Journal of Cellular Biochemistry 43:185–198 (1990)

Suppression of Protein Kinase C and the Stimulation of Glucocorticoid Receptor Synthesis by Dexamethasone in Human Fibroblasts Derived From Tumor Tissue

Preston Gadson, Judy McCoy, Ann Charlotte Wikström, and Jan-Ake Gustafsson

Department of Anatomy, Medical College of Georgia School of Medicine, Augusta, Georgia 30912-2000 (P.G., J.M.); Karolinska Institutet, Institutionen För Medicinsk Näringslara, S-14186 Huddinge, Sweden (A.C.W., J.-A.G)

Exposure of fibroblasts derived from keloid tissues, desmoid and dermal tissue from individuals with Gardner's syndrome (GS) to dexamethasone resulted in the suppression of protein kinase C (PKC) activity and [^{3}H]thymidine incorporation into DNA, and a 20-fold induction of glutamine synthetase activity. Treatment of GS and keloid fibroblasts with 0.1 μM dexamethasone for 36 h increased glucocorticoid receptor (GR) synthesis, as determined by [^{35}S]methionine labeling and immunoprecipitation with a monoclonal antibody to the human GR. The suppression of PKC activity by dexmethasone was shown to result from a loss of protein mass as determined by immunoblotting using an antibody to PKC type III. In contrast to these results, exposure of fibroblasts isolated from normal tissues to dexamethasone did not result in the suppression PKC and [^{3}H]thymidine incorporation, there was only a sixfold induction of glutamine synthetase, and a decrease of GR synthesis. As no primary receptor binding defect could be detected, the altered response of tumor cells to steroid-occupied receptor indicates a partial post-receptor binding defect in GS and keloid cells.

Key words: protein kinase C, glucocorticoid, dexamethasone, fibroblast, tumor tissue

The growth of fibroblasts has been shown to be under the control of numerous factors, including steroid hormones [1–10]. Desmoid and keloid tumors are of special interest in the search for the molecular mechanism by which glucocorticoids participate in the regulation of cell growth and cell division. Desmoids are benign tumors defined as a large mass of unusually firm scarlike connective tissue resulting from active proliferation of fibroblasts. This is a relatively rare tumor in the general population but occurs with high frequency in patients with Gardner's syndrome (GS) [11,12]. Gardner's syndrome is a genetic variant of a disease which causes adenomatosis of the colon and rectum (ACR). Desmoids are similar to keloids; keloids are benign tumors that arise

Received April 25, 1989; accepted January 10, 1990.

during wound healing in genetically predisposed individuals [1]. In vivo, keloids are characterized by prolonged proliferation of fibroblasts and overproduction of collagen and proteoglycans [1,13–15]. Cultured fibroblasts derived from desmoid and keloid tissue differ from fibroblasts derived from normal scar and dermis in their response to physiological levels of hydrocortisone: growth of normal fibroblasts is stimulated while desmoid and keloid fibroblasts are unaffected or inhibited. Collagen synthesis is inhibited in normal fibroblasts but not in keloid-derived cells [1]. The Na^+-dependent A system, which preferentially transports proline, glycine and amino acid analogue 2-(methyl amino) isobutyric acid (MeAIB) is induced twofold in normal fibroblasts and tenfold in keloid cells [16]. It has been shown that cultured fibroblasts from normal and keloid tissues contain the same number of glucocorticoid binding sites with no apparent differences in glucocorticoid affinity for receptor or nuclear bound glucocorticoid-receptor complexes [17].

To characterize further the differences between normal and tumor-derived cells in their response to glucocorticoids, we examined the dexamethasone effect on several glucocorticoid regulated phenotypes. In the present study, we show that in tumor-derived fibroblasts, dexamethasone stimulates glucocorticoid receptor synthesis and suppresses protein kinase C levels coincident with an inhibition of DNA synthesis. The altered response to the glucocorticoid-receptor complex in these cells may be closely linked to the mutations that lead to keloid formation and Gardner's syndrome.

MATERIALS AND METHODS

Materials

Dexamethasone, testosterone, estradiol-17β, phosphatidylserine (PS), histone HI (Type IIIS), EGTA, EDTA, 12-0-tetradecanoylphorbol-13-acetate, protein-A-Sepharose, molecular weight markers, were obtained from Sigma (St. Louis, MO).

Nutrient mixture F10, FBS, and trypsin were purchased from GIBCCO. [^{35}S]methionine (1,000 Ci/mmol), [methyl-^{3}H]thymidine (20 Ci/mmol), [^{3}H]triamcinolone acetonide (50 Ci/mmol) α-[1-^{14}C]methylamino, isobutyric acid (MeAIB) (53.5 mCi/mmol) were obtained from New England Nuclear (Boston, MA). [γ-^{32}P]ATP 25 Ci/mmol was purchased from ICN (Irvine, CA). The immunoblotting for PKC was done by Susan Jaken at the W. Alton Jones Health Science Center, Lake Placid, New York [15].

Cell Culture

The source of keloid and normal tissue, and methods of isolation, propagation, and freezing of fibroblasts have been reported previously [1,16]. Fibroblasts from individuals with Gardner's syndrome (CRL 1610, 1614, 1643, and 1659) were obtained from American Type Culture Collection (ATCC). G_1 and G_2 are clones isolated from desmoid cell 1659, K_1 and K_2 are clones from keloid strains 12 and 50 [1,16,17], respectively; N_1 is from normal strain 131 and N_2 is a clone from strain 103 [1,16,17]. The melanoma cell line HTB 64 and normal fibroblasts HTB 102 were purchased from ATCC. Fibroblast cultures were initiated from frozen cell collections and grown as monolayers in 100 mM plastic dishes (Fisher Scientific) at 37°C in an atmosphere of air and CO_2 adjusted to maintain a pH of 7.4 under 100% relative humidity. Cultures were fed daily with F-10 medium supplemented with 5% FBS.

