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THE ROLE OF CALMODULIN IN HUMAN RENAL CELL CARCINOMA

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Calmodulin is a ubiquitous intracellular calcium binding protein which has been shown to be associated with cell cycling. Previous studies using animal tumor models have suggested a positive correlation between tumor calmodulin content and rate of tumor growth. We studied the role of calmodulin in renal cell carcinoma (RCC) cell lines and compared this with short term normal fetal kidney cell lines. The effects of calmodulin inhibition was determined using the calmodulin inhibitor W13 (Naphthalene-sulfonamide) and its less active partner W12. Cell size, calmodulin content and inhibition studies using W13 did not reveal any simple correlations for the RCC cell lines, although the RCC lines did have a higher content than the fetal kidney cell lines. Calmodulin content determination of RCC and normal adult kidney tissue failed to show any difference. We conclude that, contrary to previous reports using animal models, there is no simple relationship between tumor growth rates and calmodulin content for human RCC.

Recently it has been reported that the cellular concentration of calmodulin increases during the cell cycle, suggesting a role for it in the regulation of the cycling process (1). In Chinese Hamster ovary K cells a sudden increase in cellular calmodulin levels occurs at the G1/S phase boundary (2). Virally transformed cells have also been shown to have increased calmodulin levels (3). This increase is due to an increased rate of synthesis rather than any change in calmodulin degradation. In vitro studies have shown that tumor cells have a decreased requirement for calcium in the culture media (4). Thus, calcium and calmodulin appeared to be involved in the growth of normal and tumor cells.

MATERIALS AND METHODS

TISSUE AND CELL LINES: RCC and non-tumor renal tissue were obtained at surgery and immediately frozen and stored at -70°C. Non tumor tissue was defined as tissue obtained from the kidney containing the RCC, but far removed from the site of the tumor. Fetal tissue was obtained from therapeutic abortions or still births and similarly stored. The RCC cell

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lines 769P, and 1072F were derived in our laboratory (5). SK-RCC-6 was a gift from Dr. Bander, Memorial Sloan Kettering Institute, New York, NY. Short term cell lines were derived from the same fetal tissue as was used for the calmodulin assay. The cell lines were prepared as previously described (6). Generation times for the short term cell lines were estimated by seeding a known number of first passage cells and counting the number reached at the next passage.

CALMODULIN INHIBITORS: The inhibitors used in these experiments were n-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide, hydrochloride (W13), together with its less active partner W12 (CAABCo, Houston, Texas). W12 contains one less chlorine atom at C-5 than the active compound W13. Previous studies have shown that the W compounds bind to calmodulin in a calcium dependent manner, with the less active compound having about 5 times less binding affinity (2,7). As the hydrophobicity of these compounds is similar, their interaction with cell membranes should also be similar, making them excellent probes for evaluating calmodulin function.

CALMODULIN ASSAY: Tumors were suspended in 125 mM Borate buffer containing 1 mM EGTA, and 75 mM NaCl (pH 8.4) and homogenized in a Brinkman Polytron Homogenizer (setting 8 for 80 seconds). Aliquots were then removed for protein determination according to Lowry (8), using BSA as standard. Following homogenization, the sample was centrifuged at 105,000 g for one hour, the supernatant removed and retained. The precipitate was resuspended in homogenization buffer and again centrifuged at 105,000g for one hour. The supernatant was again removed and combined with the first. The precipitate was resuspended in homogenization buffer and both samples were then treated at 90°C for 5 minutes after which they were rapidly cooled to 4°C in a methanol dry ice bath. Further aliquots were taken for protein determination. In this manner the supernatants were defined as the cytosolic fraction and the precipitate as the particulate fraction. Samples were then assayed in a radioimmunoassay as previously performed by us (9) (CAABCo, Houston, Texas). All results were performed in a single assay. The intra-assay coefficient of variation was 5-8%.

