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THE REGULATION OF INTESTINAL EPITHELIAL CELL PROLIFERATION BY 1,25-DIHYDROXYVITAMIN D_3 AND TRANSFORMING GROWTH FACTOR BETA

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Thesis submitted to the Department of Biochemistry in partial fulfillment of the requirements for the degree of Master's of Science

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ISBN 0-612-00578-X

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ABSTRACT

The effects of the active metabolite of vitamin D_3, $1,25(OH)_2D_3$, and of transforming growth factor β1, TGFβ1, on the proliferation and differentiation of an epithelial cell line derived from the rat small intestine(IEC-6) were investigated. IEC-6 cells contain the vitamin D receptor, which is upregulated by serum. TGFβ1 has no effect on the activities of two markers of differentiation, alkaline phosphatase and sucrase. However $1,25(OH)_2D_3$ elevates alkaline phosphatase activity 8.9-fold within 72 hours. Both $1,25(OH)_2D_3$ and TGFβ1 inhibit DNA synthesis in a concentration-dependent manner. Within 24 hours, 0.8 ng/ml TGFβ1 and 500 nM $1,25(OH)_2D_3$ reduce ^{3}H-thymidine incorporation by 55% and 60% respectively. A transient time course of DNA synthesis results from TGFβ1 treatment while $1,25(OH)_2D_3$ treatment produces a sustained effect. The effect of $1,25(OH)_2D_3$ on DNA synthesis is partially blocked with neutralizing antibodies to TGFβ which suggest that active TGFβ is produced in response to $1,25(OH)_2D_3$. TGFβ1 induces an accumulation of cells in the G_0G_1 phase of the cell cycle while $1,25(OH)_2D_3$ does not. However both $1,25(OH)_2D_3$ and TGFβ1 inhibit the phosphorylation of the product of the retinoblastoma tumor suppressor gene. In conclusion, we have shown that $1,25(OH)_2D_3$ is a potent inhibitor of intestinal epithelial cell proliferation and have obtained indirect evidence to support a role for TGFβ in this effect. We speculate that modulation of IEC-6 cell proliferation may

involve TGFβ, whereas effects of $1,25(OH)_2D_3$ on differentiated markers, such as alkaline phosphatase, are mediated independently of TGFβ.

This thesis is dedicated to

my parents and Kevin

for their love and support

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr.JoEllen Welsh, for her guidance, advice and support.

I am grateful to Dr.Douglas J. Franks and Dr.Martin Tenniswood for the use of their cell culture facilities during the course of this work. I would also like to thank Dr.Franks for his assistance with the cell cycle analysis.

I appreciate all the time that Dr.Jean Himms-Hagen allowed me to spend using her DNA fluorometer.

I wish to acknowledge Dr.Maura Simboli-Campbell for her guidance with the chemiluminescence.

The advice I received from my advisory committee was much appreciated. Thank-you Dr.Alvin Chan, Dr.Anthony Krantis and Dr.Fraser Scott.

Thank-you Julie Normand, Joanne Barlow, Bob Gricken and Art Lysionok for all your assistance.

To my lab buddies Manjula Donepudi and AnneMarie Gagnon: thank-you for your advice, your friendship, your encouragement and for all the good times.

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$1,25(OH)_2D_3$	1,25-dihydroxyvitamin D_3
$24,25(OH)_2D_3$	24,25-dihydroxyvitamin D_3
$25(OH)D_3$	25-hydroxyvitamin D_3
ALP	alkaline phosphatase
ANOVA	analysis of variance
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BSA	bovine serum albumin
cdk	cyclin-dependent kinase
cDNA	complementary deoxyribonucleic acid
CO_2	carbon dioxide
cpm	counts per minute
DBP	vitamin D binding protein
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetracetic acid
EGF	epidermal growth factor
EtOH	ethanol
FBS	fetal bovine serum
fmol	femtomole
G_0	quiescent state of cells
G_1	first gap phase of the cell cycle
G_2\M	second gap phase of the cell cycle\mitosis
glc	glucose

HCl	hydrochloric acid
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethane-sulfonic acid]
hr	hour(s)
IEC	intestinal epithelial cell
KCl	potassium chloride
kDa	kilodalton
KH_2PO_4	potassium phosphate, monobasic
LAP	latency associated peptide
LTBP	latent TGFβ binding protein
$MgCl_2$	magnesium chloride
min	minutes
Mv1Lu	mink lung epithelial cells
NaCl	sodium chloride
NaF	sodium fluoride
Na_2HPO_4	di-sodium hydrogen orthophosphate
NBT	nitro blue tetrazolium
$p27^{Kip1}$	cdk inhibitory protein 1
PBS	phosphate-buffered saline
PCA	perchloric acid
PMSF	phenylmethylsulfonyl fluoride
PNP	para-nitrophenol
PNPP	para-nitrophenyl phosphate
pRB	retinoblastoma gene product
pRB^{phos}	hyperphosphorylated retinoblastoma gene product
prot	protein

S	synthesis phase of the cell cycle
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
TGFβ	transforming growth factor beta
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
Trizma Base	Tris(hydroxymethyl)aminomethane
Tween 20	polyoxyethylenesorbitan monolaurate
VDR	vitamin D receptor
VDRE	vitamin D response element
vol	volume

1. INTRODUCTION

1.1 Intestinal Growth

The small intestinal epithelium represents a rapidly renewing tissue with an average turnover time of two to three days. There is a segregation of cells in the epithelium, with mitotically active cells in the crypt and mature, mitotically inactive enterocytes up the villus. These terminally differentiated cells, which function in absorption, digestion and secretion, have characteristic enzyme activities, transport activities and a distinct cell morphology. Cells extruded at the villus tip are continually replaced by cells originating in the crypt that lose their proliferative ability and then migrate up the villus concommitantly taking on features of the mature enterocyte(Johnson,1981). Due to the dynamic nature of this process, fine control over the mechanisms involved in the growth of the intestinal epithelium is required.

Some of the factors involved in regulating the proliferation and differentiation of the small intestinal epithelium have been identified. Positive growth regulators include gastrin, epidermal growth factor(EGF) and insulin(Johnson,1981; Kurokowa *et al.*,1987). There is also evidence to suggest that 1,25-dihydroxyvitaminD_3[1,25$(OH)_2D_3$] regulates the growth of the small intestine *in vivo*(Birge and Alpers,1973). Transforming growth factor-beta (TGFβ) has been

identified as a negative growth regulator *in vitro*(Kurokowa *et al.*,1987; Barnard *et al.*,1989). Extracellular matrix components are also believed to play key roles(Carroll *et al.*,1988; Hahn,1990; Olson *et al.*,1991). Consequently, a combination of growth factors, hormones and extracellular matrix elicits finely-tuned control to maintain the crucial balance of proliferation, migration and extrusion. This thesis will focus on the potential of $1,25(OH)_2D_3$ and TGFβ to modulate intestinal epithelial cell proliferation and differentiation.

1.2 Vitamin D

Vitamin D was first discovered in the early 1920's as an accessory food substance with antirachitic activity. Later in that decade it became apparent that vitamin D is important for calcium homeostasis and the normal development of bone(DeLuca,1984; Norman,1992). In 1932, the chemical structure of the vitamin was deduced, identifying it as a seco-steroid. This signifies that the B ring of the steroid-like structure is broken, producing a conformationally active A ring which distinguishes vitamin D from other steroids. Since then the mechanism of action through the vitamin D receptor(VDR) has been elucidated and numerous functions of the vitamin have been identified(Norman,1992).

1.3 Metabolism and Synthesis of Vitamin D

Under the action of ultraviolet light, 7-

dehydrocholesterol, found in the skin, is converted to vitamin D_3. Alternatively, vitamin D_3 can be obtained from the diet. Vitamin D_3, bound to the vitamin D binding protein(DBP) in the blood, is transported to the liver where it is hydroxylated in the C-25 position to yield 25-hydroxyvitamin D_3[25(OH)D_3]. A subsequent hydroxylation in the kidney at the 1α position produces the biologically active form of the molecule, 1,25-dihydroxyvitamin D_3 or 1,25(OH)$_2D_3$. Alternatively, the action of the 24-hydroxylase on 25(OH)D_3 can produce 24,25(OH)$_2D_3$(DeLuca,1984; Henry *et al.*,1992). The 1α hydroxylation is the major regulated step in the biosynthesis of 1,25(OH)$_2D_3$. In the absence of vitamin D, the 1α-hydroxylase acts at maximal levels and very little 24,25(OH)$_2D_3$ is produced. With vitamin D repletion, the 1α-hydroxylase is repressed, the 24-hydroxylase is induced and 24,25(OH)$_2D_3$ is the predominant metabolite(Henry,1992). Further metabolism of 1,25(OH)$_2D_3$ by C-24 hydroxylation or side chain cleavage serves to regulate the turnover time and thus the circulating levels of 1,25(OH)$_2D_3$(Eisman,1983).

1.4 Vitamin D Receptor

The initial discovery of a cellular receptor for vitamin D was made in avian intestinal mucosa(Brumbaugh and Haussler,1974). Since then, studies have demonstrated that the vitamin D receptor(VDR) has a widespread distribution, being identified in over 28 tissues and many cancer cell

lines(Norman *et al.*,1992). The VDR shows species-specific molecular weights ranging from 50-60kDa(Pike,1991). The cloning of the VDR identified it as a member of the steroid/thyroid/retinoic acid superfamily of steroid hormone receptors(Burmester *et al.*,1988). Thus like other members of this family, the VDR binds DNA through its amino-terminal region while the ligand-binding domain(LBD) is found at the carboxy terminal(Evans,1988). The affinity of the VDR for metabolites of vitamin D_3 is of the order $1,25(OH)_2D_3$ > $25(OH)D_3$ > $24,25(OH)_2D_3$(Eisman,1983).

$1,25(OH)_2D_3$ is delivered to target organs bound to DBP(vitamin D binding protein), is taken up by target cells and consequently binds the nuclear VDR(Norman *et al.*,1992). Heterodimerization of the VDR-ligand complex with a nuclear accessory factor or the retinoid X receptor is required for high affinity binding to the vitamin D response elements(VDREs) found in the promoters of vitamin D responsive genes(Kliewer *et al.*,1992; Ross *et al.*,1992). VDREs have been identified in several promoters including the osteocalcin gene, the calbindin D9K gene and more recently, the 24-hydroxylase gene(Morrison *et al.*,1989; Darwish and DeLuca,1992; Ohyama *et al.*,1994). Binding of the hormone-receptor complex to the VDRE subsequently induces changes in transcriptional activity. This results in an increase or decrease in the steady-state mRNA levels of vitamin D-dependent proteins, which elicit the biological effects

attributed to vitamin D(Norman *et al.*,1992). More recently, non-genomic effects of $1,25(OH)_2D_3$ have been described(Norman *et al.*,1992).

1.5 Vitamin D Receptor Regulation

In addition to tight control over the production of $1,25(OH)_2D_3$ from $25(OH)D_3$, regulation of $1,25(OH)_2D_3$ action occurs at the level of the VDR. Developmental changes in VDR expression have been observed(Halloran and DeLuca,1981). The appearance of VDR gene expression in the rat intestine during the third postnatal week, correlates with the onset of active calcium transport and responsiveness to $1,25(OH)_2D_3$ at weaning(Huang *et al.*,1989).

The levels of the VDR also vary with the proliferative or differentiated status of the cell. Generally, proliferating or dividing cells contain higher levels of VDR protein or mRNA compared to their confluent or differentiated counterparts(Chen and Feldman,1981; Krishnan and Feldman,1991; Zhao and Feldman,1993).

Heterologous regulation of VDR protein and/or gene expression occurs in response to glucocorticoids, EGF, insulin, and phorbol esters(Massaro *et al.*,1983; Bruns *et al.*,1989; Krishnan and Feldman,1991; Simboli-Campbell *et al.*,1992).

Homologous up-regulation by $1,25(OH)_2D_3$ has been observed *in vivo*, in cultured cell lines and in recombinant mammalian

and yeast systems(Strom *et al.*,1989; Wiese *et al.*,1992; Santiso-Mere *et al.*,1993). The data suggest that the effects of $1,25(OH)_2D_3$ on the VDR include transcriptional activation of gene expression(Strom *et al.*,1989), phosphorylation(Brown and DeLuca,1990; Hsieh *et al.*,1991; Jurutka *et al.*,1993), and ligand-induced stabilization(Wiese *et al.*,1992; Santiso-Mere *et al.*,1993).

1.6 Biological Actions of $1,25(OH)_2D_3$

The classical role of $1,25(OH)_2D_3$ is its regulation of calcium and phosphorus transport and homeostasis. This action serves to maintain plasma calcium and phosphorus levels in a range suitable for bone mineralization and is primarily accomplished through the stimulation of intestinal calcium and phosphate absorption(DeLuca,1984). A deficiency of vitamin D in childhood leads to the disease rickets, in which the bones are softened and deformed(Passmore and Eastwood,1986).

$1,25(OH)_2D_3$ also has effects on cellular proliferation and differentiation. $1,25(OH)_2D_3$ inhibits the proliferation of a variety of cells including keratinocytes, normal and malignant prostatic cells, the human myeloid leukemic cell line, HL-60, and breast cancer cells(Matsumoto *et al.*,1990; Peehl *et al.*,1994; Miyaura *et al.*,1981; Reitsma *et al.*,1983; Chouvet *et al.*,1986). Furthermore, $1,25(OH)_2D_3$ induces markers of differentiation in epidermal keratinocyes, HL-60 cells and HT-29 cells, a colon cancer cell line(Hosomi *et al.*,1983; Reitsma

et al.,1983; Zhao and Feldman,1993). These studies indicate that $1,25(OH)_2D_3$ is a potent modulator of cell growth *in vitro* and may act similarly *in vivo*. Several studies have investigated the possible mechanisms underlying the effects of $1,25(OH)_2D_3$ on proliferation and differentiation.

1.7(A)$1,25(OH)_2D_3$ and c-myc

The c-myc proto-oncogene is important for promoting the progression of cells to the S phase(Kelly *et al.*,1983). In human keratinocytes and HL-60 cells there is a decrease in c-myc gene expression in response to $1,25(OH)_2D_3$ which is associated with an inhibition of proliferation(Reitsma *et al.*,1983; Matsumoto *et al.*,1990). Furthermore, one study suggests that the effect of $1,25(OH)_2D_3$ in induction of differentiation of HL-60 cells is independent of its effects on c-myc(Taoka *et al.*,1993). Therefore, $1,25(OH)_2D_3$ may mediate effects on cellular proliferation, but not differentiation, by down-regulation of c-myc.

1.7(B)$1,25(CH)_2D_3$ and TGFβ

There is also evidence to suggest that $1,25(OH)_2D_3$-induced effects on cellular proliferation and differentiation may be mediated or modulated by TGFβ. In human bone cells, both normal and malignant, $1,25(OH)_2D_3$ and TGFβ1 act synergistically to increase alkaline phosphatase activity. In contrast, TGFβ1 blunts the increase in the steady-state mRNA level of

osteocalcin observed in response to $1,25(OH)_2D_3$(Bonewald *et al.*,1992; Wergedal *et al.*,1992). In human keratinocytes, $1,25(OH)_2D_3$-mediated inhibition of proliferation is partially blocked by neutralizing antibodies to TGFβ(Kim *et al.*,1992). Furthermore, $1,25(OH)_2D_3$ increases the steady-state mRNA level of TGFβ1 in keratinocytes and elevates the secretion of TGFβ from bone cells(Kim *et al.*,1992; Petkovich *et al.*,1987).

1.8 Transforming Growth Factor β

TGFβ represents a superfamily of peptides which regulate growth, differentiation and morphogenesis. This multifunctional family includes TGFβs, inhibins, activins, Müllerian inhibiting substance and bone morphogenetic protein(Roberts and Sporn,1990; Massagué *et al.*,1992).