DNA Synthesis Assays

On day seven after subculture, 90% confluent monolayers in 35 mm culture dishes were rinsed with F-10 and subjected to a 24 h low-serum block in F-10 plus 0.5% FBS. Cultures were then fed F-10 plus 2% FBS in the presence or absence of dexamethasone and/or TPA. Approximately 18 h after refeeding, [^{3}H]thymidine was added for 4 h and cultures were rinsed twice with phosphate-buffered saline (PBS), extracted with ice-cold 5% (wt/vol) trichloroacetic acid, and rinsed twice with distilled water. The cell monolayer was solubilized by incubating for 2 h at 37°C in 0.2 M NaOH, and radioactivity was determined by liquid scintillation counting in Scintiverse II (Fisher Scientific). The incorporated [^{3}H]thymidine was normalized by DNA content per 35 mm dish. DNA determinations were performed on 35 mm dishes using the Burton assay [18].

Glutamine Synthetase Assay

Glutamine synthetase activity was assayed on cytosol from confluent cultures grown for 3 days in the presence or absence of 0.1 μM dexamethasone by the method of Thorndike and Reif-Lehrer [19]. The specific activity of glutamine synthetase is expressed as the micromoles of gamma-glutamyl hydroxamate formed at 37°C $min^{-1}mg^{-1}$ protein.

Amino Acid Uptake

Uptake of [1-^{14}C]MeAIB, a specific nonmetabolized substrate of system A transport, was measured in confluent fibroblast monolayers (5–7 μg of DNA/dish) as previously described [16,17]. Replicate cultures were incubated for 15 min at 37°C with F-10 containing 0.2 μCi/ml [1-^{14}C]MeAIB, conditions that give initial rates of uptake. Uptake was stopped by aspirating of the incubation medium and washing twice in ice cold F-10. Acid-soluble pools were extracted with 5% trichloroacetic acid and radioactivity was counted by scintillation spectrophotometry.

Fractionation of Fibroblasts and Protein Kinase C Assay

Fibroblast monolayers (5×10^8 cells) were washed three times in phosphate-buffered saline (Ca^{+2}- and Mg^{+2}-free) and removed from dishes by scraping. The cell suspensions were centrifuged at 800g for 10 min and washed once with PBS. The resulting cell pellet was resuspended in ice-cold buffer (20 mM Tris/HCl pH 7.5, 2 mM EDTA, 2 mM EGTA, 2 mM dithiothreitol, 0.1% triton X-100 (TEEDT), and 1 mM phenylmethylsulfonylfluoride (PMSF). These extractions have been shown to solubilize cytoplasmic and particulate PKC. Prior to centrifugation at 100,000g for 1 h, the extracts were sonicated at five one second bursts using a Fisher model 300 sonicator. Aliquots of supernatant were loaded onto a 0.5 ml DEAE cellulose column equilibrated with homgenization buffer (without 0.1% triton X-100). The columns were washed with 10 ml of buffer and eluted with 2 ml of buffer made 0.15 M NaCl. The enzyme was assayed at 30°C in a final reaction volume of 100 μl containing (final concentrations) [^{32}P]ATP (specific activity 200 cpm/pmol), 10 mM Mg Cl_2, 0.5 mM EDTA, 0.5 mM EGTA, 0.4 mg/ml histone III S in 20 mM Tris/HCl (pH 7.5). In most cases assays were performed in the presence and absence of 100 μM phosphatidylserine and 1.5 mM $CaCl_2$. Samples were incubated for 10 min at 30°C and aliquots were either spotted on Whatman p81 phosphocellulose paper or electrophoresed on slab gels containing 10%

acrylamide, 0.2% bisacrylamide, and 1% SDS. Protein was measured on duplicate samples by the Bradford assay using bovine serum albumin as a standard [20].

In another study, PKC activity was measured in supernatant and extracted particulate fractions. The cell suspensions were homogenized in 2 ml of ice-cold buffer (20 mM Tris/HCl pH 7.5, 2 mM EDTA, 2 mM EGTA, 0.25 M Sucrose using a glass micro tissue grinder. The crude homogenates were centrifuged at 100,000g for 1 h at 4°C. The supernatant was loaded onto a DEAE cellulose column and processed as described above. PKC was extracted from the particulate fraction in TEEDT buffer, followed by centrifugation at 15,000g for 30 min at 4°C. The supernatant was analyzed for PKC activity.

[^{3}H]TA Binding to Monolayers

Specific binding of [^{3}H]TA to high-affinity glucocorticoid receptors was performed on intact monolayers as previously described [17].

GR Down Regulation

Fibroblast monolayers (5×10^6 cells) were grown for 5 days in F-10 plus 5% FBS at 37°C. Confluent monolayers were then incubated in the presence and absence of 0.1 μM dexamethasone for 36 h. The dexamethasone was removed from the monolayers by four 30 min washes with F-10 medium at 37°C. We determined that 2 h is long enough to wash the dexamethasone from inside the cells. Duplicate monolayer cultures were incubated with 20 nM [^{3}H]TA in the presence and absence of 5 μM nonradiolabeled TA for 1 h at 37°C. Following the incubation the monolayer cultures were processed as described above.

Metabolic Radiolabeling

Confluent monolayers (5×10^6 cells) were grown in the presence and absence of 0.1 μM dexamethasone for 32 h. These monolayers were pulse labeled with 25 μCi/ml [^{35}S]methionine for 4 h in methionine-free medium containing 5% dialyzed fetal bovine serum. The cells were then washed with PBS and scaped in 20 mM Tris (pH 7.5), 1 mM EDTA, 0.15 M NaCl, 0.1% Tween, 0.5% deoxycholate, 1 mM PMSF. The cell extracts were spun at 100,000g for 1 h, and the supernatant was placed in fresh tubes containing 1:100 dilutions of anti-GR antibody, incubated at 4°C for 18 h. Immune complexes were absorbed to protein A-Sepharose. The complexes were washed six times with lysis buffer containing 1% tween and absorbed complexes were eluted by boiling in SDS/β-mercaptoethanol, and the eluted proteins were separated on 10% polyacrylamide gels and visualized by autoradiography.

