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The Effect of Trifluoperazine, a Calmodulin Antagonist, on the Growth of Normal and Malignant Epidermal Keratinocytes in Culture

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Abstract—*Calmodulin, a cytoplasmic calcium binding protein, is present in concentrations two- to four-fold higher in malignant cells compared to normal cells. In an effort to learn the significance of these elevated levels, we examined the effect of calmodulin blockage on the growth of normal and malignant keratinocytes* in vitro. *The level of calmodulin in SCC12.B2, a line of keratinocytes derived from an epidermal squamous cell carcinoma (SCC), was about 3.5 times greater than in normal, human newborn foreskin keratinocytes. When exposed to trifluoperazine (TFP), an inhibitor of calmodulin, cell growth was reduced primarily in the cultures of normal keratinocytes. This growth inhibition resulted from two changes in the replicating population of cells, namely an increase in cell cycle length and an increase in rate of cell cycle withdrawal. Cell cycle withdrawal is the irreversible arrest of the cell cycle and is an early event in keratinocyte terminal differentiation. There was no measurable effect on the cell cycle time or withdrawal rate in SCC12.B2. The increased resistance to growth arrest in SCC cells may be a consequence of the elevated level of calmodulin in these cells.*

INTRODUCTION

CALMODULIN is an intracellular calcium binding protein that is involved in the numerous reactions in which calcium plays an essential role [1, 2]. Comparisons of the levels of calmodulin in normal and malignant cells in culture have repeatedly shown elevated levels in malignant cells. For example, the level of calmodulin in cultured human fibroblasts and rat kidney cells is elevated two- to four-fold when these cells are transformed with a DNA or an RNA tumor virus [3, 4]. The differences seen *in vitro* are reflected by similar results in normal and malignant tissue. For example, Morris hepatoma cells [5–7] and human mammary carcinoma tissue [8] have elevated calmodulin levels compared to the normal tissue.

The significance of the elevated calmodulin levels in malignant cells is unclear. Since calmodulin has been shown to be vital to a number of events that relate to cell replication [9, 10], it is possible that the elevated calmodulin levels in malignant cells reflect in some way a key aspect of the malignant phenotype. One way to gauge the importance of calmodulin in the expression of the malignant phenotype is to observe the effect of calmodulin blockage on the behavior of malignant cells. TFP is an effective and specific inhibitor of calmodulin at doses within the IC_{50} range. In this range, TFP does not appear to cause nonspecific membrane effects [11]. Wei *et al.* [12] as well as Kikuchi *et al.* [13] inhibited calmodulin with TFP in human breast and ovarian cancer cells respectively and found that cell replication was arrested. However, the effect of calmodulin blockers was not tested on normal controls making it difficult to gauge the significance of these results.

The purpose of this study is to determine if the growth of malignant cells in culture is affected by calmodulin inhibition in a different manner than normal cells. A key aspect of such a study is the choice of cell type that will allow a meaningful comparison of normal versus malignant. Keratinocytes from normal human epidermis can be grown in culture to form a stratified, squamous, incompletely keratinizing epithelium that resembles in a number of aspects the original epidermis [14]. Cells in the basal compartment constitute the germinative population and give rise to cells which undergo terminal differentiation. These latter cells withdraw

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from the cell cycle and express a number of markers of keratinocyte differentiation including synthesis of involucrin [15] and formation of cornified cell envelopes [16]. In normal keratinocytes, calmodulin is concentrated along the plasma membrane and in close association with keratin filament bundles and desmosomes (Grief F, Sasaki H, Garant PR, Soroff HS, Taichman LB, submitted for publication). When calmodulin is inhibited with TFP, the rate of cell replication is reduced and the rate at which replicating cells shift to the nonreplicating compartment is also increased. Keratinocytes have also been isolated from naturally occurring squamous cell carcinomas (SCC) of the skin [17]. In culture, SCC keratinocytes are immortalized, do not stratify, do not appear to undergo cell cycle withdrawal and do not express a number of markers characteristic of differentiating keratinocytes [18]. When introduced into a suitable animal host, they do however form expanding tumors indicating their malignant behavior. We have analyzed the effect of TFP on the growth of normal epidermal keratinocytes and SCC12.B2—a line of malignant keratinocytes derived originally from naturally occuring squamous carcinoma of the skin [17]. In contrast to the results of Wei *et al.* [12] and Kikuchi *et al.* [13], we have found that TFP in the IC_{50} dose range specifically inhibited the growth of normal keratinocytes and was without effect on the SCC cells.

