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COMMENTARY

PROTEIN KINASE C: A KEY FACTOR IN THE REGULATION OF TUMOR CELL ADHESION TO THE ENDOTHELIUM

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The metastatic process, as typified by the spread of tumor cells via the blood stream, is a complex multi-step phenomenon [1] which has been described as a cascade involving local tissue invasion, detachment of malignant cells from the primary tumor, intravasation, transport within the blood stream, lodging in a vessel at a distant site, extravasation, growth to form the secondary tumor, and generalization. Some possible contributory elements to the expression of metastatic behaviour by a tumor cell include alteration in growth, cell communication, angiogenesis, motility, deformability, invasiveness, adhesiveness, enzyme production, immunogenicity and differentiation.

To date, there is no simple phenotypic characteristic whose exclusive expression explains metastatic behaviour. Nevertheless, several lines of evidence point towards adhesive phenomena as mediating some crucial steps in the malignant spread of particular tumor types. There are several possible stages at which cell adhesiveness may influence the outcome of the metastatic cascade. Decreased homotypic adhesiveness, for example, may promote detachment from the primary tumor, whereas decreased heterotypic adhesiveness may contribute to escape from host defence cells. On the other hand, decreased cell-to-cell adhesion may limit metastatic potential by inhibiting the formation of emboli which are believed to promote malignant spread by fostering lodging in small blood vessels.

Not surprisingly, there is no universal relationship between metastatic behaviour and cell adhesiveness but the molecular dissection of the adhesive events involved in these processes has only just begun. It clearly indicates that several different determinants are likely to be involved. Among these various events, cell aggregation, cell attachment, exocytosis and cell motility are all calcium-regulated processes [2, 3]. Furthermore, phosphorylation of protein receptors often induces conformational changes in receptors, which can affect binding characteristics for the ligands [3], and integrins have been shown to be substrates for both protein tyrosine kinase [4] and protein kinase C (PKC)† [5, 6]. The latter enzyme is a calcium-activated and phospholipid-dependent kinase, which also requires diacylglycerol for full catalytic activity [7]. PKC is the major intracellular receptor for the phorbol ester tumor promoters, which bind to and activate the enzyme in a manner analogous to diacylglycerol [8, 9].

Tumor-promoting phorbol esters and compounds that activate calcium mobilization have been shown to increase metastasis *in vitro* and in experimental animal models and also to enhance the adherence potential of tumor cells [10–17]. Moreover, although of a rather non-selective nature, PKC inhibitors have revealed anti-adhesive and anti-metastatic properties both *in vitro* and *in vivo* [13, 14, 18]. These data suggest that it may be possible to limit the dissemination of tumor cells through interference with PKC, implying that this enzyme plays a key role in determining the metastatic phenotype.

Although the precise understanding of the real involvement of PKC in the metastatic process is still in its infancy, we shall summarize briefly some of the more interesting features.

THE PKC PATHWAY IN TUMOR MALIGNANCY

Given the central role of the calcium/PKC pathway in the regulation of cellular growth [2, 7, 19], and given the pivotal importance of cell proliferation in neoplastic disease, it seems intuitively obvious that PKC must somehow play a major part in affecting cancer spread. PKC plays an important role in cell-surface signal transduction and controls a wide number of physiological processes including cellular growth and differentiation as well as tumor promotion [2], and several recent findings indicate an important role for PKC in the regulation of cellular growth. Certain growth factors such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) or interleukin-2 (IL-2) mediate their mitogenic effects in part through the cascade of phosphatidyl-inositol hydrolysis [20, 21]. The generation of

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† Abbreviations: CEC, capillary endothelial cells; ELAM-1, endothelial-leukocyte adhesion molecule-1; FGF, fibroblast growth factor; HUVEC, human umbilical vein endothelial cell; ICAM-1, intercellular adhesion molecule-1; IFN-γ, interferon-γ; IL-1β, interleukin-1β; LPS, lipopolysaccharide; PDGF, platelet derived growth factor; PKC, protein kinase C; TNF, tumor necrosis factor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; and VCAM-1, vascular cell adhesion molecule-1.