Alhough TGFs were first identified as being produced by retrovirally transformed cells, the name transforming growth factor is actually a misnomer as TGFβ is produced by normal cells and tissues(Roberts *et al.*,1981) and does not cause oncogenic transformation(Massagué *et al.*,1992). The first method used to assay the activity of TGFβ was based on the ability of TGFβ to induce colony formation and growth of rat kidney fibroblasts in soft agar in the presence of EGF. This distinguished the activity of TGFβ from that of TGFα, since the latter is not potentiated by EGF in the soft agar assay and competes with EGF for binding to the EGF receptor(Roberts *et al.*,1981).

1.9 TGFβ Structure

The mature, biologically active form of TGFβ is a 25 kDa homodimer of two 112 amimo acid chains. However, TGFβ is initially secreted, from nearly all cell types, as a biologically inert latent complex that is unable to bind its receptor. This small latent complex is composed of a 75kDa homodimer of a latency associated peptide(LAP) noncovalently associated with the mature TGFβ. LAP is required for the proper folding and assembly of the disulfide bonds of the bioactive peptide(Massagué *et al.*,1992). In some cells an additional protein termed the latent TGFβ binding protein(LTBP) is disulfide-bonded to LAP giving rise to a large latent complex(Roberts and Sporn,1990). Several other as yet unidentified components make up different large latent complexes in glioblastoma cells, although their significance is unknown(Olofsson *et al.*,1992).

1.10 TGFβ Isoforms

The prototype TGFβ protein was first extracted from human platelets and the isolation of a human cDNA soon followed(Assoian *et al.*,1983; Derynck *et al.*,1985). In 1987, the existence of more than one form of TGFβ was reported(Cheifetz *et al.*,1987). Three mammalian genes have been identified, producing TGFβ1, TGFβ2 and TGFβ3. TGFβ4 and TGFβ5 genes have been identified in chicken and *Xenopus laevis* genomes respectively(Roberts and Sporn,1990). Furthermore,

TGFβ1.2 and TGFβ2.3 heterodimers have been discovered(Cheifetz *et al.*,1987; Ogawa *et al.*,1992).

The amino acid homology in the bioactive domain of TGFβ1, TGFβ2 and TGFβ3 ranges between 71% to 76%, with nine conserved cysteines. One disulfide bond exists between the two subunits of the homodimer, while the other 8 cysteines are involved in intrasubunit disulfide bonds. Conservation between species for each isoform is >98% suggesting that the differences in each isoform are critical for that isoform's biological function(Roberts and Sporn,1990; Massagué *et al.*,1992).

1.11 TGFβ Activation

Since most cells contain the TGFβ signaling receptors(see section 1.12), a physiological control mechanism to regulate the activation of latent TGFβ must exist to prevent overstimulation of the receptors by active TGFβ. Activation of the latent complex to release the mature bioactive TGFβ requires dissociation of TGFβ from LAP(Massagué *et al.*,1992). TGFβ can be activated *in vitro* by acidification, alkalinization, deglycosylating agents, and the proteolytic action of plasmin or collagenase(Roberts and Sporn,1990; Massagué *et al.*,1992). It has been suggested that the LTBP is involved in activation of latent TGFβ(Flaumenhaft *et al.*,1993), however the mechanism of activation of TGFβ *in vivo* is unknown.

1.12 TGFβ Receptors

Although TGFβ interacts with several distinct membrane proteins, three integral membrane proteins that bind TGFβ, referred to as receptor I, receptor II and betaglycan(receptor III) are found in most normal and transformed cells. Both receptors I and II, of 53kDa and 75kDa respectively, have been cloned and each contain a functional serine/threonine kinase(Ebner *et al.*,1993; Attisano *et al.*,1993; Franzén *et al.*,1993; Bassing *et al.*,1994a; Lin *et al.*,1992). Since growth factor receptors usually contain tyrosine kinase domains, the TGFβ receptors can be considered a novel class of growth factor receptors(Massagué,1992).

Evidence for a heteromeric receptor signaling complex comes from studies in receptor defective mutants of mink lung cells(Wrana *et al.*,1992) Receptor I can only bind ligand in the presence of receptor II. Receptor II can bind ligand alone, however the presence of receptor I is necessary for signaling by receptor II(Wrana *et al.*,1992). Immunoprecipitation studies confirmed that receptors I and II form a heteromeric receptor complex. This complex is sufficient to mediate gene expression and growth inhibition in response to the TGFβ ligands(Wrana *et al.*,1992; Moustakas *et al.*,1993; Attisano *et al.*,1993; Franzén *et al.*,1993; Bassing *et al.*,1994b). Receptors I and II bind TGFβ1 and TGFβ3 with a higher affinity than TGFβ2(Massagué,1992).

Betaglycan is a 300 kDa membrane-anchored proteoglycan

which can be cleaved to release the ectodomain into the medium. This free ectodomain is referred to as soluble betaglycan. Betaglycan binds TGFβ in its ectodomain, however membrane-anchored betaglycan lacks a discernible cytoplasmic signaling device (Massagué,1992). The distribution of betaglycan in cells is more limited compared to that of receptor I and II, however it is the most abundant receptor subtype when present(Massagué *et al.*,1992). All three mammalian isoforms of TGFβ bind equivalently to betaglycan(Roberts and Sporn,1990).

Membrane-bound betaglycan forms a complex with receptor II in the presence of ligand(López-Casillas *et al.*,1993; Moustakas *et al.*,1993). Receptor II binds TGFβ bound to membrane betaglycan better than TGFβ free in the medium(López-Casillas *et al.*,1993). In contrast, soluble betaglycan sequesters TGFβ and interferes with the ligand's access to the receptors(López-Casillas *et al.*,1994). Thus betaglycan acts as a modulator of TGFβ access to its signaling receptors.

Although much has been learned recently about the interaction of TGFβ and its receptors, events downstream of the membrane have yet to be elucidated to complete our understanding of the signaling process.

1.13 TGFβ and Gene Expression

Actions of TGFβ at the molecular level have been reported. TGFβ1 upregulates its own expression by increasing

the expression of nuclear factors Jun and Fos which act through an AP-1 element in the TGFβ1 promoter. Other genes affected by TGFβ include extracellular matrix components and integrin receptors. Although there is no one consensus TGFβ response element, TGFβ acts through NF1 binding sites, AP-1 binding sites and cyclic AMP response elements(Massagué *et al.*,1992).

1.14 TGFβ Functions

TGFβ is multifunctional, having a diverse array of effects encompassing nearly all cells. The use of transgenic mice has shown that disruption of the TGFβ1 alleles results in the development of a rapid wasting syndrome with subsequent premature death due to massive inflammatory lesions in many organs(Shull *et al.*,1992; Kulkarni *et al.*,1993). Generally the effects of TGFβ can be categorized into 4 groups: [1] development; [2] tissue repair and healing; [3] extracellular matrix regulation; and [4] proliferation and differentiation(Roberts and Sporn,1990; Massagué *et al.*,1992). The latter two functions will be discussed in more detail.

1.15 Extracellular Matrix Regulation by TGFβ

The net effect of TGFβ on the extracellular matrix is to upregulate cell adhesion(Massagué *et al.*,1992). This is accomplished in three ways. First, TGFβ increases the expression of extracellular matrix components such as

fibronectin, collagen and osteopontin. Second, TGFβ decreases the expression of proteases involved in extracellular matrix degradation(ie.plasminogen activator) and increases the synthesis of inhibitors of these proteases(ie. plasminogen activator inhibitor). Finally, TGFβ increases the transcription and processing of integrins, the cell adhesion molecules which form a link between the cell and the extracellular matrix. These effects of TGFβ underlie its role on cell adhesion, cell migration, development and tissue repair(Roberts and Sporn,1990; Massagué *et al.*,1992). Furthermore, there appears to be a negative feedback loop whereby the absence of a proper extracellular matrix underlying cells induces TGFβ1 gene expression(Streuli *et al.*,1993). However an excess production of extracellular matrix due to TGFβ overexpression is associated with fibrotic disorders such as glomerulonephritis(Border *et al.*,1992; Massagué *et al.*,1992).

1.16 Growth Control

1.16(A) G_1 Progression

Progression through the eukaryotic cell cycle is regulated by cyclin-dependent kinases(cdks). To be active, these kinases require regulatory subunits called cyclins which are synthesized and degraded at different times during the cell cycle. Phosphorylation and dephosphorylation events also regulate cdk activites(Nigg,1993). Since new members of the

cylcin/cdk family continue to be found, only a tentative scheme can be described for their roles during G_1 and at the G_1/S transition. The D type cyclins appear early in G_1 , cyclin E is rate-limiting for G_1 exit and cyclin A activity is required for S phase. The D type cyclins associate with cdk2, cdk4 and cdk5. Although both cyclin A and E regulate the activity of cdk2 during S phase and G_1 phase respectively (Nigg,1993; Sherr,1993), other proteins may regulate G_1 progression.

1.16(B) Retinoblastoma Protein

The inactivation of both copies of the retinoblastoma(RB) gene favours the appearance of eye tumors, as well as some osteosarcomas, breast carcinomas and small cell lung carcinomas. These findings led to the identification of RB as a tumor suppressor gene(Weinberg,1991; Hollingsworth *et al.*,1993).

The product of the RB gene is a 105 kDa nuclear phosphoprotein whose phosphorylation state oscillates during the cell cycle(Weinberg,1991). In G_0/G_1 an un- or underphosphorylated form of RB predominates. A series of phosphorylation steps begins prior to the G_1/S phase transition inducing RB hyperphosphorylation, which persists through S, G_2 and for most of mitosis, where dephosphorylation ultimately occurs(Buchkovich *et al.*,1989; DeCaprio *et al.*,1989; DeCaprio *et al.*,1992; Hollingsworth *et*

al.,1993). The presence of different forms of RB during specific phases of the cell cycle indicates that RB might have a role in cell cycle regulation. This is consistent with the demonstration that injection of purified RB can cause cells to arrest in G_1(Goodrich *et al.*,1991).

Some evidence exists to suggest how RB may function as a growth suppressor. The hypophosphorylated or G_1 form of RB binds the cellular transcription factor E2F, whose activity increases in the cell in response to mitogens(Chellappan *et al.*,1991; Weinberg,1991). Thus it is possible that by binding E2F, RB prevents the transcription of E2F responsive genes whose products may be required for S phase(Hollingsworth *et al.*,1993). Coexpression of RB and cyclin D2 in RB-negative cells leads to RB phosphorylation and prevents the appearance of the RB-E2F complex, thus suggesting that phosphorylation may indeed inactivate RB(Ewen *et al.*,1993a). Both cdk2 and cdk4 have been identified as possible RB kinases(Hinds *et al.*,1992; Ewen *et al.*,1993a).

1.16(C) TGFβ - Effects on Proliferation and Differentiation

TGFβ regulates differentiation of myoblasts, preadipocytes, osteoblasts, haematopoietic cells and other cell types *in vitro*. These effects can be positive or negative depending on the cell type(Massagué *et al.*,1992).

TGFβ induces reversible growth inhibition of epithelial, endothelial, fibroblast, neuronal and hematopoietic

cells(Massagué *et al.*,1992). TGFβ inhibits or arrests the progression of cells through the G_1 phase of the cell cycle. This is associated with suppression of RB phosphorylation thus maintaining RB in its growth suppressor state(Laiho *et al.*,1990). In human keratinocytes TGFβ blocks the expression of cyclin E(Geng and Weinberg,1993). In mink lung epithelial cells, TGFβ1 prevents the formation of active cyclin E-cdk2 complexes and decreases the expression of cdk4 in G_1(Koff *et al.*,1993; Ewen *et al.*,1993b). Since cdk4 is believed to act earlier in G_1 than cdk2, it has been proposed that the decreased expression of cdk4 might consequently hinder the activation of cdk2(Ewen *et al.*,1993b).

Recently a cdk inhibitory protein termed $p27^{Kip1}$, has been identified in contact-inhibited and TGFβ-growth arrested cells, which binds cyclin E-cdk2 complexes *in vitro*, rendering the kinase inactive. It is found in a latent form in proliferating cells(Polyak *et al.*,1994a). Overexpression of $p27^{Kip1}$ inhibits cell entry into S phase(Polyak *et al.*,1994b; Toyoshima and Hunter,1994). Furthermore it was found that binding of $p27^{Kip1}$ to cyclin D2-cdk4 complexes could restore cyclin E-cdk2 activity. It was proposed that cyclin D2-cdk4 complexes sequester $p27^{Kip1}$, prevent its binding to cyclin E-cdk2 complexes and allow cdk2 to be active. In TGFβ-growth arrested cells, there is less cdk4 to sequester $p27^{Kip1}$, which might explain the lack of cdk2 activity(Polyak *et al.*,1994a). Thus a clearer understanding of how TGFβ induces G_1 arrest is

emerging.

1.17 The Cell System

As stated in Sections 1.6 and 1.16(C) both $1,25(OH)_2D_3$ and TGFβ modulate cellular proliferation and differentiation of a variety of cell types. The intestine represents a major target tissue for vitamin D. As well as stimulating intestinal calcium absorption, $1,25(OH)_2D_3$ increases the expression of several proteins in the intestine, including alkaline phosphatase and calbindin D9K(Morrissey *et al.*,1978). The VDR has been localized by immunocytochemistry in the small intestine with highest levels in the crypt and the lowest in the villus(Clemens *et al.*,1988). Studies in the intestine have shown that vitamin D deficient rats have a villus length 70 to 80% that of their normal controls and the villus length returns to normal upon vitamin D repletion(Birge and Alpers,1973).

TGFβ1 has been demonstrated to inhibit intestinal epithelial cell proliferation *in vitro* and has been localized to the epithelium *in vivo*(Barnard *et al.*,1989; Kurokowa *et al.*,1987; Pelton *et al.*,1991; Barnard *et al.*,1993).

These studies were designed to investigate the ability of $1,25(OH)_2D_3$ and TGFβ1 to modulate intestinal epithelial cell proliferation and differentiation and to study their potential interaction in mediating these effects.

For our investigations, a cell culture system was chosen,

since animal studies are complicated by the calcemic effects of $1,25(OH)_2D_3$. Primary culture was not feasible as cells isolated from the gastrointestinal mucosa have a limited survival time(Kedinger *et al.*,1987). Thus our studies were conducted in the IEC-6 cell line, a normal, nontransformed, well-characterized epithelial cell line derived from the rat small intestinal crypt(Quaroni *et al.*,1979; Quaroni and May,1980). IEC-6 cells contain the VDR and are responsive to TGFβ1(Adams *et al.*,1982; Barnard *et al.*,1989). Our overall goal was to characterize the effects of $1,25(OH)_2D_3$ and TGFβ1 on IEC-6 cell proliferation and differentiation. Although we specifically studied the regulation of proliferation of small intestinal cells, an understanding of normal growth control is essential to understanding how this regulation may be disturbed in cancer cells.

