Vol. 204, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1994 Pages 264-272

Modulation of Human Melanoma Cell Metastasis and Adhesion May Involve Integrin Phosphorylation Mediated Through Protein Kinase C

Jennifer A. Dumont* and Alan J. Bitonti

Marion Merrell Dow Research Institute, 2110 E. Galbraith Road
Cincinnati, OH 45215

Received August 19, 1994

SUMMARY: A correlation between changes in protein kinase C (PKC) activity and tumor metastasis has been reported previously with several murine tumor cell lines. Treatment of a human metastatic melanoma cell line, M24met, with phorbol ester, phorbol-12-myristate-13-acetate (PMA), followed by injection into the tail vein of scid mice doubled pulmonary metastasis. Adhesion of M24met cells exposed to PMA, was enhanced to collagens I and IV, but not to laminin or fibronectin, suggesting a change in specific adhesion receptors on the tumor cells. Treatment of M24met cells with PMA did not affect *de novo* synthesis of integrin subunits (α_2, α_3, β_1) known to form collagen receptors. However, PMA stimulated the phosphorylation of integrin subunits α_3 and β_1 on serine. Therefore, PMA effects on metastasis and cell adhesion may occur through PKC-mediated phosphorylation of integrins.

INTRODUCTION Metastasis is the major cause of treatment failure and mortality in cancer patients [1]. To metastasize, cells must escape from the primary tumor, invade the local host tissue to enter the circulation, adhere to vascular endothelium and ultimately extravasate [2]. Blockage of any one of these steps will theoretically prevent the formation of a metastatic colony.

Cell adhesion receptors such as integrins, have been implicated as important factors in metastasis [3,4]. These adhesion receptors are heterodimeric cell surface transmembrane proteins comprised of an α and a β subunit that bind adhesion proteins such as fibronectin, fibrinogen, collagen and laminin [5,6]. A role for integrins in

*FAX: (513) 948-6337.

0006-291X/94 $5.00

metastasis was suggested in experiments in which synthetic peptides containing the RGD sequence (arginine-glycine-aspartic acid), which constitutes the receptor recognition site [7], were co-injected with tumor cells with a resultant decrease in pulmonary metastasis in an experimental murine model [8]. Transfection of cDNA for α_2 integrin (VLA-2) into rhabdomyosarcoma cells, which normally do not express the $\alpha_2\beta_1$ integrin and are not metastatic, resulted in the acquisition of a metastatic phenotype [9]. A role for integrins in metastasis has thus been established, but the mechanistic details regarding function and regulation remain elusive.

Phosphorylation/dephosphorylation may alter integrin-ligand binding affinities [10,11], and it is possible that this may be a mechanism for altered adhesive properties of metastatic cells. A role for protein kinase C (PKC) in metastasis has been implicated in several studies [12-14]. A strong correlation between basal levels of membrane-bound PKC and the metastatic potential of tumor cells has been described [12]. Highly metastatic tumor cells had greater levels of membrane-associated PKC activity when compared to tumor cells of lower metastatic capacity. Treatment of B16 murine melanoma cells with PMA for 1 h caused translocation of PKC to the membrane and increased metastasis to the lungs in an experimental model. Prolonged treatment (24 h) with PMA down-regulated PKC with a concomitant decrease in metastatic potential. Because membrane-bound PKC is known to phosphorylate membrane associated proteins [15], it is possible that through phosphorylation of membrane proteins (possibly integrins), adhesion may be regulated [12].

We have recently demonstrated that treatment of B16F1 murine melanoma cells with the PKC inhibitors diminished the metastatic capacity of the cells, inhibited adhesion to specific adhesion proteins (fibrinogen and collagen IV) found in serum and the basement membrane/ECM, respectively, and also showed preliminary effects on phosphorylation of unidentified proteins [16]. To further define the role of PKC in metastasis and to make these studies relevant to human cancer, the effect of PMA has been studied using a metastatic human melanoma cell line, M24met [17].

