what is the end result of this intricate cascade? Previously mentioned is that TGF-β1 has the capacity to both stimulate and inhibit cell division and proliferation. Not surprisingly, research over the past five years has begun to elucidate that multiple interactive mechanisms exist by which TGF-β1 ultimately affects cell cycle kinetics. While the complex details of these pathways are in the process of being characterized, some of the fundamental concepts follow, as reviewed by Saltis, 1996: In mammalian cells, the G1 phase of the cell cycle is regulated by a family of cyclin-dependent kinases (primarily CDK2, 4, and 6) as well as by specific CDK activators, the cyclins (mainly cyclin D and E). One of the most important substrates of G1 CDKs in regulating the cell cycle is the retinoblastoma gene product, "pRb." pRb, if not phosphorylated, inhibits cell cycle progression, while phosphorylation reverses that inhibition. It has been demonstrated that in cells where TGF-β1 *stimulates* cell division (mesenchymal cells), retinoblastoma protein is phosphorylated and releases a transcription factor "E2F." E2F is transported to the nucleus where it increases the transcription of genes that prepare the cell to enter the S

phase. In cell types where growth is *inhibited* by TGF-β1 (epithelial, endothelial, and hematopoietic cells), the activation of the CDK2-cyclin E complex by CDK4-cyclin D is inhibited, and pRb remains unphosphorylated. E2F therefore remains bound and unavailable for initiation of transcription, thus blocking cell division.

Once again, the overriding message from all of this is that the effects of TGF-β1 on the physiologic functions of many cell types are multiple and varied, and may be explained by alterations in production, secretion, and subsequent activation of latent TGF-β, by changes in TGF-β receptor expression, and by alterations in post-receptor signaling. In addition to understanding the role of TGF-β in maintaining healthy tissues, the investigation of *dysregulation* of TGF-β actions may be the key to specific pathologic processes in humans. Already, researchers have postulated that dysregulation of TGF-β is involved with the development of congenital defects, carcinogenesis, and chronic inflammatory and fibrotic diseases.

Both research projects with which I was involved during medical school addressed the involvement of TGF-β1 in the pathogenesis of fibrotic diseases. In one project, the disease process of interest included connective tissue disorders in general, exemplified by Dupuytren's contracture of the hand, while the second project concerned the process of vascular restenosis (Yang, Spudich et. al, 1995). What follows will provide an overview of the first of these two diseases and the questions addressed in my research on this topic.

The most extensively studied effect of TGF-β1 is its stimulatory effect on extracellular matrix proteins. This occurs by multiple pathways: first, TGF-β1 enhances collagen gene expression in a number of cell types including fibroblasts and osteoblasts. Simultaneously, TGF-β1 is known to inhibit the production of proteases that degrade the matrix, and moreover, it increases the levels of protease inhibitors (Edwards et al., 1987; Laiho et al., 1986). Further studies have demonstrated that TGF-β1 upregulates the expression of integrin receptors, thereby causing cells to more tightly adhere to the matrix. Collectively, these actions result in increased deposition and accumulation of ECM surrounding cells in a given tissue.

In the physiologic wound-healing process, the laying down of new ECM is fundamental to scar development. One can imagine, however, that dysregulation of this process resulting in excessive matrix secretion could cause scar hypertrophy and/or severe tissue contraction and fibrosis. Such a process has already been described in the pathogenesis of glomerulosclerosis and tubulointerstitial fibrosis in the kidney, where excess ECM accumulates in the glomeruli and tubules, destroying the organ's filtration capacity.

Dupuytren's contracture, formally called nodular palmar fibromatosis, is a condition first described 150 years ago as involving apparent fibrotic changes in the dermis and palmar fascia of the hand. The disease predominantly affects elderly male Caucasians, may have a hereditary component, and is strongly associated with diabetes, alcoholism,

cigarette smoking and HIV infection. It has since been postulated that increased synthesis of ECM, along with an acquisition by fibroblasts of a smooth muscle-like phenotype, are responsible for the resultant contracture and compromise in utility of the affected hand. Studies to date have demonstrated an increased total number of fibroblasts in Dupuytren's contracture (six- to forty-fold), a more disorganized pattern of collagen fibrils, and the fibroblasts to be clustered about narrowed microvessels. Finally, while type I collagen is predominant in the ECM of both normal and diseased palmar fascia, the ratio of type III to type I collagen is increased in Dupuytren's contracture. Murrell et al. (1991) determined that this ratio is not due to any intrinsic abnormality in collagen production by Dupuytren's cells; rather, the increased fibroblast density appears to inhibit type I collagen specifically which would explain the altered type III to type I ratio.

Baird et al. (1993) examined tissue samples from patients with and without Dupuytren's contracture, using reverse transcriptase/PCR to compare the relative amounts of various peptide regulatory factors expressed. These investigators found TGF-β mRNA in significantly higher amounts in the diseased tissue as compared to healthy tissue. Alioto et al. (1994) cultured cells from both normal palmar and Dupuytren's fascia, added TGF-β, and compared the cytokine's effect on proliferation rate and collagen production between the two cell types. They concluded that TGF-β is a potent stimulator of collagen synthesis in both cells types, but not mitogenic for fibroblasts that are either presently growing or not growth arrested, unlike the growth factors FGF and PDGF. They also noted that at baseline, Dupuytren's cells are more metabolically active and more sensitive

to all the growth factors they tested. These results are consistent with the hypothesis that TGF-β may contribute to the pathogenesis of this disease process.

Further, Kloen et al. (1995) found the presence of all three TGF-β receptor types on primary cultures of Dupuytren cells and normal dermal fibroblasts. Interestingly, their findings showed the type I receptor to be expressed significantly less, or, to have a lower affinity for TGF-β1, than the type II receptor in Dupuytren cultures. In comparison, there was virtually no difference between binding of TGF-β1 to these two receptor types in normal fibroblasts. In contrast to Alioto's results, Kloen's group found that addition of TGF-β1 did stimulate DNA synthesis in Dupuytren cells. However, in concurrence with the previous results, there was little mitogenic response to TGF-β1 in normal fibroblasts. Unexpectedly, Kloen also found that normal fibroblasts produced more TGF-β than diseased cells.

Also fascinating is the study by Tomasek and Rayan (1995) demonstrating greater expression of alpha-smooth muscle actin in Dupuytren's fibroblasts than in control tissue. Further, using a collagen lattice contraction assay, they found a positive correlation between this increased actin expression and an increase in contractile force in Dupuytren's cells. This data suggests that Dupuytren's fibroblasts can acquire something of a smooth muscle-like phenotype which may contribute to the contractility of the surrounding tissue.

As previously stated, Dupuytren's contracture is associated with a number of clinical predisposing factors such as diabetes, smoking, alcoholism, and HIV infection. The actual mechanism by which these risk factors may cause palmar fibrosis is unclear, but some studies have hypothesized the involvement of free radicals which I will not discuss here. In 1989, Mattson et al. coordinated a prospective study among Veterans Administration patients to evaluate previously reported retrospective associations between connective tissue disorders and yet another factor, antiepileptic medications. In this study, 10 (6%) of 178 patients who were treated for six months or more with a single barbiturate (phenobarbital or primidone) developed connective tissue disorders, with 7 of the 10 problems occurring within the first year of treatment. This data proved to be of statistical significance as compared to findings from 228 patients receiving other drugs. The connective tissue disorders developed included Dupuytren's contractures, frozen shoulder, Peyronie's disease (penile fibromatosis), and generalized joint pain.

Given the data from Mattson's study and the understood stimulatory effect of TGF-β1 on ECM production, my principal investigator, Dr. Michael Centrella, and I set out to answer a few logical questions. First, do barbiturates specifically stimulate ECM production in dermal fibroblasts? Second, do barbiturates stimulate fibroblast proliferation? Third, if the answer is "yes" to either of these questions, is TGF-β1 a mediator of these outcomes? And fourth, if TGF-β1 is involved, is the receptor profile – that is, the ratio between TGF-β receptor types I, II, and betaglycan – modulated as part of the cell signaling mechanism?