diacylglycerol followed by phosphorylation/dephosphorylation reactions constitutes a series of steps which enable a mitogenic signal, among other pathways, to travel from the plasma membrane to the nucleus. A large number of substrates phosphorylated by PKC have been described [7] but none of them was found to be directly and exclusively growth related. Nevertheless, recent findings revealed the existence of the cellular protein AP-1 whose transcription stimulatory activity was thought to be mediated via phosphorylation by PKC [22]. PKC was found to phosphorylate DNA methyltransferase, an enzyme which acts in the nucleus and alters gene expression by changing DNA methylation patterns [23]. In addition, in *ras*- and *sis*-transformed normal rat kidney cells, elevated levels of diacylglycerol were shown to correlate with persistent activation of PKC [24]. This observation led to the concept that one mechanism responsible for the transformed phenotype is an alteration in the steady-state levels of diacylglycerol [25]. Furthermore, in fibroblasts transfected with the PKC-1 isozyme, altered gene expression occurred leading to enhanced tumorigenicity [26], resulting in a tumor-promoting effect similar to that of phorbol esters [27].

Several PKC inhibitors have been detected and are capable of inhibiting PKC-mediated cellular responses such as the expression of ornithine decarboxylase [28] or superoxide generation by cells of the immune system [29]. Among them, staurosporine, an alkaloid isolated from microbial sources [30], exerted the most potent inhibitory effect on PKC activity but revealed non-selective inhibition. Staurosporine exerted anti-proliferative effects on various cell lines *in vitro* [30] as did another selective derivative (CGP 41251) which exerted anti-proliferative effects on various cell lines both *in vitro* and *in vivo* [31].

Recent data from our laboratory using the selective PKC inhibitor chelerythrine showed an association between PKC inhibition and antiproliferative activity of the murine lymphocytic leukemia cell line L1210 [32], therefore suggesting an association between PKC inhibition and anti-tumor activity.

PKC ACTIVITY AND TUMOR CELL ADHESION *IN VIVO*

Recently, a strong correlation was found between the basal levels of membrane-bound PKC and the ability of B16 melanoma cell sublines to metastatize to the lung after i.v. injection [10]. Moreover, short treatment of such melanoma cells with the tumor promoter phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) brought about a concordant increase in metastatic capability and PKC translocation to the plasma membrane, whereas prolonged treatment of cells with TPA (a procedure known to bring about PKC down-regulation [33]), caused a significant decrease in metastatic activity [10]. From these results, it was concluded that the activation of PKC, as defined by membrane localisation, had a marked stimulatory effect on the process of haematogenous spread [10]. Such observations confirmed earlier experiments showing that pre-treatment of Lewis lung carcinoma cells with TPA resulted in an increase of their metastatic activity [15]. This effect was closely correlated with increased phosphorylation of a specific intracellular protein. Similarly, spontaneous metastasis of SP1 mouse mammary adenocarcinoma cells, which do not normally metastatize, was induced by treatment with TPA or the calcium ionophore A23187 [34].

All of these data suggest that activation of PKC plays a role in determining the metastatic phenotype of tumor cells and it was therefore reasoned that inhibitors of PKC might also modulate metastasis. Several inhibitors of PKC have been detected and are capable of inhibiting PKC-mediated cellular responses [35]. Among them staurosporine [30] exerted the most potent inhibitory effect on PKC but revealed non-selective inhibition. In a recent study, Dumont *et al.* [18] showed that treatment of B16-F1 murine melanoma cells with non-selective PKC inhibitors like staurosporine, H7 or MDL 27,032 for 24 hr in culture and subsequent i.v. injection of the cells into mice resulted in almost complete inhibition of lung metastasis. The 50% inhibitory concentration value for metastasis with MDL 27,032 was similar to that obtained with the inhibition of B16-F1 membrane-associated PKC. MDL 27,032 was more potent than H-7 for the inhibition of metastasis but was significantly less potent than staurosporine.

Although the results obtained in these studies have been of a clear-cut nature, the situation with regard to PKC and the malignancy of tumor cells may be rather more complicated than even this brief presentation of the known facts would suggest. It has been shown that PKC is not a single entity but has a variety of isoforms which may vary in tissue distribution and which may be differentially regulated and expressed [36, 37], and it could well be that variations in isoform activities are responsible for the changes in metastatic behaviour observed in the various tumor lines. Unfortunately, at the present time very little data exist to support this concept but the tools for investigating it are rapidly becoming available. Recent transfection experiments with cDNA clones of PKC incorporated in expression vectors have shown that overproduction of either of the two enzyme isoforms (PKCγ or PKCβ) resulted in disordered growth and tumorigenicity in fibroblasts [26, 38]. While the *in vivo* results presented were not overwhelming, they do show the way that the role of PKC in metastasis could be investigated in future studies. Thus, expression vectors containing the genes encoding the various PKC isoforms could be introduced into tumor cell lines of defined metastatic ability to see how these alterations in PKC biochemistry affect subsequent malignancy. Overall, these *in vivo* studies strengthen the concept that membrane-bound PKC is a key regulator of cell-surface properties that influence metastasis but the mechanism of action remains hypothetical.