Western Blotting

Cells were harvested in PBS and lysed by boiling in 2% SDS contain 20 mM Tris (pH 7.5) 1 mM EDTA, 2 mM β-mercaptoethanol. The cell lysates were centrifuged at 100,000g for 1 h. Proteins were fractionated on 10% polyacrylamide gels and transfered to nitrocellulose membrane (Schleicher and Schuell). Following transfer, the membranes were placed in blocking buffer (Tris-buffered saline containing 1% gelatin) for 2 h at room temperature. The blots were probed for 2 h at 22°C with anti-GR antibody diluted 1:100. After washing the blots were probed with anti-species IgG gold conjugate (Bio Rad). Molecular weights were determined using prestained markers. Human GR

migrates a 94,000 dalton protein in this system. The characterization of monoclonal antibody to the human GR (Mab-7) has been published [21,22]. The specificity of the monoclonal antibody to protein kinase type III has been described elsewhere [23,24].

Collection of Conditioned Medium

Seven days after subculture, monolayers were rinse with F-10 and incubated for 30 h in F-10 plus 2% FBS in the presence and absence of 0.1 μM dexamethasone. Conditioned medium was collected, centrifuged at 800g for 10 min, bovine serum albumin was added to 2 mg/ml, [^{3}H]TA was added to 10 picomolar, and 10 ml of medium was treated with 0.25 g of dextran-coated charcoal. This amount of charcoal removes all [^{3}H]TA, and the medium treated in this manner does not alter basal levels of glutamine synthetase activity. The medium was resterilized by filtering through a 0.2 μM filter (Corning) and frozen until time of assay. As a control, F-10 plus 2% FBS was also treated with dextran-coated charcoal.

RESULTS

The Effect of Dexamethasone on DNA Synthesis and PKC Activity

To understand the mechanism by which dexamethasone regulates the growth of fibroblasts, [^{3}H]thymidine incorporation and PKC assays were carried out. Fibroblasts were incubated with various concentrations of dexamethasone for 18 h and then pulsed for 4 h with [^{3}H]thymidine. These data clearly show that dexamethasone caused a significant inhibition in [^{3}H]thymidine incorporation in GS (G_1) and keloid cells (K_1). In contrast, dexamethasone caused a twofold stimulation in [^{3}H]thymidine incorporation in normal cells (Fig. 1). The dose response for growth inhibition were similar to concentrations of [^{3}H]glucocorticoid required for comparable levels of hormone binding (data not shown) [17].

The activation of PKC appears to be an important step in mitogenesis. Moreover, the effects of PDGF and other growth factors on the growth of fibroblasts can be replaced by the activation of PKC [25,26]. We decided to determine whether glucocorticoids affect PKC levels in normal and tumor-derived fibroblasts.

It is firmly established that the stimulation of PKC activity in intact cells with TPA causes a shift in the subcellular location of the enzyme from the cytosol to a membranous locus. We tested the ability of PKC in each of our cells to respond to TPA. The TPA effect on PKC caused a shift in cytosolic PKC activity to the membrane pellet in normal, GS, and keloid cells with no differences in the percent of total enzyme shifted (Table I).

Analogous studies to ascertain if dexamethasone had any effect on PKC revealed that in GS and keloid cells incubated with 0.1 μM dexamethasone for 18 h there was a significant suppression of PKC activity. There was a slight stimulation of total PKC activity in normal cells grown in the presence of dexamethasone. Next, we wanted to determine the effect of dexamethasone and TPA on [^{3}H]thymidine incorporation. The results shown in Table II indicate that 10 nM TPA stimulated the incorporation of [^{3}H]thymidine into DNA 3–8-fold depending on the cell type examined. These data also show that there were slightly higher increases in [^{3}H]thymidine incorporation in normal cells. When GS and keloid cells were pretreated for 2 h with dexamethasone prior to the addition of 10 nM TPA, TPA restored [^{3}H]thymidine incorporation to basal levels. The amount of [^{3}H]thymidine incorporation in normal cells grown in TPA and dexametha-

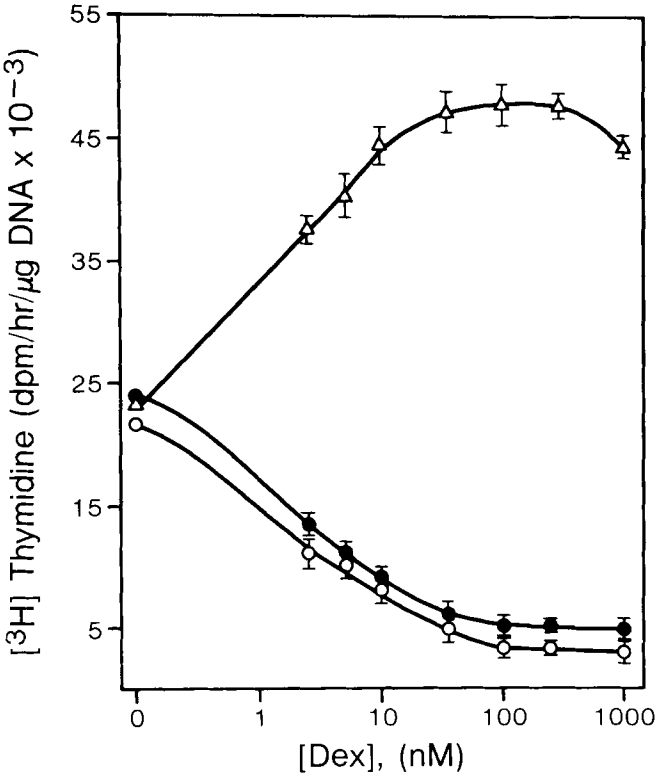

Fig. 1. The effect of dexamethasone on DNA synthesis in normal and tumor-derived cells. Normal (△) and tumor cells (G1, ○; K1, ●) were plated as described in Materials and Methods. Cells were incubated with various concentrations of dexamethasone for 18 h and pulsed 4 h with [^{3}H]thymidine. The amount of [^{3}H]thymidine incorporated into trichloroacetic acid-precipitable material in 4 h was used to measure the rate of DNA synthesis. All values are expressed as ± standard error of six determinations.

