Clin. Exp. Metastasis, 1995, **13,** 134–140

Role of phospholipase D in laminin-induced production of gelatinase A (MMP-2) in metastatic cells

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(*Received 3 June 1994; accepted in revised form 20 December 1994*)

Metastatic spread depends critically upon the invasiveness of tumor cells, i.e. their ability to breach basement membranes by elaborating and secreting specific proteolytic enzymes such as gelatinase A (MMP-2). Laminin is a major constituent of the extracellular matrix that can trigger production of MMP-2 in metastatic cells, but not in non-metastatic cells. The present study was designed to examine the role of phospholipase D (PLD) and its product, phosphatidic acid, in the intracellular signal transduction mechanisms that mediate induction of MMP-2 by laminin. Here we show that stimulation of tumor cells with laminin results in a time- and dose-dependent activation of PLD. Laminin-induced production of MMP-2 is attenuated by 1-butanol, a competitive substrate of PLD that reduces PLD-catalyzed production of PA. Moreover, phosphatidic acid itself can induce production of MMP-2 in metastatic tumor cells. MMP-2 can also be induced by exposing the cells to exogenous bacterial PLD. Elevated cellular phosphatidic acid induces MMP-2 in metastatic *ras*-transformed 3T3 fibroblasts but, like laminin, fails to do so in normal cells. These data indicate that laminin-induced activation of PLD and consequent generation of phosphatidic acid are involved in a signal propagation pathway leading to induction of MMP-2 and enhanced invasiveness of metastatic tumor cells.

Keywords: cancer, collagenase IV, invasiveness, metastasis, phosphatidic acid, signal transduction

Introduction

The metastatic cascade is a complex process which involves the dissemination of tumor cells from the primary tumor and formation of secondary foci at distal site(s). This process requires locomotion and attachment of tumor cells to the subendothelial matrix, release of lytic enzymes, local degradation of the matrix and, ultimately, migration of cells through the vessel wall [1–6]. There is a strong correlation between the ability of cells to traverse basement membranes *in vitro* and their metastatic potential *in vivo* [7]. The major structural component of basement membranes, providing the chief barrier to migration of cells, is collagen IV [8–9]. MMP-2, a neutral metalloproteinase, is the key enzyme in degradation of basement membranes [10–13]. Laminin, the main non-collagenous glycoprotein, has been implicated in cell attachment, cell spreading, mitogenesis, neurite outgrowth, morphogenesis and cell motility [14]. In certain metastatic cells, but not in normal cells, there is an increase in collagenase production in response to laminin [15, 16]. However, the intracellular mechanisms that transduce laminin's action to MMP-2 production have remained unknown. Phospholipase D (PLD) activation has been demonstrated in numerous cell types upon stimulation by external signals such as hormones, growth factors and neurotransmitters [17–20]. We now present evidence that laminin activates PLD and that the resultant generation of phosphatidic acid is causally related to the subsequent laminin-induced production of MMP-2.

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Materials and methods

HT1080 cells, derived from a metastatic lesion of a human fibrosarcoma and NIH-3T3 murine fibroblasts, were obtained from the American Type Culture Collection. B16F10 and B16B16 cells, derived from a murine melanoma, were provided by Dr I. J. Fidler, MD Anderson Research Center, University of Texas, Houston, TX. Human foreskin fibroblasts were provided by Dr Y. Yarden, Weizmann Institute, Israel. The cells were maintained under an atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) or minimal essential medium (MEM), supplemented with 10% or 5% fetal calf serum, glutamine, vitamins, nonessential amino acids and antibiotics depending on the cell type (Biological Industries, Kibbutz Beth HaEmek, Israel). Prior to their use in the various experiments, the cells were transferred to serum-free conditions (0.1% bovine serum albumin, Sigma, St Louis, MO) for 18 h.