METHODS AND MATERIALS

Chemicals. Collagen IV (human placenta), leupeptin, phenylmethylsulfonyl fluoride, histone H1 (type III-S), phosphatidylserine, 1,2-diolein (1,2-dioleoyl-sn-glycerol) and phorbol-12-myristate-13-acetate were purchased from Sigma; laminin (human) was from Telios Pharmaceuticals, Inc.; fibronectin (human serum) from Boehringer Mannheim; collagen I (human) and anti-integrin α_3 monoclonal antibody (clone PIB5) were from Collaborative Biomedical Products; anti-integrin β_1 monoclonal antibody (clone AT29/12.P.38) was from Research Diagnostics, Inc.; [γ^{32}P]-ATP (10 Ci/mmol) and [^{32}P]-orthophosphate (8500-9120 Ci/mmol) were from New England Nuclear.

Cell Culture. M24met human melanoma cells were graciously provided by Drs. Ralph A. Reisfeld and Barbara M. Mueller at the Scripps Institute [17]. The cells were grown in RPMI 1640 medium supplemented with 10 % FBS and removed from flasks with EDTA (0.5 mM EDTA, 0.15 M NaCl, 0.02 M HEPES).

Experimental Metastasis Assay. The assay was carried out as described by Fidler [18]. Subconfluent M24met cells were harvested, washed by suspension in Hanks Balanced Salt Solution (HBSS) and centrifugation, then counted after dilution with trypan blue to estimate cell viability. A single cell suspension of 10^5 cells in 0.2 ml HBSS was injected via the tail vein into 6 to 8 week old female scid mice (C.B-17/IcrTac-scidfDF, from Taconic). Three weeks later, the lungs were excised and the number of metastatic nodules were counted after staining for 24 h in Bouin's solution [saturated aqueous picric acid - 40 % formaldehyde - glacial acetic acid (15:5:1 by volume)].

Preparation and Determination of Cytosolic and Membrane-Associated Protein Kinase C. Cytosolic and membrane-associated PKC were prepared and the activity quantitated as described [16,19]. Aliquots of the enzyme were stored at -80° C and were stable for at least 3 months. PKC activity was quantitated by measuring the transfer of ^{32}P from [$\gamma^{32}P$]-ATP to histone H1.

Adhesion of Tumor Cells to Immobilized Proteins. Adhesion of tumor cells to immobilized proteins was assayed as described previously (16) with the modification that these tumor cells were harvested with 0.5 mM EDTA containing 0.15 M NaCl and 0.02 M Hepes (15 min at 37°C).

Metabolic Labelling and Immunoprecipitations. Cells were added to 100 mm tissue culture dishes, allowed to grow for 48 h, incubated for 60 min in phosphate-free RPMI containing 5 % FBS and then labelled with 1 mCi/ml [^{32}P]-orthophosphoric acid for 90 min at 37° C. Cell monolayers were rinsed twice with PBS, then solubilized with ice cold lysis buffer [50 mM Tris, pH 8.0, 150 mM NaCl, 0.02 % NaN_3, 0.1 % sodium dodecyl sulfate (SDS), 1 % Nonidet P-40, 0.5 % deoxycholate, 1 µg/ml aprotinin, 0.6 mM phenylmethylsulfonyl fluoride, 0.5 mM Na_3VO_4, 50 mM NaF and 30 mM Na_2HPO_4] and incubated for 20 min on ice. The samples were subjected to centrifugation at 500 x g for 15 min at 4° C to remove debris, followed by removal of any remaining insoluble material from the supernatant using ultracentrifugation (60,000 x g for 20 min). Equivalent amounts of protein (BioRad DC protein assay) were mixed with 15 µl/ml protein A or G-plus agarose (Oncogene Science) and 10 µg/ml anti-integrin antibody and mixed for 16 h at 4°C. The immunoprecipitate was collected by sedimentation using centrifugation at 440 x g for 15 min at 4°C and washed 3 times with lysis buffer as above. Proteins were solubilized with 50 µl sample buffer, boiled for 5 min and then separated by electrophoresis on SDS-polyacrylamide gels. The gels were dried, then exposed to Kodak X-OMAT film. Quantitation of phosphorylation was performed using densitometry.