CELL GROWTH EVALUATION: Cells were plated at 20,000 - 40,000 cells/well in 24 well plates. After allowing the cells to attach overnight, the media was removed and replaced with either control media (RMPI-1640, 10%199 supplemented with 5% fetal Bovine serum and 2 mM glutamine) or media containing W12 or W13. On days 0, 1 and 2 the cells from wells at each concentration of inhibitor together with controls were trypsinized and counted. Viability was determined by trypan blue exclusion. Thus, by initially plating 3 wells at each concentration, and counting 1 well daily, it is possible to generate a growth curve. Cell size was determined by trypsinizing the cells, cytocentrifuging them onto a glass slide (Shandon Southern Instruments, Inc., Sewickley, PA). The cells were stained with 1% crystal violet after fixation in 95% ethanol for 5 minutes. Cell size was then determined using an Optomax System IV Image Analyser (Optomax Inc., Hollis, New Hampshire).

RESULTS

The effects of calmodulin inhibitors on tumor cell line growth are shown in Figure 1. In 1a, 46 μM W13 led to complete cell death of SK-6 cells, while causing only growth reduction with 769P (1b) and 1072F (1c). Further, this growth retardation was greater for 769P than 1072F. Figure 1d shows the inhibition of growth by W13 for the three cell lines. The concentrations required to reduce the cell counts to half that for the control (IC50), calculated from each of the experiments were 26 $\pm$ 0.52