To address these questions, we decided to model our study after another completed in our laboratory where Centrella et al. (1991) investigated the effect of glucocorticoid on bone matrix collagen synthesis. The premise of this prior study was that steroid-induced bone loss may be mediated in part by changes in local TGF-β actions in skeletal tissue. By pre-exposing osteoblast-enriched cell cultures to cortisol they demonstrated a 40-50% reduction of the stimulatory effects of TGF-β1 on DNA and collagen synthesis. Additional binding studies showed that exposure to cortisol resulted in decreased TGF-β1 binding to type I and type II receptor complexes, whereas binding to betaglycan increased, suggesting increased extracellular storage of TGF-β1. The authors concluded that glucocorticoids can decrease the anabolic effects of TGF-β1 in bone, in part by a redistribution of its binding toward ECM storage sites.

Similarly, for the current experiment we chose to use an *in vitro* cell culture model of rat dermal fibroblasts (readily available and used for other experiments in our laboratory). Our first aim was to determine whether phenobarbital (one of the barbiturates from Mattson's study) exposure causes an increase in the biological effects of TGF-β1 in these cells. We planned to assess overall fibroblast proliferation by measuring [^{3}H]thymidine incorporation as a gauge of DNA synthesis. Relative collagen and non-collagen protein synthesis was to be measured by assaying [^{3}H]proline incorporation and differential sensitivity to digestion with purified collagenase. Our second aim was to determine what effect phenobarbital has on TGF-β receptor expression in rat dermal

fibroblasts. Specifically, we wanted to determine whether phenobarbital exposure alters the levels of TGF-β1 binding to type I and type II receptors and betaglycan. TGF-β receptors were to be quantified through binding studies using ^{125}I-TGF-β1, and by Western blot staining with specific antisera. This study will help clarify the relationship, if any exists, between the dynamic maintenance of extracellular matrix and barbiturate exposure in dermal tissue.

MATERIALS & METHODS

All experiments were conducted by myself, with the exception of the initial dermal fibroblast cell cultures. In our laboratory, cell cultures have been routinely prepared by one individual to maintain a consistent model. Therefore I used cell cultures prepared by Sandra Casinghino who has done so for more than 10 years.

Cell Culture and Treatments

Primary dermal fibroblast cultures were prepared from scalp skin, taken directly above the parietal bones of 22-day-old rat fetuses [stock designation Crl:CD(SD)BR, derived from Sprague-Dawley rats, Charles River Breeding Laboratories, Raleigh, NC]. Dermal/epidermal tissue from 10 rat fetuses was removed and digested together at 37°C for 1 hour with 1000 units (10 ml) collagenase (Type 2, Worthington Biochemical Company). Undigested tissue was discarded, and isolated cells were pelleted by centrifugation for 4 minutes at 4000 rpm. The supernatant, containing the collagenase, was discarded, and cells were resuspended in Dulbecco's modified Eagle's medium containing 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (pH 7.2), 100 μg of ascorbic acid per ml, penicillin and streptomycin, and 10% fetal bovine serum (FBS). Cells were plated in 75cm^2 flasks and incubated at 37°C in a humidified incubator with 95% O_2-5% CO_2. When cultures reached confluence the cells were trypsinized and plated into 6-, 12-, or 24-well plates for experiments. For all studies, cells from passage 1 were used. Prior to each treatment, cells were grown to confluence

in medium containing 10% FBS and were then re-fed identical medium lacking FBS ("serum-free medium"). Twenty-four hours later, they were re-fed serum-free medium containing various agents for the times indicated for each experiment.

Test Agents

Cell culture reagents were obtained from GIBCO (Grand Island, NY).

The TGF-β1 used in these studies was a recombinant simian preparation identical in amino acid sequence to human TGF-β1 (examined in collaboration with Bristol-Myers Squibb, Inc., Seattle, WA), which exhibits binding and biological characteristics indistinguishable from our lab's earlier studies with other native or recombinant preparations. TGF-β1 was dissolved in 0.05 M HCl containing 4 mg of bovine serum albumin (BSA) per ml to obtain a concentration of 8 nM and was stored at 4°C.

Cortisol (hydrocortisone [Sigma]) was dissolved in 95% ethanol to obtain a concentration of 10 mM and was diluted as needed in serum-free culture medium prior to use.

Phenobarbital was dissolved in 12.6% warm water/87.3% DMEM to obtain a concentration of 3 mM and was diluted as needed in serum-free culture medium prior to use.

Cell Replication

Vehicle or test agent was added in serum-free medium, and cultures were incubated for additional intervals, designated for each figure. Effects on DNA synthesis were measured by pulse-labeling cultures with [*methyl*-^{3}H]thymidine (5 μCi/ml, 80 Ci/mmol; Dupont

NEN) during the last two hours of treatment. [^{3}H]thymidine incorporation into DNA was determined by cell lysis with 0.1M sodium dodecyl sulfate-0.1N sodium hydroxide, collection of the material precipitated by 10% trichloroacetic acid, and scintillation counting.

Protein Synthesis

Effects on collagen and non-collagen protein (NCP) synthesis were measured by pulse-labeling cultures with 12.5 μCi of [2,3-^{3}H]proline (2.5 Ci/mmol; Dupont NEN, Boston, Mass.) per ml for the last two hours of treatment. Cells were lysed by freeze-thawing; lysates were collected in 0.5% Triton X-100 (Sigma Chemical Co., St. Louis, MO), precipitated with 10% trichloroacetic acid, and chilled; and the acid-precipitable material was collected by centrifugation. The precipitates were acetone extracted, dried, re-solubilized in 0.5M acetic acid, and neutralized with 0.5M sodium hydroxide. [^{3}H]proline incorporation into collagenase-digestible protein (CDP) and NCP was measured by using collagenase purified free of nonspecific protease activity (Worthington Biochemicals) and is shown as the total amount of [^{3}H]proline incorporated per culture. Percent collagen synthesis (PCS) was calculated after correcting for the relative abundance of proline in CDP and NCP.

Binding Studies

TGF-β1 was radioiodinated with chloramine T to a specific gravity of 4000-4500 Ci/mmol. Radioligand was separated from unincorporated ^{125}I by gel filtration on

Sephadex G-50 in a solution of 0.1 M acetic acid and 4 mg/ml bovine serum albumin (BSA). Binding was examined by incubation with serum-free medium containing 4 mg/ml BSA (binding medium) and 50-150 pM ^{125}I-TGF-β1 for three hours at 4°C. To visualize TGF-β binding complexes, cultures were rinsed with chilled binding medium, cross-linked with 0.2 mM disuccinimidyl suberate (Pierce Chemical Co., Rockford, IL), and extracted in a phosphate buffered saline solution containing 1% Triton X-100. A standard amount of protein (100 ng) was loaded in each lane, fractionated by electrophoresis on polyacrylamide gels (5-10%), and examined by autoradiography. Densitometry was assessed using a ScanMan densitometer and SigmaGel® (Jandel, San Rafael, CA).

Receptor Analysis

For Western blot analysis (total receptor protein quantification), cultures were extracted in a phosphate buffered saline solution containing 1% Triton X-100, 0.02 mM calcium chloride and 0.2 mM magnesium chloride, and extracts were fractionated by polyacrylamide gel electrophoresis (5-10%) with 100 ng protein loaded in each lane. Extracts were then blotted onto Immobilon P membranes (Millipore Corp.), blocked with a 5% (w/v) de-fatted milk solution, probed with anti-TGF-βR specific antibody preparations (Santa Cruz Biotechnology, Inc.), and visualized with SuperSignal™ Western blotting detection reagents (Pierce).