Phosphoamino Acid Analysis. Phosphoamino acid analysis of ^{32}P-labelled integrin subunits was determined by the double electrophoresis method [20,21]. The bands were excised from the dried gel, hydrated with 600 µl 50 mM $NaHCO_3$ containing 0.1 % SDS and 5 % β-mercaptoethanol, crushed and then incubated on a rotating mixer overnight at 37° C. BSA (30 µg) was added as a carrier, proteins were precipitated with 15 % trichoroacetic acid for 1 h on ice, and sedimented in a Speed-Vac. The protein pellet was washed once with ice cold ethanol, dried and then hydrolyzed by gaseous phase 6N HCl containing 10 µl phenol for 2 h at 110°C. Samples were lyophilized, hydrated with water and spotted on cellulose TLC plates (10 x 10 cm) along with 3 µg each of O-phospho-DL-serine, O-phospho-DL-threonine and O-phospho-DL-tyrosine. Elecrophoresis in the first dimension was for 22 min at 1500 V with pH 1.9 buffer (7.8 % acetic acid, 2.5 % formic acid), the plate was rotated 90° and then run in the second dimension for 17 min at 1000 V with pH 3.5 buffer (5 % acetic acid, 0.5 % pyridine). After drying, the plates were sprayed with ninhydrin to locate the amino acid standards and then exposed to Kodak X-OMAT film to detect radiolabelled phospho-amino acids.

Table 1 Effect of PMA on M24met cell experimental metastasis

Treatment	Number of lung metastases	Mean ± SE	% Control
None	4,4,7,10,16,63,72,80,95,110	46 ± 13	100
100 nM PMA	9,22,58,105,112,128, 137,143,174,186	107 ± 19*	233

* significantly different ($p < 0.05$).

Subconfluent M24met cells were treated with PMA for 30 min at 37°C. Cells were harvested with an EDTA solution and injected intravenously into scid mice. Three weeks later, the number of metastatic colonies were counted.

RESULTS AND DISCUSSION

Augmentation of experimental metastasis by PMA has not been demonstrated previously for human tumor cells. The present study shows that PMA more than doubled experimental metastasis of M24met human melanoma cells to the lungs of scid mice (Table 1). Membrane PKC activity in M24met cells treated with PMA was increased, while cytosol enzyme activity was decreased compared to untreated cells (Table 2). These results are consistent with studies by Gopalakrishna, et al. in which PMA was shown to increase hematogenous metastasis of murine B16F1 cells (12).

Adhesion to individual proteins was investigated since this represents a critical step in metastasis. Exposure of M24met cells to 100 nM PMA increased adhesion to collagens I and IV with minimal effect on adhesion to either laminin or fibronectin (Table 3). These data suggest a specific effect on collagen receptors on the tumor cells. Collagen IV is a major component of the basement membrane, and adhesion to this protein may be critical in movement of tumor cells past the endothelial layer and through the basement membrane/ECM (2). Collagen I also exists in the subendothelial matrix. Because collagen IV is a major component of the basement membrane/ECM , it was postulated that drug treatment may affect tumor cell adhesive properties either by decreasing levels of cell surface receptors for collagens or by altering the affinity of the tumor cell receptor for its ligand possibly through direct or indirect phosphorylation by PKC. Phosphorylation of cell surface receptor molecules is known to affect the affinity of

Table 2 Increase in Protein kinase C activity by PMA

	Protein kinase C pmol/mg protein/min	
Treatment	Membrane	Cytosol
None	4598	12,765
PMA, 15 min	21,218	1701
PMA, 30 min	13,986	884

M24met cells were incubated with 100 nM PMA for 15 or 30 min. Membrane-associated and cytosolic PKC were prepared and PKC activity was assayed as described in 'Methods and Materials'.