Increased association of PKC with nuclear-cytoskeletal components in TPA-treated cells has been reported [39] together with phosphorylation of some cell surface-associated and cytoskeletal proteins by PKC [2]. It is therefore assumed that PKC, by phosphorylating membrane/cytoskeletal proteins, regulates cell-surface attachment properties,

exocytosis and cell mobility. These cellular events together influence attachment of tumor cells to vascular endothelium/basement membrane and invasion of the blood vessel wall. This enzyme also may promote formation of larger tumor cell aggregates (self, platelet, lymphocytes) that are more easily trapped in pulmonary microcapillaries [40].

Such specific points have been addressed in detail in *in vitro* studies aimed at investigating the effects of various compounds (proteolytic enzymes, inflammatory mediators, tumor promoting phorbol ester) on tumor cell-endothelial cell interactions.

PKC ACTIVITY AND TUMOR CELL ADHESION *IN VITRO*

Experiments with cultured endothelial cells have led to several conclusions about tumor cell adhesion that appear to be applicable in multiple species and in multiple vascular beds First the expression of tumor-binding sites on the endothelial cell surface is low under "control" or "physiological" conditions, i.e. when cultured endothelial cells are confluent, quiescent and unstimulated by exogenous agents. Secondly, when treated with specific exogenous agents such as phorbol esters, cytokines or lipids, endothelial cells exhibit increased adhesiveness, and many studies have foccused on the ability of these compounds to alter the adhesive properties of endothelium. These studies suggested an interesting analogy between the recruitment of inflammatory cells at the sites of injury or infection and metastasis, and led to the conclusion that similar adhesive proteins and inducing factors may be involved.

Effect of TPA on the adhesive properties of tumor cells in vitro

A number of studies have demonstrated that phorbol esters modulate the adhesive behaviour of various cell types on different substrates such as the monolayer of endothelial cells, plastic culture dishes, adhesive proteins or scrubbed nylon fibers. Indeed, pretreatment of mouse brain microvascular endothelial cells with TPA increased the binding of P-815 mastocytoma cells, therefore mimicking the effect of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) or endotoxin (LPS) [41]. Similar results were obtained after treatment of non-adherent Walker-256 carcinosarcoma cells with TPA which caused these cells to become adherent to non-cellular foreign surfaces such as nylon fibers and plastic culture dishes and to monolayers of endothelial cells [16]. These findings strongly suggested that the adherence response induced by TPA was very similar to the adherence response induced by chemotactic peptides such as f-Met-Leu-Phe (f-MLP) which also induced a rapid, reversible, foreign surface adherence response in Walker-256 cells [42]. Surprisingly, both chemotactic- and TPA-induced adherence were blocked by 2-deoxyglucose [42, 43], colchicine and cytochalasin B, but not by cycloheximide [16], suggesting that a common pathway was involved in both chemotactic factor- and TPA-induced tumor cell adherence. Most interestingly, these authors suggested that such similarities indicated that the underlying mechanism by which the tumor cells respond to chemotactic factors and to agents such as TPA is very similar to the mechanism used by the leukocytes.

In a recent study, Brown [17] showed that stimulation of CHO 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. This work suggested that PKC may be involved in regulating fibronectin-mediated adhesion by modifying fibronectin receptor function. Other components of the adhesion cascade, such as adhesion plaque proteins, also appear to be affected by phorbol ester stimulation of cells. Vinculin is phosphorylated by PKC in response to phorbol esters [44], and talin, which interacts with the fibronectin receptor, may also be a target for phorbol action [45].