RESULTS

The purpose of our studies was to assess the affect of the barbiturate phenobarbital on rat dermal fibroblasts *in vitro*. The parameters we chose to evaluate after phenobarbital exposure included DNA synthesis as a measure of cell proliferation, collagen synthesis as a measure of overall extracellular matrix production, and the quantity of and TGF-β1 binding to the three TGF-β receptor subtypes commonly found on mammalian cells.

DNA Synthesis

Our first objective was to determine whether phenobarbital affects DNA synthesis in these fibroblasts. As previously reported (Centrella et al., 1991), treatment of fetal rat osteoblast cultures with TGF-β1 for 23 hours induces a dose-related biphasic stimulatory effect on DNA synthesis, with peak stimulation occuring at 1 ng/ml TGF-β1. Therefore, in order to determine the TGF-β1 dose which maximally stimulates our rat dermal fibroblasts, we treated our cell cultures for 24 hours with TGF-β1 concentrations varying from 0.03 to 3.0 ng/ml, subsequently evaluating each treatment group for [^{3}H]thymidine incorporation. We found maximal stimulation of DNA synthesis at a TGF-β1 concentration of 0.1 ng/ml in our cells, at which concentration mitogenesis was enhanced about 2.5-fold (Fig. 1).

Also previously reported in osteoblasts (Centrella et al., 1991) is the finding that 100 nM cortisol reduces basal DNA synthesis rates and suppresses the known mitogenic effect of

TGF-β1 at 1 ng/ml and lower concentrations. Exposure of our fibroblasts to this same

concentration of cortisol produced similar outcomes: basal DNA synthesis rates were

reduced as compared to untreated cells, and the observed mitogenic effect of 0.1 ng/ml

TGF-β1 was suppressed by 85% (Fig. 2). No notable effects of 30 to 300 μM

phenobarbital were observed on basal DNA synthesis rates, and TGF-β1 appears to

stimulate mitogenesis to the same extent with or without these concentrations of 30 to 300

μM phenobarbital. Both basal and TGF-β1-stimulated DNA synthesis were inhibited by 3

mM phenobarbital by 25 - 30%.

Collagen Synthesis

Our second objective was to determine whether phenobarbital affects collagen synthesis in

rat dermal fibroblasts. TGF-β1 is a well-known enhancer of type I collagen synthesis in

fibroblasts (see Border & Noble, 1995). As previously described for osteoblasts

(Centrella et al., 1991), collagen synthesis is enhanced by TGF-β1 concentrations ranging

from 12 to 1200 pM in a steadily increasing fashion with maximal effect near 120 pM and

with amount of synthesis remaining fairly constant at higher doses TGF-β1. Given the

similarities between the TGF-β1 standard curves for fetal rat osteoblasts and dermal

fibroblasts with respect to DNA synthesis, and the reliable stimulation of collagen

synthesis in osteoblasts at all tested TGF-β1 concentrations, we did not carry out our own

dose-response curve with respect to collagen synthesis. However, in separate

experiments, when TGF-β1 was tested at 2.5 ng/ml, collagen synthesis increased by 50%,

while 10 ng/ml produced a 2.0 to 2.5-fold increase. The results reported here reflect those studies carried out at a concentration of 10 ng/ml.

At baseline, 10 ng/ml TGF-β1 increased collagen-digestible-protein (CDP) synthesis approximately twofold (Fig. 3). 100 nM cortisol reduced basal CDP synthesis and decreased the stimulatory influence of TGF-β1 by about 66%. Phenobarbital exposure resulted in minimal variation of CDP synthesis both without and with TGF-β1 treatment as compared to untreated samples at phenobarbital concentrations of 3 to 300 μM. At 3 mM phenobarbital, it inhibited both basal and TGF-β1-stimulated collagen synthesis. Phenobarbital similarly had no apparent affect on non-collagen protein (NCP) synthesis in our cells except for at the highest dose of 3mM, where it was inhibitory as for the CDP samples (Fig. 4). 100 mM cortisol only minimally inhibited basal NCP synthesis, but appeared to cause a 10 to 50% inhibition of the stimulatory effect of 10 ng/ml TGF-β1 in two independent studies. However, the changes in CDP and NCP were not as evident in experiments carried out with 2.5 ng/ml TGF-β1.

Receptor Assays

Our third objective was to investigate the response of TGF-β receptor types I, II, and III (betaglycan) to phenobarbital exposure. Our approach was two-fold: after phenobarbital treatment, we first examined the extent of ligand binding to each receptor subtype, and second, the total amounts of each receptor protein in our cells. Ligand binding was measured using ^{125}I-TGF-β1, chemical cross-linking, and polyacrylamide gel

electrophoresis as described in Materials and Methods. Individual absolute receptor amounts were quantified using polyacrylamide gel electrophoresis and by probing with antibodies specific for each receptor subtype as described in Materials and Methods. Quantification of bands detected by autoradiography was carried out by densitometric analysis of intensity times total area. Densitometry for all bands from a given experiment was carried out at one sitting, so that the readings should accurately represent relative amounts of ^{125}I-TGF-β1 or antibody binding to phenobarbital-exposed cells versus control cells.

As in previous studies (Kloen et al. 1995), ^{125}I-TGF-β1 binding was detected primarily within complexes migrating at molecular weights of 65 kDa, 73 kDa, and >200 kDa, analogous to the types I, II, and betaglycan binding sites, respectively. For antibody binding, migration was at 53 kDa, 70 kDa, and >200 kDa due to the absence of complexed ligand. Treatment with 100 nM cortisol for 24 h prior to the addition of ^{125}I-TGF-β1 resulted in an 86% reduction in labeling of the type I receptor, a 42% reduction in binding to the type II receptor, and a 33% reduction in binding to betaglycan (Figs. 5-7). 24-h treatment with 30 μM phenobarbital (closest to physiologic concentration) similarly resulted in reduction in ^{125}I-TGF-β1 binding to the three receptor subtypes, by 75%, 31%, and 57% for I, II, and betaglycan respectively. 3 mM phenobarbital was the only concentration of the drug which produced an increase in binding, by 30%, 56%, and 60% for I, II, and betaglycan. With the exception of one condition (300 μM phenobarbital : Type I receptor), a general trend was observed that as phenobarbital dose increased, ^{125}I-

TGF-β1 binding to all three receptor types steadily increased from below control levels to above.

In the antibody studies, treatment with 100 nM cortisol for 24 h resulted in only a 10% reduction in total cellular Type I receptor protein, while simultaneously leading to a 53% increase in total cellular Type II receptor protein (Figs. 8-9). Total cellular betaglycan was not assessed as the anti-betaglycan antibody that was available did not react with our protein blots. 30 μM phenobarbital resulted in a 29% reduction in total cellular Type I receptor protein, and a 14% reduction in Type II receptor protein. 24-h incubation with 3 mM phenobarbital resulted in a 71% reduction in Type I receptor protein, while causing an increase of 42% in Type II receptor protein. Overall, increasing concentrations of phenobarbital appeared to cause a progressive decrease in Type I receptor protein amounts. Additionally, phenobarbital concentrations increasing from 30 μM to 3mM appeared to gradually increase Type II receptor protein amounts; however the effects at 3 μM did not fit this trend. These results are collected in Table 1.