Table 3 Effect of PMA on adhesion of M24met cells to immobilized proteins

Adhesion Protein	Treatment	Total Number of Adherent Cells*	% Control
Collagen I	none	20132 ± 1472	100
	PMA	38637 ± 1582	192
Collagen IV	none	5549 ± 636	100
	PMA	10602 ± 1119	191
Laminin	none	36954 ± 1312	100
	PMA	37717 ± 1134	102
Fibronectin	none	31396 ± 686	100
	PMA	41684 ± 1935	133

* mean ± S.E.

M24met cells were labelled metabolically with [^{3}H]-thymidine for 24 h at 37°C. Cell monolayers were exposed to PMA for 30 min, then harvested and added to protein-coated wells and incubated for 60 min at 37°C. Non-adherent cells were removed by gentle washing. Adherent cells were quantitated by liquid scintillation counting.

certain receptors for their ligands [22]. Molecules known to be receptors for collagens are members of the integrin family of cell adhesion molecules (i.e. $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$). We examined the synthesis of each of these subunits (M24met cells are known to express

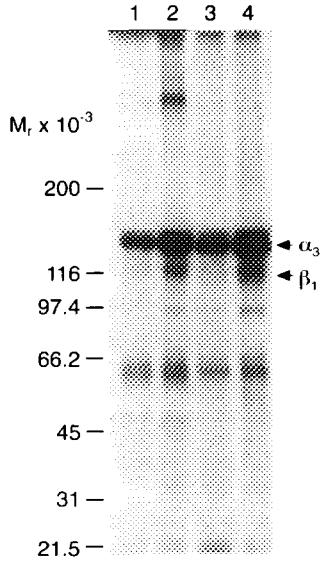

Figure 1. Integrin phosphorylation in M24met cells. Cells were labelled with [^{32}P]-orthophosphate for 90 min and PMA (100 nM) was added during the final 30 min of the phosphate labelling period to some cells. Cell lysates were immunoprecipitated with monoclonal antibodies to α_3 and β_1 integrins followed by separation of proteins by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (4 -12 % gradient gels) as described in "Materials and Methods". Electrophoresis was under non-reducing conditions. *Lanes 1-2*, immunoprecipitation with anti-α_3 integrin; *lanes 3-4*, immunoprecipitation with anti-β_1 integrin. *Lanes 1 and 3* represent control cells; *lanes 2 and 4*, cells treated with 100 nM PMA (30 min).

α_2, α_3, and β_1 integrins; personal communication, Barbara Mueller and Anthony Montgomery, the Scripps Institute) and found them to be unchanged in response to drug treatment. The α_1 integrin was undetectable in control M24met cells. Several integrin