OBJECTIVES

1. To characterize the effect of $1,25(OH)_2D_3$ on proliferation and differentiation of IEC-6 cells.

2. To characterize the effect of TGFβ1 on proliferation and differentiation of IEC-6 cells.

3. To investigate any potential association between the results obtained from objectives 1 and 2 above.

3. MATERIALS AND METHODS

3.1 Cell Culture

3.1(A) IEC-6 Cells

IEC-6 cells were obtained from the American Type Culture Collection(ATCC,Rockville,MD). This cell line was established by Quaroni *et al.*(1979) from the small intestine of adult rats. IEC-6 cells were maintained in Dulbecco's Modified Eagle Medium(DMEM,Life Technologies,Burlington,ON) containing 5% heat-inactivated fetal bovine serum (FBS,ICN,Mississauga,ON), glucose(4.5g/l), bovine insulin(0.1 Units/ml, Collaborative Biomedical Products,Bedford,MA), streptomycin sulfate(50μg/ml) and penicillin G(50U/ml) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cells were routinely subcultured using 0.25% trypsin-0.03% EDTA every 6-7 days at a density of 4500cells/cm^2 with one change of media. Cells were used between passages 15-20. Subconfluent cells were exposed to serum-free, low-glucose(1 g/l) media for 24 hours prior to treatment with $1,25(OH)_2D_3$(Biomol Research Laboratories,Inc.,Plymouth Meeting,PA) in absolute ethanol or human platelet TGFβ1(Calbiochem,San Diego,CA) in 4mM HCl.

3.1(B) Mv 1 Lu Cells

The mink lung epithelial cell line, Mv 1 Lu, established from the lungs of the Aleutian mink, was obtained from

ATCC(Rockville,MD). Mv 1 Lu cells were passaged at a density of $5x10^5$ cells per 80 cm^2 flask every 3-4 days in DMEM with 10% FBS(Life Technologies,Burlington,ON). They were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C and used between passages 45-47.

3.2 Vitamin D Receptor Analysis

3.2(A) Ligand Binding

IEC-6 cells were grown to subconfluence and serum-starved for 24 hr. The cells were then fluid changed into DMEM with 5% FBS and the appropriate treatment. VDR abundance was measured by equilibrium binding of ^3H-1,25$(OH)_2D_3$. Briefly, subconfluent IEC-6 cells were sonicated in 5 volumes of hypertonic TKED buffer (10mM Tris-HCl(pH 7.4), 300mM KCl, 1mM EDTA, 5mM DTT, 10mM sodium molybdate, and 200µg/ml trypsin inhibitor). The suspensions were centrifuged at 105,000xg for 1 hr at 4°C in a Beckman L8-55M ultracentrifuge(Beckman Instruments [Canada] Inc.,Mississauga,ON)and the resulting cytosol was assayed for protein content. Scatchard analysis(see Figure A1 in Appendix) demonstrated that 0.2 nM ^3H-1,25$(OH)_2D_3$ was sufficient to detect binding with less than 30% non-specific binding. 200µg of cytosolic protein was incubated with 0.2nM 1α25-dihydroxy[26,27-methyl-^3H]cholecalciferol (130-180 Ci/mmol,Amersham Life Science,Oakville,ON) in the presence or absence of 100nM of nonradioactive 1,25$(OH)_2D_3$ at 4°C overnight. Free ligand was removed by binding to dextran-

coated charcoal. The hormone bound to the receptor was quantified by counting in a Beckman LS 1701 β liquid scintillation counter(Beckman Instruments [Canada] Inc.,Mississauga,ON). Specific binding was determined by subtracting the nonspecific binding of ^{3}H-1,25$(OH)_2D_3$ from the total binding. VDR levels were expressed as fmol ^{3}H-1,25$(OH)_2D_3$ bound per mg protein.

3.2(B) Vitamin D Receptor Immunoblotting

TKED-extracted cytosolic protein(see 3.2[A]) was passed through a Sephadex G-25 dry column to remove salt and then separated on 10% SDS-PAGE at 130 volts. Proteins were transferred to nitrocellulose at 0.5 amps for 40 minutes utilizing the Tyler ET-20 electrophoretic transfer system(Tyler Research Instruments,Edmonton,AB). The membrane was blocked in 4% skim milk in PBS(137mM NaCl, 3mM KCl, 8mM Na_2HPO_4, 1.5mM KH_2PO_4, pH 7.4) for 2 hr. Rat anti-vitamin D receptor monoclonal antibody(Chemicon,Temecula,CA) was then added at a 1:100 dilution in PBS/skim milk for 1.5 hr at room temperature. After washing in 0.1% Tween 20 in PBS, an alkaline phosphatase-conjugated goat anti-rat secondary antibody, diluted 1:5000 in PBS/skim milk(Jackson ImmunoResearch Laboratories,Inc.,West Grove,PA) was added for 1 hr. After washing, the alkaline phosphatase substrates, nitro blue tetrazolium(NBT) and 5-bromo-4-chloro-3-indolyl-phosphate(BCIP) (both from Promega Corporation,Madison,WI),

were added in AP buffer(100mM Tris[pH 9.5], 100mM NaCl and 5mM $MgCl_2$) for colour development.

3.3 Enzyme Assays

IEC-6 cells were grown to confluence and treated in serum-free media or DMEM with 5% FBS. Alkaline phosphatase activity was determined in IEC-6 cell homogenates as the production of p-nitrophenol(PNP) from p-nitrophenyl phosphate(PNPP) in 0.625M AMP buffer(product number R06480,BDH,Inc.,Mississauga,ON) at pH 10.2 as described by Neafsey and Schwartz(1977). The production of PNP in IEC-6 cells was assessed at 410 nm over 10 minutes. Sucrase activity was measured colorimetrically as the liberation of glucose from sucrose at 450 nm utilizing products from Sigma Diagnostics Procedure No.510(Sigma Chemical Company, St.Louis,MO) based on a glucose oxidase/peroxidase determination.

3.4 ^{3}H-Thymidine Incorporation

3.4(A) IEC-6 Cells

Cells were grown in 35 mm dishes to 60-70% confluence prior to addition of serum-free media. The cells were incubated with [methyl-^{3}H]-thymidine(0.5μCi/ml, 70-85Ci/mmol, Amersham Life Science, Oakville,ON) for 16 hours prior to harvest. In some experiments, a pan-specific TGFβ neutralizing antibody(5μg/ml in PBS,AB-100-NA,R&D Systems,Minneapolis,MN) was added at the time of treatment. Initial studies indicated that 5μg/ml of

the antibody was sufficient to partially block the $1,25(OH)_2D_3$-mediated effects on DNA synthesis. Cells were washed 2x with PBS(137mM NaCl,3mM KCl, 8mM Na_2HPO_4, 1.5mM KH_2PO_4, pH 7.4) and fixed with 10%acetic acid:10%formaldehyde:80%PBS for 30 minutes. The cells were subsequently washed with H_2O then incubated at 4°C for 15 minutes with 0.5N perchloric acid(PCA). Following two washes with PCA, the DNA was solubilized by incubation of the cells with 2 ml of PCA at 90°C for 20 minutes. The amount of radioactivity incorporated into DNA was quantified in a Beckman LS 1701 β liquid scintillation counter. DNA content,as assessed by Hoechst 33258 reagent binding, was quantified in a TKO 100 fluorometer(Hoefer Scientific Instruments,San Francisco,CA) following the instructions provided with the instrument.

3.4(B) Mv 1 Lu Cells

DNA synthesis was measured based on the method of Danielpour *et al.*(1989b). The Mv 1 Lu cells were plated at a density of $1x10^4$ cells per well of a 96-well flat-bottomed microtiter plate in 100 µl of Assay Buffer(DMEM,0.2% FBS,10mM HEPES). Cells were allowed to attach for 4 hr in a 37°C humidified CO_2 incubator. The media was aspirated and replaced with 100 µl of TGFβ1 standard solutions(see Figure A2 in Appendix) and 100 µl of serially diluted media samples and incubated for 20 hr. These solutions were replaced with 100 µl of ^3H-thymidine(1µCi/ml) in Assay Buffer and incubated for 4 hr at

37°C. The plates were then washed twice with PBS,and the cells were fixed with a 3:1 vol/vol solution of MeOH:Acetic Acid for 1 hr. The cells were washed with 80% MeOH twice, incubated with 0.5% trypsin(100 µl) for 30 min at 37°C and then were solubilized with an additional 100µl of 1% SDS. The amount of cell-associated radioactivity was determined by scintillation counting.

3.5 Cell Cycle Analysis

Cells were grown to subconfluence in 100 mm plates. After serum-starvation for 24 hours, the cells were fluid changed into DMEM with 5% FBS and the appropriate treatment. Cells were washed in PBS containing 2.5 mM EDTA and fixed/ permeabilized in 75% EtOH for up to 2 days at -20°C. 4-6x10^5 fixed cells were stained for 15 min with the Coulter DNA-Prep Stain reagent, containing propidium iodide and RNase. Cell cycle analysis was performed within 2 hr on a Coulter Epics XL flow cytometer (Coulter Corporation,Miami,FL). The proportion of cells in each phase of the cell cycle was determined using the Multicycle AV software.

3.6 TGFβ Bioassay

Conditioned media was prepared following the method of Danielpour *et al.*(1989a). Briefly, subconfluent monolayers were washed with PBS and serum-free media containing 100µg/ml BSA fraction V was added to the cells. After 6 hr, fresh BSA-

containing media was added along with the treatments. Following a 16 hr incubation, fresh media was again added and allowed to condition for 24 hr. Media was collected, protease inhibitors(50µg/ml PMSF, 2µg/ml leupeptin and 5µg/ml aprotinin) were added and cell monolayers were counted. Media was acid activated to pH 2.0 according to Lawrence(1991). TGFβ was quantified in the conditioned media with reference to a standard curve of inhibition of DNA synthesis in Mv1Lu cells(see 3.4[B]).

3.7 Retinoblastoma Protein Immunoblotting

IEC-6 cells were growth-arrested by placing in serum-free media for 24 hr. Fresh media with 5% FBS and treatments was then added. Cytosol was prepared by lysing the cells in 50mM Tris-HCl(pH 8.0), 120mM NaCl, 0.5%Nonidet P-40, 100mM NaF, 50µg/ml aprotinin and 0.6mM PMSF as described by Laiho *et al.*(1990). Proteins were separated on 7.5% SDS-PAGE and transferred to nitrocellulose. Membranes were blocked with 4% skim milk in PBS for 2 hr, then incubated with 1µg/ml mouse anti-human retinoblastoma(RB) protein monoclonal antibody(Rb-PMG3-245,PharMingen,San Diego,CA) in PBS/skim milk for 2 hr. After washing, peroxidase-conjugated goat anti-mouse IgG(1:1000 in PBS/skim milk, Jackson ImmunoResearch Laboratories,Inc.,West Grove,PA) was added for 1 hr. Rb was detected with enhanced chemiluminescence utilizing products from Amersham(Oakville,ON,).

3.8 Protein Assay

Protein was measured using the Bio-Rad protein assay(Bio-Rad Laboratories,Inc.,Mississauga,ON).

3.9 Statistical Analysis

Statistical evaluations were performed with the Instat software program. ANOVA was used to detect differences between the means of three or more groups, with subsequent analysis utilizing the Bonferroni t test to determine the level of significance. To test for differences between two groups an unpaired t test was used. Linear regression was employed to generate the standard curve for the TGFβ bioassay. A p value of less than 0.05 was considered significant.

3.10 Materials

Cell culture supplies were obtained from Life Technologies(Burlington,ON). Chemicals(ie. ethanol,acetic acid, salts) were from BDH,Inc.(Toronto,ON). All other reagents(ie. protease inhibitors, acrylamide, Tris) were from Sigma Chemical Company(St.Louis,MO) unless otherwise stated.

RESULTS

4.1 Vitamin D Receptor Measurement

To confirm the presence of the VDR in IEC-6 cells, the VDR was assessed by a ligand binding technique in near-confluent cells. The level of the VDR was 5.9±2.1 fmol/mg protein in vehicle treated cells(Figure 4.1), with no significant change in response to treatment with 500nM $1,25(OH)_2D_3$(4.4±2.9) or 2 ng/ml TGFβ1(7.0±2.2) for 24 hours.

To assess whether the level of the VDR varied with the state of cell proliferation, extracts of proliferating and quiescent cells were immunoblotted with a monclonal antibody to the VDR(Figure 4.2). This antibody detected a single band on the immunoblot which migrated between the 45 and 66 kDa standards. No non-specific binding was detected(not shown). The intensity of this band, representing the VDR protein(molecular weight=55 kDa), was higher in proliferating cells, grown in 5% serum(lane 2), compared to the quiescent, serum-free cells(lane 1).

4.2 Assessment of Markers of Intestinal Differentiation

To investigate the ability of TGFβ1 and $1,25(OH)_2D_3$ to modulate differentiation of IEC-6 cells, two enzymatic markers of enterocytic differentiation, sucrase and alkaline phosphatase activities, were studied. These experiments were performed in serum-free conditions to prevent serum

FIGURE 4.1 VITAMIN D RECEPTOR LEVELS IN IEC-6 CELLS

Near-confluent IEC-6 cells in serum-containing media were treated with 500 nM $1,25(OH)_2D_3$, 2 ng/ml TGFβ1 or appropriate vehicles for 24 hr prior to harvest. Cells were lysed in hypertonic buffer and cytosol was assayed for specific [^{3}H]-$1,25(OH)_2D_3$ binding as described in Methods. Results are reported as mean±SEM(n=3).