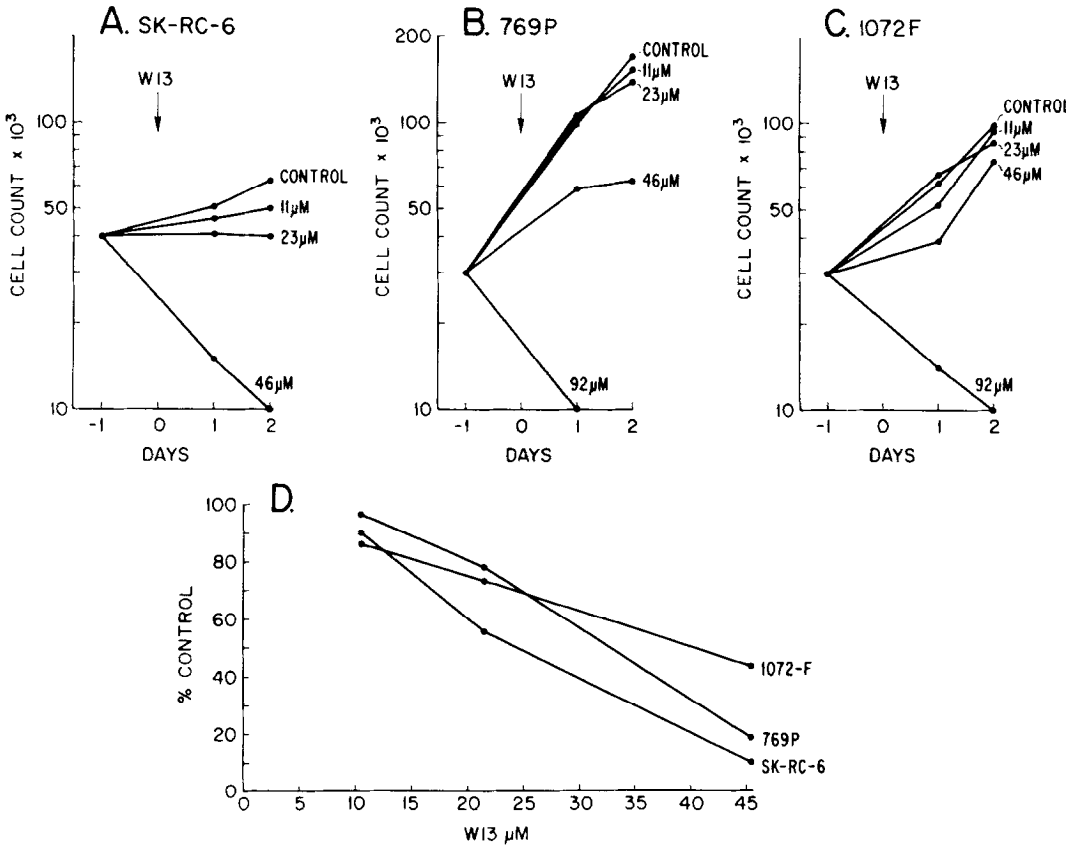

Figure 1: A, B, C: The effect of calmodulin inhibitor W13 on cell growth. The results show examples from one experiment each. The experiments were performed on three occasions.

D: The effect of W13 on cell growth, expressed as a percentage of control, after 48 hours of cultivation (mean of 3 experiments).

μM for SK-6, 35.2 ± 3.7 μM for 769P, and 42 ± 7 μM for 1072F (mean ± SEM). In comparison, the IC50's for W12 treatment were 80 μM for SK-RCC-6, 120 μM for 769P, and 220 μM for 1072F (data not shown). These values are 3-5 times the concentration required for W13, demonstrating the specific calmodulin inhibiting effect of W13.

Determination of the cellular calmodulin content for the RCC cell lines revealed significantly more calmodulin per mg protein than the fetal kidney cell lines (Table 1), $p < 0.05$, yet on an absolute basis (content per cell), the fetal kidney levels fell within the mid range for the RCC lines. When the calmodulin levels for the cytoplasmic and particulate

TABLE 1
Cell Line Characterization

CELL LINE	CELL SIZE PICTURE UNITS	GENERATION TIME (HRS)	PROTEIN/CELL pg	CALMODULIN pg/cell	CALMODULIN µg/mg PROTEIN Total	Cytosolic	Particulate	C/P[a]
FETAL KIDNEY								
FK.1	-	38-64	840	1.16	1.38	1.60	0.70	6.95
FK.2	-	-	790	1.13	1.43	1.74	0.62	7.08
Mean ± SEM					1.41±.03[b]	1.67±.07[b]		
RENAL TUMORS								
1072F	2200±100	42	1130	2.05	1.81	2.33	0.45	13.67
SK-6	1730±133	41	730	1.69	2.32	2.79	0.93	8.31
769P	1250±114	28	410	0.84	2.05	2.70	0.63	9.66
Mean ± SEM					2.06±.15	2.61±.14		

[a] Cytosolic/particulate ratio. Absolute content per cell.
[b] Significance $p < 0.05$ (Student t-test) compared with renal tumors.

compartments were analyzed, the RCC lines had elevated cytoplasmic levels, compared with the fetal lines, $p < 0.05$, but the particulate levels and the absolute cytoplasmic/particulate ratio were similar.

Comparison of cell growth rates with calmodulin content and cell size revealed that 1072F, which had the largest cell size, highest calmodulin level, and required the most W13 for inhibition, was the slowest growing cell line. This trend was not found for 769P and SK-RCC-6. No difference was seen in calmodulin content between these cell lines, when expressed per mg protein, for any of the parameters used, despite the generation times varying by 50% from slowest to fastest, and the cell size, similarly by 76%.

The tissue calmodulin levels are shown in Table II. Tissues No. 1 and 2 for RCC and non-tumor kidney were obtained from the same patients. Both demonstrated an increased level of calmodulin for the non-tumor kidney over the RCC itself. Non-tumor kidney tissue had higher levels of total, cytosolic, and particulate calmodulin; but these were small and statistically insignificant.