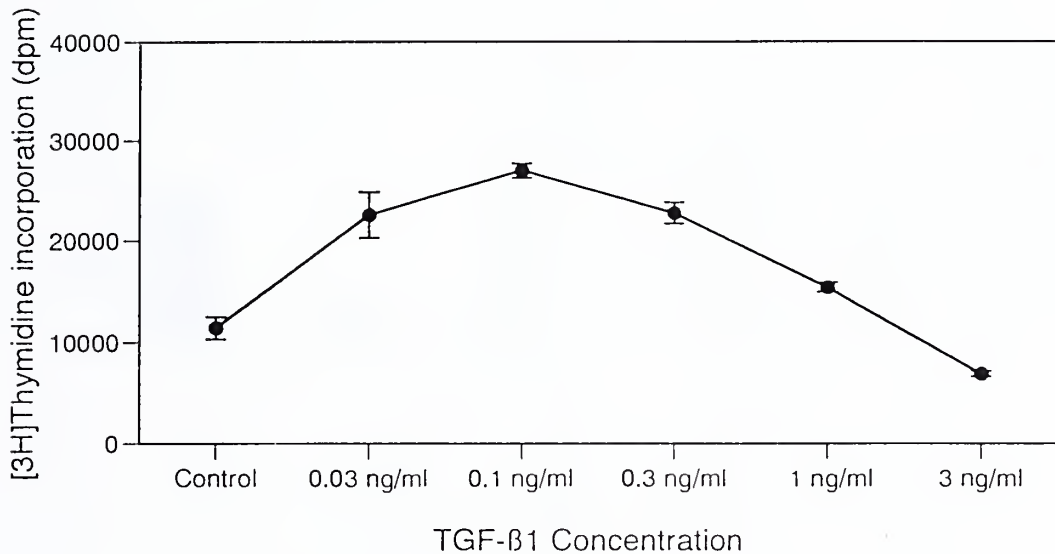# TGF-ß1 Dose-Response Curve in Rat Dermal Fibroblasts
(DNA Synthesis)

FIG. 1: Effect of TGF-ß1 on DNA synthesis rates in dermal fibroblast cultures from fetal rat scalp. Serum-deprived confluent cell cultures were incubated for 24 h with the concentrations of TGF-ß1 shown. DNA synthesis was measured by pulse-labeling with [^{3}H]thymidine for the last 2 h of culture as described in Materials and Methods. Data are the means +/-SE of results of one experiment including six replicate culture wells per condition. DNA synthesis was significantly enhanced by TGF-ß1 concentrations at or below 1.0 ng/ml, with a peak of stimulation at 0.1 ng/ml.

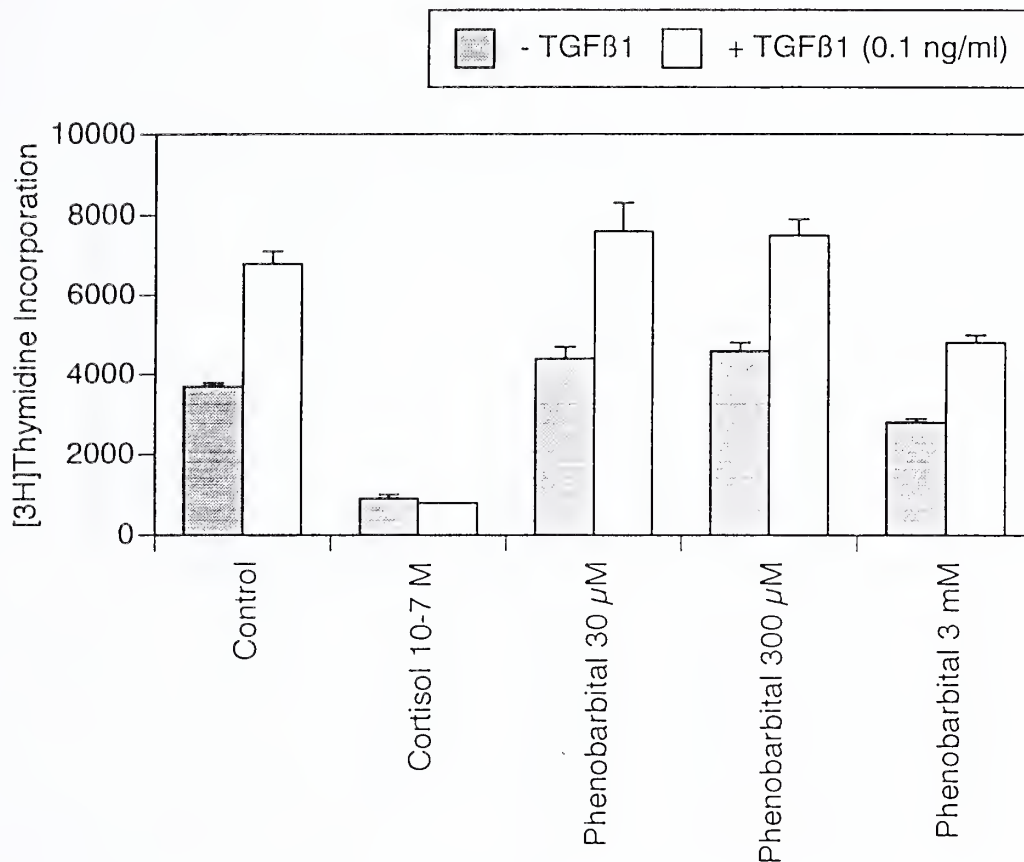# Response by Rat Dermal Fibroblasts to Phenobarbital
(DNA synthesis)

FIG. 2: Effect of phenobarbital and cortisol on DNA synthesis rates in untreated and TGF-ß1-stimulated dermal fibroblast cultures from fetal rat scalp. Serum-deprived confluent cell cultures were incubated for 24 h without or with the concentrations of phenobarbital or cortisol shown, followed by a 24-h treatment with 0.1 ng/ml TGF-ß1. DNA synthesis was measured by pulse-labeling with [^{3}H]thymidine for the last 2 h of culture and analyzed as described in Materials and Methods. Data are the means +/-SE of results of one representative experiment including six replicate culture wells per condition. Both basal DNA synthesis and the stimulatory effect of 0.1 ng/ml TGF-B1 were inhibited by 100 nM cortisol. Phenobarbital had little effect on basal DNA synthesis at concentrations of 30 μM and 300 μM, and TGF-ß1 tended to stimulate to the same extent without or with phenobarbital. DNA synthesis (both +/- TGF-ß1) was inhibited by 3 mM phenobarbital.

Response by Rat Dermal Fibroblasts to Phenobarbital
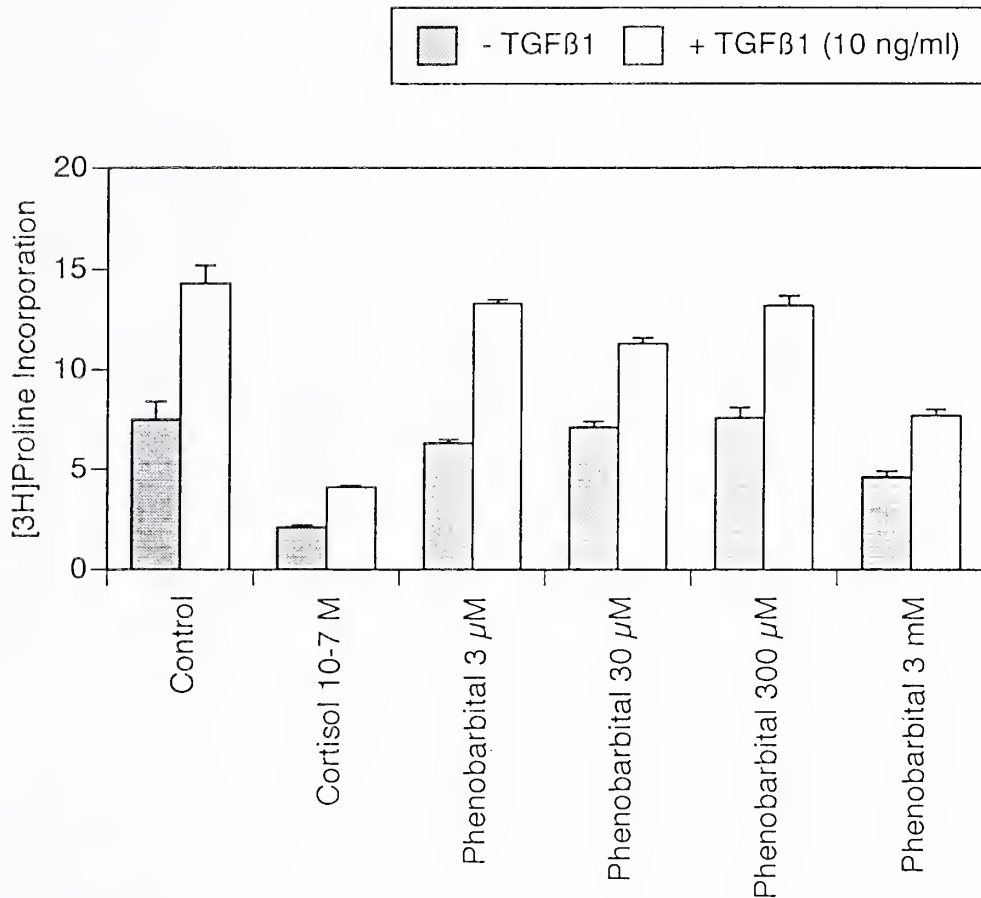### (Collagen synthesis)