Collagenolytic activity was determined on substrate-impregnated gels as previously described [12], with minor modifications. Briefly, samples of culture media were separated on gelatin-impregnated (1 mg/ml, Difco, Detroit, MI), SDS-8% polyacrylamide gels under non-reducing conditions, followed by 30 min shaking in 2.5% Triton X-100 (BDH, UK). The gels were then incubated for 16 h at 37°C in 50 mM Tris, 0.2 M NaCl, 5 mM $CaCl_2$, 0.02% Brij 35 (w/v) at pH 7.6. At the end of the incubation, the gels were stained with 0.5% Coomassie G 250 (Bio-Rad, Richmond, CA) in methanol/acetic acid/H_2O (30:10:60). The intensity of the various bands was determined on a computerized densitometer (Molecular Dynamics type 300A).

Total RNA (10–20 μg) was separated on 1% agarose gels, transferred to nylon membranes, and probed with a random primer (Amersham kit, Amersham, Arlington Heights, IL) ^{32}P-labeled cDNA probe, kindly provided by Dr G. I. Goldberg, Washington University, St Louis.

Phospholipid phosphatidyl moieties were labeled by pre-incubating the cells for 24 h with [^{3}H]oleic acid (5 μCi/60-mm dish; Du Pont-New England Nuclear) in DMEM supplemented with 0.1% bovine serum albumin. At the beginning of the treatment schedules, 1-propanol (0.5%) was added to the appropriate dishes. Following the various treatment schedules, the culture dishes were placed on ice and rinsed with ice-cold phosphate-buffered saline (PBS). Cold methanol (1 ml) was added to each dish, cells were scraped and transferred to glass tubes. Chloroform (1 ml) and 1N HCl, 1 mM EDTA solution (1 ml) were added, tubes were vigorously mixed and centrifuged (10 min, 2500 rpm). The lower phase containing the extracted lipids was separated and dried under vacuum. The lipid extract was separated by thin layer chromatography (TLC) on silica gel LK6 glass plates (Whatman, Maidstone, UK) using the organic phase of a mixture of ethyl acetate/2,2,4-trimethyl pentane/acetic acid/water (13:2:3:10) as the mobile phase. The plates were sprayed with EN^{3}HANCE (Du Pont-New England Nuclear) and exposed for 7–10 days at −70°C. Bands corresponding to phosphatidylpropanol standard were identified, scraped and counted. The TLC background was determined in parallel with the experimental samples by running extracts from pre-labeled but untreated and propanol-free cultures. These values were subtracted from the [^{3}H]-phosphatidylpropanol values in all experiments.

Results

MMP-2 was characterized as a neutral metalloproteinase, Mr ~ 70 kDa [10–13] that shares homology with a number of metalloproteinases including interstitial collagenase [21], stromeolysin [22, 23] and a recently described 92 kDa proteinase [24]. The enzyme was shown to be produced by vascular endothelial cells in response to angiogenic factors [25] and by cells known to cross basement membrane barriers such as polymorphonuclear leukocytes [26], stimulated macrophages [27] and stimulated Sertoli cells [47]. HT-1080 cells produce two collagenolytic enzymes, MMP-2 and MMP-9. Figure 1A shows that laminin induces MMP-2 collagenolytic activity (72 kDa) in metastatic human fibrosarcoma HT1080 cells and in two metastatic mouse melanoma cell lines, B16F10 and B16B16, but not in non-metastatic mouse NIH 3T3 cells or normal human foreskin fibroblasts. The accumulation of this collagenase is time- and dose-dependent in HT1080 cells (Figure 1B) as well as in the other cell lines tested (not shown). The increased activity correlates with increased expression of MMP-2 mRNA levels following 6 h incubation with various concentrations of laminin. To detect PLD activation, we examined the effect of laminin on accumulation of phosphatidylpropanol, a specific product of the PLD-catalyzed trans-phosphatidylation reaction rather than measurement of the unstable natural product, phosphatidic acid, in T1080 cells [17]. A significant increase in phosphatidylpropanol accumultion (up to five-fold) in response to laminin concentrations in the range of 10–40 μg/ml was observed (Figure 2A). As PLD activation is thought to subserve a signaling role in eukaryotic cells [7] it