MATERIAL AND METHODS

Epidermal keratinocytes from human foreskin were cultured essentially as described by Rheinwald and Green [19] in a semi-defined medium [20]. The malignant keratinocytes were an established line of cells (SCC12.B2) from a naturally occurring, human, epidermal squamous cell carcinoma [17]. Media for SCC12.B2 cells consisted of fetal calf serum (10%), Dulbecco's modified Eagle's medium and hydrocortisone (0.4 μg/ml). TFP (Stelazine, Smith Kline & French) was dissolved fresh in medium before use. Mitomycin C and bromodeoxyuridine (BrdUrd) were purchased from Sigma, trypsin (0.02%) from Worthington, and culture dishes from Falcon. Cell counts were performed on a Coulter Counter model ZB1.

Normal keratinocytes were seeded into 10 and 60 mm diameter culture dishes at 3×10^4 and 3×10^5 cells per dish, respectively. These dishes had been seeded with mitomycin-inactivated 3T3 cells 24 h previously. SCC12.B2 were seeded into 10 and 60 mm culture dishes at 10^4 and 10^5 cells per dish, respectively. Cultures were routinely fed every third day. All cell counts were done in triplicate and the average value was plotted.

Details of the double label assay have been published elsewhere [21] and are illustrated schematically in Fig. 1. Some modifications were introduced

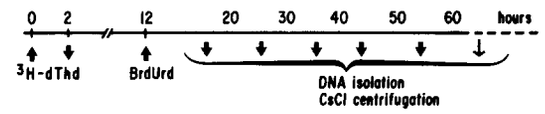

Fig. 1. Schematic illustration of the double label assay. Details of this assay have been published [21]. *Briefly, multiple cultures are simultaneously pulse labeled with* [^{3}H]*dThd for* 2 h *and then labeled continuously with BrdUrd beginning at* 12 h. *At various times thereafter, DNA was extracted from individual cultures and the percentage double label DNA measured by* CsCl *density gradient centrifugation. Typical results are shown in Fig.* 2.

to simplify the procedure. Briefly, from 0 to 2 h, multiple cultures of normal and malignant keratinocytes were labeled with [^{3}H]dTdr (thymidine[methyl 1,2′-^{3}H], 101 Ci/mmol, New England Nuclear) and at 12 h, the cultures were fed media containing BrdUrd for the remainder of the experiment. The doses of [^{3}H]dThd and BrdUrd were 2 and 50 μg/ml for normal cells, respectively and 1 and 10 μg/ml for SCC cells respectively. At selected times, DNA from individual cultures was isolated and centrifuged to equilibrium in cesium chloride density gradients. Fractions (250 μl) were collected directly into vials and the radioactivity in each fraction was measured in scintillation fluid (National Diagnostics). A plot of the counts per minute in each fraction versus fraction number produced two peaks, one indicating DNA singly labeled with [^{3}H]dThd, and a second indicating DNA doubly labeled with [^{3}H]dThd and BrdUrd (Fig. 2). SCC cells were shown in previous trials to be sensitive to BrdUrd and as such it may inflate withdrawal rates. To correct for any toxic effects of BrdUrd plateau levels of SCC and normal keratinocytes were plotted against different concentrations of BrdUrd and the best fit line was extrapolated to zero concentration of BrdUrd. All cultures with TFP and BrdUrd were handled under reduced light. All experiments were performed at least twice. Each set of experiments (control + TFP treated cells) were performed on cells obtained from either, a new foreskin or, a different batch of SCC.12B2 cells.