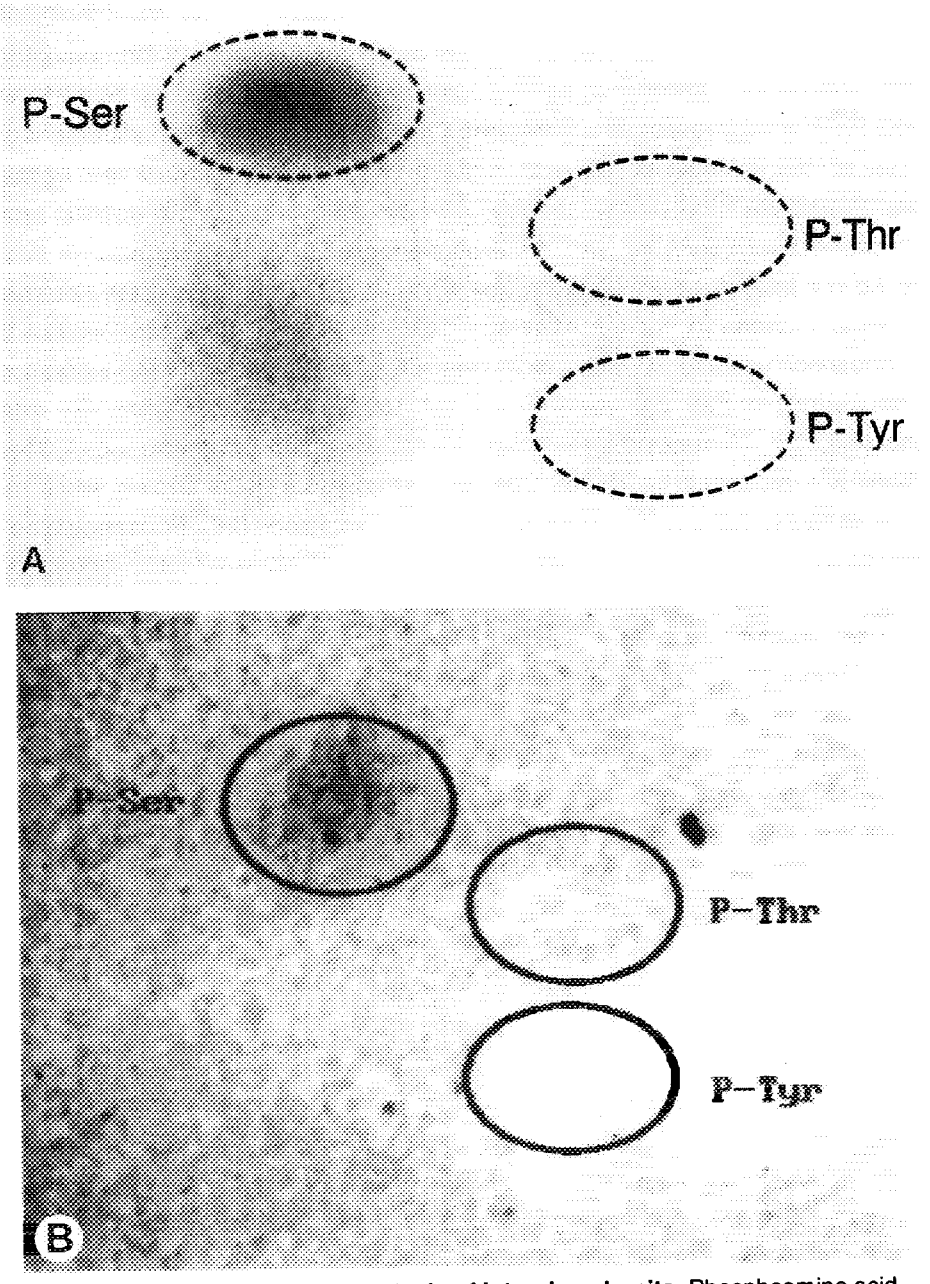

Figure 2. Phosphoamino acid analysis of integrin subunits. Phosphoamino acid analysis was performed by double electrophoresis as described in "Methods and Materials" on bands excised from the polyacrylamide gel depicted in Figure 1. Non-radioactive phosphoamino acid standards are marked and labelled.(A) Phosphoamino acids derived from α_3 band excised from lane 2. (B) Phosphoamino acids derived from β_1 band excised from lane 2.