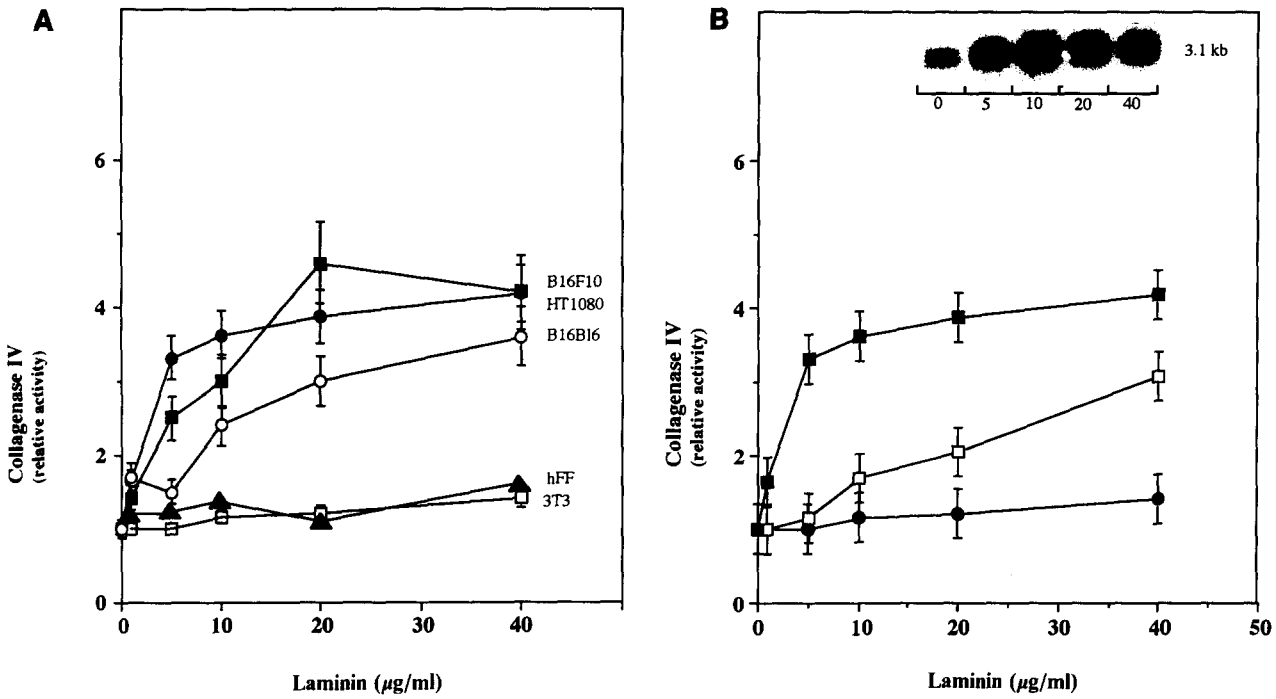

Figure 1. Effect of laminin on MMP-2 production. (A) The various cell lines were exposed to laminin at the indicated concentrations for 6 h. Collagenase activity is presented relative to unstimulated levels for each cell line. (B) HT1080 cells were exposed to laminin at the indicated concentrations for 2 h (●), 4 h (□) or 6 h (■). MMP-2 activity is presented relative to unstimulated levels. Inset: expression of MMP-2 mRNA at 6 h in response to laminin. Experiments were run in duplicate and were repeated at least twice.