RESULTS

Calmodulin levels in cultured keratinocytes

Calmodulin levels have been reported to be higher in malignant than in normal cells. However, no data exist for calmodulin levels in normal and malignant keratinocytes in culture. Calmodulin levels were kindly determined by Dr. Anthony Means (Baylor College of Medicine, Houston, Texas) using a radioimmunoassay [22] on duplicate samples of subconfluent and confluent, normal keratinocytes and SCC12.B2. The results show that the levels of calmodulin were about three and one half times higher in

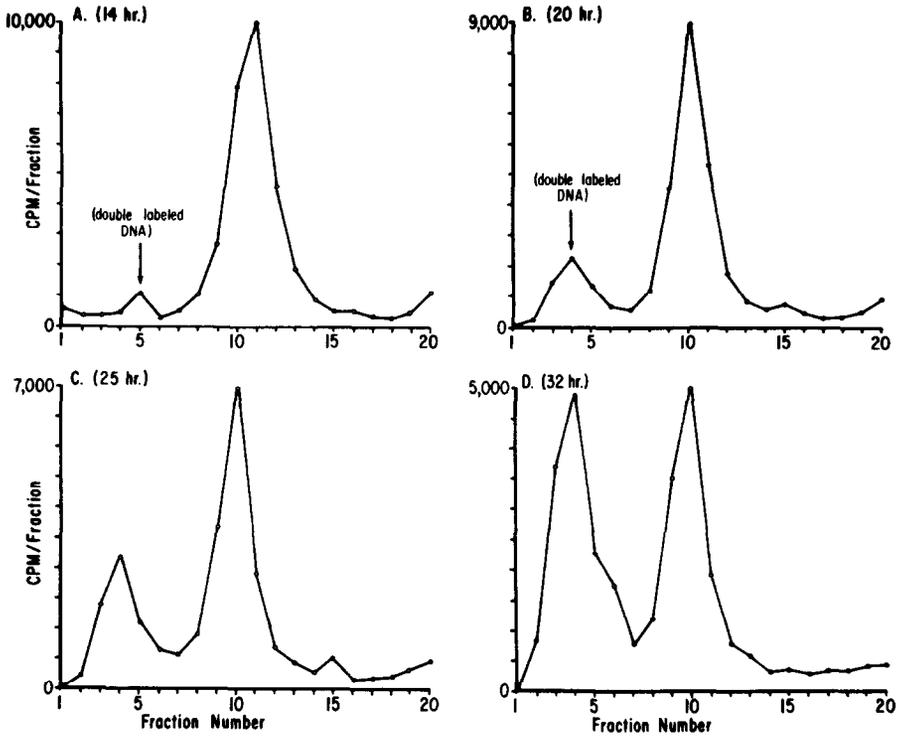

Fig. 2. CsCl *density gradient centrifugation of DNA samples generated in the double label assay. Multiple cultures of normal keratinocytes were labeled as described in Fig. 1. At the times indicated in brackets, DNA was extracted from a culture and centrifuged to equilibrium in* CsCl. *Two bands of* ^{3}H*-labeled DNA are present: The larger band on the right, or less dense DNA, contains no BrdUrd. The band of DNA on the left, or more dense DNA, is doubly labeled with BrdUrd. The amount of doubly labeled DNA was determined by measuring the area of the more dense band relative to the combined area of the two bands and these values were plotted as a function of time in Fig. 5.*

Table 1

Camodulin levels	ng/10^6 cells	μg/mg cell protein
Normal keratinocytes		
Subconfluent	457	1.2
Confluent	433	0.95
SCC12.B2		
Subconfluent	1573	3.2
Confluent	680	1.2

Calmodulin levels were determined by radioimmunoassay [22] by Dr. Anthony Means (Baylor College of Medicine). Subconfluent cultures were about 50% confluent at the time of harvesting. Confluence was determined by growth arrest of sister cultures. Duplicate cultures were measured and the average is noted in the table.

subconfluent malignant keratinocytes than in their normal counterparts and only about twice as high in confluent SCC cells (Table 1).

