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TUMOR CELL ADHERENCE TO CULTURED CAPILLARY ENDOTHELIAL CELLS IS PROMOTED BY ACTIVATORS OF PROTEIN KINASE C

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Abstract—Stimulation of cells with protein kinase C (PKC)-specific activators such as phorbol esters increased in a reversible manner the rate of adherence of [^{3}H]leucine-labelled L1210 cells to cultured bovine cerebral cortex capillary endothelial cells (CEC). This effect was not specific for L1210 cells since 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) strongly increased the binding of various other tumor cell lines. Phorbol esters increased the rate of L1210 cell adhesion to CEC by enhancing their binding capacity without affecting the apparent affinity of L1210 cells for CEC. This stimulation was specific to the phorbol analogs which activate PKC since it was not effected by 4α-phorbol didecanoate, known to be inactive for PKC. Down-regulation experiments showed that adhesion enhancement was entirely attributable to an effect on tumor cells without contribution of CEC intracellular PKC. PKC inhibitors like staurosporine, sphingosine and H-7 showed strong antagonistic activity towards TPA-induced L1210 cell adherence to CEC (IC_{50} = 0.5 nM, 160 nM and 10 μM, respectively). Adhesive proteins such as vitronectin, fibrinogen, fibronectin and the tetrapeptide RGDS, an active sequence from their cell-binding domains, exhibited potent, dose-dependent inhibition of PKC-induced tumor cell adhesion.

Hematogenous metastasis is initiated by the arrest of circulating tumor cells in the microcapillaries, their attachment to the vascular endothelium and/or the basement membrane and subsequent invasion of the blood vessel wall [1, 2]. While the sequence of events in the adhesion cascade has been well described, the biochemical events involved in the various steps are as yet not clearly defined.

Calcium and phospholipid-dependent protein kinase (protein kinase C, PKC†) plays a crucial role in the regulation of calcium-dependent cellular functions [3] and has been reported to participate in the modulation of the adhesive behavior of cells [4–6]. Indeed, lymphoid cells [4, 5] and Chinese hamster ovary cells [6] respond to specific stimulation of PKC with tumor-promoting phorbol esters by exhibiting increased binding to adhesive proteins *in vitro*. Recently, Gopalakrishna and Barsky [7] found a strong correlation between the levels of membrane-bound PKC and the ability of B-16 melanoma cell sublines to metastasize to the lung after intravenous injection. As these authors suggested that PKC plays a key role in promoting the hematogenous spread of cancer in the body, our work consisted of evaluating the exact influence of intracellular PKC activity on the adherence of tumor cells to confluent bovine cerebral cortex capillary endoethelial cell (CEC) monolayers *in vitro*.

MATERIALS AND METHODS

Cells. The lymphocytic mouse leukemia L1210 cells were originally obtained from the American type tissue culture collection (Ref. No. CCL 219) and grown in suspension with RPMI 1640 medium and 10% heat-inactivated foetal calf serum (FCS) in 5% CO_2 incubators at 37°. The cells were passaged (usually every 3 days) when they reached a concentration of 10^6 cells/mL by diluting them to a concentration of 2×10^5 cells/mL. All experiments were conducted on cells in early log phase ($4.5–5 \times 10^5$ cells/mL) to minimize experimental variations which occur with proliferative status.

Capillary endothelial cells were isolated from bovine cerebral cortex, cloned and cultured as described by Gospodarowicz *et al.* [8]. Cells were grown in DMEM supplemented with 10% FCS, 4 mM glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin sulphate and 1 ng/mL bovine pituitary fibroblast growth factor (Amersham International, Amersham, U.K.) (added every other day) until cultures were confluent ($4–6 \times 10^5$ cells/cm^2). CEC normally grew to confluence in 5–6 days and the cells were used at fifth to ninth passage. Endothelial cell cultures were passaged weekly at a split ratio of 1:4. CEC were identified as vascular endothelial cells by the presence of factor VIII-related antigen as previously described [9]. More than 99% of the cultures were always factor VIII-positive.

