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Effects of Activators and Inhibitors of Protein Kinase C On X-ray Induced Malignant Transformation *in vitro*

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We have examined the effects on X-ray induced malignant transformation *in vitro* of a number of activators and inhibitors of protein kinase C (PKC). Several of these substances were found to enhance or inhibit transformation, and the extent of the effects on transformation were found to be consistent with the potencies of the substances in activating or inhibiting PKC. Additionally, the observed transformation enhancement was found to be reversed by the presence of the anticarcinogenic protease inhibitors antipain or the Bowman–Birk inhibitor. These results suggest that activation of protein kinase C may be involved in the mechanism of *in vitro* X-ray induced malignant transformation.
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INTRODUCTION

NUMEROUS EXTRACELLULAR messengers have been shown to activate cells by signal transduction acting through membrane-associated inositol lipid breakdown pathways [1]. A significant role for these pathways in regulation of cell growth and division has been suggested by the fact that these messengers include several polypeptide hormones and growth factors [1]. There is now considerable evidence that inositol lipid metabolism may be involved in malignant transformation as well, as a number of oncogene products have been shown to resemble either growth factor-related components of these pathways [2–4] or enzymes which participate in producing such components [5, 6].

Additionally, the enzyme protein kinase C (PKC), which is activated by inositol lipid breakdown and which contributes to the signal transduction process [7], has been shown to be the cellular receptor for the tumour promotor 12-0-tetradecanoyl-phorbol-13-acetate (TPA) [8].

Given the growing evidence that inositol lipid-related pathways may be involved in the process of malignant transformation, we have decided to investigate the effects of activators and inhibitors of these pathways on radiation-induced transformation in the C3H10T1/2 cell line. This report details the effects on 4.0 Gy- or 6.0 Gy-induced transformation of three inhibitors of PKC, trifluoperizine (TFP) [9], *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W-7) [9] and 1-(5-isoquinolinesulfonyl)-2-methyl-piperazine (H-7) [10], two activators of PKC, the diacylglycerol analogues *sn*-1,2-dioctanoylglycerol (DG-8) and *sn*-1,2-didecanoylglycerol (DG-10) [11, 12], and an activator of PKC as well as of other cellular activities, the calcium ionophore A23187 [13]. Additionally, we have examined the effects of the latter three compounds when administered in the presence of the anticarcinogenic protease inhibitors antipain and the Bowman–Birk inhibitor (BBI), both of which have been shown to be effective in suppressing radiation-induced transformation in this system [14–16].

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MATERIALS AND METHODS

The C3H/10T1/2 transformation assay has been used extensiveiy in our laboratory for radiation transformation experiments. Details of our experimental techiques for radiation transformation experiments using 10T1/2 cells with protease inhibitors and promoting agents have been described in detail elsewhere [14, 16]. Irradiation was performed with a Philips MG-100 industrial unit (100 kV, 10 mA) with 0.795 mm aluminium filtration, yielding a dose rate of 0.835 G/min. Stock cultures were maintained in 60 mm petri dishes and were passed by subculturing at a 1:20 dilution every 7 days. The cells used were in passages 9–14. They were grown in a humidified 5% CO_2 atmosphere at 37°C in Eagle's basal medium supplemented with 10% heat-inactivated fetal bovine serum and gentamicin. (In all transformation experiments, the concentration of serum was reduced to 5% on day 10 and was maintained at that concentration throughout the remainder of the 6-week assay period.) Plating efficiencies were determined from three plates seeded with a cell density one-fifth that of plates used for the transformation assay; these cultures were terminated at 10 days. The various treatment toxicities were considered in the design of the experiments. It was planned that dishes to be used for the transformation assay would contain ~300 viable cells per dish. Types 2 and 3 foci were scored as transformants. Transformation results have been analysed in terms of the fraction of dishes with transformed foci, a method of analysis recommended for C3H10T1/2 cell transformation assays [19]. If the results presented are analysed in terms of transformation frequency per surviving cell, the same conclusions can be drawn; this method has not been utilised, however, as cell density has been shown to influence frequency per surviving cell measurements [19–21].

The concentrations and sources of the chemical compounds used in these studies were: TPA (lot 031, Consolidated Midland Co.) 0.1 μg/ml, BBI and antipain were used at the concentrations indicated in Tables 1–3. Antipain was purchased from the Sigma Chemical Co. The purified Bowman–Birk protease inhibitor was prepared by us using a modification of the procedure originally described by Birk; this modified procedure has been described

Table 1. Effect of trifluoperazine dihydrochloride (TFP), an inhibitor of protein kinase C, on radiation transformation in vitro

Treatment	Plating efficiency [% (S.E.)]	Total no. of viable cells at risk	Total no. of foci	Fraction of dishes containing transformed foci (types 2 and 3)*†
Controls—no treatment	33.8 (7.6)	9392	0	0/25
6.0 Gy	2.3 (0.4)	22603	23	22/75 = 0.29
6.0 Gy + TFP 1 μmol/l	1.5 (0.3)	15900	6	5/70 = 0.07