Effect of TFP on keratinocyte growth

When normal epidermal keratinocytes are seeded in culture dishes containing inactivated 3T3 feeder cells, single keratinocytes attach and replicate to produce expanding colonies [19]. At the center of the colonies, stratification takes place. With time the rate of growth decreases and after about 21 days in culture, the dish contains a confluent, stratified squamous epithelium. Although there is no further increase in cell number per dish during this confluent phase, there is a steady state loss of cells by desquamation at the surface matched by cell renewal in the basal layer [23]. These cultures resemble normal epidermis more closely than the subconfluent cultures.

To determine the effect of TFP on keratinocyte replication, daily cell counts were performed on normal and malignant cultures exposed to varying doses of the drug. In subconfluent cultures of normal keratinocytes, TFP at doses of 2.5–5.0 μM reduced growth rates as well as final numbers of cells per dish (Fig. 3, left). At doses 10 μM or higher, there was a clear loss of cells from the culture. In confluent cultures, doses of 2.5–5 μM TFP led to reduced numbers of cells per culture but the numbers tended to remain relatively constant at this reduced density. Doses of TFP 10 μM or higher led to progressive loss of cells from confluent cultures (Fig. 3, right). Thus, doses of TFP of 10 μM or higher lead to a loss of cells from the culture indicative of cell death. At lower doses, there is disruption of the normal

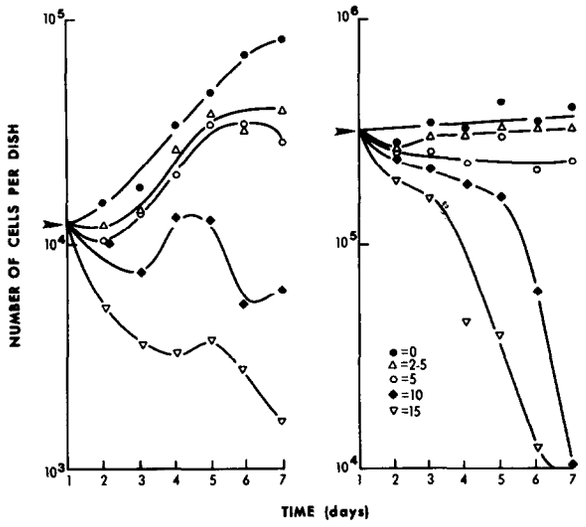

Fig. 3. Growth of normal keratinocytes in the presence of TFP. Left side: multiple 60 mm dishes seeded 5 days previously with 3 × 10^5 keratinocytes were fed medium containing various concentrations of TFP. Daily cell counts were performed on duplicate cultures and the average cell number per dish plotted. Right side: sister cultures of keratinocytes were allowed to continue in the absence of the drug for an additional 10 days and when confluent, were fed medium containing varying concentrations of TFP. Daily cell counts were performed. The concentration of TFP (μM) in the medium is indicated in the figure on the right.