Melanoma cells [10] and lymphoid cells [46, 47] respond to stimulation with phorbol esters by exhibiting an increase in adhesion either to other cells or to adhesive ligands, and we recently demonstrated that stimulation of the lymphocytic mouse leukemia L1210 cells with phorbol esters increased in a reversible manner their rate of adherence to cultured bovine brain cortex capillary endothelial cells [13, 14]. This effect was not specific for L1210 cells since TPA strongly increased the binding of other tumor cell lines to capillary endothelial cells (CEC). Phorbol esters increased the rate of tumor cell adhesion to CEC by enhancing their binding capacity without affecting their apparent affinity for the endothelium. Most surprisingly, down-regulation experiments showed that adhesion enhancement was entirely attributable to an effect on tumor cells without contribution of CEC intracellular PKC. In this respect, the action of phorbol esters in enhancing tumor cell binding was different from the action of other potent inducers of cell adherence such as thrombin, LPS, IL-1 β and interferon-γ (IFN-γ) which enhanced cell binding by altering the endothelial cell adhesive properties but had no detectable direct effect on the adherent cell adhesiveness [48–51]. The biochemical feature of this difference remained unclear but phorbol esters have been already shown to induce a conformational change in cell surface receptors on tumor cells, thereby facilitating increased adherence to endothelial cells [17, 47].

To investigate the functional importance of individual adhesive glycoproteins, we examined the competitive effect of adhesive proteins on PKC-stimulated tumor cell adherence to CEC monolayers and showed that the increased adherence was strongly affected by RGDS-containing adhesive proteins whereas 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 [52]. Since it is now well known that both the fibronectin/vitronectin cell attachment peptide RGDS and YIGSR antagonize tumor cell colonization *in vivo* [53], our data suggested that it is likely that these peptides block tumor cell colonization via alternative biochemical pathways.

In the human monoblastoid cell line U-937, originally derived from a patient with histiocytic

lymphoma [54], phorbol esters inhibit DNA synthesis and induce maturation along the monocytic differentiation pathway [55]. Although the inhibition of DNA synthesis occurred at doses lower than those required to induce translocation of PKC [56], treatment with tumor-promoting phorbol esters induced adherence of the cells to plastic, endothelial cells or the extracellular matrix proteins [10–18, 10, 51, 57]. The adherence process showed characteristics typical of the adhesion of leukocytes and was inhibited by a monoclonal antibody to the leukocyte adhesion molecule CD18 [57].

In a recent study, Cavender *et al.* [11] showed that phorbol ester-stimulated U-937 cell adhesion to endothelial cells was due to an action on the tumor cells. It was also demonstrated that phorbol ester-induced U-937 cell adhesion to endothelial cell monolayers was mediated through an increase in avidity of the existing LFA-1 molecules (CD11a/CD18) and was not due to an increased number of those molecules. In the absence of phorbol ester, a small proportion of the U-937 cells adhered to fibronectin, and almost none bound to either laminin or denatured type 1 collagen, whereas the addition of phorbol esters resulted in an increase in the adhesion of U-937 cells to fibronectin, with only a slight increase in the proportion of cells which bound to laminin or gelatin. Additional adhesion assays performed in the presence of a pentapeptide containing the amino acid sequence RGD, which is part of one of the cell-binding domains of fibronectin, demonstrated that the RGD-containing peptide almost totally blocked the phorbol ester-induced adhesion of U-937 cells to fibronectin without any inhibitory effect on the phorbol ester-induced binding of U-937 cells to endothelial cells.

It should be noted that U-937 cells express VLA-4 and VLA-5 cell surface integrin molecules with fibronectin-binding activity [58, 59], but to date none of these adhesive molecules have been shown to be affected following phorbol ester treatment of these cells.

Effect of PKC inhibitors on tumor cell adhesion to the endothelium

Invasion of an artificial basement membrane matrix by human bladder carcinoma cells has been shown to be sensitive to inhibition of PKC by staurosporine [60], and Dumont *et al.* [18] recently showed that MDL 27,032, an analogue of staurosporine, inhibits the binding of B16-F1 cells to collagen IV, to fibrinogen and to confluent vascular endothelial cells but not to either fibronectin or laninin. This observation suggested that the drug, a non-specific PKC inhibitor, had an effect only on specific cell-adhesion receptors. While the role of binding of cells to either collagen IV or fibrinogen is not clearly understood, collagen IV is one of the most abundant proteins in the subendothelium, and binding to this protein may be crucial to the penetration of the metastatic cell through the matrix.

Work from our laboratory showed that PKC inhibitors like staurosporine, H-7 or sphingosine exhibited potent, dose-dependent inhibition of TPA-induced L1210 cell adherence to CEC [13, 14]. An as yet unpublished observation demonstrated that chelerythrine, a selective, potent PKC inhibitor [32], also showed strong antagonistic activity towards TPA-induced L1210 cell adherence to CEC. As suggested above, all of these studies confirmed that TPA-enhanced tumor cell adhesion was entirely attributable to an effect on tumor cells without contribution of CEC intracellular PKC.