FIG. 3: Effect of phenobarbital and cortisol on collagen synthesis rates in untreated and TGF-ß1-stimulated dermal fibroblast cultures from fetal rat scalp. Serum-deprived confluent cell cultures were incubated for 24 h without or with the concentrations of phenobarbital or cortisol shown, followed by a 24-h treatment with 10 ng/ml TGF-ß1. Collagen synthesis was measured by pulse-labeling with [^{3}H]proline for the last 2 h of culture and analyzed as described in Materials and Methods. Data are the means +/-SE of results of one representative experiment including six replicate culture wells per condition. Both basal collagen synthesis and the stimulatory effect of 10 ng/ml TGF-ß1 were inhibited by 100 nM cortisol. Phenobarbital had little effect on basal collagen synthesis at concentrations of 3 µM, 30 µM and 300 µM. 10 ng/ml TGF-ß1 tended to stimulate to the same extent without or with phenobarbital. Collagen synthesis (both +/-TGF-ß1) was inhibited by 3 mM phenobarbital.

Response by Rat Dermal Fibroblasts to Phenobarbital
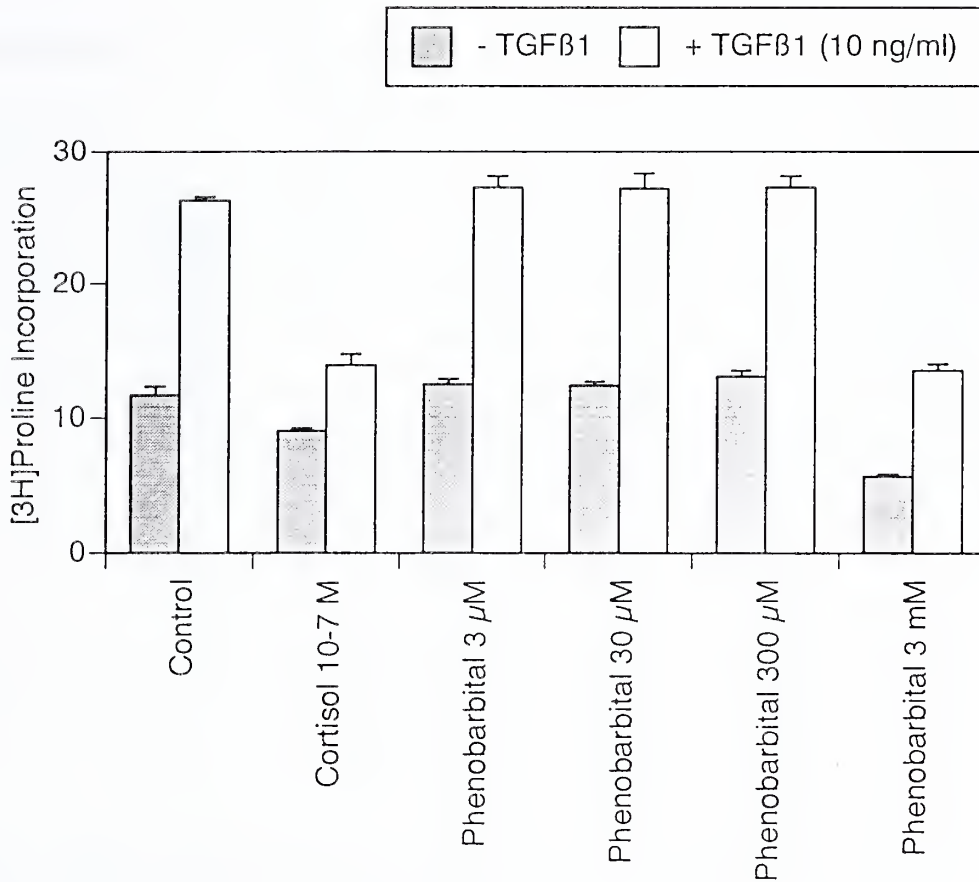## (Non-collagen protein synthesis)

FIG. 4: Effect of phenobarbital and cortisol on non-collagen protein synthesis rates in untreated and TGF-ß1-stimulated dermal fibroblast cultures from fetal rat scalp. Cells were prepared and treated as described for Fig. 3, and non-collagen protein synthesis was measured by pulse-labeling with [^{3}H]proline for the last 2 h of culture and analyzed as described in Materials and Methods. Data are the means +/-SE of results of one representative experiment including six replicate culture wells per condition. This experiment was carried out simultaneously and using the same original cell cultures as for the experiment depicit in Fig.3. As with collagen synthesis, both basal non-collagen synthesis and the stimulatory effect of 10 ng/ml TGF-ß1 were inhibited by 100 nM cortisol. Phenobarbital had little effect on basal non-collagen synthesis at concentrations of 3 µM, 30 µM and 300 µM. 10 ng/ml TGF-ß1 tended to stimulate to the same extent without or with phenobarbital. Non-collagen protein synthesis (both +/-TGF-ß1) was inhibited by 3 mM phenobarbital.

^{125}I-TGFß: Type I Receptor Binding in Rat Dermal Fibroblasts after Phenobarbital Exposure (Western Blot)
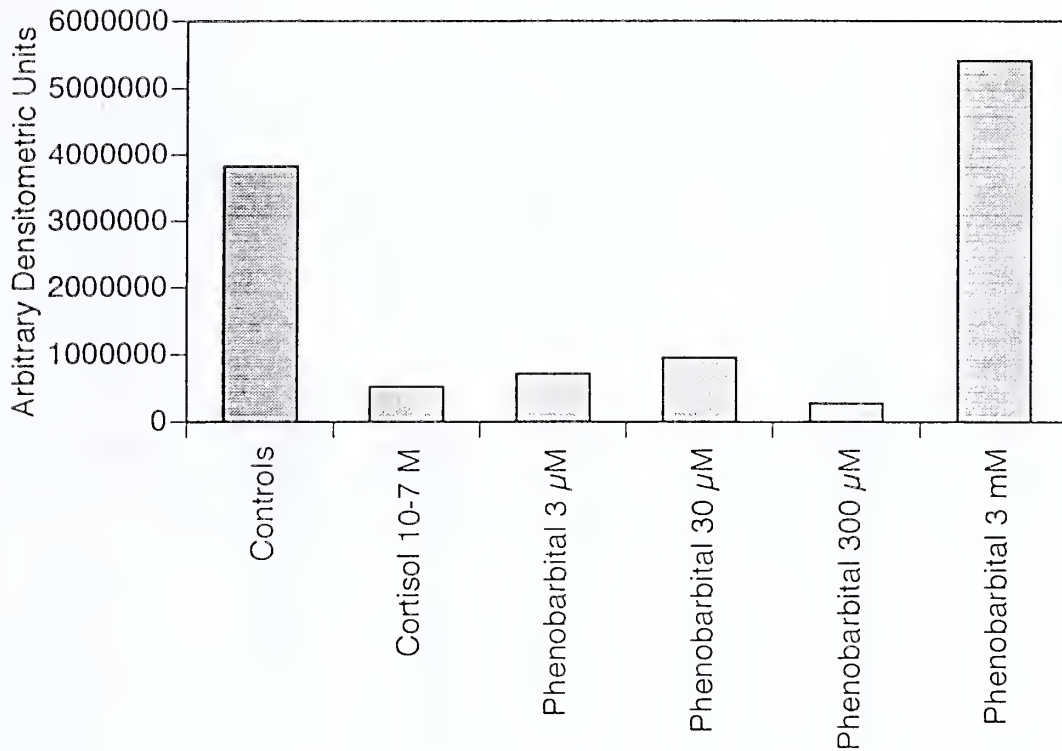