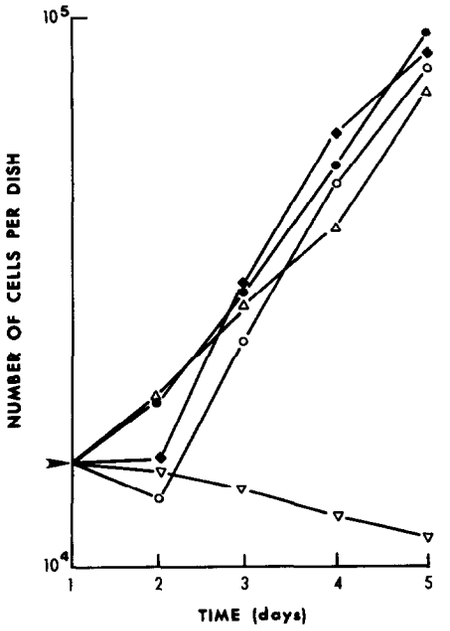

Fig. 4. Growth of SCC12.B2 in the presence of TFP. Multiple 60 mm dishes of SCC12.B2 containing 3 × 10^4 cells were fed medium supplemented with various amounts of TFP. The concentration of TFP (μM) is noted in Fig. 3 (right). Daily cell counts were performed on duplicate dishes and the average plotted.

growth pattern but the basic parameters of renewal are maintained.

SCC cells are known to fail to withdraw from the cell cycle [18] and are presumed to expand constantly. In order to try and mimic *in vivo* conditions, similar experiments were performed only with subconfluent cutures of SCC.B2 cells. Non-treated cells grew exponentially with a doubling time of 39 h (Fig. 4). At doses of TFP of 2.5–5.0 μM, there was an initial retardation of growth but thereafter a return to normal growth kinetics. However, at 15 μM and higher, there was an irreversible decline in cell number indicating a loss of cells from the dish (data regarding doses of TFP higher than 15 μM are not shown). Thus, the growth of normal and malignant keratinocytes was clearly affected by the presence of TFP in the media in a dose related manner. However, malignant keratinocytes tolerated higher doses of TFP than normal keratinocytes after an initial period of reduced growth.

Keratinocytes in culture and *in vivo* undergo a process of terminal differentiation in the formation of stratified layers of epithelium. Keratinocytes that enter this program undergo an irreversibe arrest of cell replication. There are two ways TFP could have slowed down the growth of keratinocytes in culture: (1) in the cells that undergo repeated rounds of replication, the duration of the cell cycle could have been prolonged or (2) cells in the replicating compartment may have been induced to undergo cell cycle withdrawal at a higher rate than usual. To investigate these possibilities, we utilized a newly described double label assay (Fig. 1).

The double label assay measures two aspects of keratinocyte growth, the length of time between two successive S phases (i.e. the cell cycle time) and the percentage of cells which, having completed one cycle of replication, undergo a second cycle [24]. The assay quantitates the amount of pulse labeled [^{3}H]DNA that becomes doubly labeled with the density analog, BrdUrd. The accumulation of double labeled DNA was monitored by CsCl density gradient centrifugation (Fig. 2). Such DNA is derived from cells which underwent two successive cycles of replication. The percentage of doubly labeled DNA was calculated and plotted as a function of time (Fig. 5). From 12 to 20 h there was an increase in double labeled DNA followed by a plateau. The percentage of ^{3}H-labeled DNA in this plateau is the percentage of cells which having replicated once in the presence of [^{3}H]dThd replicate again in BrdUrd. The time taken to reach 50% plateau level is the average cell cycle time. Cells that replicated in the presence of ^{3}H-labeled cells but failed to replicate in the presence of BrdUrd are considered to have withdrawn from the cell cycle. The rate of cell cycle withdrawal is indicated by the difference between the plateau and 100%.

Based on the results in Fig. 5, it is evident that in normal keratinocytes, TFP at a concentration of 5 μM increased the cell cycle time from 22 to 27 h and increased the percentage of ^{3}H-labeled cells