TABLE I. Effect of TPA on PKC Activity in Normal and Tumor Cells. Cells Were Incubated With and Without (Control) 10 nM TPA for 30 min at 37°*

	PKC activity (pmol ATP/mg/min)					
	N_1		G_1		K_1	
Cell type	Cytoplasm	Particulate	Cytoplasm	Particulate	Cytoplasm	Particulate
Control	470 ± 85	172 ± 34	361 ± 52	108 ± 44	620 ± 71	185 ± 42
TPA	97 ± 26	608 ± 71	81 ± 23	557 ± 87	139 ± 41	771 ± 83

*The samples were processed as described in Materials and Methods. All data are the mean ± standard error of three separate determinations.

sone was equal to that achieved by TPA alone. In addition, these results suggest that chronic treatment of fibroblasts with TPA down regulated PKC, a result that is similar to that reported by other investigators [27,28]. However, unlike the suppression of PKC levels in GS and keloid cells by dexamethasone, DNA synthesis was increased in all fibroblasts grown in the presence of TPA. Moreover, the results indicate that the TPA effects on DNA synthesis were initiated prior to the eventual loss of PKC.

TABLE II. Effect of Dexamethasone and TPA on DNA Synthesis and PKC Activity*

Cell type	DNA synthesis [^{3}H]thymidine dpm/hr/μg DNA $\times 10^{-3}$			
	Control	Dex	TPA	TPA/Dex
N1	25 ± 3	48 ± 5	146 ± 23	151 ± 31
N2	22 ± 4	45 ± 7	171 ± 28	183 ± 35
K1	26 ± 5	4 ± 0.5	77 ± 15	30 ± 11
K2	21 ± 3	5 ± 0.8	88 ± 24	25 ± 4
G1	22 ± 5	8 ± 0.5	92 ± 21	24 ± 9
G2	27 ± 3	10 ± 0.7	89 ± 20	35 ± 8
Fetal-1502	34 ± 5	22 ± 3	138 ± 42	
Normal-HT3102	25 ± 2	47 ± 4	145 ± 36	
Melanoma-HTB64	49 ± 6	30 ± 5		
	PKC activity (ATP pmol/mg/min)			
	Control	Dex	TPA	TPA/Dex
N1	422 ± 51	537 ± 44	126 ± 20	66 ± 19
N2	481 ± 36	545 ± 26	139 ± 29	57 ± 12
K1	693 ± 46	186 ± 42	152 ± 37	63 ± 10
K2	651 ± 48	209 ± 37	135 ± 30	54 ± 10
G1	496 ± 57	240 ± 31	122 ± 33	51 ± 18
G2	513 ± 61	188 ± 40	140 ± 24	60 ± 19
Fetal-1502	685 ± 37	770 ± 58		
Normal-HTB102	469 ± 38	510 ± 36		
Melanoma-HTB64	882 ± 62	642 ± 37		

*Confluent cultures of fibroblasts derived from normal, keloid, GS, fetal, and melanoma tissue were subjected to a low serum block and fed 95% F-10 + 5% serum for 18 h at 37°C in the presence and absence of 0.1 μM dexamethasone and/or 10 nM TPA. The data are expressed as mean ± the standard error from eight determinations.

Knabbe et al. [29] have demonstrated that glucocorticoids inhibit the growth of MCF-7 cells by stimulating the release of a transforming growth factor-like substance from these cells. To determine whether the effect of dexamethasone on PKC would be observed using media conditioned by GS and keloid cells grown in the presence of dexamethasone, we removed media from GS, keloid, and normal cells grown in the presence and absence of 0.1 μM dexamethasone. Dextran-coated charcoal was used to remove the glucocorticoid from the media. Normal, GS, and keloid cells that had not been treated with dexamethasone were incubated with conditioned media from cells grown in the presence or absence of glucocorticoid. As shown in Table III, the suppression of total PKC activity was mediated by a substance released in the media of GS and keloid cells grown in the presence of 0.1 μM dexamethasone (CMD). Moreover, the CMD from keloid cells increased PKC activity in GS and normal cells. The inability of CMD from keloid fibroblasts to suppress PKC activity in GS cells may reflect differences in the origin of the cultured cells. Unlike keloid cells the GS fibroblasts used in this study were isolated from abdominal muscle tissue. Preliminary evidence from our laboratory suggests that CMD from keloid fibroblasts suppresses PKC activity in dermal fibroblasts isolated from individuals with GS (data not shown). For both GS and keloid cells the loss of PKC activity following exposure of cells to dexamethasone was due to a loss of the corresponding protein. We prepared whole homogenates from GS, keloid, and normal cells grown for 18 h in the presence and absence of dexamethasone. The data in

TABLE III. Effects of Conditioned Media (CM) on PKC Activity*

Source of (CM)	PKC activity (pmol ATP/mg/min)		
	Control (CM)	Dex (CM)	Cell type
N1	450 ± 32	591 ± 47	N1
	538 ± 61	703 ± 51	K1
	409 ± 38	572 ± 53	G1
K1	725 ± 79	206 ± 41	K1
	484 ± 65	577 ± 59	N1
	340 ± 53	673 ± 77	G1
G1	375 ± 46	128 ± 43	G1
	496 ± 54	579 ± 68	N1

*Condition media was isolated from normal and tumor derived fibroblasts grown in the presence and absence of 0.1 μM dexamethasone. The CM was incubated with fresh cultures for 18 h at 37°C, and PKC activity was measured as described in the text. All values are expressed as the mean ± standard error of three separate determinations.

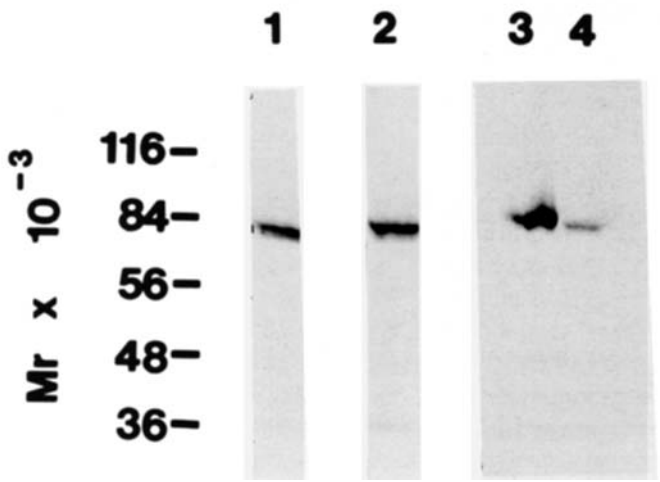

Fig. 2. Use of monoclonal antibody to detect protein kinase C in normal and tumor-derived fibroblasts. Cells were grown for 18 h in the presence and absence of dexamethasone. In lanes 1–4 120 μg of whole cell lysates were analyzed by Western blot and probed with type 3 PKC IgG [24]. Lanes 1, 3 represent extract from cells grown in the absence of dexamethasone. Lanes 2, 4 are samples from cells grown for 18 h in the presence of dexamethasone. **Lanes 1, 2:** Normal (N_1) dermis. **Lanes 3, 4:** Keloid (K_1) fibroblasts.

