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DMSO INCREASES TYROSINE RESIDUE PHOSPHORYLATION IN MEMBRANES FROM MURINE ERYTHROLEUKEMIA CELLS

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SUMMARY: Phosphorylation of membranes from murine erythroleukemia cells was performed in the presence and absence of the polar solvent dimethyl sulfoxide. Quantitation of the phosphoamino acid content revealed that DMSO stimulated phosphotyrosine accumulation by three-fold; serine and threonine phosphorylation decreased significantly. We had previously shown that DMSO stimulated tyrosine residue phosphorylation of the hepatic epidermal growth factor receptor. EGF had little effect in MEL membranes; therefore, DMSO results in accumulation of phosphotyrosine in cell membranes that do not exhibit significant EGF-dependent phosphorylation.

Exposure of Friend murine erythroleukemia (MEL) cells to the highly polar solvent, DMSO, for one cell cycle results in commitment to terminal differentiation (1). Within 3-5 days, more than 75% of the cells stop proliferating and express membrane and intracellular proteins characteristic of erythrocytes (2,3). The mechanism of DMSO action is unknown, but recent evidence suggests that changes in membrane function and properties (e.g., altered ion flux) play a pivotal role in differentiation (4-7). We have observed that DMSO alters phosphorylation in rat liver membranes (8). The most dramatic effect of DMSO was selective stimulation of the hepatic EGF receptor tyrosine residue kinase. A more general decrease in membrane serine and threonine phosphorylation was also observed (8,9). Since alteration in protein phosphorylation is one bio-

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Abbreviations: DMSO, Dimethyl sulfoxide; EGF, epidermal growth factor; MEL, murine erythroleukemia; P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine.

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chemical mechanism by which membrane function can be regulated, we tested whether DMSO would stimulate MEL membrane tyrosine residue kinase activity.

METHODS

MEL cells (GM086C obtained from NIGMS Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, NJ) were grown to a density of 3 x 10^6/ml in Eagle's MEM medium supplemented with essential amino acids, vitamins, two times non-essential amino acids, and 15% unheat-treated fetal calf serum. The cells were washed with .15 M NaCl, resuspended in 10 mM phosphate buffer with 1 mM EDTA, pH 7.4, and disrupted with a Brinkman Polytron apparatus (setting 7.5, two 15 sec bursts). An equal volume of 10 mM phosphate buffer with 1 mM EDTA, 0.5 M sucrose, pH 7.4 was added, and the homogenate was centrifuged at 1000 rpm for 5 min in a Sorvall RC-3 centrifuge. A crude membrane fraction, prepared by centrifugation of the supernatant at 105,000 x g, was resuspended in 20 mM Pipes buffer, pH 7.0 and phosphorylated (1 mg protein per reaction) in the presence of 30 mM $MgCl_2$ as previously described (8-10). The reaction was initiated by adding 1, 5 or 10 μM ATP (25-50 μCi γ^{32}P-ATP) after a 10 min, 0°C preincubation with various concentrations of DMSO or 1 μg/ml EGF. The reaction was terminated with 50% trichlorocetic acid, and the precipitates were washed, dried and hydrolyzed at 110°C. Two-dimensional phosphoamino acid separation with added P-Ser, P-Thr and P-Tyr standards was performed by the method of Hunter and Sefton (11) as previously described (10). Following autoradiography, phosphoamino acids were quantitated by scraping, eluting and scintillation counting. The proportion of γ^{32}P-ATP remaining at the end of the reaction was quantitated by withdrawing an aliquot prior to trichlorocetic acid precipitation. Following precipitation with perchloric acid, the supernatant was neutralized and chromatographed on cellulose PEI plates with lithium chloride buffer. The ATP spot was identified by uv absorption of added standard ATP and was scraped and counted. Statistical significance was determined by paired t analysis.

RESULTS

Figure 1 demonstrates the effect of EGF and DMSO on phosphoamino acid content of MEL cell membranes. DMSO clearly increased P-Tyr, while EGF caused little or no change. Figure 2 shows the dose-dependent change in phosphoamino acid content of membranes incubated with various concentrations of DMSO. A progressive increase in P-Tyr occurred while a concomitant fall in P-Ser and P-Thr was observed. Cumulative data from nine experiments revealed that 15-20% DMSO caused a 3.2 ± 0.5 fold increase in P-Tyr incorporation (mean ± S.E.M., $p<0.001$). This represented a change in the % P-Tyr abundance (P-Tyr divided by the incorporation of all three phosphoamino acids) from 0.15 ± .09% to 1.45 ± 0.32% ($p<.001$). Absolute P-Ser and P-Thr content fell by 56 ± 2% and 45 ± 3% respectively ($p<.05$). In contrast, EGF did not alter serine or threonine phosphorylation and the effect on tyrosine phosphorylation was variable. In seven experiments, the P-Tyr content of membranes incubated with EGF was 42% ± 18% above control membranes incubated with water of phosphate-buffere