subunits (e.g. α_6, β_1, β_2, β_3, β_4 and β_5) have been reported to be substrates for phosphorylation [11, 23-26]. Some of these integrins have been shown to be substrates for phosphorylation by PKC [23-25] and others for protein tyrosine kinase [27]. In addition, phosphorylation of some of these subunits (e.g. β_2 and Band 3 of avian integrin which is homologous to β_1 integrin) has been described to alter the affinity of the receptor for its ligand [10, 25]. Integrin subunits which could potentially form receptors for collagens were analyzed for their ability to become phosphorylated by labelling whole cells with [^{32}P]-orthophosphoric acid followed by immunoprecipitation with monoclonal antibodies to integrin subunits. We examined the effect of PMA on these cells because it is known to activate PKC which subsequently phosphorylates several specific proteins [28]. Treatment with PMA increased the phosphorylation of both α_3 and β_1 subunits (Figure 1), but had no effect on phosphorylation of the α_2 subunit (data not shown). Phosphorylation of the β_1 subunit in untreated cells was barely detectable, while a basal level of phosphorylation for α_3 was readily measured. Treatment with PMA increased the phosphorylation of the α_3 integrin 10 fold over untreated, while β_1 integrin phosphorylation was increased a similar amount (quantitation by densitometry). That the α_3 subunit is a substrate for phosphorylation represents a novel finding, while the β_1 subunit is already known to be a substrate for phosphorylation [26]. Phosphoamino acid analysis of both α_3 and β_1 subunits from M24met activated by PMA revealed that increase in phosphorylation was on serine (Figures 2A and B), suggesting that phosphorylation may occur either directly by PKC, or indirectly by other serine/threonine kinases. These data suggest that elevated phosphorylation of $\alpha_3\beta_1$ by PMA may explain the increase in metastasis through activation of PKC.

To confirm that $\alpha_3\beta_1$ could act as a receptor for collagens on M24met cells, antibody blocking studies were performed. A monoclonal antibody to α_3 integrin blocked adhesion to collagen I (51 %), whereas, treatment with monoclonal anti-β_1 integrin blocked adhesion to both collagen IV and collagen I by approximately 30 % (Table 4). However, the combination of the two antibodies resulted in a greater decrease in adhesion to collagen IV (60%) and collagen I (99 %). Addition of both antibodies to M24met cells blocked adhesion to laminin less than 29 %, but some inhibition is not unreasonable since the β_1 subunit can serve as a receptor for this protein when associated with different α subunits [29]. It has been reported that expression of the integrin, $\alpha_3\beta_1$, correlates with cutaneous malignant melanoma tumor progression, increasing along with progression [30]. Therefore, it is logical that modulation of the adhesive function of this integrin would affect the malignant phenotype of the cell.

The importance of our studies linking metastasis more closely to PKC, adhesion and integrin phosphorylation in human cancer will only become clear after further experimentation and eventual testing of compounds inhibitory to PKC in the clinics for antimetastatic effects. Further advances in the development of compounds for the treatment and/or prevention of metastasis may be facilitated by elucidation of the role for adhesion molecules and their receptors in metastasis and the involvement of PKC.

Table 4 Effect of Monoclonal Antibodies to α_3 and β_1 Integrins on Adhesion of M24met Cells to Immobilized Proteins

Adhesion Protein	Antibody, 1 mg/ml	Total number of adherent cells*	% Control
Collagen I	none	8456 ± 647	100
	α_3	4176 ± 1770	49
	β_1	5880 ± 1168	70
	$\alpha_3 + \beta_1$	125 ± 107	1.5
Collagen IV	none	13756 ± 673	100
	α_3	15111 ± 350	110
	β_1	9784 ± 1826	71
	$\alpha_3 + \beta_1$	5644 ± 883	41
Laminin	none	23317 ± 476	100
	α_3	19081 ± 2373	82
	β_1	28654 ± 815	123
	$\alpha_3 + \beta_1$	16634 ± 1415	71
Fibronectin	none	28111 ± 1008	100
	α_3	36590 ± 432	130
	β_1	33500 ± 589	119
	$\alpha_3 + \beta_1$	31725 ± 4281	113

* mean ± S.E.

M24met cells were metabolically labelled with [^{3}H]-thymidine for 24 h at 37°C. The cells were harvested and preincubated with monoclonal antibody to α_3 or β_1 integrin separately or in combination for 15 min at 37° C. The cells were then added to protein-coated wells (in triplicate) and incubated for 45 min at 37° C. Non-adherent cells were removed by washing and adherent cells were quantitated by liquid scintillation counting.