Measurement of L1210 cell binding to CEC. CEC were removed from the flasks with trypsin 0.02% and EDTA 0.05%, centrifuged and suspended in culture medium at a concentration of 4×10^4 cells/mL. Aliquots of 1 mL were cultured for 48 hr in flat-bottomed 24-well cluster plates (Nunclon) precoated with human fibronectin (Sigma, France). After the endothelial monolayer was confirmed with an inverted microscope, the CEC were washed twice

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† Abbreviations: PKC, protein kinase C; DMEM, Dulbecco's modified Eagle medium; TPA, 12-*O*-tetradecanoyl phorbol 13-acetate; PDBu, phorbol-12, 13-dibutyrate; CEC, capillary endothelial cell.

with DMEM + 0.2% bovine serum albumin (BSA) (adhesion buffer).

For adhesion studies, L1210 cells were metabolically labelled with L-[4,5-^{3}H]leucine (120 Ci/mmol) (Amersham International) (2 μCi/mL) for 48 hr. At the end of the labelling period [^{3}H]leucine-labelled L1210 cells were counted, centrifuged at 800 *g* for 7 min at 20° and washed by resuspending them in 10 mL DMEM + 0.2% BSA. L1210 cells were pelleted again using the same centrifugation conditions and resuspended in an appropriate volume of adhesion buffer to yield a final concentration of 2×10^5 cells/mL. The binding assay was performed for various periods of time at 37° by adding 1 mL of [^{3}H]leucine-labelled L1210 cells to confluent monolayers of CEC. Control buffer, 12-*O*-tetradecanoyl phorbol 13-acetate (TPA), phorbol-12,13-dibutyrate (PDBu) and 4α-phorbol didecanoate (4α-PDD, Sigma) solubilized in DMEM + 0.2% BSA were added simultaneously or preincubated with either L1210 cells or CEC depending on the experimental scheme. In all experiments, cell viability was greater than 95% as determined by trypan blue exclusion. After the incubation period (usually 120 min) non-adherent L1210 cells were removed, the monolayers were washed twice with 1 mL of DMEM + 0.2% BSA and the adherent radiolabelled L1210 cells and the CEC were solubilized in 1% Triton X-100, transferred to scintillation vials and counted. The fraction of adherent L1210 cells was then quantitated. Each test was done in triplicate. For down-regulation experiments, CEC or L1210 cells were preincubated with 200 nM TPA for 48 hr prior to measurement of L1210 cell adhesion to CEC. Depletion of PKC into the cells was controlled by binding of [20(n)-^{3}H]phorbol-12, 13-dibutyrate ([^{3}H]PDBu, 28.2 Ci/mmol) (Amersham International) as already described [10].

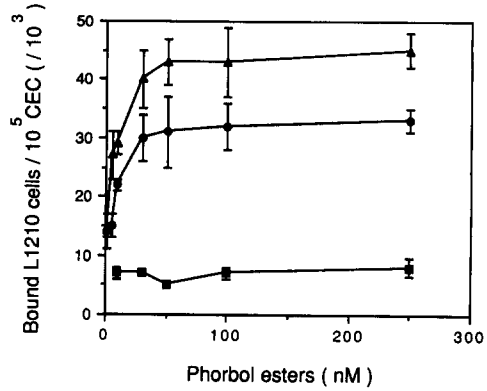

Fig. 1. Dose–response of phorbol ester-induced enhancement of L1210 cell adhesion to CEC. 12-*O*-Tetradecanoyl phorbol 13-acetate (TPA) (▲), phorbol-12, 13-dibutyrate (PDBu) (●) or 4α-phorbol didecanoate (4α-PDD) (■), a phorbol ester known to be inactive for PKC, were added to CEC monolayers immediately after addition of [^{3}H]leucine-labelled L1210 cells (2×10^5 cells/mL). The cells were incubated for 120 min at 37°. Values are means ± SD (N = 9).

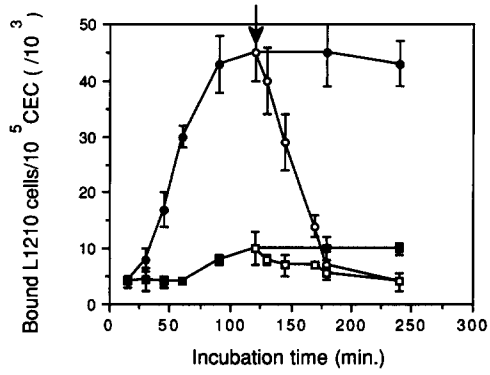

Fig. 2. Time-dependent adhesion of L1210 cells to confluent CEC. [^{3}H]Leucine-labelled L1210 cells (2×10^5 cells/mL) were incubated at 37° for the indicated periods of time with confluent CEC in the presence of either TPA (250 nM) (●) or the carrier (DMSO) (■). The arrow indicates the time at which a 100-fold excess of unlabelled L1210 cells was added (○, □). Values are means ± SD (N = 9).