On the monocytic cell line U-937, increased adhesion caused by exogenous stimulators, including α-thrombin, endotoxin, IL-1β and TPA, was fully inhibited by the PKC inhibitor H-7, but not by its structural analog HA1004. The latter drug is a more potent inhibitor of cyclic AMP- and cyclic GMP-dependent protein kinases than of PKC [61].

These results further ascertained the fundamental role of PKC in tumor cell adherence to the endothelium and suggested that PKC inhibitors may be of a possible use to ultimately limit the dissemination of tumor cells through interference with specific stages in the metastatic cascade.

Importance of PKC on cytokine-induced tumor cell adhesion in vitro

Cytokines are a broad class of proteins which play important roles in inflammatory and immunological processes. Each cytokine can be produced by more than one type of cell and displays multiple effects on various cells and tissues. Because they can have stimulatory effects on inflammatory cells, there has been considerable interest in their ability to stimulate host defence against tumors [41, 62]. In addition, some cytokines have been shown to affect tumor behaviour directly. Previous studies have also demonstrated that cytokine treatment of endothelial cells results in increased tumor cell binding via induction of specific adherence proteins on the surface of endothelial cells that bind to tumor cells [62, 63]. These proteins include endothelial leukocyte adhesion molecule-1 (ELAM-1) and INCAM-110, which has subsequently been shown to be an alternatively spliced variant of the T-cell binding protein vascular cell adhesion molecule-1 (VCAM-1) [64]. Additionally, histopathologic studies of melanoma have also suggested that the invasiveness of this tumor correlates with the expression of the adhesion molecule intercellular adhesion molecule-1 (ICAM-1) [65].

TNF-α, a protein secreted mainly by activated macrophages, was originally defined by its antitumor activity in animals [66] and was also shown to have tumor cytostatic and cytolytic effects *in vitro* while sparing normal cells [67]. Although cytokine treatment has shown some success in experimental *in vivo* models, its application in cancer therapy is still controversial.

The broad spectrum of TNF activities includes, among others, its action on the endothelium. For example, TNF causes the endothelium to express procoaguant activity but it has also been shown to stimulate or induce the expression of cell adhesion molecules such as ICAM-1 or ELAM-1 on the endothelial cell surface [68].

There is, however, limited information on whether TNF or cytokines in general can affect the binding of neoplastic cells to the endothelium in a manner similar to that observed in leukocyte–endothelium

interactions. The binding of human melanoma cell lines, colorectal carcinoma cell lines and mastocytoma cells to endothelial cells was shown to increase following treatment of the endothelium with TNF or IL-1β [41, 62, 69] but to date little is known about the intracellular pathways involved in either the regulation of cell adhesion molecule expression or the mechanism of action of TNF or IL-1β.

The involvement of PKC was proposed for IFN-γ-mediated stimulation of ELAM-1 and ICAM-1 on human umbilical vein endothelial cells (HUVEC) [70, 71], and Lane *et al.* [72, 73] found that activators and inhibitors of PKC could affect ICAM-1 expression which suggested that PKC was involved. Several studies indicated that PKC also played a role in TNF signal transduction in a number of cell types including lymphoid cell lines where TNF caused a transient activation and translocation of PKC [74] or in melanoma cells where TNF-mediated gene expression was found to be regulated in a positive manner by PKC [75].

In recent studies, Bereta *et al.* [12, 41] examined the role of various protein kinases in TNF signal transduction leading to increased binding of P-815 mastocytoma cells to the endothelium, but these studies remained inconclusive with regard to the real implication of PKC in TNF-enhanced cell binding.

Role of PKC in tumor cell adhesion induced by various compounds

The results presented recently [76] with rat Walker carcinosarcoma W-256 cells and in a previous study with Lewis lung carcinoma cells [77] clearly indicated that a lipoxygenase metabolite of arachidonic acid, 12-(*S*)-hydroxyeicosatetraenoic acid (12-(*S*)-HETE), produced by normal cells as well as tumor cells [78, 79], mimicked the effect of TPA on tumor cell adhesion to the endothelium. This enhancement was mediated by activation of tumor cell PKC as shown by the inhibitory effect of PKC inhibitors such as H-7 and especially calphostin C and the inhibitory effect of PKC down-regulation [80].