Figure 2 indicate that there was a significant loss of total PKC immunoreactive protein from tumor cells grown in the presence of dexamethasone. No such change in PKC protein was observed in normal cells grown in the presence of this glucocorticoid.

A time course showing the dexamethasone effect on the suppression of total PKC activity in keloid cells is shown in Figure 3, there was approximately a 70% loss of activity after an 18 h exposure to dexamethasone. In GS cells there was a 50% loss of total PKC activity in response to dexamethasone over the same time course as shown for keloid fibroblasts (Table II). However, as shown in Figure 3 the PKC activity returned to basal levels at 48 h.

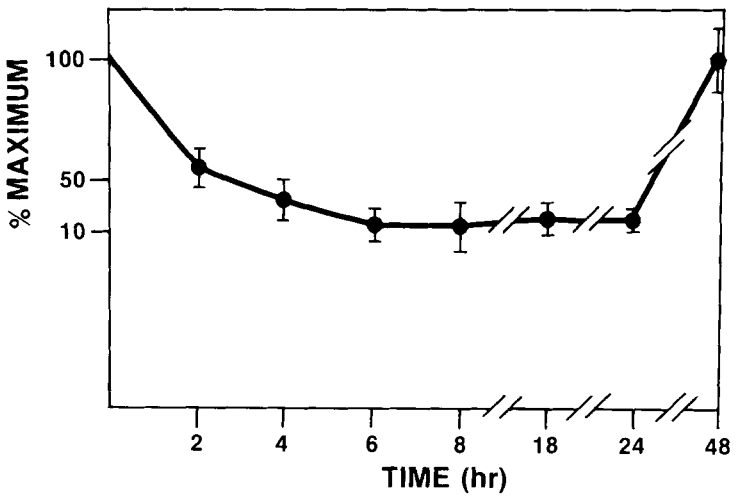

Fig. 3. Time course for the effect of dexamethasone on total PKC activity. Cells were grown in the presence and absence 0.1 μM dexamethasone for various lengths of time. Total PKC activity was assayed as described in Material and Methods. The data are reported as the mean ± standard error of four determinations.

Recent evidence indicates that fibroblasts isolated from fetal tissue behave similarly to keloid fibroblasts in response to glucocorticoid. Hydrocortisone has been shown to inhibit or have no effect on DNA synthesis in fetal and keloid fibroblasts [1,30]. In Table II we show that DNA synthesis in a fetal cell line was inhibited by dexamethasone, however, the data also show that there was a small increase in total PKC activity. In addition, dexamethasone inhibited DNA synthesis and suppressed PKC activity in a melanoma cell line. It should be noted that this cell line has 7,000 GR sites per cell, which is several-fold less than the 100,000 GR binding sites in human fibroblasts. All of these responses appear to be specific for dexamethasone since testosterone and estradiol 17β at concentrations as high as 1 μM were unable to elicit similar effects in normal, GS, and keloid cells (data not shown).

The Effect of Dexamethasone on MeAIB Transport, Glutamine Synthetase, and GR Synthesis

Previously, it has been shown that cultured fibroblasts derived from keloids differ from fibroblasts derived from normal scar or dermis in their response to physiological levels of hydrocortisone [1,16]. To determine whether or not the altered response to glucocorticoids is limited to system A transport, collagen synthesis, and cell growth, we tested the ability of dexamethasone to alter the synthesis of two other glucocorticoid-inducible proteins; glutamine synthetase and the glucocorticoid receptor. As shown in Figure 4, dexamethasone induced glutamine synthetase activity approximately 20-fold in GS and keloid cells (indicated by T), while glutamine synthetase was induced only sixfold in normal cells.

To examine GR regulation in GS, keloid, and normal fibroblasts in culture, all cells were treated for 32 h in the presence and absence of 0.1 μM dexamethasone and pulse labeled with 25 μCi/ml [^{35}S]methionine for 4 h at 37°C. Cytosol prepared from cells was incubated for 18 h with anti-receptor antibody. Proteins were then absorbed to protein A

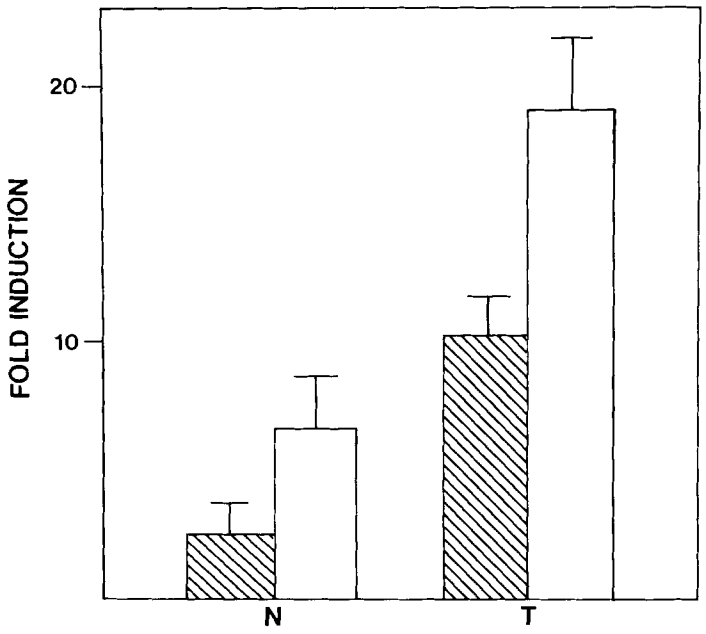

Fig. 4. The effect of dexamethasone on system A transport and glutamine synthetase activity. Normal (N) and tumor-derived (T) cells were grown in the presence and absence of 0.1 μM dexamethasone. Initial rates of system A transport (nmol/mg DNA/15 min: **stripe bar**) were measured as described in Materials and Methods. The initial rates of system A transport in normal cells for control and dexamethasone treated cultures were 2.3 ± 0.3 and 5.9 ± 0.4, and 1.4 ± 0.2 and 15.1 ± 0.9 for tumor cells, respectively. Glutamine synthetase activities (units/mg protein min) (**open bar**) in normal fibroblasts were 0.3 ± 0.02 for control and 2.2 ± 0.04 for dexamethasone treated cells. The glutamine synthetase activity values for tumor cells grown in the absence and presence of dexamethasone were 0.24 ± 0.03 and 6.6 ± 1.7, respectively.