RESULTS

Phorbol esters increase L1210 cell adhesion to CEC monolayers

Addition of the tumor-promoters, phorbol esters TPA and PDBu, strongly increased the binding of L1210 cells to the CEC monolayer (Fig. 1). Increased binding was observed at a concentration of 5 nM for both phorbol esters with maximal stimulation of adhesion at 50 nM. The dose–response curves for TPA and PDBu, and the slightly lower potency of the latter, are similar to results found with these agents in other systems [11, 12]. The inactive ester 4α-phorbol didecanoate had no significant effect on the adhesion of L1210 cells. The increased binding induced by the two active phorbol esters was observed by phase-contrast microscopy to be due to L1210 cell adhesion to CEC monolayers rather than L1210 cell–L1210 cell aggregation.

The kinetics of L1210 cell adhesion to confluent CEC are shown in Fig. 2. After an initial lag period of 15 min, the stimulated cells adhered rapidly, with more than four times the number of TPA-stimulated cells adhering after 120 min incubation than control cells. The binding achieved within 120 min at 37° was reversible since most of the specifically-bound L1210 cells could be displaced by a 100-fold excess of unlabelled L1210 cells.

Studies of saturation binding of L1210 cells to confluent CEC in the presence of 250 nM TPA revealed that the specific binding was saturable and reached a maximum at around 2–3 $\times 10^5$ cells/mL (Fig. 3A). Scatchard analysis of data from plotting the bound/free ratio of the labelled L1210 cells as a function of the concentration of CEC-bound L1210 cells revealed the presence of a single class of non-interacting binding sites on CEC (Fig. 3B). The equilibrium dissociation constant (K_D) and the maximal binding capacity (B_{max}) of L1210 cells were 1.3×10^5 L1210 cells and 5.35×10^4 L1210 cells/10^5 CEC, respectively.

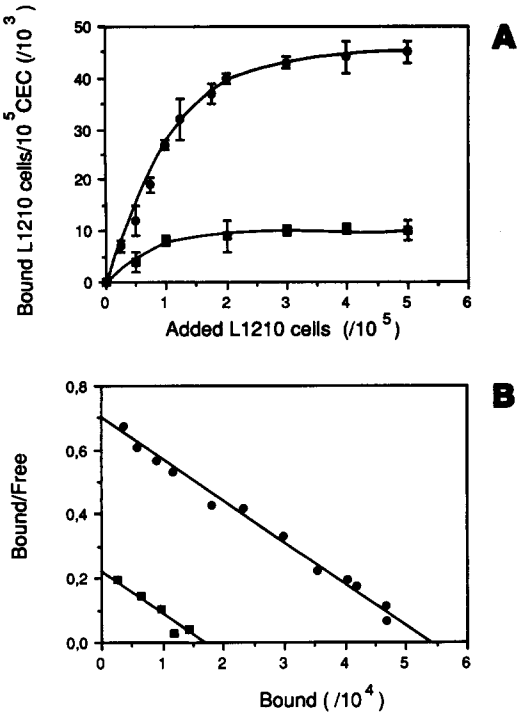

Fig. 3. Saturability of L1210 cell binding to CEC. (A) L1210 cell binding to CEC as a function of L1210 cell concentration. Increasing concentrations of [^{3}H]leucine-labelled L1210 cells were incubated with confluent CEC for 120 min in the absence (■) or presence (●) of TPA (250 nM). Values are means ± SD (N = 9). (B) Scatchard plots of L1210 cell binding to CEC. Scatchard plots were calculated after incubation of L1210 cells with CEC in the absence (■) or presence (●) of TPA (250 nM). Each data point was calculated from a data point in (A).