*Combined results of three separate experiments showing similar trends in the data.
†Statistical analysis (χ^2): group 2 vs. 3, $P<0.001$.

in detail elsewhere [15]. TPA was dissolved in acetone (the final concentration of acetone in the media was 0.5%, a concentration of acetone shown previously to have no effect in the C3H/10T1/2 transformation assay system). Antipain and the Bowman–Birk protease inhibitors were dissolved in water. Other chemicals utilised were obtained as follows: H-7 and W-7 were from Cal. Biochem. (La Jolla), TFP was from Aldrich, A23187 was from Sigma and DG-8 and DG-10 were from Serdary Research Labs, Inc. (Ontario). For all transformation experiments, compounds were added immediately after the radiation exposure and were replenished after each medium change at the concentrations indicated in Tables 1–3. All compounds were initially studied at the highest non-toxic concentrations for C3H10T1/2 cells (as determined in preliminary toxicity studies).

RESULTS AND DISCUSSION

Table 1 indicates that TFP significantly reduces X-ray induced transformation in C3H10T1/2 cells. Other inhibitors of PKC,

Table 2. Effects of H-7 and W-7 on radiation transformation with and without enhancement by TPA

Treatment*	Plating efficiency (%)	Total no. of viable cells	Total no. of foci observed (types 2 and 3)	Fraction of dishes containing transformed foci (types 2 and 3)†
Controls	42.0	12600	0	0/30
4 Gy	7.0	20580	4	4/49 = 0.08
4 Gy + TPA	6.8	19176	162	45/47 = 0.96
4 Gy + TPA + H-7	6.1	6222	19	7/17 = 0.41
4 Gy + TPA + W-7	6.3	16522	117	45/49 = 0.92
6 Gy	1.8	12960	16	16/48 = 0.33
6 Gy + H-7	1.6	11710	23	19/49 = 0.39
6 Gy + W-7	1.4	10080	12	12/48 = 0.25

*The concentrations of H-7 and W-7 utilised in these experiments were 3 μmol/l.
†Statistical analysis (χ^2): group 2 vs. 3, $P<0.001$; 3 vs. 4, $P<0.001$; 3 vs. 5, $P>0.05$; 6 vs. 7, 8, $P>0.05$.

Table 3. Effect of 10 μmol/l W-7, an inhibitor of protein kinase C, on radiation transformation in vitro

Treatment	Plating efficiency (%)	Total no. of viable cells	No. of foci (types 2 and 3)	Fraction of dishes containing transformed foci	
				Type 3	Types 2 and 3†
Controls—no treatment	41.6	4488	1	0/12	1/12
6.0 Gy	1.1	2530	16	8/23 = 0.35	12/23 = 0.52
6.0 Gy + W-7* (10 μmol/l)	0.3	600‡	17	3/20 = 0.15	11/20 = 0.55

*W-7 = *N*-(16-aminohexyl)-5-chloro-1-napthalene sulphonamide.
†Statistical analysis (χ^2): group 2 vs. 3, $P>0.05$.
‡The toxicity of W-7 at 10 μmol/l resulted in a lower cell density than ordinarily utilised in such experiments. This is not expected to affect the yield of transformants, however, as discussed in detail elsewhere [20, 21].

specifically, H-7 and W-7, were not capable of suppressing radiation transformation when applied at 3 μmol/l, a non-toxic concentration of these agents in the cells utilised, as shown in Table 2. To determine whether a higher concentration of W-7 might have an effect, a toxic concentration (10 μmol/l) of W-7 was studied in the system; this higher concentration of W-7 was also observed to have no effect on radiation transformation *in vitro*, as shown in Table 3. In this experiment, due to the toxicity of the 10 μmol/l W-7, only 30 viable cells per dish resulted in the dishes receiving 6 G and 10 μmol/l W-7; 30 cells per dish is considerably lower than the approximately 300 cells per dish usually present in such experiments. Although this cell density is low, our previous work has shown that cell density does not influence the final yield of radiation induced transformants, as discussed in detail elsewhere [20, 21]. Our results with these different inhibitors of PKC are not surprising considering the observation of Helfman *et al.* [19], that TFP is a more potent inhibitor than W-7 (on a molar basis) of PKC-dependent phosphorylation in human neutrophils.

While neither H-7 or W-7 were capable of affecting radiation transformation, H-7 suppressed the enhancement of radiation transformation by TPA in a highly significant fashion, as shown in Table 2. The mechanism of inhibition of PKC is different for the compounds; H-7 has been reported to act at the phospholipid cofactor site, while W-7 acts at Ca^{2+}-calmodulin sites [10]. W-7 is a potent calmodulin antagonist which acts as a weak inhibitor of many protein kinases at high concentrations, while H-7 is a more selective inhibitor of PKC which inhibits the enzyme at lower concentrations [10]. Thus, our results are consistent with the relative ability of these compounds to inhibit PKC.