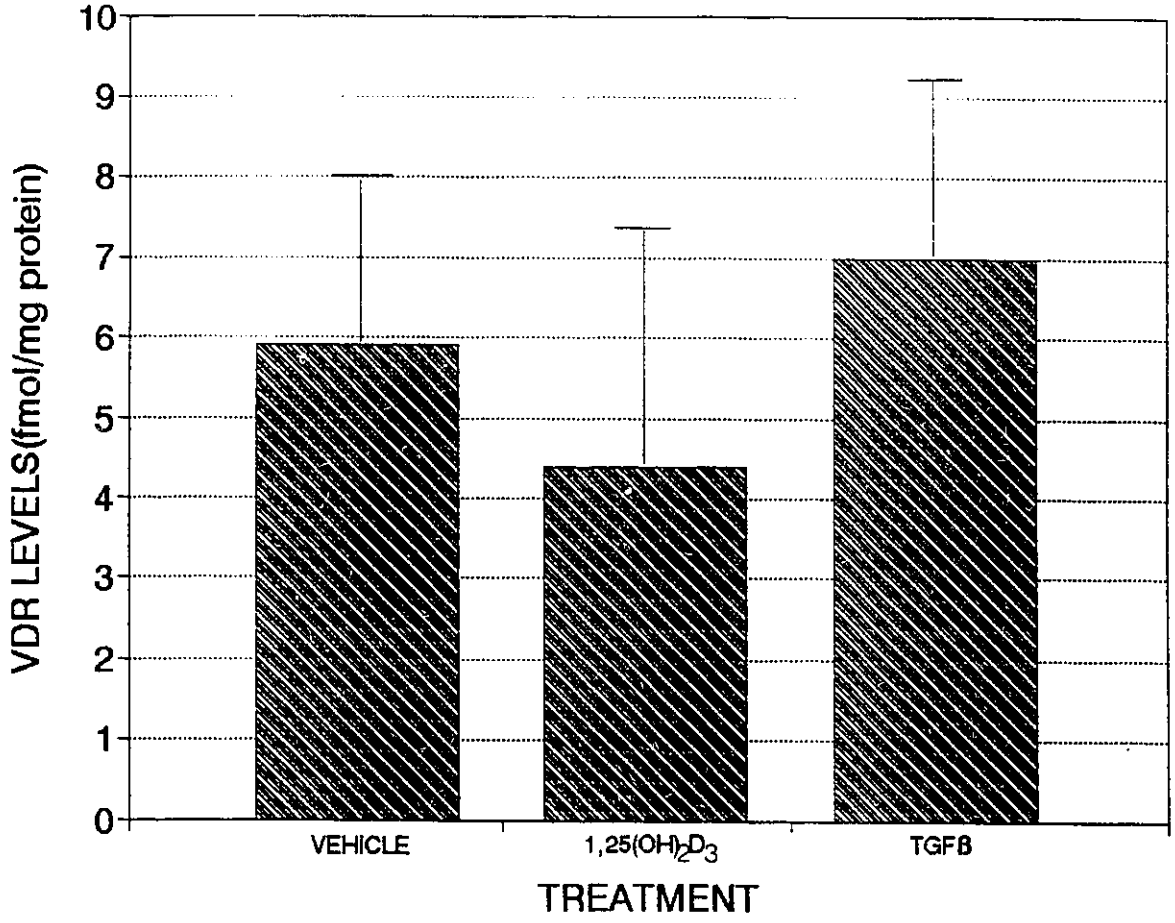
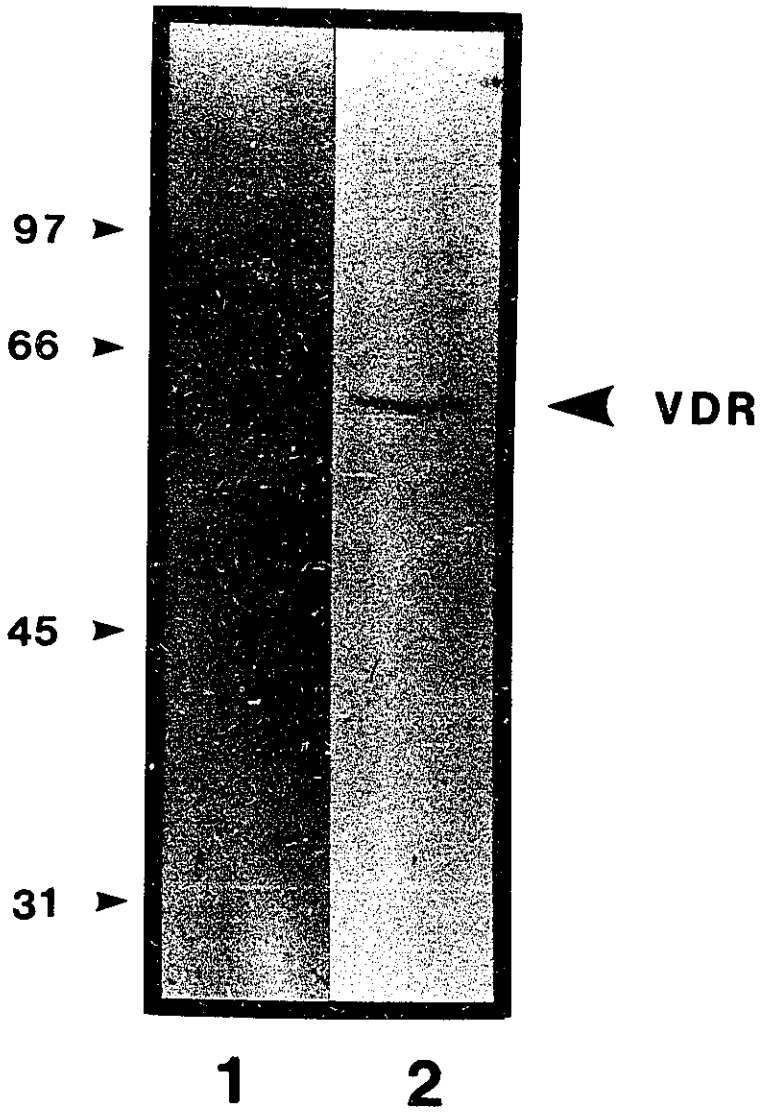

10
9
8
7
6
5
4
3
2
1
0
VDR LEVELS(fmol/mg protein)
VEHICLE
1,25(OH)2D3
TGFB
TREATMENT

FIGURE 4.2 IMMUNOBLOT OF VITAMIN D RECEPTOR PROTEIN LEVELS IN PROLIFERATING VS QUIESCENT IEC-6 CELLS

Near-confluent IEC-6 cells were made quiescent(lane 1) by withdrawing serum for the final 24 hr of incubation. Proliferating IEC-6 cells(lane 2) were serum-starved for 24 hr and then growth-stimulated with 5% FBS for the final 24 hr of incubation. The VDR was isolated in hypertonic buffer and 170 µg of cytosolic protein was separated by SDS-PAGE. The VDR was detected by immunoblotting as described in Methods. Non-specific binding was assessed by PBS/skim milk in place of the primary antibody and showed no binding. Molecular weight standards, in kDa, are indicated on the left.


97
66
45
31
VDR
1
2

contamination by DBP, TGFβ or $1,25(OH)_2D_3$. Treatment of IEC-6 cells with TGFβ1(0.8 ng/ml) for 48 hours had no significant effect on either alkaline phosphatase or sucrase activity(Table 4.1). Concentrations of up to 2 ng/ml TGFβ1 for 48 hours still had no effect on either enzyme activity(data not shown). $1,25(OH)_2D_3$ also had no effect on sucrase activity at any time(up to 72 hours) or concentration studied(up to 1 μM) or when the cells were grown in serum(Table 4.1 and data not shown). However, 100nM $1,25(OH)_2D_3$ significantly increased alkaline phosphatase activity 2.8-fold after 48 hours. This effect was found to be concentration-dependent(see Figure A3 in Appendix)and more dramatic when cells were treated in the presence of 5% serum(Figure 4.3). In serum-containing media, the increase in response to 100nM $1,25(OH)_2D_3$ at 48 hours was 7.7-fold. By 72 hours, the level of alkaline phosphatase activity was 8.9-fold above control.

The combination of TGFβ1(2 ng/ml) with $1,25(OH)_2D_3$(100 nM) did not alter the effects of $1,25(OH)_2D_3$ alone on sucrase or alkaline phosphatase activity(data not shown).

In summary, although $1,25(OH)_2D_3$ increased alkaline phosphatase activity, it did not modulate sucrase activity and TGFβ1 had no effect on either enzyme. Thus under the conditions used in these studies, neither agent was capable of inducing complete differentiation of IEC-6 cells as defined by these two markers.

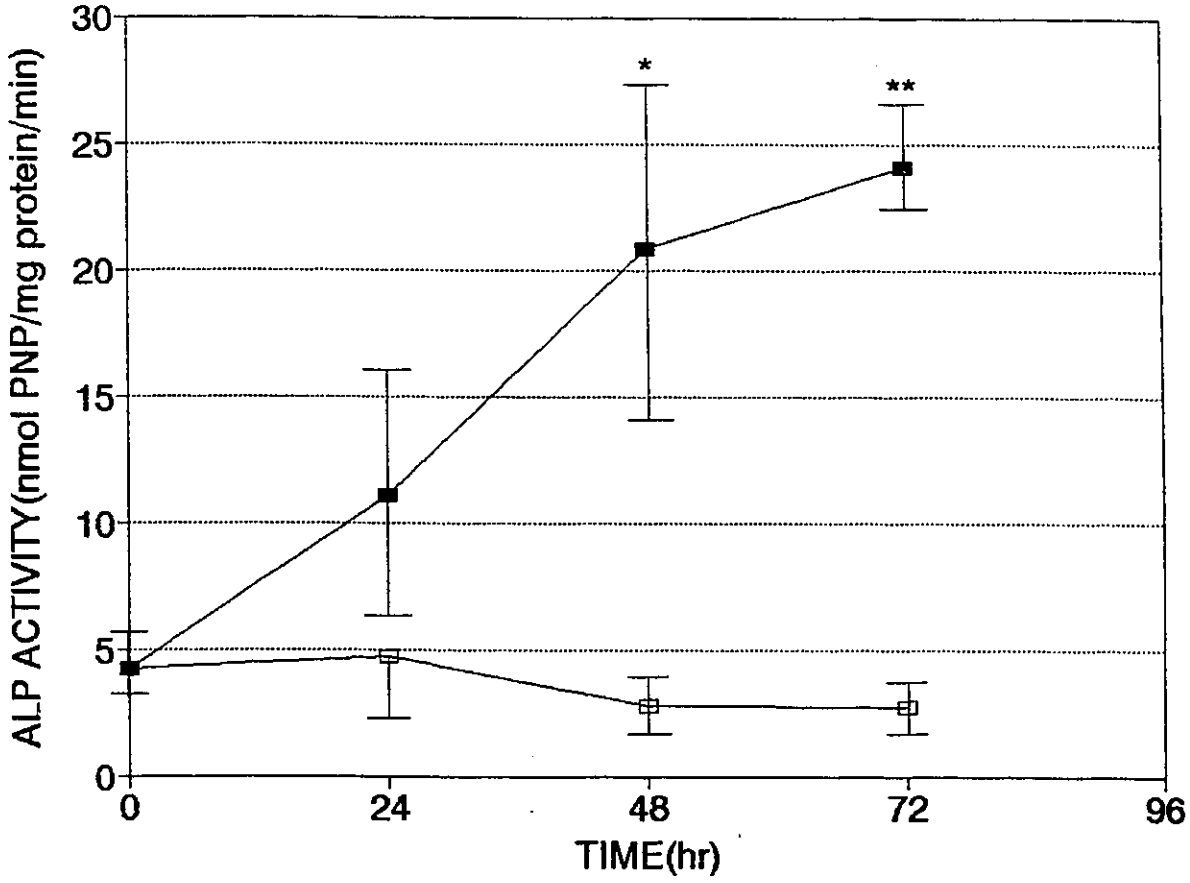
TABLE 4.1 ALKALINE PHOSPHATASE AND SUCRASE ACTIVITIES IN RESPONSE TO $1,25(OH)_2D_3$ AND TGFβ1

Subconfluent, serum-free IEC-6 cells were treated with 100 nM $1,25(OH)_2D_3$, 0.8 ng/ml TGFβ1 or vehicle for 48 hr and assayed for alkaline phosphatase and sucrase activities. Results are reported as mean±SEM(n=3). Statistical significance was determined with unpaired t test(*$p<0.05$).

TREATMENT	ALKALINE PHOSPHATASE ACTIVITY (nmolPNP/mg prot/min)	SUCRASE ACTIVITY (μg glc/mg prot/min)
Ethanol	2.8±0.68	0.33±0.13
$1,25(OH)_2D_3$	8.0±1.90*	0.43±0.19
HCl	1.1±0.39	0.21±0.15
TGFβ1	1.0±0.74	0.23±0.04

FIGURE 4.3 TIME COURSE OF THE EFFECT OF 1,25(OH)$_2$D$_3$ ON ALKALINE PHOSPHATASE ACTIVITY

Near-confluent IEC-6 cells were treated with 100 nM 1,25(OH)$_2$D$_3$(■) or vehicle(□) in serum-containing media for the indicated times. Alkaline phosphatase activity was determined as the production of p-nitrophenol from p-nitrophenyl phosphate at pH 10.2. Results are plotted as mean±SEM of three trials. Statistical significance was determined with unpaired t test(*$p<0.05$;**$p<0.01$).


30
25
20
15
10
5
0
ALP ACTIVITY(nmol PNP/mg protein/min)
*
**
0
24
48
72
96
TIME(hr)

4.3 Effects on Cellular Proliferation

The next step was to investigate the potential of TGFβ1 and $1,25(OH)_2D_3$ to modulate IEC-6 proliferation. ^{3}H-thymidine incorporation into DNA was employed as an index of cell proliferation and experiments were performed with subconfluent cells in serum-free media.

A concentration-dependent decrease in DNA synthesis was observed when cells were treated for 24 hours with increasing concentrations of TGFβ1(Figure 4.4). A half-maximal concentration of inhibition was observed with 0.8 ng/ml TGFβ1 while almost complete growth arrest was observed with concentrations of 3-4 ng/ml. A time course of DNA synthesis using 0.8 ng/ml TGFβ1 showed a transient effect(Figure 4.5). The level of ^{3}H-thymidine incorporation gradually declined to reach a minimal level at about 40 hours, where DNA synthesis in TGFβ1 treated cells was 22% of vehicle treated cells. The cells then recovered from the growth inhibition, although even by 72 hours the level of DNA synthesis was still significantly lower in treated cells compared to control(73% of vehicle).

$1,25(OH)_2D_3$ also produced a concentration-dependent decrease in DNA synthesis of IEC-6 cells(Figure 4.6). With concentrations of 500nM and 1000nM $1,25(OH)_2D_3$, DNA synthesis was significantly decreased, to 34% and 16% of control respectively, at 24 hours. Lower concentrations of $1,25(OH)_2D_3$ were without effect. A time course of DNA synthesis using 500nM $1,25(OH)_2D_3$(Figure 4.7) demonstrated a gradual decline

FIGURE 4.4 EFFECT OF TGFβ1 ON DNA SYNTHESIS

Subconfluent, serum-free IEC-6 cells were treated with increasing concentrations of TGFβ1 or vehicle and ^{3}H-thymidine incorporation was measured during the final 16 hr of treatment. Results are plotted as mean±SEM(n=5) and are representative of 3 similar experiments. Statistical significance was determined by unpaired t test(a=$p<0.05$,b=$p<0.01$,c=$p<0.001$).

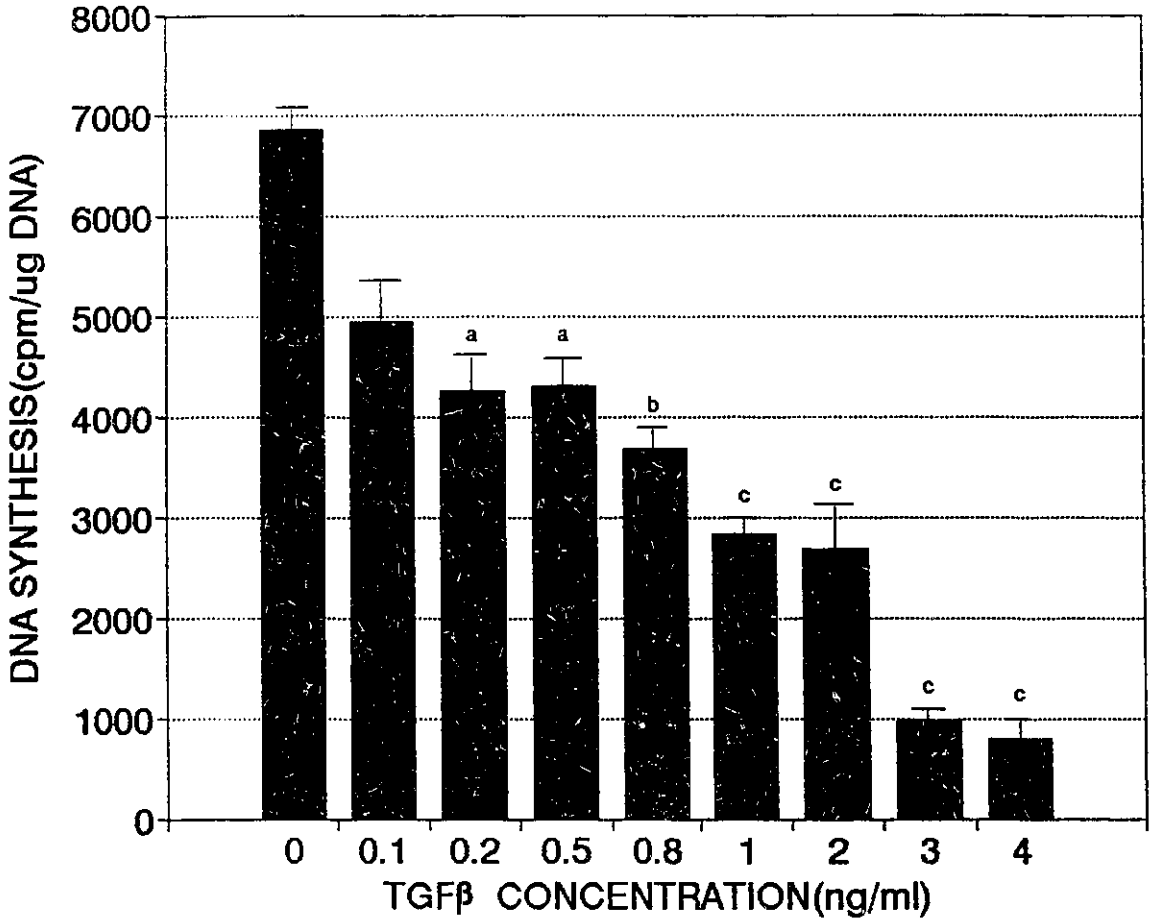

8000
7000
6000
5000
4000
3000
2000
1000
0
DNA SYNTHESIS(cpm/ug DNA)
a
a
b
c
c
c
c
0
0.1
0.2
0.5
0.8
1
2
3
4
TGFβ CONCENTRATION(ng/ml)

FIGURE 4.5 TIME COURSE OF TGFβ1 EFFECT ON DNA SYNTHESIS

Subconfluent, serum-free IEC-6 cells were treated with 0.8 ng/ml TGFβ1(■) or vehicle(□) for the indicated times. ^{3}H-thymidine incorporation was measured during the final 16 hr of treatment. Results are plotted as mean±SEM(n=3) and are representative of 3 similar experiments. Statistical significance was assessed with unpaired t test(*$p<0.05$,**$p<0.01$).