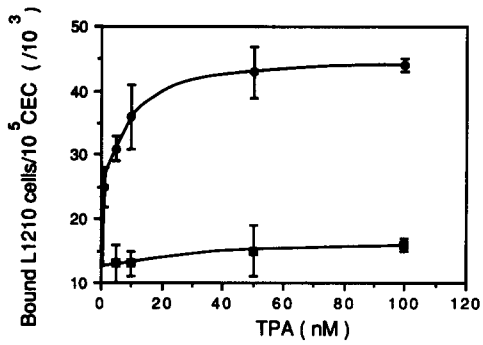

Fig. 4. Effect of preincubation of L1210 cells or CEC with TPA on L1210 cell adhesion. [^{3}H]Leucine-labelled L1210 cells (●) or CEC (■) were preincubated for 60 min with increasing concentrations of TPA prior to co-incubation. Cells were then washed and the adhesion assay was conducted in the standard manner, adding L1210 cells to CEC monolayers for 120 min without addition of TPA. Values are means ± SD (N = 9).

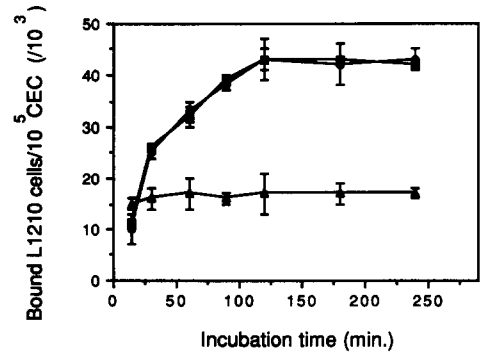

Fig. 5. Down-regulation of L1210 cell-adhesion to CEC. [^{3}H]Leucine-labelled L1210 cells (▲) or CEC (■) were preincubated with TPA (200 nM, 48 hr). Cells were then washed and co-incubated with the corresponding untreated cells for the indicated periods of time in the presence of TPA (250 nM) (▲, ■) or the carrier (DMSO) (●). Values are means ± SD (N = 6).

In the absence of TPA, the binding of L1210 cells to CEC was also saturable but Scatchard analysis of data revealed that, although K_D was nearly identical to the K_D obtained with cells incubated with TPA ($K_D = 1.2 \times 10^5$ L1210 cells), maximum binding capacity was three-fold lower than the B_{max} observed previously ($B_{max} = 1.7 \times 10^4$ L1210 cells/10^5 CEC). Hill coefficients (n_H) were close to unity, indicating that L1210 cells were binding to a single class of non-interacting binding sites whether or not TPA was enhancing the rate of cell adhesion. It is of importance to note that there was no evidence that serum-coated plastic dishes could support L1210 cell adhesion, even with TPA concentrations as high as 500 nM (not shown).

Phorbol esters increase L1210 cell adhesion by action on tumor cells

TPA was employed in an experiment in which either L1210 cells or CEC were preincubated prior to the binding experiment. The results demonstrated that the effect of a 60 min preincubation of tumor cells or CEC with increasing concentrations of TPA resulted in cell adhesion which was entirely attributable to an effect on L1210 cells and no action on the CEC (Fig. 4).

In an attempt to certify such an observation, we performed down-regulation experiments. It is now well known that PKC can be down-regulated by exposing the cells to high concentrations of TPA for prolonged periods [13]. Accordingly, cells were seeded into flasks and allowed to grow for 48 hr in culture medium (complete with supplements) with 200 nM TPA. CEC were then washed extensively with DMEM + 0.2% BSA, released from flasks with trypsin/EDTA and allowed to attach for 2 hr. Untreated labelled L1210 cells were then added to the wells and allowed to bind under treatment with 250 nM TPA for 120 min. Similarly, L1210 cells preincubated for 48 hr with 200 nM TPA were co-incubated with untreated CEC. Figure 5 shows that PKC-depletion of CEC did not affect L1210 binding.