However, 12-(*S*)-HETE may not be the only effector mediating this heterotypic cell-to-cell interaction because data demonstrated that 12-(*S*)-HETE-enhanced adhesion was inhibited by 13-(*S*)-hydroxyoctadecadienoic acid (13-(*S*)-HODE) [76]. This latter monohydroxy fatty acid is a lipoxygenase metabolite of linoleic acid which was originally identified as a platelet chemorepellant factor produced by endothelial cells [81]. Later studies demonstrated that some tumor cells produced 13-(*S*)-HODE and that the ratio of 13-(*S*)-HODE/HETEs produced by tumor and endothelial cells appears to modulate the adhesive properties of these cells [76, 78].

It was observed that 12-(*S*)-HETE mimicked the effect of TPA on tumor cell adhesion [77] and Liu *et al.* [76] suggested the possibility of an alternative mechanism for PKC activation by this hydroxy fatty acid. Most interestingly, as second messengers, 12-(*S*)-HETE/13-(*S*)-HODE may provide yet another mechanism for bidirectional regulation of PKC and subsequent stimulation or inhibition of tumor cell adhesion to endothelium.

The regulation protein thrombin has been shown to stimulate multiple endothelial cell functions, including the production of PDGF, platelet-activating factor (PAF) and neutrophil adhesion [82, 83] and was also shown to cause increased binding of the monocytic cell line U-937 [51] to cultured endothelial cells through a PKC-dependent mechanism.

PKC ACTIVITY AND THE EXPRESSION OF CELL ADHESION RECEPTORS

Integrins

The role of integrins in the development of cancer is an area of active investigation. The integrins are a family of non-covalently associated heterodimer complexes, all of which are cell surface receptors involved in cell adhesion processes, and most of which recognize the sequence Arg-Gly-Asp (RGD) in their ligands [84,85]. Until recently, it was thought that the integrin family consisted of three subfamilies, each characterized by a specific β chain which was capable of associating with several different α subunits [85]. Because extracellular matrix fibronectin fibrils are organized in alignment with intracellular actin filaments [86], and because integrin receptors have been shown to be localized in and around cellular adhesion plaques [87, 88], it has been suggested that the cytoplasmic domains of the integrin receptors bind to cytoskeletal proteins and that the integrins serve to link these extracellular and intracellular structural elements. A variety of evidence supports this suggestion although little is known about the direct basis or the regulation of these interactions. Many stimuli that activate membrane-associated protein kinases lead to significant morphological changes. For example, tumor promoters which activate protein kinase C cause dramatic shape changes in adherent cells [5]. Such changes may be brought about through the phosphorylation of the integrins.

Phosphorylation has been shown to regulate the function of a variety of cellular proteins [89] and phosphorylation of surface receptors is commonly involved in the regulation of affinity for ligands as well as enzymatic activities and other processes, e.g. internalization [5].

The β subunit of the chicken CSAT antigen complex, a mixture of receptors belonging to the integrin family of cell adhesion receptors [90], becomes phosphorylated on a tyrosine residue in cells transformed by retroviruses encoding protein-tyrosine kinases [4]. The common β subunit of another integrin subfamily, the leukocyte adhesion receptors, leukocyte function associated antigen-1 (LFA-1), Mac-1 and P150/95, becomes phosphorylated when cells are treated with TPA [91].

In a recent work, Freed *et al.* [5] described the TPA-induced phosphorylation of a novel β subunit (designated as βs) which was precipitated by anti-VNR antibodies. This protein was associated with an α subunit (αv) and was present on the surface of MG-63 human osteosarcoma cells, AG 1523 human fibroblasts and human endothelial cells, forming a functional RGD receptor.

In U-937 cells, treatment with TPA markedly increased CD11a, CD11b and CD18 antigen

expression, suggesting that PKC may play a major role in regulating the expression of these surface antigens [92]. However, regulation of Mac-1 expression by C5a was shown to be independent of PKC activation [93].

Nevertheless, the functional effects of these phosphorylations have not yet been clearly demonstrated but phosphorylations would be likely to have a large influence on the adhesive properties of the integrin receptors.

Luminal cell adhesion molecules

During inflammation and wound healing, one important function of the endothelium is to direct the flow of cellular blood elements into the appropriate tissue compartments, and to date there is good evidence that tumor cells utilize similar processes to undergo metastatization. These white blood cell, platelet and tumor cell interactions are mediated through an ever-growing number of cell-to-cell adhesion molecules but, because this topic has been the subject of many recent reviews [94, 95], it will be summarized only briefly here.