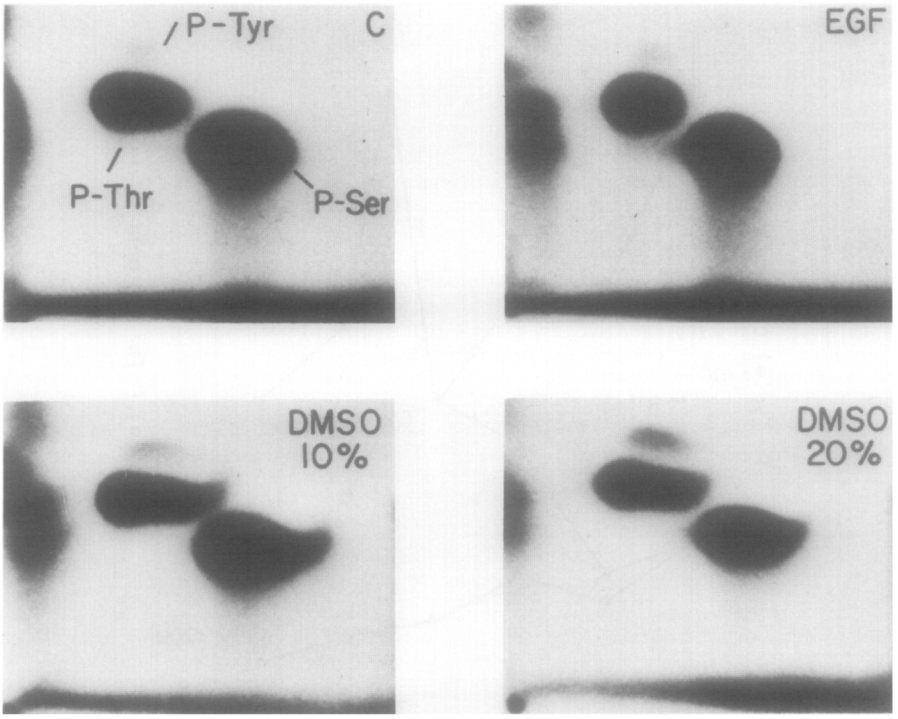

Figure 1 Phosphoamino Acid Analysis
After phosphorylation with H_2O control; 1 µg/ml EGF; 10% DMSO; and 20% DMSO, hydrolysis and separation of phosphoamino acids was performed. The autoradiogram was developed after a 24 hour exposure at -70°C using a DuPont Cronex intensifying screen.

saline ~ 0.1% albumin. The increase was not significant ($p>0.10$). Little or no ^{125}I-EGF binding was detected in these membranes, nor was any EGF-dependent phosphorylation of the 170,000 dalton EGF receptor seen after polyacrylamide gel electrophoresis and autoradiography (10) (data not shown).

The possibility that the rise in P-Tyr was simply a reciprocal change due to a decrease in serine and threonine phosphorylation with the attendant changes in ATP availability was addressed in three types of experiments. First, the increase in P-Tyr in the presence of DMSO was similar at 1, 5 and 10 µM ATP. Second, the proportion of ATP remaining at the end of the 1 min, 0°C phosphorylation reaction was 70-80%; it was not changed by either EGF or DMSO. Lastly, Table 1 shows the results of incubating DMSO and a control solvent, acetone, with membranes from two strains of MEL cells prepared and assayed together (GM086C and 6A11A isolated by Axelrod and coworkers (12)). Under these conditions,

