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AUTOCRINE REGULATION OF GROWTH: I. GLUCOCORTICOID INHIBITION IS OVERCOME BY EXOGENOUS PLATELET DERIVED GROWTH FACTOR

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The ductus deferens smooth muscle tumor cell line (DDT_1MF-2) expresses c-sis proto-oncogene mRNA transcripts which encode at least one subunit of the potent mitogenic agent, platelet derived growth factor (PDGF). These cells also synthesize and secrete a protein which is immunologically identical to this growth factor. Therefore, PDGF is implicated in the autocrine regulation of DDT_1MF-2 cell proliferation. While androgens also stimulate proliferation and induce an augmentation in androgen receptor levels in DDT_1MF-2 cells, glucocorticoids inhibit both events and arrest cells in the G1 phase of the cell cycle. Addition of PDGF overcomes the glucocorticoid cell cycle arrest, but does not diminish the suppressive action on androgen receptor concentration. These findings are consistent with a mechanism by which glucocorticoids regulate DDT_1MF-2 cell proliferation through modulation of PDGF expression which is independent of the glucocorticoid effects on androgen receptor concentrations.

While there is a clear association between expression of oncogenes and tumorigenesis, the molecular biology of this phenomenon is poorly understood (1-3). The recent and exciting discovery that the c-sis proto-oncogene encodes platelet-derived growth factor (PDGF) suggested, however, that the development of a neoplastic state may be related to abnormal synthesis of particular growth factors (4-6). Furthermore, since NIH-3T3 cells can be transformed by a human c-sis cDNA clone (7), it is apparent that PDGF could participate in the steps leading to carcinogenesis. That the ductus deferens smooth muscle tumor cell line (DDT_1MF-2) expresses c-sis mRNA (8,9) and synthesizes a protein which immunologically is identical to PDGF (10), in itself, implicates this growth factor in the autocrine regulation of DDT_1MF-2 cell proliferation.

Interestingly, this cell line also contains receptors for and is differentially sensitive to androgens and glucocorticoids (11-14). In the presence of androgens, proliferation is markedly stimulated and the concentration of androgen receptors is

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elevated (12-14). Addition of glucocorticoids, however, dramatically inhibits both of these androgen-mediated events and arrests the cells in the G1 phase of the cell cycle (12-14). Since PDGF appears to initiate cell cycle traverse of G0-G1 arrested cells by rendering them competent to respond to other growth factors present in platelet-poor plasma (15), it was apparent that the mechanism of the glucocorticoid-induced cell cycle arrest may be intrinsically related to effects on PDGF function.

Herein, we document that the addition of physiological levels of PDGF releases DDT_1MF-2 cells arrested in G1 by glucocorticoids. However, the suppressive action on androgen receptor concentration was not diminished. These results suggest that glucocorticoids regulate proliferation of DDT_1MF-2 cells by modulating PDGF expression through effects which are independent of the glucocorticoid effects on androgen receptor concentrations.

MATERIALS AND METHODS

Materials: All tissue culture reagents were obtained from GIBCO Laboratories, Grand Island Biological Co., Grand Island, NY. Platelet derived growth factor (receptor grade, 95% pure) was purchased from Seragen, Inc., Boston, MA. Epidermal growth factor (receptor grade, 98% pure) and insulin (98% pure) were obtained from Collaborative Research, Inc., Lexington, MA. [17-methyl-^{3}H]-methyltrienolone (R1881; 87 Ci/mmol), a synthetic ligand with high affinity for the androgen receptor, was purchased from New England Nuclear, Boston, MA. All other chemicals and reagents were purchased from Sigma Chemical Co., St. Louis, MO.

Cell Culture Conditions: DDT_1MF-2 cells were routinely grown as monolayers on 100 mm petri dishes in a humidified, 5% CO_2-95% air atmosphere at 37°C. The high-glucose variant of Dulbecco's modified Eagle's medium (DME) supplemented with 5% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 µg/ml) and testosterone (10 nM) was used to maintain the cultures and was replenished every 48h. Cells were harvested and passaged by exposure to trypsin (0.05%) and EDTA (0.02%).

Flow Cell Cytometry: This method is a modification of Vindelov's "pH 10 procedure" for rapid preparation and staining of cell nuclei as indicated in Ortho Instruments Protocols No. 28, and as previously described (12). Briefly, cells were harvested by trypsinization and resuspended in Tris-HCl buffer (100 nM Tris [Hydroxymethyl] aminomethane, 100 nM NaCl, 1% Triton-X-100, pH 10) containing 0.004% RNase and 20 µM ethidium bromide). Samples were stored at 4°C prior to analysis on an Ortho Diagnostic System 2150 (Ortho Diagnostic Systems, Westwood, MA) fitted with a 5W argon laser. The number of cells present in each phase of the cell cycle were obtained by fitting a mathematical model to the histograms generated.