Adhesion receptors of the immunoglobulin superfamily. The immunoglobulin superfamily includes a large number of related molecules, most of which function in cell-to-cell recognition or adhesion [96]. Platelet-endothelial cell adhesion molecule-1 (PECAM-1) is a recently identified member of the immunoglobulin superfamily that is present on the surface of platelets, some white blood cells, and endothelial cells [97]. In addition to PECAM-1, three other members of this family are present on endothelial or epithelial cells and play a role in white blood cell adhesion but also probably in tumor metastasis. ICAM-1 is a 90 kDa, inducible surface glycoprotein that is one of the ligands of LFA-1 and Mac-1, the $\beta 2$ (or CD18) integrins expressed by white blood cells [98]. Although present at low levels on both endothelium and epithelium under normal conditions, this protein is markedly regulated by a variety of inflammatory stimuli such as endotoxin, IL-1β and TNF-α [95]. It appears that up-regulation of ICAM-1 is important in both the adhesion and migration of circulating neutrophils and monocytes into inflamed tissues.

A second, related ligand for LFA-1 has been identified and termed ICAM-2 [98]. This molecule is smaller than ICAM-1 and appears to be constitutively expressed on endothelial cells. Its role in the inflammatory process and in tumor metastasis is still unknown.

Another immunoglobulin superfamily member that interacts with an integrin has recently been identified and cloned. This molecule, termed VCAM-1, is also an inducible surface glycoprotein found on endothelial cells [99]. The ligand for VCAM-1 is the $\alpha 4\beta 1$ (or VLA-4) integrin [100]. VCAM-1 is up-regulated by the same inflammatory mediators as ICAM-1 over the same time course. The primary role of VCAM-1, however, appears to be in the adhesion of lymphocytes to activated endothelium at sites of inflammation.

LEC-CAMs. ELAM-1 and granule-associated membrane protein 140 (GMP-140) are members of a second family of inducible cell surface proteins present on the surface of endothelial cells called the selectins or LEC-CAMs (lectin-like cell adhesion molecules) [101]. ELAM-1 is a 115-kDa protein found only on the surface of endothelial cells that have been activated with agents such as endotoxin, IL-1β, IFN-γ, or TNF-α [102]. Increased surface expression is dependent on protein synthesis. ELAM-1 appears on the cell surface more rapidly than does ICAM-1 or VCAM-1. Until very recently, the ligand for ELAM-1 remained a mystery; however, there is now strong evidence that ELAM-1 binds to a carbohydrate group called the sialyl-Lewis X determinant, a structure found on cell-surface glycoproteins and glycolipids of neutrophils, monocytes, and some tumor cells [103].

The other member of the LEC-CAM family, GMP-140 or PADGEM (platelet activation-dependent granule external membrane protein), is found on platelets, as well as on endothelial cells [104]. The regulation of this protein is quite different from ELAM-1. GMP-140 is stored in preformed granules, along with von Willebrand's factor, within endothelial cell Weibel-Palade bodies. Upon stimulation with a unique set of activating agents (i.e. histamine, thrombin, and PAF), GMP-140 is rapidly moved to the endothelial cell surface where it mediates adhesion of neutrophils [105]. Like ELAM-1, the ligand for GMP-140 also appears to be a carbohydrate group. In this case, it is the non-sialylated Lewis X determinant found on the surface of the white blood cells and recognized by antibodies against CD15 [106].

Tumor cell adherence to adhesion receptors and the involvement of PKC. The intracellular signaling events which participate in the up-regulation of ICAM-1, VCAM-1 or ELAM-1 are not fully understood. In a study aimed at investigating the metabolic pathways which mediate up-regulation of ELAM-1 in endothelial cells, down-regulation of PKC activity induced by exposure of HUVEC to TPA as well as preincubation with staurosporine or H-7 prevented TNF-, IL-1β- or LPS-dependent up-regulation of ELAM-1 and ICAM-1 or HUVEC [107]. These authors concluded that activation of PKC played a major role in inflammatory mediator-induced ICAM-1 and ELAM-1 expression in endothelial cells.