The fetal kidney tissue FK1 and FK2 were used to obtain the short term fetal cell lines. The total calmodulin (per mg protein) was three times higher for the tissue than for the cell lines, while the C/P ratios were similar. Comparison of the fetal tissue with the non tumor and RCC tissue

TABLE 2
Tissue Calmodulin Levels

TISSUE	CALMODULIN (μg/mg PROTEIN) TOTAL	CYTOSOLIC	PARTICULATE	CYTOSOLIC/PARTICULATE
RENAL CELL CARCINOMA				
1.	1.71	2.12	0.30	24.28
2.	2.73	3.57	0.58	15.79
3.	1.98	2.91	0.31	16.54
Mean±SEM	2.14±.31[a]	2.90±.42	0.4±0.09[a]	18.9±2.7[a]
NON TUMOR KIDNEY				
1.	1.94	2.48	0.35	20.60
2.	4.99	7.82	0.64	19.07
3.	1.98	2.36	0.39	25.77
4.	4.22	5.21	1.56	9.02
Mean±SEM	3.28±0.76	4.5±1.3	0.74±0.28	18.6±3.5
FETAL KIDNEY				
1.	3.15	5.78	0.62	9.03
2.	5.48	1.01	12.04	5.73
3.	6.54	1.23	12.58	7.53
4.	2.22	1.63	7.54	1.95
5.	5.97	7.79	2.59	5.56[a]
Mean±SEM	4.67±0.84[a]	3.5±1.4	7.1±2.4[a]	6.0±1.2[a]

[a]Significance $p < 0.05$ (Student t-test) compared with renal tumor tissue.

demonstrated a significant elevation for all parameters of calmodulin measurement over the RCC levels, except the cytosolic levels.

DISCUSSION

Previous studies using animal tumor models have suggested a positive correlation between tumor calmodulin content and rate of tumor growth. This study presents data suggesting that cellular calmodulin content has no simple relationship with either malignant transformation, or tumor growth rates in human RCC. RCC cell lines had a higher calmodulin content than the fetal kidney lines, but only relative to protein content and not on a content per cell basis. No difference was seen for C/P ratios or cell calcium content (data not shown). Further, inhibition of growth by W13, did not have any clear cut relationship with cell growth rates or calmodulin content. We observed that at low concentrations (W13 < 4 μg/ml, W12 < 16 μg/ml) the cells had higher growth rates than the controls. This phenomenon is most likely due to non-specific, non calmodulin mediated membrane effects, as membrane stimulation per se tends to cause cell division. This data is contrary to previous studies using non human material. In one study, Morris hepatomas had increasing amounts

of calmodulin associated positively with growth rates (10). This association was restricted to transformed cells as regenerating liver did not have a similar increase. In another study, there was an increased C/P ratio for the tumors over the normal tissues (11). Chafouleas, et al. (3) found that virally transformed cells had a greater rate of calmodulin production over the "normal" cells, together with a higher absolute content. These data appear contrary to our findings, though none is exactly comparable.

Normal kidney had slightly higher levels than the RCC, while the fetal kidney levels were almost twice those of the RCC. There were no differences in the cytosolic fractions between tissues, but the fetal kidney had particulate levels 10 times that for RCC or normal. This led to higher C/P ratios for the RCC and normal tissue, in contrast to previous hepatoma studies (11). Comparison of the tissue C/P values with those for the cell lines showed a good correlation for the fetal kidney, while the RCC cell lines had lower levels than the tissue.

With the demonstration of numerous other calcium or calmodulin binding proteins, (12,13,14), including one (Oncomodulin) (15) which has only been found in malignant tissues, it seems plausible that the differences seen between ours and other studies is due to variations in the content of these other calcium binding proteins. In a recent study (16), the same group that evaluated the rat kidney cells (3) subsequently found, that changes in calmodulin acceptor proteins, and not calmodulin itself, were associated with viral transformation.

In conclusion, we have found that calmodulin inhibitors are cytotoxic for human RCC but that unlike the case in some animal tumor models, there is no simple relationship between calmodulin and tumor cell growth. In addition, our studies show that extrapolations from "in vitro" studies to the clinical situation must be made with extreme caution.

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