sepharose and analyzed by SDS-polyacrylamide gel electrophoresis. Figure 5 shows an autoradiogram of the gel. Glucocorticoid receptor synthesis was stimulated in GS cells grown in the presence of dexamethasone. In contrast, there was a dramatic decrease in the synthesis of GR in normal cells grown in the presence of dexamethasone. In the absence of steroid, GS and normal cells contain similar amounts of immunoreactive GR as determine by immunoblotting and [^{3}H]glucocorticoid binding assays (data not shown). Also, the data in Figure 5 indicate that in the absence of dexamethasone the rate of GR synthesis was the same in normal and GS tumor cells.

DISCUSSION

The results presented here demonstrate that fibroblasts isolated from keloid and GS tissue differ from fibroblasts of normal dermis by their ability to suppress PKC and [^{3}H]thymidine incorporation and failure to decrease the synthesis of the glucocorticoid receptor in the presence of dexamethasone.

Previous studies have shown that in response to in vitro or in vivo treatments of glucocorticoids the GR levels can either increase or decrease depending on the cell type. These changes in GR levels have been shown to result from a difference in the half-life of the GR protein and steady state levels of GR mRNA [31–35]. The inability of GR to

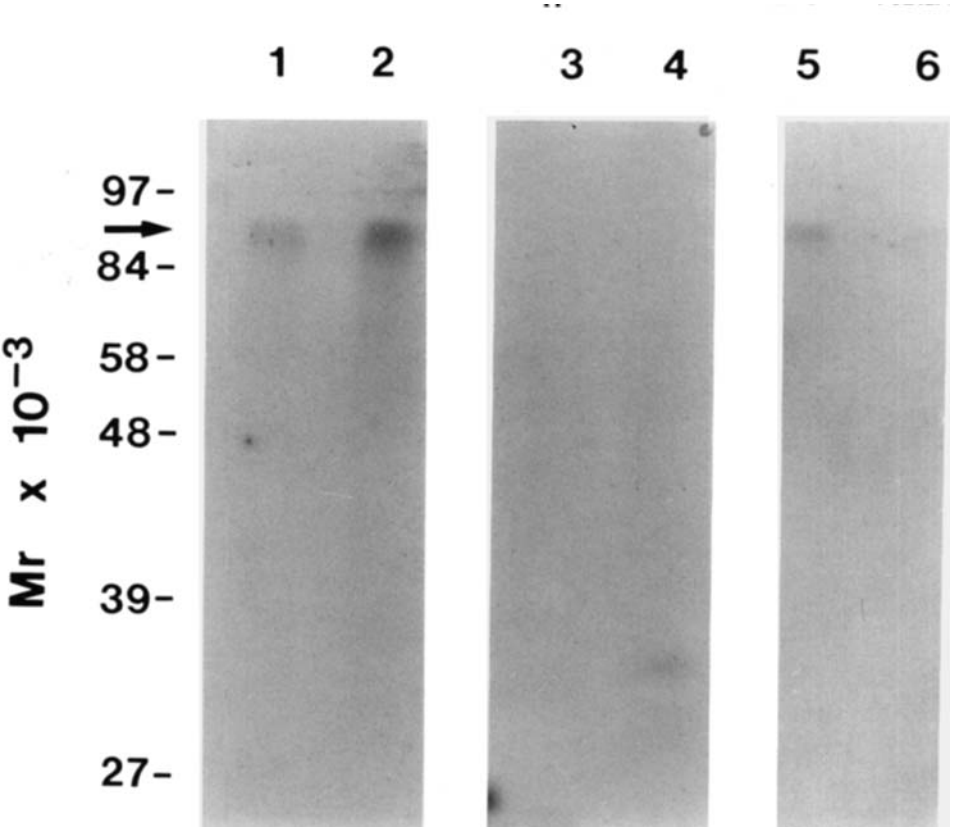

Fig. 5. Representative sample of the effect of dexamethasone on GR synthesis. ^{35}S-methionine-labeled cytosol was prepared from normal and tumor-derived fibroblasts and incubated for 18 h with anti-GR IgG or nonimmune IgG. Antibody-protein complexes were then absorbed to protein A-Sepharose. Protein A-Sepharose pellets were washed six times prior to gel electrophoresis. An autoradiogram of the gel, exposed to film for 5 days, is shown. **Lanes 1 and 5:** G_1 and N_1 grown for 36 h in the absence of dexamethasone. **Lanes 2 and 6:** G_1 and N_1 cells grown for 36 h in the presence of dexamethasone. **Lanes 3 and 4:** Represent immunoprecipitations with nonimmune serum from control G_1 and N_1 extracts, respectively. Molecular weight markers are phosphorylase B (97,400 Mr), fructose-6-phosphate kinase (84,000 Mr), Pyruvate kinase (58,000 Mr), fumarase (48,000 Mr), lactate dehydrogenase (39,000 Mr), and trisephosphate isomerase (27,000 Mr).

down regulate its own synthesis in tumor-derived fibroblasts may be due to the dexamethasone mediated suppression of PKC. Several recent studies document that PKC activation can have profound effects on steroid and thyroid hormone receptors in cultured cells. Boyle and Van der Walt [36] have reported that the activation of PKC by phorbol esters increases the total number of progresterone binding sites and decreases the DNA binding capacity of progesterone-receptor complexes in T47D breast cancer cells. Furthermore, Kido et al. [37,38] have shown that PKC activation enhanced the induction of tyrosine aminotransferase and ornithine decarboxylase by glucocorticoids in rat hepatocytes. Their finding show that PKC activation may be crucial to the translocation of glucocorticoid receptor-complexes to the nucleus. It has also been shown that the thyroid hormone receptor can be phosphorylated by the activation of PKC and cAMP-dependent protein kinase [39]. Collectively, these data strongly suggest that PKC

activation has a major role in steroid and thyroid hormone action; however, the exact consequences of these phosphorylation events on steroid and thyroid hormone receptors will require additional studies.