Whole Cell Binding Assay: Total intracellular androgen receptors were quantitated as previously described (13,16). Confluent monolayers were maintained on DME containing 2% charcoal-extracted FBS for 48h. Cells were then washed and the medium replaced with serum-free medium supplemented with 1 nM [^{3}H]-R1881. Non-specific binding was determined by competition with a 100-fold excess of radioinert steroid. Since these cells do not possess progestin binding sites for which R1881 also has affinity, it was not found necessary to compensate for this by adding other steroids to saturate these sites. The effect of glucocorticoids was determined by the addition of 10 nM triamcinolone acetonide. Cells were washed to remove free steroid and extracted with ethanol to determine bound radioactive ligand.

DNA Assay: Following ethanol extraction, the remaining cell pellets were retained for DNA determination using a modification of the Burton technique (17,18).

RESULTS AND DISCUSSION

The effect of PDGF on DDT_1MF-2 cells arrested in G1 by treatment with the synthetic glucocorticoid triamcinolone acetonide (TA) was examined by monitoring the release of these cells from G1 into S-phase by flow cell cytometry. The reversibility of this block can be demonstrated by thorough washing of the cells to remove TA and addition of fresh medium. If the fresh medium was supplemented with TA this release was inhibited by approximately 50%. Simultaneous treatment with PDGF in the presence of TA, however, allowed the remaining quiescent cells to exit G1 and enter S in a dose dependent manner (Fig. 1). These results suggested a direct relationship between PDGF and glucocorticoids in the regulation of DDT_1MF-2 cell proliferation.

The presence of endogenous PDGF in fresh medium could also account for the ability of some cells to escape the G1 block following replenishment with fresh medium, regardless of the presence of TA. Alternatively, this phenomenon may be

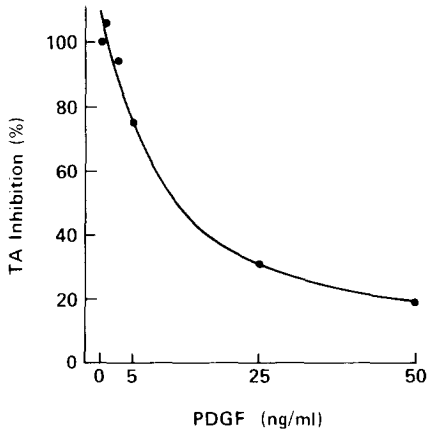

Figure 1. DDT_1MF-2 cells (2.5×10^5) were plated in 100 mm culture dishes in 10 ml DME supplemented with 2% FBS and 10 nM testosterone. After 24h, cultures were replenished with similar medium containing 10 nM triamcinolone acetonide (TA) for 48h. The medium was then aspirated and the cells washed twice in Hanks balanced salt solution (HBSS). Cells were then incubated in 5 ml fresh media in the presence or absence of 10 nM TA for 18h. To those cultures treated with TA was also added 0-50 ng/ml PDGF. Cells were then analyzed by flow cell cytometry. Data is given as a percentage of those cells remaining in G1 compared to cells grown in the presence of TA. 100% inhibition represents the difference between TA arrested cells and those released from the G1 block by removing TA. Each data point represents the mean of duplicate determinations for which the coefficient of variation does not exceed 5%.

TABLE 1

Effect of cycloheximide on glucocorticoid-induced cell cycle arrest

Treatment	Cells in each phase of cycle (%)		
	G1	S	G2+M
Control	56.3 ± 1.2	29.7 ± 0.6	14.0 ± 1.0
Cycloheximide	68.7 ± 1.2	21.0 ± 1.0	10.3 ± 0.6
TA	75.3 ± 0.6	9.3 ± 0.6	15.4 ± 1.2
TA + Cycloheximide	65.7 ± 1.2	22.0 ± 0.0	12.3 ± 1.2

DDT_1MF-2 cells (5×10^5) were plated in 100 mm culture dishes in 10 ml DME supplemented with 2% FBS and 10 nM testosterone. After 24h, cultures were replenished with identical medium containing either 10 nM TA or 1 μg/ml cycloheximide. Following a 6h incubation at 37°C, cells were analyzed by flow cell cytometry as described in Materials and Methods. Data represent the mean and standard deviation of triplicate determinations.

due to a lag in the time required for TA to evoke a critical level of possible repressor proteins in the fresh medium. Evidence for the existence of such repressors arises from studies in which TA induced the synthesis of several new proteins which were not detectable during the first few hours of stimulation (19). These proteins are important in mediating the effect of glucocorticoids, since cells grown in the presence of TA and cycloheximide for 6h do not accumulate in G1 as rapidly as those treated with TA alone (Table 1). Furthermore, partial release of cells in the presence of TA does not occur if the medium is not replenished. Therefore, the effect of PDGF on TA arrested cells was examined by adding the growth factor directly to cultures containing TA "pre-conditioned" medium, where release of cells from G1 could be directly attributable to the addition of PDGF. Following treatment with TA for 48h, greater than 80% of the cell population was located in G1, with only 6% in S-phase. When PDGF was added to identical cultures after 30h exposure to TA, a dose-dependent release of cells from G1 was observed over the next 18h (Table 2). The maximum effect of PDGF was observed at a concentration of 50 ng/ml, where greater than 80% of the G1 arrested cells were released compared to controls in which the glucocorticoid had been removed by washing. This was accompanied by a 2.5-fold increase in the total number of cells in S, and a smaller elevation of cells in G2 and mitosis (Table 2).