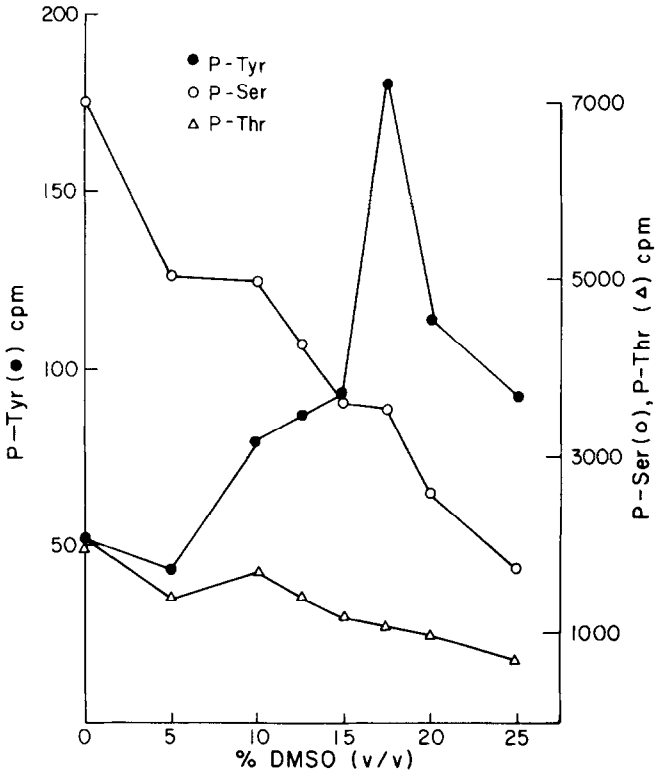

Figure 2 Phosphoamino Acid Analysis of Membranes Incubated with Various Concentrations of DMSO

Phosphoamino acid spots were identified by ninhydrin staining. The three spots were scraped, eluted and quantitated by scintillation counting.

acetone decreased P-Ser incorporation to the same extent that DMSO did but there was no comparable rise in P-Tyr content.

Table 1 Phosphoamino acid analysis (cpm per individual spot) of membranes from two strains of MEL cells phosphorylated with H_2O, acetone (20%), or DMSO (17.5%).

	STRAIN	P-Ser	P-Thr	P-Tyr
H_2O	GM086	27010	8412	52
	6A11A	36089	10702	57
Acetone	GM086	17500	4423	70
	6A11A	15111	4113	64
DMSO	GM086	15724	6233	254
	6A11A	17627	7466	249

DISCUSSION

We have previously shown that DMSO selectively stimulates tyrosine residue phosphorylation of the hepatic EGF receptor. When crude membrane fractions from liver are analyzed, EGF and DMSO are equally effective in increasing P-Tyr. Since the two stimuli are not additive, we concluded that they affect the same tyrosine residue kinase (8,9). In contrast, DMSO increases P-Tyr in MEL membranes in the absence of significant EGF-dependent tyrosine kinase activity. The data do not completely exclude EGF-dependent EGF receptor phosphorylation in MEL cells, but if present, it represents a small percentage of the tyrosine phosphorylation stimulated by DMSO. Thus, DMSO causes P-Tyr accumulation by at least two tyrosine residue kinases, the EGF receptor kinase and a distinct activity in MEL cell membranes. The mechanism by which DMSO stimulates tyrosine incorporation was not elucidated by these experiments. Three possibilities exist: DMSO directly activates the MEL membrane tyrosine kinase; the solvent alters the conformation of substrates making them more susceptible to tyrosine residue phosphorylation; or DMSO retards tyrosine residue dephosphorylation. DMSO does not alter tyrosine dephosphorylation in liver membranes (unpublished results); therefore, it seems more likely that one of the first two explanations is correct.

Does DMSO stimulation of tyrosine residue phosphorylation play a role in MEL differentiation? The concentrations of DMSO needed to maximally stimulate tyrosine phosphorylation (15-20%) are higher than those used for differentiation in intact MEL cells (1-3%). These higher concentrations would be toxic in the prolonged incubation. However, if smaller changes in tyrosine phosphorylation are caused by 1-3% DMSO at 37^{o}C in intact cells they might be sustained for the 10-12 hours required for commitment. It has been suggested that regulation of ATPase activity (a 40% decrease) is important in the differentiation of MEL cells (5-7). DMSO affects Na+/K+ ATPase-mediated transport in intact MEL cells at 1-3% (5). However, in order to produce acute changes in ATPase activity in isolated membranes 10-20% DMSO is required (13,14). Therefore, the expression of the biologic effects in intact cells may occur at concen-

trations of DMSO lower than those needed to detect the effect in membranes. This is analogous to the effects of EGF; much higher concentrations are needed to detect EGF-dependent phosphorylation in membranes than are needed to stimulate cell division (15-17). Alternately, the decreases in serine and threonine phosphorylation may mediate the effects of DMSO. Further experiments will be needed to determine what role alterations in membrane phosphorylation play in DMSO action.

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