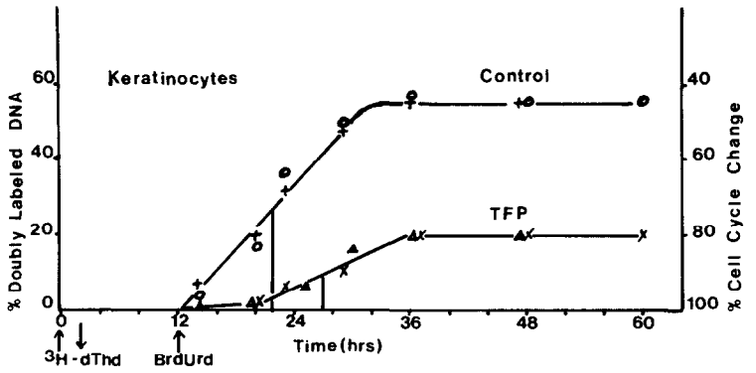

Fig. 5. Results of the double label assay performed on normal keratinocytes exposed to TFP (5 μM). Multiple cultures of subconfluent cultures of normal keratinocytes were labeled as illustrated in Fig. 1. The percentage of double labeled DNA was determined (as in Fig. 2). TFP (5 μM) was added at the conclusion of the [^{3}H]dThd pulse and maintained in medium thereafter. There is an initial increase followed by a plateau. The percentage of ^{3}H-labeled cells undergoing withdrawal was determined from the difference between the amount of double labeled DNA at the plateau and 100% (indicated on ordinate on the right). Vertical lines were drawn from the midpoint and intersect the abcissa at a point equal to the average cell cycle time. Each trial was repeated twice. With each set (control + TFP treated cells) originating from a different foreskin. I set: Control cultures (○---○); TFP treated cultures (×---×). II set: Control cultures (+---+); TFP treated cultures (△---△).

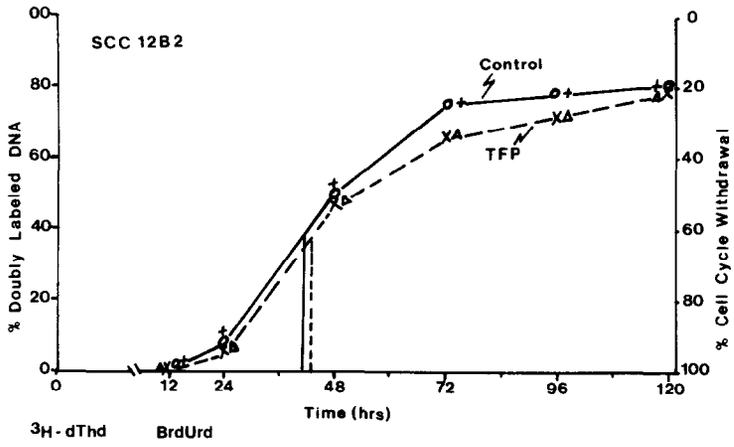

Fig. 6. Results of the double label assay performed on TFP-treated SCC12.B2. Details of this experiment are given in the legend to Fig. 5. Each trial was repeated twice. With each set (control + TFP treated cells) originating from a different batch of SCC cells. I set: Control cultures (○---○); TFP-treated cultures (×---×). II set: Control cultures (+---+); TFP-treated cultures (△---△).

that underwent cell cycle withdrawal from 45 to 80%. These values are close to or match those previously noted (Greif F, Sasaki H, Garant PR, Soroff HS, Taichman LB, submitted for publication). Thus, TFP altered both the cell cycle time and the rate at which normal cells withdrew from the replicating compartment. In untreated cultures of SCC12.B2 (Fig. 6), the cell cycle time was 42 h and the rate of withdrawal was approx. 20%. In cultures treated with 5 μM TFP the average cycle time was 43 h and the rate of withdrawal was approx. 18%. SCC cells, unlike normal keratinocytes (Fig. 7), can be induced to withdraw from the cell cycle by BrdUrd in the double labeled assay (Fig. 8). To offset this, plateau levels were measured at different concentrations of BrdUrd and the curve thus generated was extrapolated back to zero BrdUrd concentration. Using this procedure, the withdrawal rates were confirmed to be the same i.e. 15%, in SCC12.B2 cells with or without TFP treatment (Fig. 9). Thus, in SCC12.B2, TFP (15 μM) causes no change in cell cycle withdrawal or length of the cell cycle.