The presence of PDGF appears to be an essential requirement for cell cycle traverse in DDT_1MF-2 cells, however, since cell associated PDGF has a half-life of

TABLE 2

Effect of PDGF, EGF and insulin on release of TA arrested cells from the G1 phase of the cell cycle

Treatment	Cells in each phase (%)		
	G1	S	G2+M
TA	82	6	12
TA+PDGF (2.5 ng/ml)	78	7	15
TA+PDGF (25 ng/ml)	74	9	17
TA+PDGF (50 ng/ml)	68	15	17
TA/-	65	15	20
TA+EGF (25 ng/ml)	78	7	15
TA+Insulin (25 µg/ml)	77	6	17

DDT_1MF-2 cells (5 x 10^5) were plated in 100 mm culture dishes in 10 ml DME supplemented with 2% FBS and 10 nM testosterone. After 24h, cultures were replenished with 10 ml of similar media containing 10 nM TA. Thirty hours later, either PDGF (2.5-50 ng/ml), epidermal growth factor (25 ng/ml) or insulin (25 µg/ml) was added directly to the cultures. Addition of growth factors directly, avoided the need to replace the existing conditioned media. Cultures were then maintained in these media for a further 18h, after which the cells were analyzed by flow cell cytometry as described in Materials and Methods. To assess the release of the cells from the TA-induced block without growth factors, one set of cultures were washed twice in HBSS following 30h incubation in the presence of TA, and then replenished with media without TA (TA/-).

50-60 min, its supply in culture media would soon become exhausted (20,21). Hence, DDT_1MF-2 cells appear to be self-sustaining in their production of this growth factor and provide one of the few clear examples of autocrine regulation of cell growth. This implies that glucocorticoid inhibition of proliferation is mediated through a reduction in intracellular levels of PDGF rather than at its site of action, particularly since the addition of exogenous PDGF overcomes the steroid effects. Release from G1 arrest appears to be relatively specific for PDGF, since both epidermal growth factor (EGF) and insulin at high concentrations elicited only minimal responses (Table 2). These data are consistent with those of Pledger et al., who have shown that a putative intracellular messenger of PDGF is induced by competence factors and not by factors causing progression through the cell cycle (22).

We have previously shown that androgens increase both the rate of proliferation in DDT_1MF-2 cells and induce a doubling in the number of androgen receptors within

TABLE 3

Effect of PDGF on glucocorticoid inhibition of androgen receptor augmentation

Treatment	[^{3}H]-R1881 bound specifically fmol/μg DNA
[^{3}H]-R1881 (1h)	4.26±0.01
[^{3}H]-R1881 (6h)	9.34±0.16
[^{3}H]-R1881 (6h) + TA	4.84±0.05
[^{3}H]-R1881 (6h) + TA + PDGF (50 ng/ml)	5.05±0.08

Total intracellular androgen binding sites were quantitated using the whole cell binding assay as described in Materials and Methods. Cultures in duplicate, were incubated at 37°C in the presence of 1 nM [^{3}H]-R1881 ± 100 nM [^{1}H]-R1881 for either 1h or 6h. To half of the cultures was added 10 nM triamcinolone acetonide (TA) in the presence or absence of 50 ng/ml PDGF.

6h (12-14). Triamcinolone acetonide specifically inhibits both these androgen-mediated events without altering the equilibrium dissociation constant (Kd) of the androgen receptor for [^{3}H]-methyltrienolone (Kd = 0.46 nM) (13). It has recently been shown that glucocorticoid receptor synthesis in HeLa cells is preferentially associated with precise times during the cell cycle (23,24). Since the appearance of androgen receptors might be similarly determined by the cell cycle, glucocorticoids could cause a reduction in receptor concentration by blocking cells in G1 prior to the region where androgen receptors are increased. Simultaneous administration of PDGF to these cells did not, however, overcome this glucocorticoid effect (Table 3). Since PDGF does permit TA arrested cells to resume their passage through the cell cycle, it appears that the regulation of androgen receptor levels by glucocorticoids is independent of the steroids' effect on the cell cycle.

The data presented here and by others highlight the importance of PDGF in regulating the re-entry of arrested cells into the cell cycle. That PDGF overcomes the glucocorticoid-induced G1 block is highly significant, since it implicates steroids in the regulation of PDGF expression in these cells. This has now been confirmed by hybridization studies using a cDNA probe for the v-sis gene, which demonstrates that TA inhibits the appearance of cytoplasmic mRNA which encodes PDGF (8,9). These studies, therefore, provide a model for control of tumor cell proliferation through steroid regulation of oncogene expression. A full comprehension of the

mechanisms involved will undoubtedly take into account recent reports of a regulatory linkage between the function of c-sis and c-myc oncogenes (25), particularly since DDT_1MF-2 cells also express the latter oncogene. Whether these regulatory mechanisms play an important role in the normal control of proliferation remains to be determined, however, glucocorticoids have been implicated in the growth suppression of many normal tissues which might normally respond to PDGF or other growth factors.

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