FIG. 5: Effect of cortisol and phenobarbital on TGF-ß1 binding in dermal fibroblast cultures from fetal rat scalp. Serum-deprived confluent cultures were incubated for 24 h without or with 100 nM cortisol or 30 µM to 3 mM phenobarbital, rinsed, and labeled for 3 h at 4°C with 80 pM ^{125}I-TGF-ß1. The cultures were rinsed, cross-linked with disuccinimidyl suberate, and fractionated by electrophoresis through a 5-10% polyacrylamide gel, and ^{125}I-labeled binding complexes were displayed by autoradiography as described in Materials and Methods. Complexes were designated by analogy to other tissue systems, in which the betaglycan complex migrates at >200kDa, the type II complex at 70 kDa, and the type I complex at 53 kDa, identified relative to a mixture of protein standards. Densitometric analysis of bands was used to quantify ^{125}I-TGF-ß1 binding to the three receptor subtypes: type I (**Fig. 5**), type II (**Fig. 6**), and betaglycan (**Fig. 7**) for the various conditions labeled. Data displayed in all three figures are results from one experiment including six replicate culture wells per condition. Binding to the type I receptor was inhibited by cortisol exposure and by 3 µM to 300 µM phenobarbital, while 3mM phenobarbital resulted in increased binding. Equal amounts of protein (100 ng) were added to each lane.

^{125}I-TGFß : Type II Receptor Binding in Rat Dermal Fibroblasts after Phenobarbital Exposure

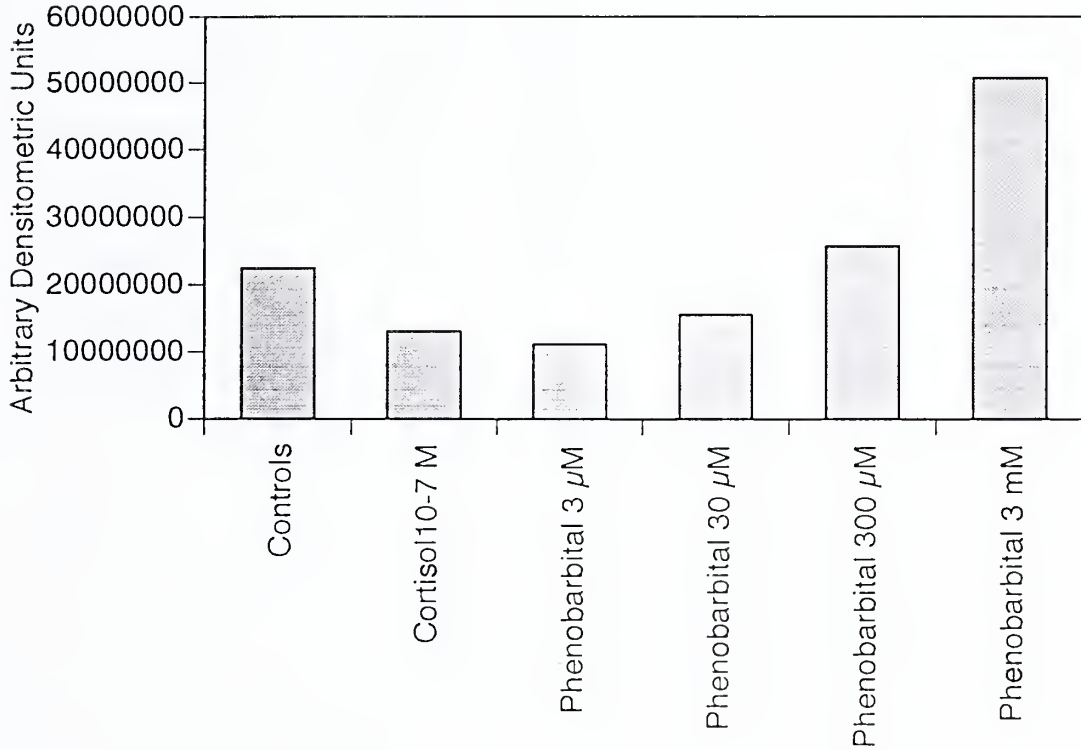**FIG. 6:** (See Fig. 5 legend for details) Binding to the TGFß type II receptor was inhibited by cortisol exposure and by 3 µM and 30 µM phenobarbital, while 3mM phenobarbital resulted in increased ^{125}I-TGF-ß1 binding. Exposure to 300 µM phenobarbital resulted in similar binding levels as found in untreated cells. Equal amounts of protein (100 ng) were loaded in each lane.

^{125}I-TGFß : Betaglycan (Type III Receptor) Binding in Rat Dermal Fibroblasts after Phenobarbital Exposure
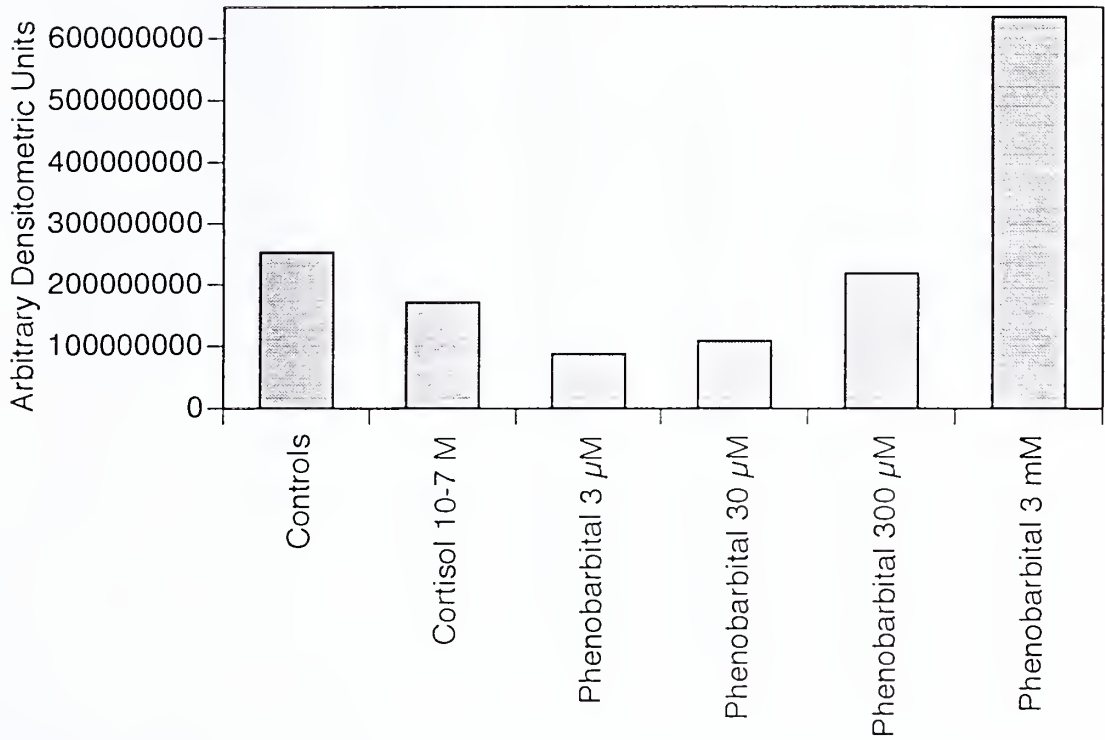

FIG. 7: (See Fig. 5 legend for details) Binding to betaglycan was inhibited by cortisol exposure and by 3 µM and 30 µM phenobarbital, while 3mM phenobarbital resulted in increased ^{125}I-TGF-ß1 binding. Exposure to 300 µM phenobarbital resulted in similar binding levels as found in untreated cells. Equal amounts of protein (100 ng) were loaded in each lane.