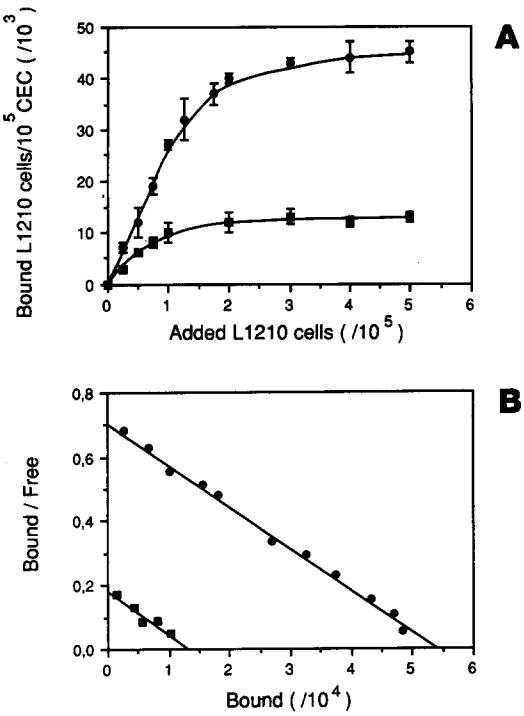

Fig. 6. Saturability of L1210 cell adhesion to CEC after down-regulation of PKC. (A) L1210 cell binding to CEC as a function of L1210 concentration. Increasing concentrations of [^{3}H]leucine-labelled L1210 cells were incubated with confluent CEC for 120 min. Adhesion experiments were performed before (●) and after (■) preincubation of L1210 cells with TPA (200 nM, 48 hr). Values are means ± SD (N = 9). (B) Scatchard plots of L1210 cell binding to CEC. Scatchard plots were calculated before (●) and after (■) preincubation of L1210 cells with TPA (200 nM, 48 hr). Each data point was calculated from a data point in (A).

However, down-regulation of PKC in tumor cells totally inhibited TPA-induced binding of L1210 cells to CEC. This observation confirmed that the adhesion-enhancing effect of TPA was exerted on the L1210 cells rather than the CEC. Saturability of L1210 cell binding to CEC after down-regulation of tumor cell-PKC (Fig. 6), revealed that after prolonged incubation with TPA, L1210 cells were still adhering to the same class of binding sites. However, although affinity of L1210 cells for the CEC-receptor sites was unchanged, the maximum binding capacity was dramatically reduced and returned to basal level (i.e. unstimulated cells) as shown previously (Fig. 3).

PKC inhibitors affect L1210 cell adhesion to CEC monolayer

Staurosporine and H-7 are both competitive, non-selective antagonists of PKC [14]. However, sphingosine has been recently characterized as a more selective inhibitor of PKC, interacting with the regulatory domain of PKC [15].

We therefore characterized the effect of these antagonists on the TPA-induced adhesive response of L1210 cells. As shown in Fig. 7, staurosporine (Fluka AG, F.R.G.), H-7 (Seikagaku Kogyo, Japan) and spingosine (Sigma) inhibited L1210 cell adhesion to CEC in a concentration-dependent manner. The concentrations required to inhibit 50% of L1210 cell binding to CEC (IC_{50}) were 0.5 nM, 10 μM and 160 nM, respectively. Within the same range of concentrations, basal adherence of L1210 cells was altered by these inhibitors to the same extent as under stimulation with TPA. IC_{50} values for staurosporine, H-7 and sphingosine were 0.7 nM, 18 μM and 120 nM, respectively. This observation indicates that even basal levels of PKC can regulate tumor cell adhesion to CEC. Cell viability, as assessed by trypan blue exclusion at high inhibitor concentration (100 μM), was the same as in the medium control; therefore, a decrease in adhesion was unlikely to be due to a cytotoxic effect of protein kinase inhibitors. Preincubation experiments performed on either CEC or tumor cells to determine which type of cell the inhibitory effect of protein kinase inhibitors was directed toward revealed, as already shown, that only L1210 cell-intracellular PKC inhibition affected adhesion to CEC monolayers (results not shown).

Exogenous adhesive proteins inhibit tumor cell adhesion to CEC

Over the past few years, various proteins have been demonstrated as being involved mainly in cell to cell adhesion processes [16–18]. Therefore, in order to characterize more precisely the mechanism of TPA-induced tumor cell adherence to the endothelium, various cell-attachment proteins were tested in our model.