DISCUSSION

In an effort to understand the significance of elevated levels of calmodulin in cultures of malignant cells as compared to their normal counterparts,

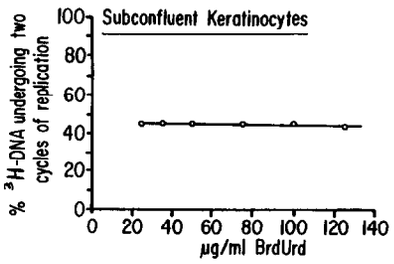

Fig. 7. Withdrawal from the cell cycle in subconfluent cultures of keratinocytes. Twenty-four 60 mm *dishes of subconfluent keratinocytes wre labeled with* [³H]*dThd at* 0–2 h. *At* 12 h, *sets of four dishes were fed different concentrations of BrdUrd. Two dishes from each set were harvested at* 36 *and* 48 h *after* [³H]*dThd addition. The plateau values for each concentration were averaged and plotted.*

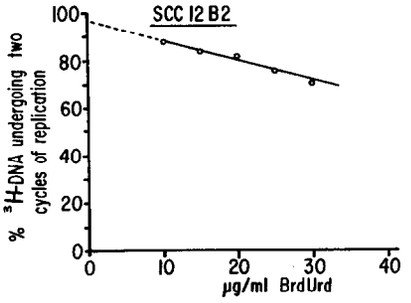

*Fig. 8. Withdrawal from the cell cycle of cultures of SCC*12.*B2. Twenty* 60 mm *dishes of subconfluent SCC*12. *B2 cells were labeled with* 1 µCi/ml [³H]*dThd for* 2 h. *At* 12 h *each set of four dishes was labeled with different concentrations of BrdUrd. Plateau values obtained for each BrdUrd concentration averaged and plotted as detailed in Fig. 7.*

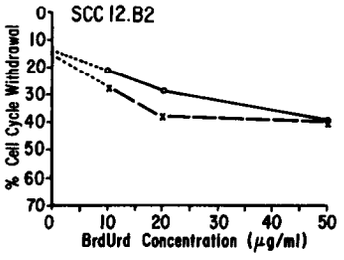

*Fig. 9. Cell cycle withdrawal in SCC*12.*B2 as a function of BrdUrd concentration. Withdrawal rates were measured in cultures of SCC*12.*B2 using* 10, 20 *and* 50 µg/ml *with and without TFP* (5 µM). *The withdrawal rate was extrapolated back to zero BrdUrd level. Control cultures* (o---o); *TFP-treated cultures* (×---×).

we have examined the effect of TFP—a calmodulin antagonist on the growth of normal and malignant epidermal keratinocytes in culture. The trial was performed on exponentially growing (subconfluent) cultures of normal keratinocytes derived from human foreskin and SCC12.B2—one of the few established cell lines derived from human SCC of the skin. Our results indicate that growth of both cell types is adversely affected by the calmodulin blocker but normal cells appear to be more sensitive to the effects than malignant cells. In cultures of normal keratinocytes treated with 5.0 µM TFP, the rate of population increase was reduced. Using a newly developed double label assay, this growth inhibition was shown to have resulted from a prolongation of the average cell cycle length from 22 to 27 h and an increase in the rate of cell cycle withdrawal from 45 to 80%. At the same concentration of TFP, SCC12.B2 cells exhibited a transient slowing in their growth kinetics and no significant changes in cell cycle length or cell cycle withdrawal.