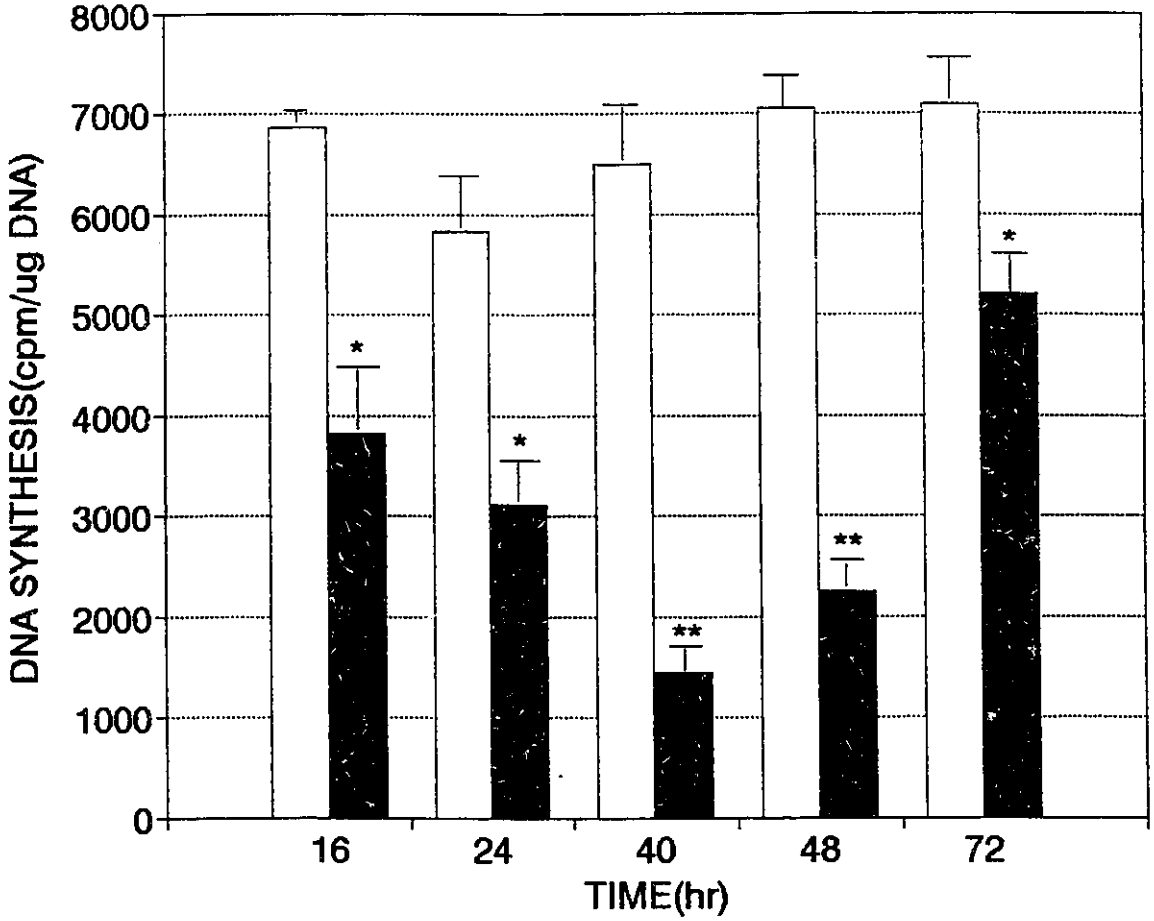

8000
7000
6000
5000
4000
3000
2000
1000
0
DNA SYNTHESIS(cpm/ug DNA)
*
*
**
**
*
16
24
40
48
72
TIME(hr)

FIGURE 4.6 EFFECT OF 1,25$(OH)_2D_3$ ON DNA SYNTHESIS IN IEC-6 CELLS IN THE PRESENCE OR ABSENCE OF TGFβ1

Subconfluent, serum-free IEC-6 cells were incubated with increasing concentrations of 1,25$(OH)_2D_3$ in the presence(□) or absence(●) of 0.8 ng/ml TGFβ1, TGFβ1 alone(■) or vehicle(▾) and DNA synthesis was measured during the final 16 hr of treatment. Results are expressed as mean±SEM of triplicate determinations and are representative of 3 similar experiments. Statistical significance was assessed by ANOVA(**$p<0.01$).

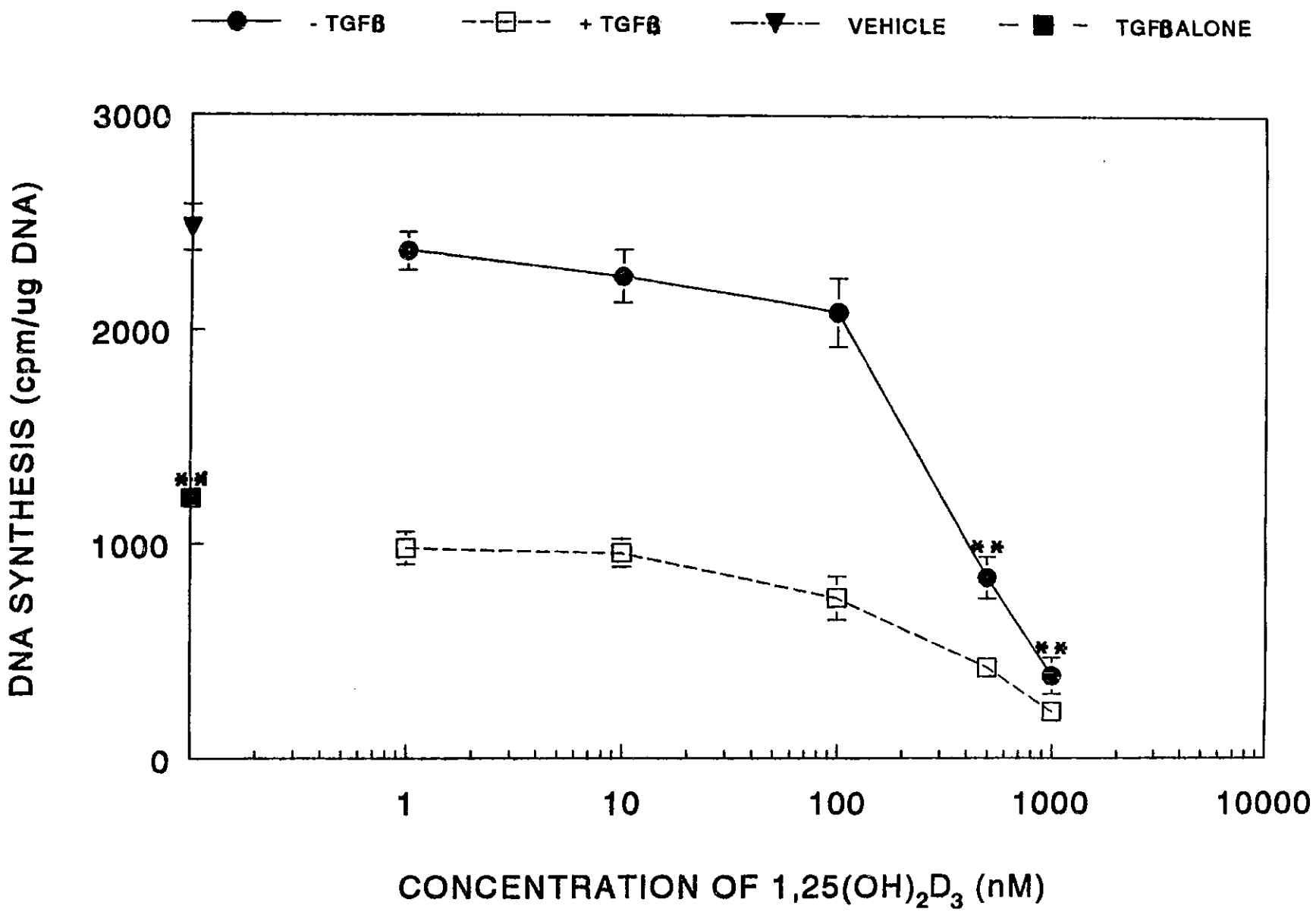
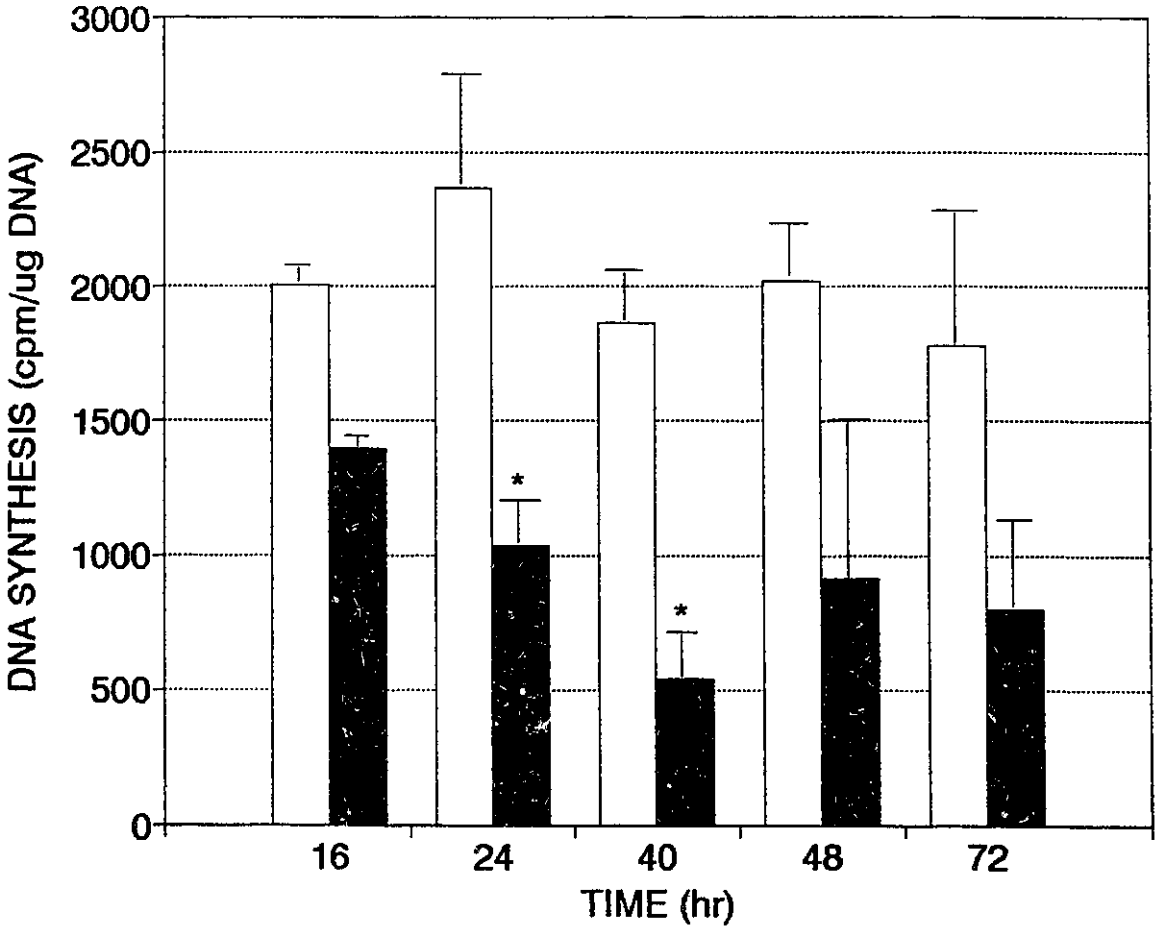

- TGFβ
+ TGFβ
VEHICLE
TGFβ ALONE
DNA SYNTHESIS (cpm/ug DNA)
3000
2000
1000
0
1
10
100
1000
10000
CONCENTRATION OF 1,25(OH)2D3 (nM)

FIGURE 4.7 TIME COURSE OF THE EFFECT OF 1,25(OH)$_2$D$_3$ ON DNA SYNTHESIS

Subconfluent, serum-free IEC-6 cells were treated with 500 nM 1,25(OH)$_2$D$_3$(■) or vehicle(□) for the indicated times. ^{3}H-thymidine incorporation was measured during the final 16 hr of treatment. Results are plotted as mean±SEM(n=3) and are representative of 3 similar experiments. Statistical significance was determined with unpaired t test(*$p<0.05$).


3000
2500
2000
1500
1000
500
0
DNA SYNTHESIS (cpm/ug DNA)
*
*
16
24
40
48
72
TIME (hr)

in ^{3}H-thymidine incorporation, and as with TGFβ1, minimal levels were observed at 40 hours(29% of control). The effect of $1,25(OH)_2D_3$ on DNA synthesis was sustained at 48 and 72 hours. This sustained effect was reproducible in other trials.

There was no further effect on DNA synthesis when the cells were treated with the highest concentrations of $1,25(OH)_2D_3$ in combination with 0.8 ng/ml TGFβ1(Figure 4.6). At lower concentrations of $1,25(OH)_2D_3$(1 and 10 nM), the effect of $1,25(OH)_2D_3$ was enhanced by 0.8 ng/ml TGFβ1 but this effect was not greater than the sum of the two agents used alone.

Since DNA synthesis only measures cells actively undergoing cell division, a second method was used to assess cell cycle kinetics. Flow cytometric analysis allowed the determination of the distribution of the entire cell population in various phases of the cell cycle.

For flow cytometry, the cells were synchronized to ensure most of the population of cells were initially quiescent. As shown in Table 4.2, 87±2.1% of the cells were in the $G_0\backslash G_1$ phase of the cell cycle after 24 hours in serum-free media. After the addition of 5% serum to the media for 24 hours, there was a significant decrease(15%) in the number of cells in $G_0\backslash G_1$ and a doubling of the cells in S phase, as well as an increase in the number of cells in $G_2\backslash M$. The inclusion of 500nM $1,25(OH)_2D_3$ along with serum addition to the IEC-6 cells caused no changes in the cell cycle distribution compared to the vehicle control at 24 hours. In contrast, TGFβ1 at

TABLE 4.2 CELL CYCLE DISTRIBUTION OF IEC-6 CELLS IN RESPONSE TO $1,25(OH)_2D_3$ AND TGFβ1

Subconfluent IEC-6 cells were serum-deprived for 24 hr before treatment with 500 nM $1,25(OH)_2D_3$, 2 ng/ml TGFβ1 or vehicle in 5% FBS for 24 or 40 hr. Cells were harvested by trypsinization, stained with propidium iodide and analyzed for cell cycle distribution by flow cytometry. Results are reported as mean±SEM of 3 independent trials. Statistical significance was determined by ANOVA(*$p<0.05$,**$p<0.01$).