Findings that both phorbol esters and mezerein, a non-phorbol stimulator of PKC, [108] were potent inducers of ICAM-1 on HUVEC [72], partly confirmed these observations which were further ascertained by the effects of staurosporine and H-7, both of which prevented the up-regulation of ICAM-1 on HUVEC in response to IL-1β, LPS, TNF-α or IFN-γ [71, 73]. In these experiments, inhibition of ICAM-1 up-regulation by staurosporine and H-7 was accompanied by inhibition of PMN adherence to HUVEC [74, 76]. Within the same study, Lane *et al.* [73] showed that prolonged exposure to TPA (leading to down-regulation of PKC) was accompanied by dose-dependent suppression of the response of the cells to a subsequent exposure to TPA or to IL-1β, suggesting a major role for PKC in HUVEC activation by IL-1β. However, the response to TNF-α and LPS was only partially inhibited by TPA preincubation, suggesting an

alternative, non-PKC-dependent pathway for TNF-α and LPS, or the existence of functionally distinct compartments of PKC within HUVEC which differ in resistance to down-regulation by TPA. Such observations which were partly confirmed by others [107, 109–111], led to the concept that ICAM-1 induction by IL-1β, TNF-α or LPS may involve PKC in a "non-exclusive" manner but findings to date must be considered neither in agreement nor conclusive.

While the regulatory mechanism for expression of GMP-140 in platelets is poorly understood, it apparently involves a complex sequence of activation processes including structural transformation and translocation of α-granules and secretion of their contents. This process is triggered by transmembrane signaling transducers including agonist receptor associated G-proteins, phospholipases A_2 and C, PKC, and 47-kDa phosphoprotein [112]. Some of these processes have been proposed to be modulated by sphingosine [113], glycosphingolipids and sphingosine derivatives [114, 115].

Importance of PKC in the control of the transcription of the adhesive proteins. To date, the molecular mechanism that controls transcription of the adhesive protein genes has not yet been elucidated, but recently Montgomery *et al.* [116] reported for the first time the DNA sequence of the 5′ flanking region of the ELAM-1 gene. This region revealed consensus binding sequences for two known transcription factors, AP-1 and NF-κB. AP-1 is the protein product of the *jun* proto-oncogene [117]. Activated AP-1 binds to a *cis*-acting element that mediates a transcriptional response to active phorbol esters such as TPA. NF-κB and a family of related proteins have been shown to increase transcription of several genes involved in immunity and inflammation [118, 119]. Several studies have demonstrated that LPS [118], TNF-α, and IL-1β [120] activate NF-κB and increase gene transcription. Although the mechanism(s) involved in NF-κB activation has not been identified completely, previous observations [121] suggest that PKC may play a key role in NF-κB activation, possibly via phosphorylation of its cytoplasmic inhibitor, I-κB [122]. However, other studies have shown that agents such as TNF-α and IL-1β activate NF-κB through PKC-independent pathways [120, 123, 124].

To elucidate the mechanisms involved in endothelial activation, Montgomery *et al.* [125] reported that ELAM-1 expression in HUVEC involved transcriptional activation of ELAM-1 gene and that ELAM-1 mRNA induction was preceded by PKC-independent activation of NF-κB but not AP-1. PKC-independent activation of NF-κB in HUVEC with TNF-α, IL-1β or LPS appeared to be associated with, but not sufficient for, activation of ELAM-1 gene transcription.

Thus, the current studies leave many questions regarding the cell biology of adhesive proteins and integrin expression unanswered, including the precise mechanism of PKC activation by the various inflammatory mediators that are suspected to alter tumor metastasis and how the activation of PKC translates into adhesive protein up-regulation and increases tumor cell adhesion. Investigations of these questions may eventually suggest pharmacologic means of altering endothelial cell–tumor cell interactions by the modulation of adhesive proteins/integrin expression.

FUTURE PROSPECTS

Modulation of PKC activity may have important applications in attenuating the endothelial cell response to specific stimuli during tumor metastasis, thereby improving patient survival. Since PKC exists as a family of isozymes (at least seven of which are now well characterized) [126], identification of a specific endothelial or tumoral PKC isozyme and the development of an appropriately specific inhibitor may make possible the deliberate and beneficial modulation of endothelial cell functions involved in tumor metastasis.

During the past 7 years, we have witnessed an explosive growth in our knowledge about the basic mechanisms of cell adhesion. Most of this information has been generated using cell culture and molecular biology techniques. The challenge for the future will be to understand how these various receptors work together *in vivo* to regulate normal physiology and how they contribute to disease. Although some of our pathophysiologic models or concepts may require modification as new adhesion-molecule ligand/receptors and cytokines are discovered, it is hoped that the proposed paradigms will serve as a basis to integrate and understand the new molecular and cellular associations, as well as providing a rational basis for the development of new therapeutic modalities.

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