Since TFP at the concentration used in this study inhibits calmodulin specifically [11], changes in calmodulin metabolism are likely to underly the kinetic changes we observed. Calmodulin levels are elevated in late G1 phase of the cell cycle and blockage of calmodulin does lead to cell cycle arrest specifically at the G1/S boundary [10, 25]. These effects may underly the prolongation of cell cycle seen in this study. How TFP induced the cell cycle withdrawal as detected by the double label assay is not known. Changes in extracellular calcium concentration are known to have profound effects on the cell cycle withdrawal in normal keratinocytes [26, 27]. When grown in low concentrations of calcium (0.02–0.1 mM), terminal differentiation is reversibly blocked. Under these conditions, cells remain in the replication phase and do not undergo cell cycle withdrawal. However, when the cultures are returned to normal calcium level, the cells undergo cell cycle withdrawal and terminal differentiation en masse. TFP does not alter this calcium-induced transition [28] whereas the calcium ionophore A23187 inhibits its occurrence [29]. It is not known if the cell cycle withdrawal associated with shifts in external calcium concentrations, or with the addition of TFP to normal cells, or with normal keratinocyte differentiation are in fact the same event. We know little about withdrawal. We do not yet know the intracellular events that constitute cell cycle withdrawal and therefore we do not know if the withdrawal seen in the different situations just mentioned are in fact the same event.

The failure of SCC cells to undergo any cell cycle changes in response to TFP could be due to two factors: (1) Malignant keratinocytes may be unable to undergo cell cycle withdrawal as part of the malignant phenotype. In malignant keratinocytes there appears to be a block to terminal differentiation. When normal keratinocytes are placed in suspension, they form cornified cell envelopes at a defined rate [16]. When SCC cells are placed in suspension, they form cornified evelopes but at a variable and reduced rate [18]. An examination of the data in Fg. 9 shows that in untreated cultures of SCC12.B2, only 15% of the cells underwent cell cycle withdrawal. These results and those soon to

be published (Greif F, Albers KM, Setzer RW, Soroff HS, Taichman LB, unpublished) indicate that SCC cells in culture exhibit little if any cell cycle withdrawal. Although terminal differentiation seems to be blocked in SCC cells, they can be stimulated to do so under appropriate conditions. When SCC cells are grown in medium devoid of vitamin A, a well defined stratum corneum develops and keratin proteins typically seen in the epidermis are synthesized [30]. Retinoic acid in low doses will also cause SCC cells to undergo cell cycle withdrawal (Greif F, Albers KM, Setzer RW, Soroff HS, Taichman LB, unpublished). Therefore, it is unlikely that SCC cells are incapable of undergoing cell cycle withdrawal. (2) The higher level of calmodulin in SCC cells might have provided some measure of resistance to calmodulin inhibition by TFP. A similar mechanism has been proposed to explain the resistance of SV40 transformed WI-38 fibroblasts to growth arrest in low calcium containing media [31]. WI-38 cells are normally inhibited when calcium concentration in the extracellular fluid is reduced whereas SV40 transformed cells are not. If higher levels of calmodulin in SCC12.B2 did provide some measure of protection against inhibition by TFP, then using higher levels of TFP should have overcome this protection. However, higher levels of TFP caused a loss of cells from the dish making it impossible to explore this idea further.

The goal in chemotherapy of malignancy is to utilize a drug that has selective toxicity for malignant cells. Most chemotherapeutic agents are toxic for all replicating cells and in this way derive selectivity for tumors with rapid growth kinetics or with a high growth fraction. The discovery that calmodulin levels are generally higher in malignant cells led to the hypothesis that malignant and normal cells might differ in their response to the calmodulin inhibitor, TFP. In fact it was proposed that TFP might specifically inhibit malignant cells [12, 13]. The results of this study refute this assertion. Normal cells are more sensitive to cell cycle arrest induced by TFP than SCC cells. It is likely that the elevated levels of calmodulin in SCC cells reflect the fact that a greater fraction of SCC cells are in the replicative cycle than normal keratinocytes. Calmodulin levels are elevated in late G1 and early S phase [10] and the increased levels of calmodulin in SCC cells may reflect the fact that a higher percentage of cells in SCC cultures are in G1/S phase than in normal keratinocyte cultures.

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