The current evidence on the mechanism of steroid hormone action indicates that the interaction of glucocorticoid receptors with specific recognition sites of responsive genes alters their rate of transcription [31]. The inhibitory effects of glucocorticoids on DNA synthesis are well known in many tissues and cultured cells [1,29,30,40–42]. However, the biochemical reactions that lead to growth suppression following the interaction of glucocorticoid with its receptors remains to be elucidated. Recent studies indicate that steroid hormone and growth factor-regulated proliferation of certain tumors cells can be correlated with the changes in expression of a combination of growth inhibitors and growth stimulators [29]. PKC has been shown to play an integral role in the proliferation of many cell types [43]. In an attempt to understand better how glucocorticoids control the growth of tumor cells, we studied the effects of glucocorticoids on PKC. The present results demonstrate that dexamethasone suppressed 50–70% of the total PKC activity. Other investigators have shown that several substances can alter PKC activity, including dexamethasone [44], sphingosine and lysosphingolipids [45], interferons [46], polyamines [47], and cyclic AMP [48]. The mechanism in which these substances affect PKC activity has not been determined.

However, Kleine et al. [44] have shown that in rat liver epithelial cells dexamethasone stimulates PKC by binding to a site on the protein that is different from that of diacylglycerols and TPA. In addition, the authors suggest that dexamethasone stimulates the growth of normal rat liver epithelial cells by the direct activation of PKC. There have been numerous reports indicating that dexamethasone increases sphingomyelin levels in 3T3 L11 cells, and this change in intracellular concentrations appears to be important in the differentiation of these preadiposite cells [49–51]. Furthermore, sphingosine has been shown to mimic the dexamethasone effect on 2-deoxyglucose uptake [52]. These data indicate that sphingolipids mediate some of the effects of glucocorticoids. It is unlikely that sphingolipids released into the media of dexamethasone treated cells is a direct mediator of the glucocorticoid effect on PKC. First, charcoal treatment would probably remove spingolipids from the conditioned media. Second, the effect of sphingolipids on PKC would not be limited to tumor-derived fibroblasts. Finally, media conditioned by keloid fibroblasts grown in the presence of dexamethasone increases PKC activity in fibroblasts from normal and GS desmoid-derived tissue. There are also similarities between the glucocorticoid effect on PKC in human fibroblasts and the effect of gamma interferon on PKC in human epipharygiama and melanoma cells [46]. It was shown that interferon β (IFN-β) and interferon γ (IFN-γ) decreased total PKC activity in these tumor cell lines. However, membranous PKC from cells treated with IFN-γ returned to basal levels after 24 h. In our study, we observed a suppression of total PKC activity in tumor-derived cells grown in the presence of dexamethasone, but the activity reappeared after 48 h. Moreover, the results indicate that the dexamethasone effect on PKC in GS and keloid cells are specific for the cell types analyzed and indirect. The TPA effect on PKC levels in normal, GS, and keloid cells is consistent with results from previous studies; which is, the cytosolic form of PKC is translocated to the membrane pellet 30 min after TPA treatment [28]. Furthermore, the immunoblots of PKC show that the PKC proteins from normal and tumor cells have the same molecular weight.

These data suggest that the structure of the PKC III is similar in all cell types examined in this report.

Russell et al. [1,30] have shown that the growth of keloid cells is either inhibited or unaffected by glucocorticoids. In the present report, when keloid and GS cells were grown in the presence of 10 nM TPA and 0.1 μM dexamethasone DNA synthesis was not inhibited. These data indicate that the presence or absence of PKC activators can determine whether or not glucocorticoids have an affect on DNA synthesis in GS and keloid cells. We also show that dexamethasone inhibited DNA synthesis in a fetal cell line without decreasing PKC activity. This suggests that changes in the levels of PKC alone may not alter DNA synthesis.

The differences between normal and tumor-derived fibroblasts have been extended to include other GR regulated phenotypes: GR synthesis, glutamine synthetase, and PKC suppression. Recent evidence indicates that dexamethasone affects type I and IV collagen at the level of gene transcription [53], and it has been proposed that the increase in collagen synthesis in keloid cells results from a failure of glucocorticoids and their receptor to negatively regulate collagen messenger ribonucleic acid levels [1,16,54,55].

In summary, the present findings demonstrate that there is a post-glucocorticoid-receptor binding lesion in fibroblasts isolated from keloid and GS tumor tissue. Consequently, the suppression of PKC by glucocorticoids may alter the response of these tumor cells to growth factors, such as PDGF and EGF. Recently, Livneh et al. [56] reported that the phosphorylation of the epidermal growth factor receptor by the activation of PKC blocked the mitogenic capacity of EGF. Thus, in the presence of glucocorticoids, fibroblasts may be more sensitive to the mitogenic effect of EGF because of the decrease in PKC levels.

ACKNOWLEDGMENTS

We thank Susan Jaken for help with the PKC immunoblotting and Dr. Robert Wrenn for assisting with the PKC assays. This work was supported by grants from the American Cancer Society No. IN 147 and the Medical College of Georgia Research Institutional Grant.