As shown in Fig. 8, exogenous adhesive proteins like fibronectin, fibrinogen and vitronectin were equipotent in inhibiting L1210 cell adhesion to CEC (IC_{50} values around 100 μg/mL). The cell-attachment site within the fibronectin, fibrinogen and vintronectin molecules has been identified as the tetrapeptide Arg-Gly-Asp-Ser (RGDS), the basic unit of a widespread cellular recognition system [19]. As already shown with other cell types [20–22], the tetrapeptide determinant RGDS (Bachem, Switzerland) showed strong inhibitory activity towards TPA-induced L1210 cell adhesion to CEC suggesting the existence of a specific recognition sequence of events triggered by selective activation of PKC. However, under the same experimental conditions laminin or the synthetic laminin pentapeptide YIGSR (Bachem), recently identified as a major site for cell binding on the laminin molecule [23], was inactive towards either TPA-stimulated or unstimulated cells.

In order to determine which part of the L1210 cell–CEC interaction was affected by the action of an adhesive protein, preincubation experiments were performed. Pretreatment of CEC with 100 μg/mL of RGDS for 1 hr inhibited markedly the binding of L1210 cells, whereas pretreatment of L1210 cells with RGDS had no effect (Table 1). Therefore, the major action of RGDS in these experiments, and of RGDS-containing cell-attachment proteins, is directed toward the CEC.

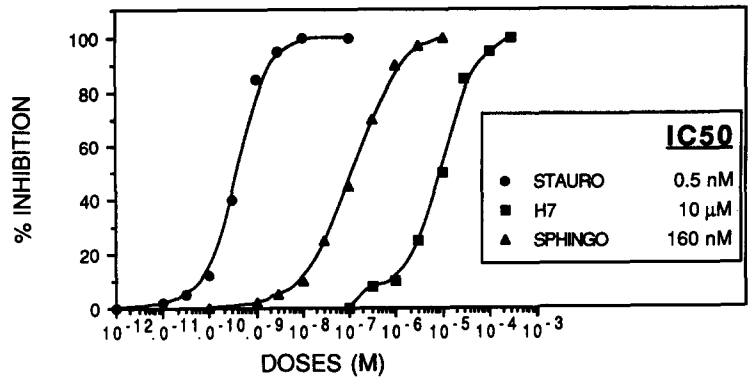

Fig. 7. Effect of protein kinase inhibitors on L1210 adhesion to CEC. Staurosporine (●), H-7 (■) or sphingosine (▲) were added to CEC monolayers immediately after addition of [^{3}H]leucine-labelled L1210 cells. The cells were incubated for 120 min in the presence of TPA (250 nM). Values are means of triplicate experiments.

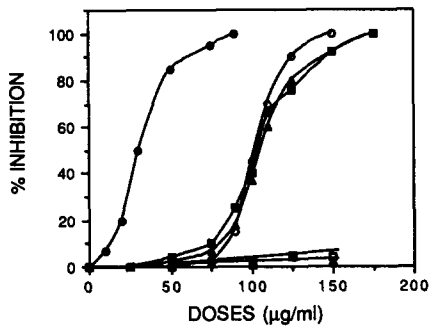

Fig. 8. Effect of adhesive proteins on L1210 cell binding to CEC. [^{3}H]Leucine-labelled L1210 cells were incubated for 120 min with increasing concentrations of fibronectin (○), the fibronectin cell-binding sequence (RGDS) (●), vitronectin (■), fibrinogen (▲), laminin (□) or the laminin cell-binding sequence YIGSR (△) in the presence of TPA (250 nM). Values are means of triplicate experiments.

Table 1. Effect of pretreatment with RGDS on L1210 cell adhesion to CEC

Pretreatment of cells		Per cent of control (mean ± SD)
CEC	L1210 cells	
—	—	100
RGDS	—	3.1 ± 0.05
—	RGDS	87 ± 6.2
RGDS	RGDS	2.2 ± 0.4

Cells were preincubated for 1 hr with RGDS (100 μg/mL) or with medium (—) as the control. Unbound RGDS was washed off and the cells were rinsed and incubated with untreated cells for 120 min in the presence of TPA (250 nM).