The results presented in Table 4 indicate that the calcium ionophore A23187 significantly increases radiation transformation yields at the higher concentration used (5×10^{-8} mol/l). This enhancement is reversed by the presence of the protease inhibitors antipain or BBI.

The PKC activator DG-8 is seen to increase transformation yields significantly, while DG-10 has no effect on transformation in this system (Table 5). It should be noted that at the DG-8 and DG-10 concentrations used in our study (20 μmol/l), Lapetina *et*

Table 4. Effect of the Ca ionophore A23187, an activator of protein kinase C, on radiation transformation, in the presence and absence of protease inhibitors

Treatment	Plating efficiency (S.E.)	Total no. of viable cells	Total no. of foci (types 2 and 3)	Fraction of dishes containing transformed foci (types 2 and 3)*†
Controls—no treatment	37.1 (3.2)	9470	0	0/27
6.0 Gy	1.7 (0.6)	15952	11	10/76 = 0.13
6.0 Gy + Ca ionophore 5×10^{-8} mol/l	0.7 (0.4)	13964	30	25/70 = 0.36
6.0 Gy + Ca ionophore 2×10^{-8} mol/l	1.7 (0.8)	10710	8	7/55 = 0.13
6.0 Gy + Ca ionophore (5×10^{-8} mol/l) + antipain 50 μg/ml	1.4 (0.5)	12640	2	2/48 = 0.04
6.0 Gy + Ca ionophore (5×10^{-8} mol/l) + BBI (1 μg/ml)	1.1 (0.4)	13580	5	5/55 = 0.09

*Combined results of three separate experiments showing similar trends in the data.
†Statistical analysis (χ^2): group 2 vs. 3, $P<0.01$; group 2 vs. 4, $P>0.05$; group 3 vs. 5 or 6, $P<0.001$.

al. [11] found that DG-8 induced more rapid PKC-dependent phosphorylation of a 40000 dalton protein in human platelets than did DG-10, and that the level of this DG-8 induced phosphorylation appeared to plateau at a relatively high value while that of DG-10-induced phosphorylation decreased immediately after reaching its peak.

Table 5 also indicates that the enhancement of transformation induced by DG-8, analogous to that induced by the calcium ionophore A23187 (Table 4), is reversed by the presence of the anticarcinogenic protease inhibitors antipain or BBI. The nature of the putative protease involved is not known at this time, although Nishizuka *et al.* have reported that PKC can be activated by limited proteolysis with an associated Ca^{2+}-dependent neutral protease, to produce a permanently active form of the enzyme (protein kinase M) [17, 18]. While the millimolar concentrations of calcium involved make the physiological significance of this activation reaction uncertain, the protease is found to be inhibited by the anticarcinogenic protease inhibitor leupeptin [22], and other Ca^{2+} dependent neutral proteases have been found to be inhibited by antipain [23, 24].

The results presented in this paper indicate that X-ray induced transformation *in vitro* can be enhanced or inhibited by PKC activators or inhibitors, and that the effects of these activators or inhibitors on transformation occur in a manner consistent with their potencies in affecting the activity of PKC. This suggests that PKC activation, and perhaps other Ca^{2+}-mediated processes, may be involved in the mechanism of X-ray induced transformation.

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Table 5. Effect of activators of protein kinase C (DG-8 and DG-10) with and without protease inhibitors on radiation transformation*

Treatment	Exp. no.	Plating efficiency (%)	Total no. of viable cells	Total no. of foci	Fraction of dishes containing transformed foci: Per experiment (type 3)	Per experiment (types 2 and 3)	Total (types 2 and 3)
Controls—no treatment	1	54.3	5724	0	0/12	0/12	1/24 = 0.04
	2	30.6	3300	1	0/12	1/12 = 0.08	
4.0 Gy	1	9.0	12420	4	0/23	4/23 = 0.17	6/43 = 0.14
	2	6.5	7800	3	0/20	2/20 = 0.10	
4.0 Gy + DG-8 (20 μmol/l)	1	8.8	10032	6	3/19 = 0.16	6/19 = 0.32	14/40 = 0.35
	2	6.9	8694	10	4/21 = 0.19	8/21 = 0.38	
4.0 Gy + DG-10 (20 μmol/l)	1	7.5	9450	3	2/21 = 0.10	3/21 = 0.14	3/21 = 0.14
4.0 Gy + DG-8 (20 μmol/l) + Antipain (50 μg/ml)	2	7.2	9504	0	0/22	0/22	0/22
4.0 Gy + DG-8 (20 μmol/l) + BBI (1.0 μg/ml)	2	5.9	7080	0	0/20	0/20	0/20

*Abbreviations: DG-8, 1,2 Dioctanonin = *sn*-1,2 dioctanylglycerol; DG-10, 1,2 Dicaprin = *sn*-1,2 didecanoylglycerol; BBI, Bowman–Birk protease inhibitor.
Statistical analysis (χ^2): group 2 vs. 3, $P>0.05$; group 2 vs 4, $P<0.05$; group 3 vs. 5, $P<0.01$; group 3 vs. 6, $P<0.01$.

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