REFERENCES

1. Russell JD, Russell SB, Trupin KM: J Cell Physiol 97:221–229, 1978.
2. Munck A, Guyre PM, Holbrook NJ: Endocr Rev 5:25–44, 1984.
3. Syms AJ, Norris JS, Smith RG: Biochem Biophys Res Commun 122:68–74, 1984.
4. Fritch DF, Kaji H: Biochimie 70:215–220, 1988.
5. Kondo H, Kasuga H, Noumura T: Exp Cell Res 158:342–348, 1985.
6. Conover CA, Rosenfeld RG, Hintz RL: J Cell Physiol 128:47–58, 1986.
7. Baker JB, Barsh GS, Carney DH, Cunningham DD: Proc Natl Acad Sci USA 75:1882–1886, 1978.
8. Goustin AS, Leof EB, Shipley GD, Moses HL: Cancer Res 46:1015–1029, 1986.
9. Scher CD, Shepeard RC, Antoniades HN, Stiles CD: Biochim Biophys Acta 560:217–241, 1979.
10. O'Keefe EJ, Pledger WJ: Mol Cell Endocrinol 31:167–186, 1983.
11. Gardner E, Richards R: Am J Hum Genet 5:139–148, 1953.
12. Kopelovich L: Mutat Res 199:369–385, 1988.
13. Uitto J: Intern Med 105:740–756, 1986.
14. Abergel RP, Pizzuro D, Meeker CA, Lask G, Matsuoka LY, Minor RR, Chu M-L, Uitto J: Invest Dermatol 85:384–390, 1985.
15. James WD, Besanceney CD, Odom RB: J Am Acad Dermatol 3:50–57, 1980.

16. Russell SB, Russell JD, Trupin JS: J Biol Chem 257:9525–9531, 1982.
17. Gadson PF, Russell JD, Russell SB: J Biol Chem 259:11236–11241, 1984.
18. Burton K: Method Enzymol 12B:163–166, 1968.
19. Thorndike J, Reif-Lehrer L: Enzyme 12:235–241, 1971.
20. Bradford MM: Anal.Biochem 72:248–254, 1976.
21. Okret S, Wikström A-C, Wrange O, Andersson B, Gustafsson J-A: Proc Natl Acad Sci USA 81:1609–1613, 1984.
22. Denis M, Wikström A-C, Gustafsson J-A: J Biol Chem 262:11803–11806, 1987.
23. Leach KL, Powers EA, McGuire JC, Dong L, Kiley S, Jaken S: J Biol Chem 263:13223–13230, 1988.
24. Jaken S, Kiley S: Proc Natl Acad Sci USA 84:4418–4422, 1987.
25. Kaibuchi K, Tsuda T, Kikuchi A, Tanimoto T, Yamashita T, Yoshimi T: 261:1187–1192, 1986.
26. Habenicht AJR, Glomset JA, King WC, Nist C, Mitchell CD, Ross R: J Biol Chem 256:12329–12335, 1981.
27. Grabriella Lacal J, Warren BS, Aaronson SA, Blumberg PM: Science 321:407–410, 1986.
28. Wolfman A, Wingrove TG, Blackshear PJ, Macara IG: J Biol Chem 16546–16552, 1987.
29. Knabbe C, Lippman ME, Wakefield LM, Flanders KC, Kasid A, Derynck R, Dickson RB: Cell 48:417–428, 1987.
30. Russell SB, Trupin KM, Rodriguez-Eaton S, Russell JD, Trupin JS: Proc Natl Acad Sci USA 85:587–591, 1988.
31. Eisen LP, Elsasser MS, Harmon JM: J Biol Chem 263:12044–12048, 1988.
32. Gustafsson J-A, Carlstedt-Duke J, Poellinger L, Okret S, Wikström A-C, Bronnegard M, Gillner M, Dong Y, Fuxe K, Cintra A, Harfstrand A, Agnati L: Endocrine Rev 8:185–234, 1987.
33. Cidlowski JA, Cidlowski NB: Endocrinology 109:1975–1982, 1981.
34. McIntyre WR, Samuels HH: J Biol Chem 260:418–427, 1985.
35. Kalinyak JE, Dorin RI, Hoffman AR, Perman AJ: J Biol Chem 262:10441–10444, 1987.
36. Boyle DM, Andre van Der Walt L: J Steroid Biochem 30:239–244, 1988.
37. Kido H, Fukusen N, Katunuma N: Biochem Biophys Res Commun 144:152–159, 1987.
38. Kido H, Fukusen N, Ishidoh K, Katunuma N: Biochem Biophys Res Commun 138:275–282, 1986.
39. Goldberg Y, Glineur C, Gesquiere J-C, Ricouart A, Sap J, Vennstrom B, Ghysdaell J: EMBO J 7:2425–2433, 1988.
40. Pratt WB: J Invest Dermatol 71:24–35, 1978.
41. Cristofalo VJ, Wallace JM, Rosner BA: In Soto GH, Ross R (eds): "Hormones and Cell Culture." Cold Spring Harbor, New York: Cold Spring Harbor Laboratory, 1979, pp 875–887.
42. Finlaz CA, Cristofalo VJ: In Boynton AL, Leffert HL (eds): "In Vitro control of Animal Cell Proliferation." New York: Academic Press, Inc., 1987, pp 203–217.
43. Nishzuka Y: Science 233:305–312, 1986.
44. Kleine LP, Whitfield JF, Boynton AL: Biochem Biophys Res Commun 135:33–40, 1986.
45. Hannun YA, Bell RM: Science 235:670–674, 1986.
46. Ito M, Takami Y, Tanabe F, Shigeta S, Tsukui K, Kawade Y: Biochem Biophys Res Commun 150:126–132, 1988.
47. D-Fang QI, Shatzman RC, Mazzel GJ, Turner RS, Raynor RL, Liao S, Kuo JF: Biochem J 213:281–288, 1983.
48. Navindrasorasak S, Brickenden A, Ball E, Sanwall BD: J Biol Chem 262:10497–10501, 1987.
49. Nelson D, Murray D, Brady J: J Clin Endocrinol Metab 54:292–295, 1982.
50. Nelson D, Murray D: Proc Natl Acad Sci USA 79:6690–6694, 1982.
51. Hannun YA, Bell RM: Science 243:500–507, 1988.
52. Nelson D, Murray D: Biochem Biophys Res Commun 138:463–467, 1986.
53. Weiner FR, Czaja MJ, Jefferson DM, Giambrone M-A, Tur-kaspa R, Reid LM, Zern MA: J Biol Chem 262:6955–6958, 1987.
54. Murray JC, Pollack SV, Pinnell SR: Acad Dermatol 4:461–470, 1981.
55. Russell SB, Trupin JS, Myers JC, Broquist AH, Smith JC, Myles ME, Russell JD: J Biol Chem 264:13730–13735, 1989.
56. Livneh E, Dull TJ, Berent E, Prywes R, Ullrich A, Schlessinger J: Mol Cell Biol 8:2302–2308, 1988.