Phorbol esters increase adhesion of various types of tumor cells to CEC

Since the lymphocytic mouse leukemia L1210 cells normally grow as a non-adherent cell line, other cell strains were evaluated for the ability of phorbol esters to enhance their binding to CEC. [^{3}H]Leucine-labelled B16-F10 murine melanoma cells (ATCC, Ref. No. CRL 6475), mouse lymphoid neoplasm P-388 (ATCC, Ref. No. CCL 46), rat carcinoma cell line Walker-256 (WRC-256, ATCC, Ref. No. CCL 38) and mouse sarcoma cell line S-180 (ATCC, Ref. No. CCL 8), were incubated with confluent CEC and 250 nM TPA. Due to a high basal level of adherence, experiments were performed for a shorter incubation period (15 min). Under the experimental conditions for L1210 cells, described in Materials and Methods, TPA significantly enhanced the binding of these cell lines to CEC (Table 2). This observation indicates that the pathway used by phorbol esters to enhance tumor cell adhesion to the endothelium does not apply to L1210 cells only, but is ubiquitous.

DISCUSSION

A fundamental event in tumor extravasation and metastasis is the adherence of tumor cells to the vascular endothelium. It is also believed that this is important in the pathophysiology of cancer [1, 2]. However, the mechanisms which regulate binding of tumor cells to the endothelium are still not understood. A relatively unexplored aspect of the question of regulation of tumor cell adhesion to endothelial cells is the series of intracellular steps, including the type and sequence of second messenger systems, which must occur during adhesion of tumor cells to binding sites on the endothelial cell surface. The present study has demonstrated that this process can be regulated by the intracellular enzyme PKC. Activation of this enzyme by phorbol esters resulted in a rapid increase in L1210 cell adherence to monolayers of bovine cerebral cortex capillary endothelial cells (CEC). This stimulatory effect may

Table 2. Effect of TPA on the adherence of various tumor cell lines to confluent CEC

Cell lines	Specific binding of tumor cells (tumor cells/10^5 CEC)
L1210 (2 hr incubation)	43,200 ± 1860
B16-F10	48,460 ± 1120
P-388	39,020 ± 810
WRC-256	41,210 ± 780
S-180	43,180 ± 520

[^{3}H]Leucine-labelled tumor cells (2×10^5 cells/mL) were incubated with confluent CEC for 15 min at 37° in the presence or absence of 250 nM TPA. Basal (unstimulated) binding was subtracted from stimulated adhesion to determine specific binding of tumor cells. L1210 cell adhesion to CEC after 2 hr incubation in the same experimental conditions is presented as a control.

Values are means ± SD of triplicates.