	Percent cells in cell cycle phases		
	$G_0\backslash G_1$	S	$G_2\backslash M$
Growth-arrested			
0 hr	87±2.1	9±1.9	4.1±0.2
Growth-stimulated (5% FBS)			
<u>24 hr</u>			
vehicle	72±1.8	18±1.1	9.7±0.7
$1,25(OH)_2D_3$	76±2.2	16±1.1	8±1.1
TGFß1	84±1.6**	9.0±0.8*	7.2±0.8
$1,25(OH)_2D_3$+TGFß1	82±1.2*	9.4±0.9*	8.4±0.2
<u>40 hr</u>			
vehicle	86±1.4	9±1.2	5.4±0.2
$1,25(OH)_2D_3$	87±1.5	7.6±1.0	5.3±0.5
TGFß1	94.3±0.8**	3.1±0.5*	2.6±0.4
$1,25(OH)_2D_3$+TGFß1	95.0±0.8**	2.6±0.2*	2.4±0.6

a concentration of 2ng/ml, prevented the growth stimulatory efffect of serum by maintaining the cell cycle distribution the same as that of the growth-arrested cells. Combined treatment of $1,25(OH)_2D_3$ and TGFβ1 produced no further changes to that of TGFβ1 alone.

By 40 hours the cells are very near confluent and are consequently contact-inhibited as evidenced by the increase in the population of cells in $G_0\backslash G_1$. After 40 hours of treatment with $1,25(OH)_2D_3$ there was still no detectable change in cell cycle distribution. With 40 hours of TGFβ1 treatment, there was a further accumulation of cells in $G_0\backslash G_1$ with 94.3±0.8% of the cells in this phase compared to only 86±1.4% in the vehicle treated cells. There was no further effect on cell cycle distribution when the two agents were used in combination for 40 hours.

4.4 $1,25(OH)_2D_3$-Induced Inhibition of Proliferation: Mediation by TGFβ

Based on the evidence summarized in Section 1.7(B) of the introduction, we hypothesized that growth inhibitory effects of $1,25(OH)_2D_3$ in IEC-6 cells may be mediated by TGFβ. Although $1,25(OH)_2D_3$ and TGFβ1 exerted different effects on cell cycle distribution, both agents were potent inhibitors of ^3H-thymidine incorporation. These findings thus provided a basis for our investigation of whether $1,25(OH)_2D_3$-induced inhibition of proliferation of IEC-6 cells might be mediated by TGFβ.

A neutralizing antibody to TGFβ was included with $1,25(OH)_2D_3$ treatment to see if the effect of $1,25(OH)_2D_3$ on DNA synthesis could be blocked by this antibody. There was no significant change in DNA synthesis of the control cells when the neutralizing antibody was included with the vehicle treatment(Figure 4.8). The neutralizing antibody significantly blunted the effect of 1μM $1,25(OH)_2D_3$ on ^{3}H-thymidine incorporation and completely blocked the effect of 500nM $1,25(OH)_2D_3$. Thus there was no significant difference in DNA synthesis between cells treated with 500 nM $1,25(OH)_2D_3$ in the presence of the neutralizing antibody and the control cells. These data suggest that active TGFβ was being produced by IEC-6 cells in response to $1,25(OH)_2D_3$ and that TGFβ was acting as an autocrine negative growth regulator.

We next attempted to measure the levels of TGFβ secreted into the IEC-6 media after 24 hour treatment with $1,25(OH)_2D_3$ or TGFβ1. Media from treated IEC-6 cells,before and after acid treatment to activate latent TGFβ, was assayed for growth inhibitory activity of mink lung epithelial cells. This standard assay is based on the extreme sensitivity of these cells to growth suppression by TGFβ. Active TGFβ was not detected in the media from $1,25(OH)_2D_3$ treated IEC-6 cells and very little was detected in the TGFβ1 and vehicle treated media(Figure 4.9). Latent TGFβ levels, detected after acid activation, were extremely variable, with high standard error. The reproducibility of this assay was poor, precluding an

FIGURE 4.8 EFFECT OF TGFβ NEUTRALIZING ANTIBODIES ON $1,25(OH)_2D_3$-INDUCED INHIBITION OF DNA SYNTHESIS

Subconfluent, serum-free IEC-6 cells were treated with $1,25(OH)_2D_3$ or vehicle for 24 hr prior to DNA synthesis measurement. In some dishes(■), 5 μg/ml of neutralizing antibody to TGFβ was added along with treatment. ^{3}H-thymidine incorporation was measured as described in Methods. Results are plotted as mean±SEM of 3 independent experiments(except for 500nM point where n=2). Statistical significance was assessed by ANOVA(*$p<0.05$).

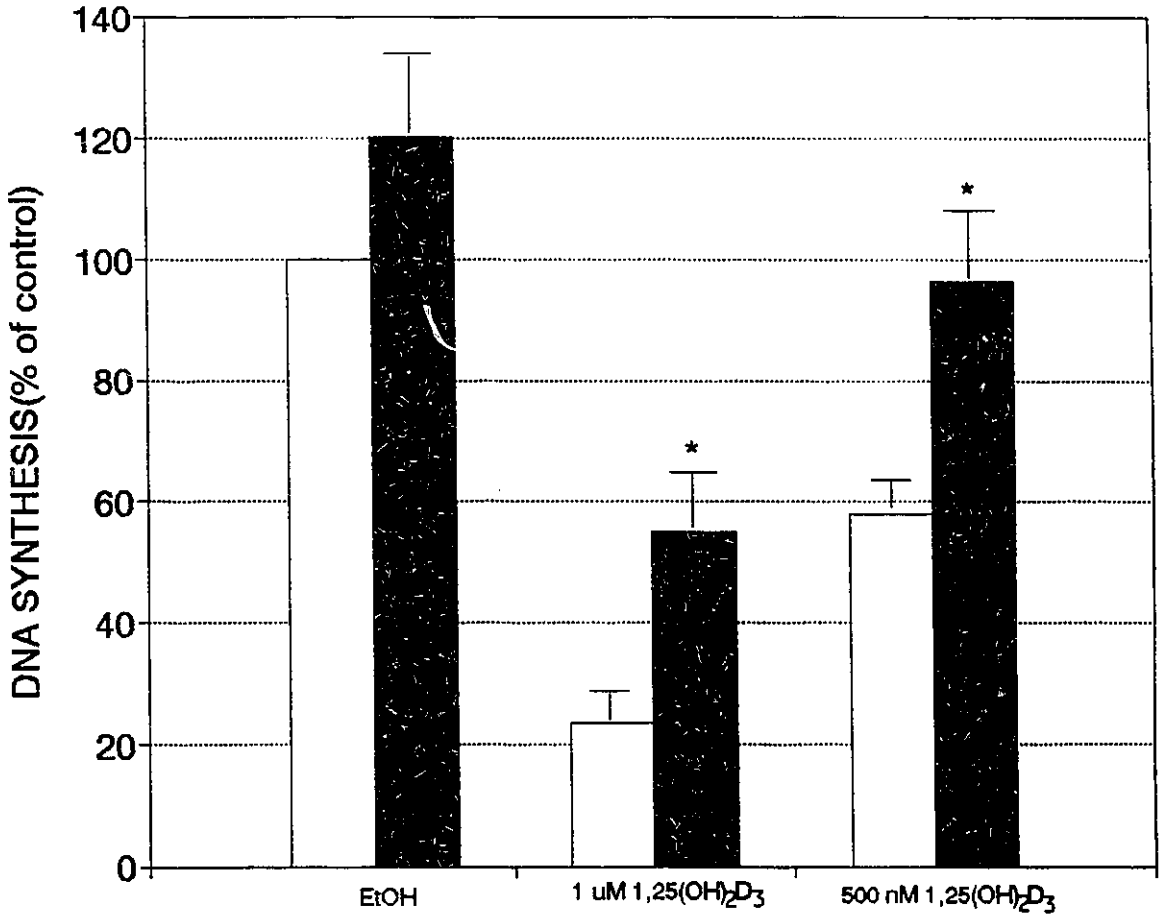
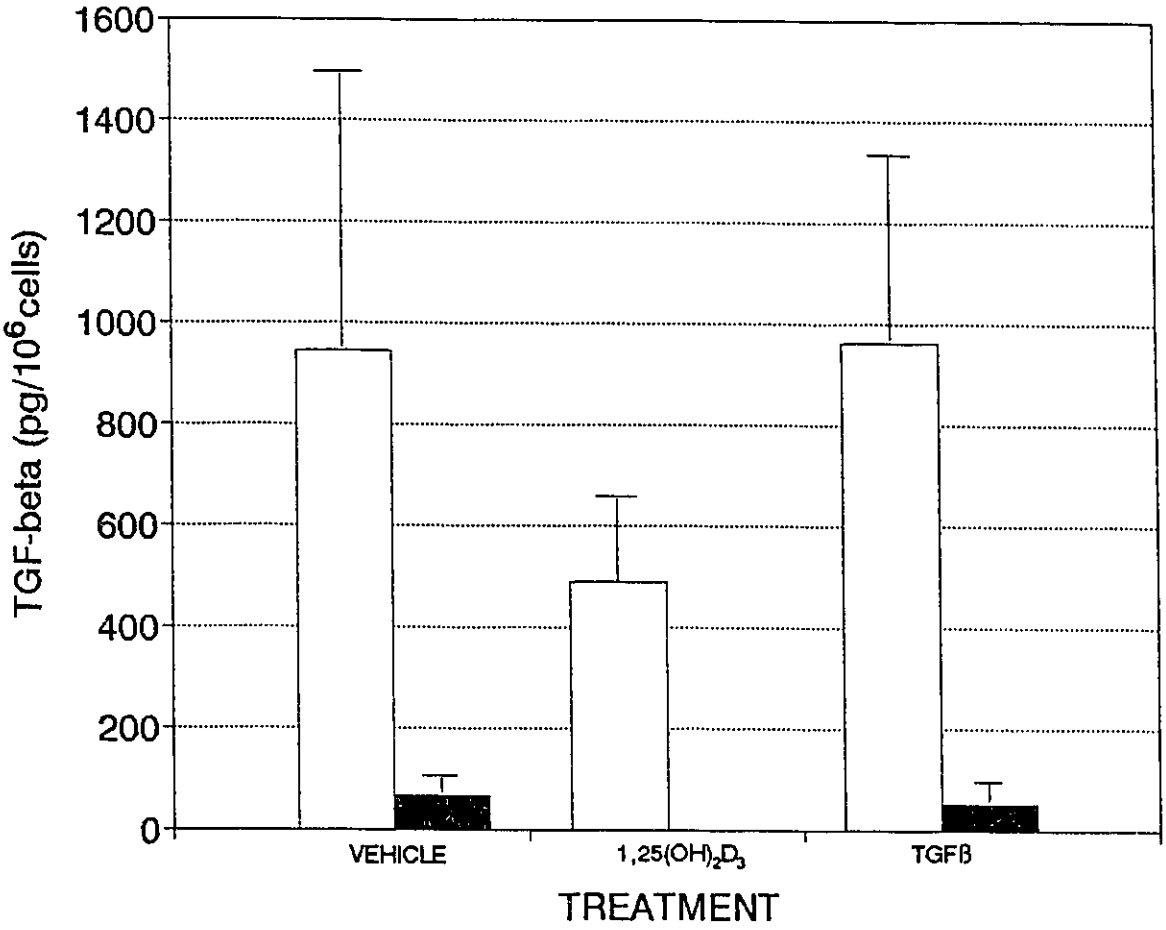

DNA SYNTHESIS(% of control)
140
120
100
80
60
40
20
0
*
*
EtOH
1 uM 1,25(OH)2D3
500 nM 1,25(OH)2D3

FIGURE 4.9 MEASUREMENT OF TGFβ IN IEC-6 CONDITIONED MEDIA

Subconfluent IEC-6 cells were treated in serum-free DMEM containing 100 μg/ml BSA V with 500 nM $1,25(OH)_2D_3$, 2 ng/ml TGFβ1 or vehicle. After 16 hr, fresh media was added and allowed to condition for 24 hr. The media was then analyzed for latent(□) and active(■) TGFβ as described in Methods. Results are plotted as mean±SEM of 2 trials(except $1,25(OH)_2D_3$-treated where n=3). Statistical significance was assessed by unpaired t test.

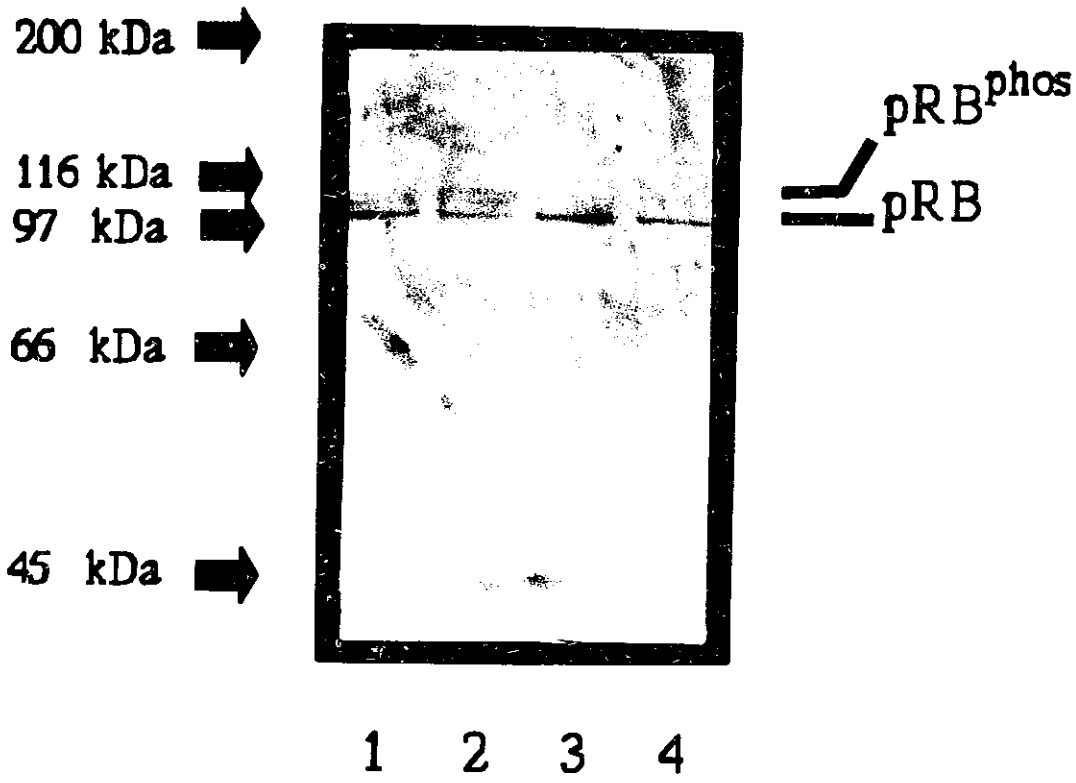

1600
1400
1200
1000
800
600
400
200
0
TGF-beta (pg/10^6cells)
VEHICLE
1,25(OH)2D3
TGFβ
TREATMENT

accurate assessment of the levels of TGFβ. Due to this inherent experimental variability, no further trials were conducted with this assay.