TGFß Type I Receptor Protein in Rat Dermal Fibroblasts after Phenobarbital Exposure (Western Blot)
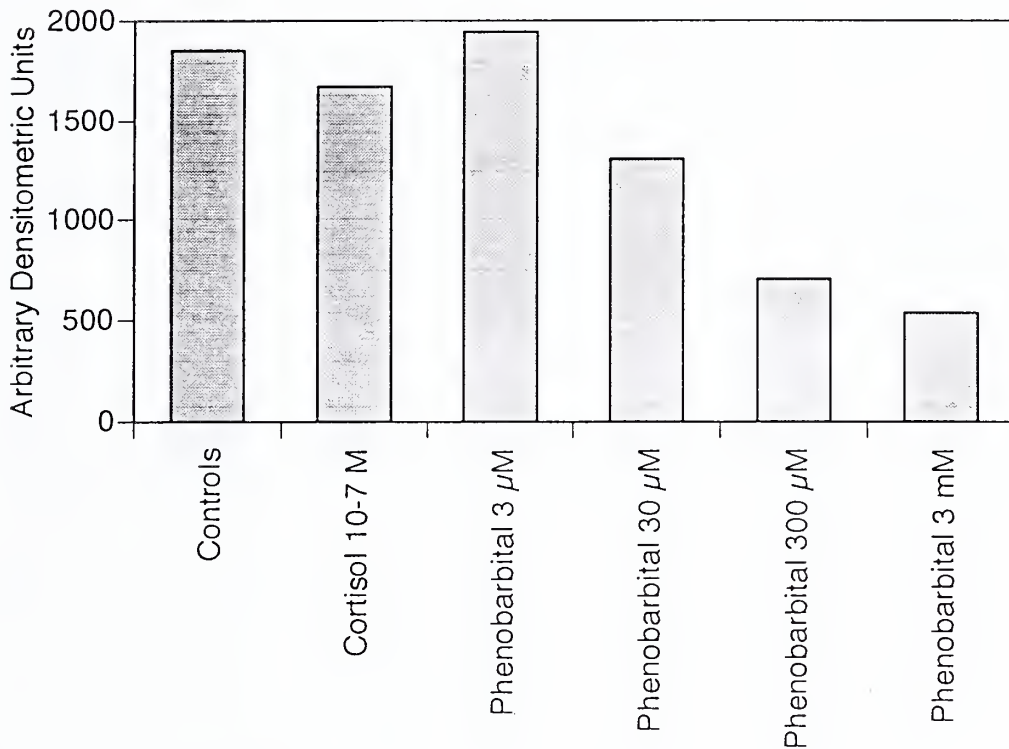

FIG. 8: Effect of cortisol and phenobarbital on total receptor protein levels in dermal fibroblast cultures from fetal rat scalp. Serum-deprived confluent cultures were incubated for 24 h without or with 100 nM cortisol or 30 µM to 3mM phenobarbital, rinsed, and fractionated by electrophoresis through a 5-10% polyacrylamide gel. Individual TGF-ß receptor subtypes were detected by probing with anti-TGF-ß-Receptor specific antibody preparations and were visualized by chemiluminescence as described in Materials and Methods. Receptor subtypes were designated by analogy to other tissue systems, in which the type II complex migrates at 70 kDa, and the type I complex at 53 kDa, identified relative to a mixture of protein standards. Densitometric analysis of bands was used to quantify specific antibody binding to two receptor subtypes: type I (**Fig. 8**) and type II (**Fig. 9**), for the various conditions labeled. Data displayed in Fig. 8 are results from one experiment including six replicate culture wells per condition. Type I receptor protein decreased after exposure to 30 µM to 3 mM phenobarbital, while neither cortisol nor 3 µM phenobarbital altered amounts of type I receptor appreciably. Equal amounts of protein (100 ng) were loaded in each lane.

TGFß Type II Receptor Protein in Rat Dermal Fibroblasts after Phenobarbital Exposure (Western Blot)
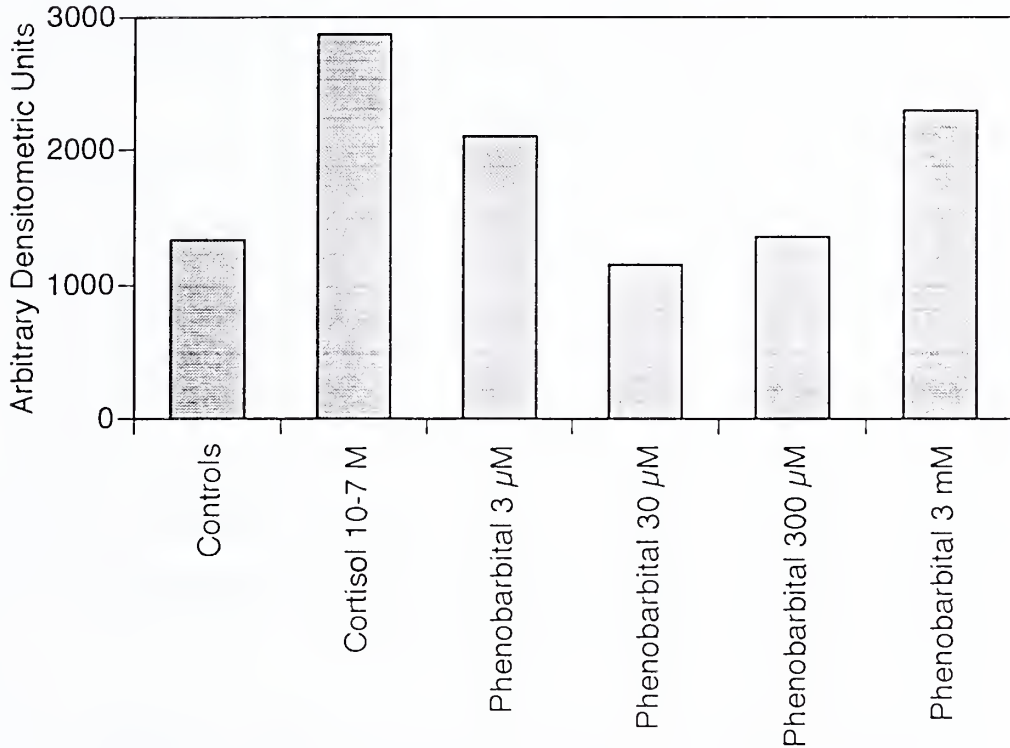
FIG. 9: (See Fig. 8 legend for details) Data displayed are averaged results from two separate experiments including six replicate culture wells per condition. Cortisol exposure resulted in a marked increase in amount of TGFß Type II receptor protein in this experiment. Type II receptor also appeared to increase at phenobarbital concentrations of 3 μM and 3 mM, but remained relatively unchanged from control levels at concentrations of 30 and 300 μM. Equal amounts of protein (100 ng) were loaded in each lane.