be attributed specifically to PKC activation since the structural analog 4α-PDD, which does not stimulate this enzyme [24], did not provoke any alteration in tumor cell adherence to CEC in comparison with unstimulated cells. Furthermore, exposure to PKC inhibitors or prolonged treatment (48 hr) of tumor cells with TPA (down-regulation) resulted in both inactivation of PKC activity (not shown) and the loss of adhesive capability. In this respect, L1210 cells behaved like CHO cells on fibronectin-coated substratum [6], where stimulation of cells with phorbol esters resulted in a modification of the fibronectin receptor leading to an apparent increase in the interaction of the receptor with fibronectin. A recent study showed that a strong correlation existed between basal levels of membrane-bound PKC and the ability of B-16 melanoma cell sublines to metastasize to the lung after intravenous injections [7]. The authors also indicated that additional stimulation of tumor cells with phorbol esters strongly increased metastasis *in vivo*. The mechanism(s) of such an effect remain unclear but, since phosphorylation of cell surface-associated proteins and cytoskeletal proteins by PKC has been shown [25], one could postulate that PKC, by phosphorylating membrane/cytoskeleton proteins will regulate cell-surface attachment properties. This effect, together with PKC activity on cell motility and enzyme exocytosis (e.g. proteases) [26], will influence the attachment of tumor cells to the vascular endothelium or the basement membrane and subsequent invasion of the blood vessel wall. In an attempt to further elucidate such a mechanism, either L1210 cells or CEC were incubated with phorbol esters for a short period of time. The results demonstrated that a 60 min preincubation with TPA resulted in cell adhesion entirely attributable to an effect on tumor cells with no action on the CEC. In this respect, therefore, the action of phorbol esters in enhancing L1210 cell binding was different to the action of other potent inducers of cell adherence such as thrombin, IL4, LPS, IL1 and IFN-γ, which enhanced cell binding by altering the endothelial cell adhesive properties but had no detectable direct effect on the adherent cell adhesiveness [27–31]. The biochemical feature of this discrepancy remains unclear but, recently, Gladwin *et al.* [32] showed that monocyte adherence to the endothelium was regulated through a PKC-dependent mechanism, primarily mediated via the expression of the Mac-1 adhesion protein on the monocyte surface. Additionally, since various mediators including fMLP, leucotriene B4, C5a, platelet-derived growth factor and tumor necrosis factor were able to induce a selective increase in cell surface expression of Mac-1 in monocytes and neutrophils [33, 34], the effect of these factors on cell adhesiveness may also be mediated through a PKC-dependent pathway. The mechanism(s) of increase in surface expression of adhesion receptors remains unclear but since the increase was induced shortly after incubation with phorbol esters, it is unlikely that it is mediated by stimulation of glycoprotein synthesis. Indeed, 18 hr treatment with cycloheximide did not significantly affect the rate of PKC-induced adhesion of L1210 cells to CEC (not shown). Therefore, stimulation of PKC may cause exocytosis of intracellular stores of adhesion glycoproteins. It has also been suggested that in neutrophils increased adherence after stimulation, which can occur independently of a quantitative change in receptors, is the result of qualitative alterations in the adhesion glycoproteins at the cell surface [35]. Therefore, phorbol esters can induce a conformational change in cell surface receptors on tumor cells, thereby facilitating increased adherence to endothelial cells. It has already been demonstrated that activation of PKC with TPA can cause phosphorylation of the α-subunit of the adhesion glycoprotein LFA-1 in leukocytes [36], inducing increased adhesion of cells; but whether similar modifications of other adhesion proteins occur in tumor cells after stimulation with phorbol esters requires further investigation. Nevertheless, recent work from our laboratory characterized a trypsin-sensitive membrane-associated protein kinase present at the surface of L1210 cells (J.M.H.; manuscript in preparation). To our knowledge, no such extracellular protein kinase has been described in tumor cells, but recently, Imada *et al.* [25] detected the presence of a membrane-associated ecto-protein kinase and its substrate in cultured Swiss 3T3 fibroblasts. Such a one phosphorylated fibronectin specifically at serine and threonine residues. According to these authors, phosphorylation of fibronectin may modulate the chemistry of extracellular matrix compounds which in turn results in a local modulation of morphogenetic movement and cell aggregation. Similarly, Pyerin *et al.* [37] partially purified extracellular protein kinase from HeLa cells and characterized it as a member of casein type II kinase. Extracellular protein phosphorylation can, therefore, potentially provide a mechanism of local modulation of morphogenetic determinants. Supporting this concept is the observation reported by Ehrlich *et al.* [38] that ecto-protein kinases catalysed phosphorylation of neural cell adhesion molecules that might contribute to altered neural cell adhesiveness.

To investigate the functional importance of individual adhesive glycoproteins, this study also

examined the competitive effect of adhesive proteins on PKC-stimulated tumor cell adherence to CEC monolayers. Previous investigations [20–23] have demonstrated that the incubation of cells with specific adhesive proteins can be used to elucidate the importance of specific adhesive glycoproteins in adherence-dependent functions, since adhesive protein binding can lead to impairment of receptor function by interaction with a functionally important site on the molecule. In this work, the increased adherence was strongly affected by RGDS-containing adhesive proteins such as fibronectin, vitronectin and fibrinogen. In contrast, PKC-stimulated adherence was not affected by laminin or the synthetic laminin pentapeptide YIGSR, recently identified as a major site for cell binding on the laminin molecule [23]. Therefore, since it is now well known that both the fibronectin/vitronectin cell attachment peptide RGDS and YIGSR antagonize tumor cell colonization *in vivo* [23, 39], our data suggest that it is likely that these peptides block tumor cell colonization via alternative biochemical pathways.

In conclusion, we have demonstrated that PKC-activation has a specific action on tumor cells, increasing their adhesiveness for endothelial cells by inducing a stable alteration in the tumor cell surface. This alteration appears to involve the expression or up-regulation of a tumor cell adhesion molecule that interacts with endothelial cells via an RGDS cell binding domain. This process may be important in recruiting tumor cells by selectively increasing their adhesiveness for the local vasculature.

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