REFERENCES

1. Sugarbaker, E. V. and Ketcham, A. S. (1977) Semin. Oncol., 4: 19-32.
2. Fidler, I. J. (1991) Br. Med. Bull., 47: 157-177.
3. Dedhar, S. (1990) BioEssays, 12: 583-590.
4. Albelda, S. M. (1993) Lab Invest., 68: 4-17.
5. Tamkun, J. W., DeSimone, D. W., Fonda, D., Patel, R. S., Buck, C., Horwitz, A. F. and Hynes, R. O. (1986) Cell, 46: 271-282.
6. Albelda, S. M. and Buck, C. A. (1990) FASEB J., 4: 2868-2880.
7. Ruoslahti, E. and Pierschbacher, M. D. (1987) Science (Washington, DC), 2: 491-497.
8. Humphries, M. J., Yamada, K. M. and Olden, K. (1988) J. Clin. Invest., 81: 782-790.
9. Chan, B. M. C., Matsuura, N., Takada, Y., Zetter, B. R. and Hemler, M. E. (1991) Science (Washington, DC), 251: 1600-1602.
10. Tapely, P., Horwitz, A., Buck, C., Duggan, K. and Rohrschneider, L. (1989) Oncogene, 4: 325-333.
11. Shaw, L. M., Messier, J. M. and Mercurio, A. M. (1990) J. Cell Biol., 110: 2167-2174.
12. Gopalakrishna, R. and Barsky, S. H. (1988) Proc. Natl. Acad. Sci. USA, 85: 612-616.
13. Takenaga, K. and Takahashi, K. (1986) Cancer Res., 46: 375-380.
14. Korczak, B., Whale, C. and Kerbel, R. S. (1989) Cancer Res., 49: 2597-2602.
15. Nishizuka, Y. (1986) Science (Washington, DC), 233: 305-312.

16. Dumont, J. A., Jones, Jr., W. D. and Bitonti, A. J. (1992) Cancer Res., 52: 1195-1200.
17. Mueller, B. M., Romerdahl, C. A., Trent, J. M. and Reisfeld, R. A. (1991) Cancer Res., 51: 2193-2198.
18. Fidler, I. J. (1975) Cancer Res., 35: 218-224.
19. Thomas, T. P., Gopalakrishna, R. and Anderson, W. B. (1987) Methods Enzymol., 141: 399-411.
20. Cooper, J. A., Sefton, B. M. and Hunter, T. (1983) Methods Enzymol., 99: 387-402.
21. Dahl, S. C. and Grabel, L. B. (1989) J. Cell Biol., 108: 183-190.
22. Sibley, D. R., Benovic, J. L., Caron, M. G. and Lefkowitz, R. J. (1987) Cell, 48: 913-922.
23. Freed, E., Gailit, J., van der Geer, P., Ruoslahti, E. and Hunter, T. (1989) The EMBO J., 8: 2955-2965.
24. Parise, L. V., Criss, A. B., Nannizzi, L. and Wardell, M. R. (1990) Blood, 75: 2363-2368.
25. Valmu, L., Autero, M., Siljander, P., Patarroyo, M. and Gahmberg, C. G. (1991)Eur. J. Immunol., 21: 2857-2862.
26. Aneskievich, B. J., Haimovich, B. and Boettiger, D. (1991) Oncogene, 6: 1381-1390.
27. Hirst, R., Horwitz, A., Buck, C. and Rohrschneider, L. (1986) Proc. Natl. Acad. Sci., USA, 83: 6470-6474.
28. Kikkawa, U. and Nishizuka, Y. (1986) Ann. Rev. Cell Biol., 2: 149-178.
29. Wayner, E. A. and Carter, W. G. (1987) J. Cell Biol., 105: 1873-1884.
30. Natali, P. G., Nicotra, M. R., Bartolazzi, A., Cavaliere, R. and Bigotti, A. (1993) Int. J. Cancer, 54: 68-72.