^{125}I-TGF-β1 Binding	% of Control		
	Type I	Type II	Type III
Cortisol 10^{-7}M	14%	58%	67%
Phenobarbital 30 μM	25%	69%	43%
Phenobarbital 3 mM	130%	156%	160%
Antibody Binding			
	Type I	Type II	
Cortisol 10^{-7}M	90%	153%	
Phenobarbital 30 μM	71%	86%	
Phenobarbital 3 mM	29%	142%	

TABLE 1: Effect of cortisol or phenobarbital treatment on ^{125}I-TGF-β1 binding to TGF-β receptor types I, II and III, and on specific anti-receptor antibody binding to receptor types I and II.

DISCUSSION

The barbiturate phenobarbital has selective anticonvulsant activity, and due to its low toxicity, high efficacy, and low cost, is still widely used to treat patients with seizure disorders. In a 1989 study, Mattson et al. described a higher incidence of connective tissue disorders in patients taking phenobarbital over a year's time than is present in the general population. This association between connective tissue diseases (including Dupuytren's contracture of the palmar fascia, "frozen shoulder," and Peyronie's disease or penile fibrosis) and phenobarbital intake has thus far not been further investigated. We know from review of the literature that the connective tissue diseases mentioned involve some component of superfluous extracellular matrix deposition with or without fibroblast hyperproliferation (LeRoy 1974; Murrell et al. 1991; Kikuchi et al. 1992; Alioto et al. 1994). Additionally, we know that TGF-β1 is a potent stimulator of extracellular matrix (ECM) production in many cell types, including fibroblasts (Centrella et al. 1987; Border & Noble 1995). In fact, a clear association has been demonstrated between dysregulation of TGF-β1 activity and fibrotic processes affecting other human organ systems. It therefore seems feasible that altered regulation of TGF-β1 activity at some level may contribute to pathogenesis of these connective tissue disorders, as further studies have suggested (Kloen et al. 1993).

Given this body of information, we set out to determine if phenobarbital has a direct effect on ECM production in and cell proliferation of fibroblasts. Stimulation of one or both of

these variables by phenobarbital would indicate a direct causal relationship in the development of Dupuytren-like disorders, which would be of clinical interest. Further, we asked whether phenobarbital altered the stimulatory effect of TGF-β1 toward collagen and DNA synthesis in these cells. TGF-β1 is one of a group of growth factors central to the maintenance of tissue homeostasis in most cell types. Modification, whether suppression or enhancement, of the cellular responsiveness to TGF-β1 could therefore result in physiologically significant outcomes. We chose to culture dermal fibroblasts from fetal rat scalp tissue because of their ready availability in our laboratory and excellent proliferative capacity. In order to establish the baseline responsiveness of our cells we set up two control groups. One group assessed normal fibroblast sensitivity to TGF-β1, in which we expected to see stimulation of both collagen and DNA synthesis with TGF-β1 treatment as demonstrated in previous studies (Kloen et al. 1995). The second control group assessed fibroblast response to a concentration of cortisol known to suppress collagen and DNA synthesis, as well as the stimulatory effect of TGF-β1, in osteoblasts (Centrella et al. 1991).

TGF-β1 proved to be stimulatory and cortisol proved to be inhibitory for both collagen and DNA synthesis in rat dermal fibroblasts. Therefore, we were able to conclude that our cell culture had produced cells sensitive and responsive to stimuli in predictable ways. Phenobarbital, when tested at concentrations of 3 to 30 μM had no clear effect on either ECM production or cell proliferation. Across all experiments, however, the highest dose of phenobarbital (3 mM) inhibited both collagen and DNA synthesis, as well as the

stimulatory effect of TGF-β1. Clinically, the concentration of phenobarbital in the bloodstream of patients receiving the drug is about 10 mg/ml, or 43 μM (MW = 232.24). Therefore, the effect of so high a concentration as 3 mM seems unlikely to be physiologically significant. It appears then, that under the described conditions, using these cells, physiologic levels of phenobarbital do not appear to have a direct effect with respect to collagen or DNA synthesis.

These results point away from our original hypothesis that the Dupuytren-like connective tissue diseases may result directly from phenobarbital-induced overproduction of ECM proteins. Further, phenobarbital does not appear to alter the fibroblasts' level of response to TGF-β1. However, it is important to remember that changes in cell behavior related to TGF-β1 may be effected via various mechanisms: one might consider alterations in production, secretion, or subsequent activation of latent TGF-β1, changes in TGF-β receptor expression, or alterations in post-receptor cell signaling. It was of interest therefore to us to evaluate whether TGF-β receptor numbers or receptor affinity for ligand changed with phenobarbital exposure.

Recall that receptor types I and II are transmembrane receptors directly responsible for propagating the TGF-β signal to attain the desired cellular effects. Betaglycan exists in both soluble and membrane-bound forms, which may correlate to storage and ligand-presenting roles, respectively. Therefore, if change in receptor profile were to account for increased matrix deposition and resultant fibrosis, we would have expected to see an

increased ratio of type I and II receptors to betaglycan. This, however, is not what our studies showed. Rather, 24-h exposure of dermal fibroblasts to clinically therapeutic levels of phenobarbital resulted in a reduction in TGF-β binding to all receptor subtypes. Further, the greatest reduction in binding occurred at the type I receptor. By looking at the results of ^{125}I-TGF-β1 binding alone, it is not possible to determine whether decreased binding results from decreased affinity of receptor for ligand, or decreased overall availability of receptor on the cell surface. Looking to the results of antibody-binding experiments to help clarify this point, we found parallel reductions in both total cellular Type I and II receptor proteins after exposure to therapeutic phenobarbital levels as compared to untreated samples. This suggests, but does not determine, that overall amounts of receptor Types I and II are decreased in cells treated with this concentration of phenobarbital. Such a decrease in receptor amount could either result from a decreased level of transcription of receptor mRNA or an increase in the rate of receptor protein turnover.

The question arises, why did we not see the results we expected to? There are a number of possible explanations. First, further repetition of all experiments (which unfortunately, time did not permit) may have led to a more accurate interpretation of the data given a greater overall sample size. Worth consideration is that Mattson's clinical study revealed a significant, but only 10% increase in incidence of Dupuytren-like diseases over controls. Therefore, it is likely that we would only see a tendency toward increased matrix production in 10% of our cells, undetectable in a small sample size. Second, we were

clearly operating under the assumption that fibrotic processes analogous to human connective tissue disorders occur in rats, which may be a misguided supposition. I, in fact, could find no literature describing fibrotic skin diseases in rats, which may mean human fibroblasts would have been necessary to culture in order to accurately demonstrate our hypothesis. Third, we modeled our experiments based on a clinical study where patients received phenobarbital chronically, over a year's time. It is quite possible that longer or repeated exposure to the drug would be necessary in order to mimic the clinical scenario described. Successful demonstration of our hypothesis might even require continued passaging of cells while exposing to phenobarbital to select for cultures with the correct conditions for study.

Further possibilities include insufficient amounts of phenobarbital receptors on the surfaces of rat dermal fibroblasts to evoke any response. The mechanism of phenobarbital effectiveness in the CNS involves action on the $GABA_A$ receptor of neurons, resulting in potentiation of synaptic inhibition. Its mechanism of action on other tissue types, however, is unclear; if, however, it binds with greater efficiency to human fibroblasts than those in rats, our inability to see its effects might be explained. Yet another possibility is that fibroblasts cultured in exclusion of other cell types may not provide a critical intermediary for response to phenobarbital. The development of the connective tissue disorders described may in fact be an indirect effect of exposure to this drug, mediated by another molecule or other molecules unavailable to our cells in culture. Future studies

might address any one of these issues, and might significantly contribute to the

understanding of the development of connective tissue disorders in this patient population.

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