A key feature of TGFβ-mediated growth inhibition is its suppression of RB protein phosphorylation(Laiho *et al.*,1990). If, as suggested by the neutralizing antibody data, active TGFβ was being produced in response to $1,25(OH)_2D_3$, it would follow that changes in the state of RB phosphorylation would be observed in $1,25(OH)_2D_3$ treated cells. A Western blot of IEC-6 cells treated for 24 hours with TGFβ1, $1,25(OH)_2D_3$ and vehicles is presented in Figure 4.10. In growth-arrested IEC-6 cells(lane 4), the predominant form of RB was the underphosphorylated, 105 kDa growth-suppressive form, although some hyperphosphorylated RB(116 kDa) was detected. The addition of serum to these quiescent cells, induced an increase in the hyperphosphorylated form, the form associated with cell division(lane 2). With either 500 nM $1,25(OH)_2D_3$ or 2 ng/ml TGFβ1 treatment(lanes 1 and 3 respectively) the level of the unphosphorylated form of RB was greater than that of the vehicle control(lane 2). A third band, presumably an intermediary form of phosphorylated RB, was also detected between the upper and lower bands in lanes 1 to 3. Therefore $1,25(OH)_2D_3$, like TGFβ1, inhibited the appearance of the phosphorylated form of RB.

FIGURE 4.10 IMMUNOBLOT OF RETINOBLASTOMA PROTEIN IN IEC-6 CELLS TREATED WITH 1,25$(OH)_2D_3$ AND TGFβ1

Subconfluent IEC-6 cells were serum-deprived for 24 hr(lane 4) before treatment in 5% FBS with 500 nM 1,25$(OH)_2D_3$(lane 1), vehicle(lane 2) or 2 ng/ml TGFβ1(lane 3). After 24 hr, cells were harvested and 100 µg of cell lysate was separated by SDS-PAGE. RB was detected by immunoblotting. pRB represents the 105 kDa underphosphorylated form of RB and pRB^{Phos} the 116 kDa hyperphosphorylated form. No non-specific binding was detected.


200 kDa
116 kDa
97 kDa
66 kDa
45 kDa
pRB^phos
pRB
1 2 3 4

5. DISCUSSION

$1,25(OH)_2D_3$ is involved in the regulation of cell proliferation of both normal and transformed cells *in vitro*(Miyaura *et al.*,1981; Reitsma *et al.*,1983; Chouvet *et al.*,1986; Matsumoto *et al.*,1990; Peehl *et al.*,1994). In our studies, we demonstrate a role for $1,25(OH)_2D_3$ in the proliferation of crypt cells of the small intestinal epithelium and extend these findings to a possible involvement of TGFβ in $1,25(OH)_2D_3$-mediated actions.

Essential to the action of $1,25(OH)_2D_3$ is a receptor through which it can mediate its biological effects. Using ligand binding techniques, we observed that the VDR is present in the IEC-6 cells at levels comparable to that reported previously(Adams *et al.*,1982). However, these levels are about 10-fold lower than that reported in IEC-6 cells by DeLuca's group(Wiese *et al.*,1992) using a different technique. Wiese and co-workers also demonstrated up-regulation of the VDR in IEC-6 cells in response to $1,25(OH)_2D_3$, which we failed to detect after 24 hours of treatment. This finding is surprising since the homologous up-regulation of the VDR is well-documented. Although the level of steady-state mRNA of the VDR is elevated in some systems in response to $1,25(OH)_2D_3$(Strom *et al.*,1989), the majority of the evidence suggests ligand-induced stabilization of the VDR protein. Thus in fibroblasts, osteosarcoma cells and recombinant yeast and

mammalian systems, the half-life of the receptor increases upon $1,25(OH)_2D_3$ treatment(Wiese *et al.*,1992; Arbour and DeLuca,1993; Santiso-Mere *et al.*,1993). Our failure to detect VDR up-regulation may be due to the method employed since equilibrium binding of ^3H-$1,25(OH)_2D_3$ measures only the unoccupied VDR(Eisman,1983). Following treatment with $1,25(OH)_2D_3$, much of the receptor is in an occupied state, and therefore an equilibrium between unlabelled $1,25(OH)_2D_3$ and radioactive $1,25(OH)_2D_3$ binding to the VDR is not established.

The levels of the VDR protein in IEC-6 cells varied with the culture conditions. Higher amounts of VDR protein were detected in proliferating cells compared to quiescent cells. Variations in VDR levels with the state of proliferation have been shown in several systems including crypt cells which contain more VDR than the villus cells, differentiated HT-29 cells with low VDR levels and bone cells where VDR levels are highest during the logarithmic phase of growth(Clemens *et al.*,1988; Zhao and Feldman,1993; Chen and Feldman,1981). Therefore, our findings obtained with IEC-6 cells compliment this general trend.

There were no changes in ^3H-$1,25(OH)_2D_3$ binding in response to 24 hour treatment with TGFβ1. TGFβ has been demonstrated to elevate VDR numbers with no change in receptor affinity in osteoblast-like cells(Schneider *et al.*,1992). Further study is required to investigate the generality of TGFβ effects on the VDR.

The presence of the VDR in IEC-6 cells indicated that they represent potential target cells for $1,25(OH)_2D_3$, and led to these studies to characterize the effect of $1,25(OH)_2D_3$ on proliferation and differentiation.

Neither $1,25(OH)_2D_3$ nor TGFβ1 induced terminal differentiation of the IEC-6 cells. Plating the IEC-6 cells on a complex mixture of extracellular matrix components(Matrigel) stops their proliferation, induces morphological changes and causes the appearance of markers of differentiation(Carroll *et al.*,1988). This is the only process through which complete differentiation of IEC-6 cells has been accomplished to date. The fact that we observed no changes in sucrase or alkaline phosphatase activity in response to TGFβ1 is in agreement with Barnard and coworkers(1989). However this contrasts with the work of others(Kurokowa *et al.*,1987), where an increase in sucrase activity was observed as early as 24 hours after treatment with TGFβ1. Thus controversy remains over the ability of TGFβ to induce certain markers of differentiation in IEC-6 cells.

In contrast to TGFβ1, $1,25(OH)_2D_3$ had a positive effect on alkaline phosphatase activity which was concentration- and time-dependent. Alkaline phosphatase is commonly used as a marker of differentiation with its activity highest at the villus tip and lowest at the crypt (Marche *et al.*,1980). $1,25(OH)_2D_3$ increases alkaline phosphatase activity *in vivo*(Morrissey *et al.*,1978) and the results observed in IEC-6

cells are consistent with these findings. This effect may be transcriptional as the two mRNA species of intestinal alkaline phosphatase both increase with *in vivo* administration of $1,25(OH)_2D_3$(Eliakim *et al.*,1990). $1,25(OH)_2D_3$ had no effect on sucrase activity. Thus $1,25(OH)_2D_3$ may have induced partial differentiation of IEC-6 cells.

The induction of differentiation of intestinal cells is preceded by the loss of proliferative ability of the crypt cells. Both $1,25(OH)_2D_3$ and TGFβ1 inhibited proliferation of intestinal epithelial cells *in vitro* in a concentration-dependent manner and with similar, but not identical, time courses. TGFβ1 has been shown to inhibit IEC-6 proliferation (Kurokowa *et al.*,1987; Barnard *et al.*,1989; Ko *et al.*,1994) and this was confirmed here. We found that the effect of TGFβ1 on DNA synthesis was transient. Since the cells appear to recover from the TGFβ1-induced growth suppression, TGFβ1 likely did not affect cell viability.

The results of the $1,25(OH)_2D_3$ time course of cell proliferation suggest a sustained effect. Such an effect might imply a toxic effect of $1,25(OH)_2D_3$. Although it is possible that the highest concentrations of $1,25(OH)_2D_3$ caused cell death, we do not believe that this occurred as few detached cells were observed floating in the media. Furthermore, similar concentrations of $1,25(OH)_2D_3$ induced increases in alkaline phosphatase activity at 48 hours, indicating that the cells remained viable. A prolonged effect would be consistent

with the nuclear receptor-mediated mechanism of action of $1,25(OH)_2D_3$. These receptors act to induce changes in gene expression, which are often sustained. If in fact $1,25(OH)_2D_3$ did cause partial differentiation of IEC-6 cells, then it is possible that the cells cannot re-enter the cell cycle. Alternatively, $1,25(OH)_2D_3$ might induce production of a growth inhibitory factor, perhaps TGFβ, which might exert its effect after 24 hours, producing a sustained decrease in cell proliferation. According to this hypothesis, $1,25(OH)_2D_3$ would exert more rapid, direct effects, such as down-regulation of the early-response gene c-myc, which would explain the initial decrease in cell proliferation. $1,25(OH)_2D_3$ decreases c-myc in keratinocytes and HL-60 cells and this is associated with suppression of proliferation(Reitsma *et al.*,1983; Matsumoto *et al.*,1990; Taoka *et al.*,1993). Further studies will be necessary to determine if $1,25(OH)_2D_3$ modulates c-myc in IEC-6 cells.

If $1,25(OH)_2D_3$ acts via increasing TGFβ secretion or activation in IEC-6 cells, then the combination of the two agents would be additive, but not synergistic, in their effect on DNA synthesis. An additive effect would normally suggest that the two agents act through different pathways. If early effects on cell proliferation were independent of TGFβ, while later effects were mediated by TGFβ, then a time delay for $1,25(OH)_2D_3$ induction of TGFβ would occur. Therefore the TGFβ1 used for treatment and the TGFβ produced in response to

$1,25(OH)_2D_3$ would not be acting at the same time on the cells and the effect would appear additive rather than synergistic.

A characteristic action of TGFβ-mediated growth suppression is its ability to inhibit the progression of cells through the G_1 phase of the cell cycle(Laiho *et al.*,1990). An accumulation of cells in G_1 in response to TGFβ1 was observed in IEC-6 cells. A higher concentration of TGFβ1 was required to observe effects on cell cycle distribution in serum-containing medium compared to the concentration of TGFβ needed for effects on DNA synthesis, performed in serum-free media. These observations are consistent with reports that α_2-macroglobulin, present in serum, can bind active TGFβ1, thereby potentially reducing its effective concentration(Roberts and Sporn,1990).

No effects of $1,25(OH)_2D_3$ on IEC-6 cell cycle distribution were observed. This is in contrast to a significant effect of $1,25(OH)_2D_3$ on thymidine incorporation, a measure of cell proliferation. Although DBP, which binds $1,25(OH)_2D_3$(DeLuca,1984) and is present in serum might have diminished the concentration of $1,25(OH)_2D_3$, effects of $1,25(OH)_2D_3$ in the presence of serum were still observed on alkaline phosphatase activity and RB phosphorylation. Furthermore the VDR was upregulated in cells growing in serum suggesting that these cells would be more sensitive to $1,25(OH)_2D_3$. The observation that $1,25(OH)_2D_3$ modulates RB phosphorylation without affecting cell cycle distribution was

initially surprising but can be explained. The SV40 large T antigen binds unphosphorylated RB and in doing so overcomes the growth-suppressive action of RB(Ludlow *et al.*,1989; Laiho *et al.*,1990). Overexpression of large T antigen in mink cells along with TGFβ1 treatment prevents RB phosphorylation however growth arrest does not occur(Laiho *et al.*,1990). Thus the presence of the growth stimulatory large T antigen overcomes the negative effect of TGFβ1. Since the experiments with $1,25(OH)_2D_3$ treatment were conducted on cells growing in serum, an excess of stimulatory factors might have prevented growth suppression even though $1,25(OH)_2D_3$ inhibited RB phosphorylation.

The effects of $1,25(OH)_2D_3$ on DNA synthesis could be partially blunted with the use of a neutralizing antibody to TGFβ. These results implied that active TGFβ was being produced in response to $1,25(OH)_2D_3$. A quantitative assessment of the levels of TGFβ secreted in the media was not successful. Although the problems encountered in these studies were with the reproducibilty of the assay there are inherent problems with the bioassay as well. It cannot distinguish between different isoforms of TGFβ and is not completely specific for TGFβ, as other factors present in the conditioned media might influence the growth of the mink lung cells. Despite these limitations this is the standard method to measure TGFβ. Although Northern blotting and Western blotting would detect differences in the mRNA or protein levels of the

different TGFβ isoforms, these methods cannot distinguish between latent and active TGFβ. Without a quantitative measure of bioactive TGFβ we have no direct evidence that $1,25(OH)_2D_3$-induced growth inhibition of IEC-6 cells involves TGFβ, however indirect evidence came from the RB immunoblotting.

TGFβ blocks cell cycle progression by inhibiting RB phosphorylation(Laiho *et al.*,1990). This is believed to result from a series of events including the suppression of cdk2-cyclin E kinase activity by $p27^{Kip1}$ and the subsequent blocking of RB phosphorylation by cdk2 or cdk4(Hinds *et al.*,1992; Ewen *et al.*,1993a; Polyak *et al.*,1994a). In IEC-6 cells, several bands were observed on the RB blot, consistent with RB phosphorylation occurring in multiple stages as cells progress through the cell cycle(DeCaprio *et al.*,1992). TGFβ1 inhibited the phosphoryation of RB, assessed as a decrease in the 116 kDa band, representing the hyperphosphorylated form. The effect of $1,25(OH)_2D_3$ was identical, consistent with the hypothesis that $1,25(OH)_2D_3$ actions in IEC-6 cells might involve TGFβ. A role for TGFβ in mediating the actions of $1,25(OH)_2D_3$ in other cell types is documented. An effect on RB phosphorylation was shown in keratinocytes where $1,25(OH)_2D_3$ prevented the appearance of the hyperphosphorylated form of RB and induced a decrease in the number of cells in S phase(Kobayashi *et al.*,1993). In bone cells and keratinocytes, the levels of the TGFβ protein and mRNA, respectively, are increased in response to $1,25(OH)_2D_3$(Petkovitch *et al.*,1987;

Kobayashi *et al.*,1993).

The effects of TGFβ1 and $1,25(OH)_2D_3$ on alkaline phoshatase activity were quite different. $1,25(OH)_2D_3$ stimulated the enzyme activity while TGFβ had absolutely no effect. $1,25(OH)_2D_3$ increases the steady-state level of mRNA of the alkaline phosphastase genes in intestine and bone(Eliakim *et al.*,1990; Kyeyune-Nyombi *et al.*,1991). In bone, this reflects an elevation in the transcriptional rate of the alkaline phosphatase gene(Kyeyune-Nyombi *et al.*,1991) therefore a direct effect of $1,25(OH)_2D_3$ on alkaline phosphatase is likely. Consequently we speculate that $1,25(OH)_2D_3$ may mediate its growth-inhibitory effects through TGFβ but that $1,25(OH)_2D_3$ exerts additional effects, such as induction of alkaline phosphatse activity, via other pathways.

If $1,25(OH)_2D_3$ is acting in part through TGFβ, then it is not likely that this effect is at the transcriptional level since no consensus steroid hormone response element has been found in the promoters of any of the TGFβ genes(Roberts and Sporn,1992). However an increase in the steady-state level of TGFβ1 mRNA has been reported in keratinocytes treated with $1,25(OH)_2D_3$(Kim *et al.*,1992). This action of $1,25(OH)_2D_3$ might be at the level of mRNA stability. In addition, $1,25(OH)_2D_3$ could affect formation of the TGFβ latent complex or activation of latent TGFβ(Roberts and Sporn,1992). No evidence is currently available to favour one mechanism over the others.

We have shown that TGFβ is a potent inhibitor of intestinal epithelial cell proliferation *in vitro*. TGFβ demonstrates a gradient of expression *in vivo* with highest levels found at the villus tip and lowest in the crypt, demonstrated at both the mRNA and protein levels(Barnard *et al.*,1989; Glick *et al.*,1991; Barnard *et al.*,1993). The localization data suggest that the primary action of TGFβ may be in the mature enterocyte rather than in the crypt cells. In a wounded monolayer of intestinal epithelial cells TGFβ1 can increase the migration of cells across the wound while still inhibiting cell proliferation(Ciacci *et al.*,1993). Thus the main role of TGFβ may be to modulate intestinal cell migration which is consistent with the effects of TGFβ on extracellular matrix and cell adhesion(Roberts and Sporn,1990; Massagué *et al.*,1992).

$1,25(OH)_2D_3$ inhibits the proliferation of intestinal epithelial crypt cells *in vitro*. Therefore $1,25(OH)_2D_3$ could be one of multiple signals converging on the crypt cells which limit their proliferation. This is consistent with *in vivo* findings where the villus length is decreased in vitamin D deficient animals(Birge and Alpers,1973). The crypt cells express high levels of the VDR and thus represent a primary target for $1,25(OH)_2D_3$ actions *in vivo*(Clemens *et al.*,1988). However, $1,25(OH)_2D_3$ is certainly not the only factor responsible for arresting crypt cell growth. With several known regulators of intestinal proliferation, such as

glucocorticoids, EGF and extracellular matrix(Quaroni and May,1980; Kurokowa *et al.*,1987; Carroll *et al.*,1988), $1,25(OH)_2D_3$ would be part of a much larger scheme, whereby a variety of compounds would act together to switch off proliferation in crypt cells at the appropriate time.

The inhibition of proliferation by $1,25(OH)_2D_3$ has recently developed clinical relevance. Psoriasis is a hyperproliferative skin disorder and topical administration of $1,25(OH)_2D_3$ provides some reversal of the condition(Kragballe,1992). The development of vitamin D analogs, such as EB 1089(Mathiasen *et al.*,1993), which are potent inhibitors of cell proliferation and do not exert hypercalcemic effects, is a rapidly expanding research area. Such analogs are targetted for cancer therapy since $1,25(OH)_2D_3$ can inhibit the proliferation of cancer cells but treatment with $1,25(OH)_2D_3$ *in vivo* is not practical due to its calcemic effects. Therefore the role of $1,25(OH)_2D_3$ as a regulator of cell growth is a crucial one and understanding further its mechanism of action is essential to its therapeutic application in disease states.

6. CONCLUSIONS

The first two objectives of this thesis were met. Both $1,25(OH)_2D_3$ and TGFβ1 were potent inhibitors of intestinal epithelial cell proliferation whereas only $1,25(OH)_2D_3$ induced partial differentiation of the cells. Indirect evidence was obtained for objective three, whereby $1,25(OH)_2D_3$-induced suppression of proliferation may be mediated by TGFβ. These studies confirm previous findings where $1,25(OH)_2D_3$ has been found to be an important modulator of cell growth. An understanding of the factors involved in the regulation of cell proliferation is essential to discovering how this regulation may be disturbed in disease states.

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APPENDIX

FIGURE A1 SCATCHARD ANALYSIS OF VITAMIN D RECEPTORS IN IEC-6 CELLS

Near confluent IEC-6 cells were lysed in hypertonic buffer and 200 µl of the resulting cytosol(1mg/ml) was incubated with increasing concentrations of [^{3}H]-1,25(OH)$_2$D$_3$ for 18 hr at 4°C before separation of bound and free hormone. Specific [^{3}H]-1,25(OH)$_2$D$_3$ binding was plotted by the method of Scatchard.

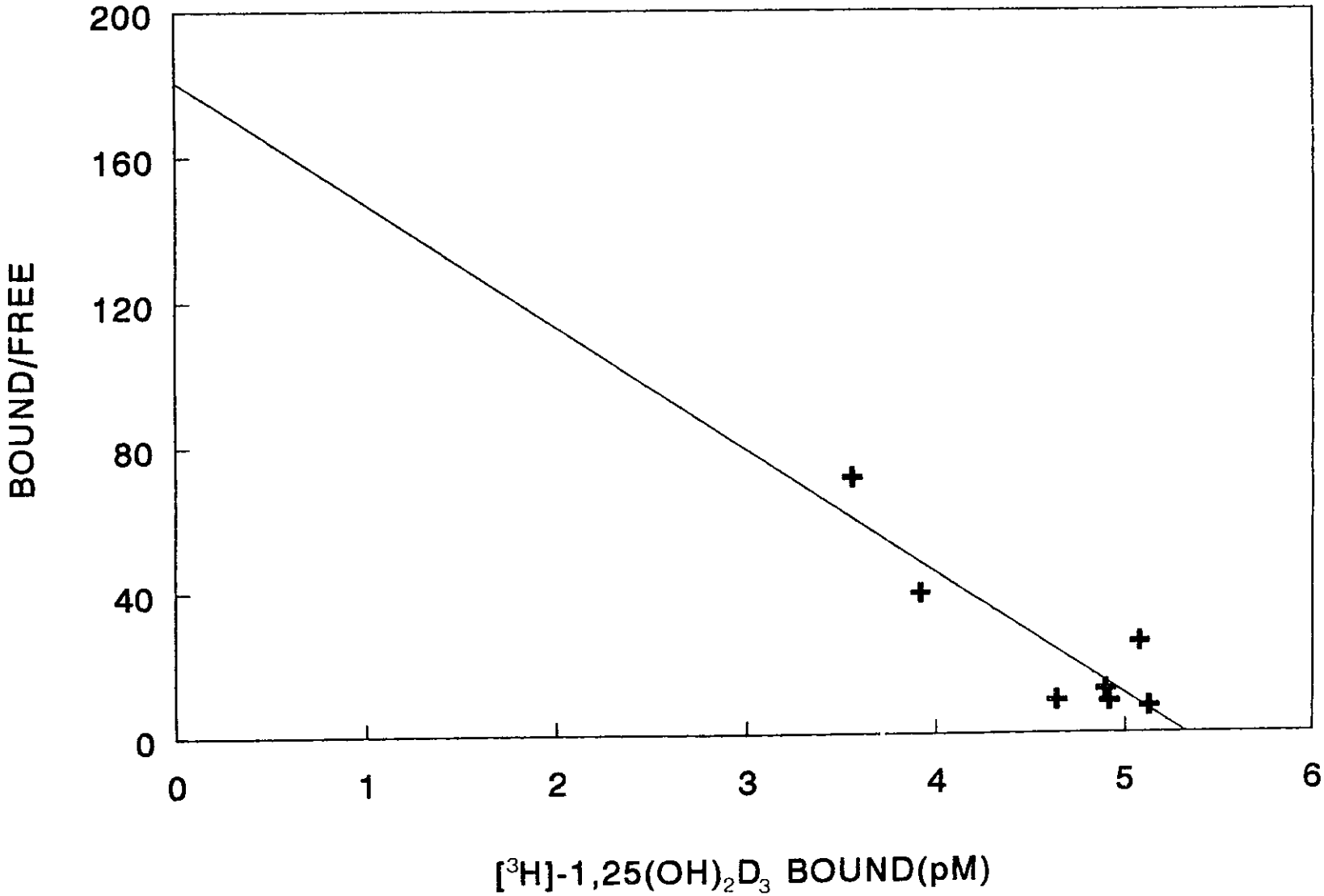

200
160
120
80
40
0
BOUND/FREE
0
1
2
3
4
5
6
[3H]-1,25(OH)2D3 BOUND(pM)

FIGURE A2 TGFβ BIOASSAY STANDARD CURVE

Mv1Lu cells were grown in 96-well microtiter plates and exposed to increasing concentrations of TGFβ1 for 24 hr. After 20 hr, ^{3}H-thymidine was added(1 μCi/ml) and 4 hr later, DNA synthesis was determined. Results are plotted by linear regression analysis and demonstrated a correlation coefficient(r) of -0.8485[r^2=0.7200].

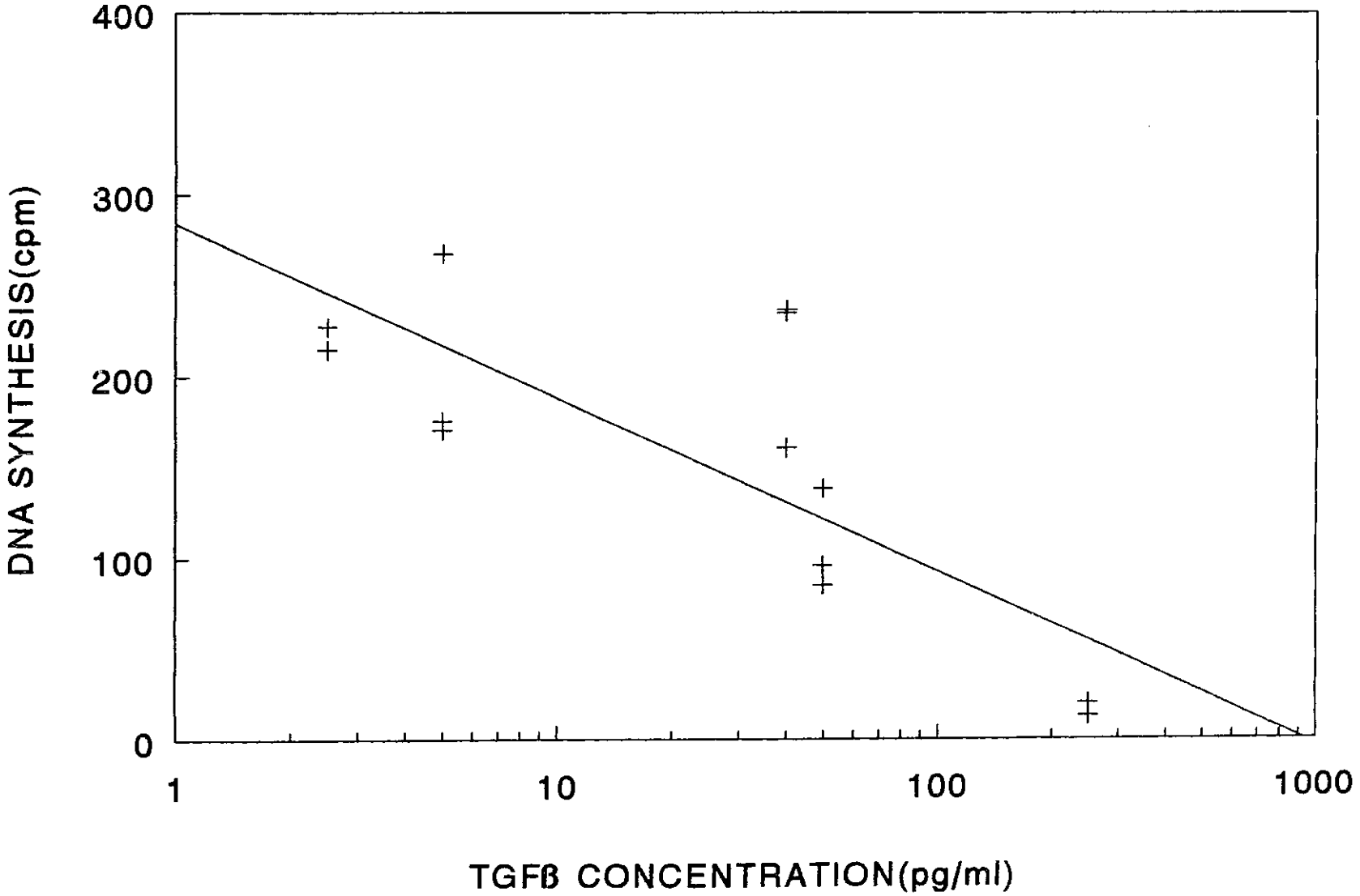
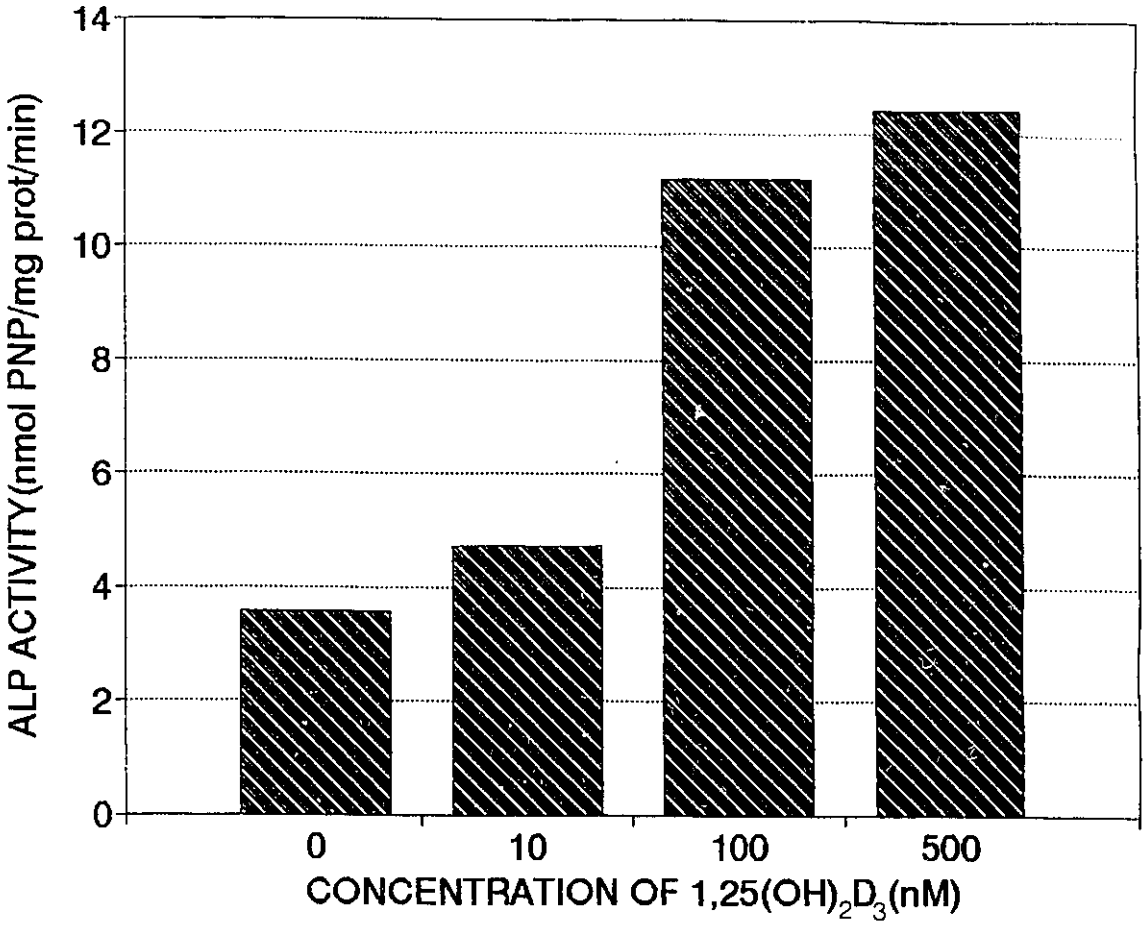

400
300
200
100
0
DNA SYNTHESIS(cpm)
1
10
100
1000
TGFβ CONCENTRATION(pg/ml)

FIGURE A3 ALKALINE PHOSPHATASE ACTIVITY OF IEC-6 CELLS IN RESPONSE TO INCREASING CONCENTRATIONS OF $1,25(OH)_2D_3$

Confluent, serum-free IEC-6 cells were harvested after 48 hr of treatment with the indicated concentrations of $1,25(OH)_2D_3$. Alkaline phosphatase activity was determined as the production of para-nitrophenol from para-nitrophenyl phosphate at pH 10.2. 100 nM $1,25(OH)_2D_3$ was sufficient to induce an increase in alkaline phosphatase activity.


14
12
10
8
6
4
2
0
ALP ACTIVITY(nmol PNP/mg prot/min)
0
10
100
500
CONCENTRATION OF 1,25(OH)2D3(nM)

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Abstracts:

Bonell,D. and Welsh,J. Interaction of $1,25(OH)_2D_3$ and TGFβ in IEC-6 cells. Presented at the Ninth Workshop on Vitamin D, Orlando, Florida, 1994.