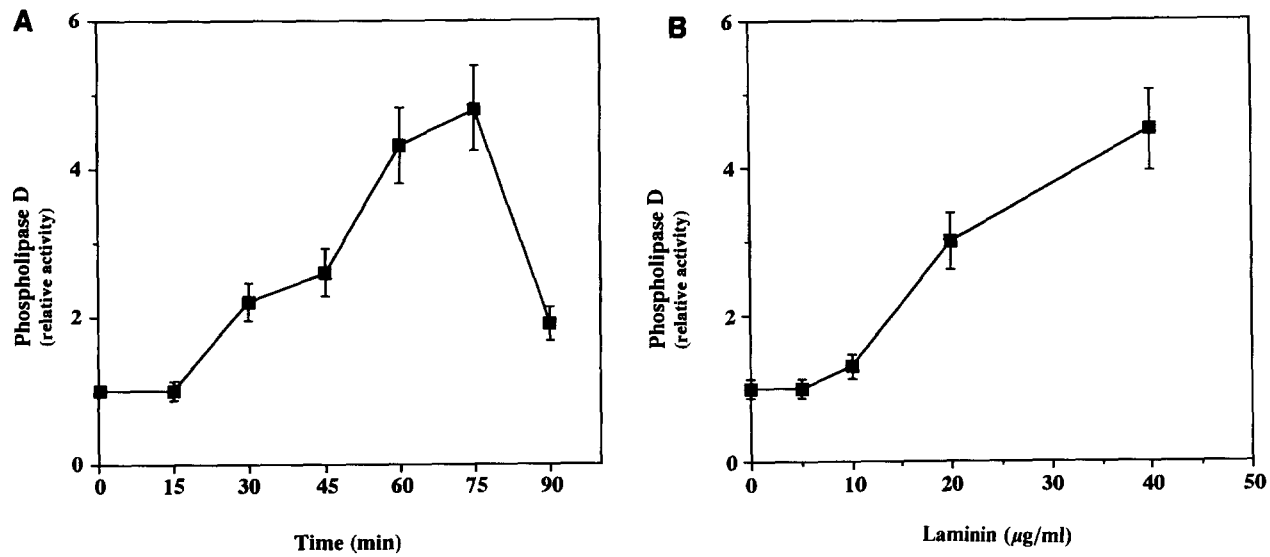

Figure 2. Effect of laminin on phospholipase D activity in HT1080 cells. (A) Cells were incubated with laminin (25 μg/m) for the indicated time. 1-Propanol (0.5%) was added 15 min prior to the termination of each incubation. Phospholipase D activity was estimated by measuring accumulation of phosphatidylpropanol [40], the specific product of the phospholipase D-catalyzed transphosphatidylation reaction, during these 15 min intervals and is expressed relative to phospholipase D activity in unstimulated cells. (B) Cells were incubated with the indicated concentrations of laminin for 60 min in the presence of 0.5% 1-propanol. Phospholipase D activity was estimated by measuring accumulation of phosphatidylpropanol and is expressed relative to phospholipase D activity in unstimulated cells. Experiments were run in duplicate and were repeated at least twice.

was important to determine the time course of its activation by laminin. Figure 2B shows that the accumulation of phosphatidylpropanol in HT1080 cells in response to stimulation by laminin is rather slow. Following a distinct lag period of at least 15 min, phosphatidylpropanol production increases and peaks at 60 min. The other cell lines tested exhibited similar profiles. Thus, PLD activation clearly precedes elevation of MMP-2 production.

To examine further the causal relationships between PLD activation and production of MMP-2, we have taken advantage of the ability of primary alcohols (e.g. 1-butanol) to serve as substrates for the trans-phosphatidylation reaction catalyzed by PLD. This reaction competes with the normal hydrolytic reaction that produces phosphatidic acid and yields instead a phosphatidic acid alkyl ester (e.g. phosphatidylbutanol). This reduces signal-induced generation of phosphatidic acid and, hence, should attenuate or abolish any processes downstream of PLD-catalyzed production of PA. Figure 3 shows that includion of 1-butanol (20 mM) in the culture medium reduces laminin-induced collagenase production. 2-Butanol which does not serve as substrate for PLD trans-phosphatidylation but may be expected to have similar non-specific effects, is much less effective against laminin-induced MMP-2 poduction.

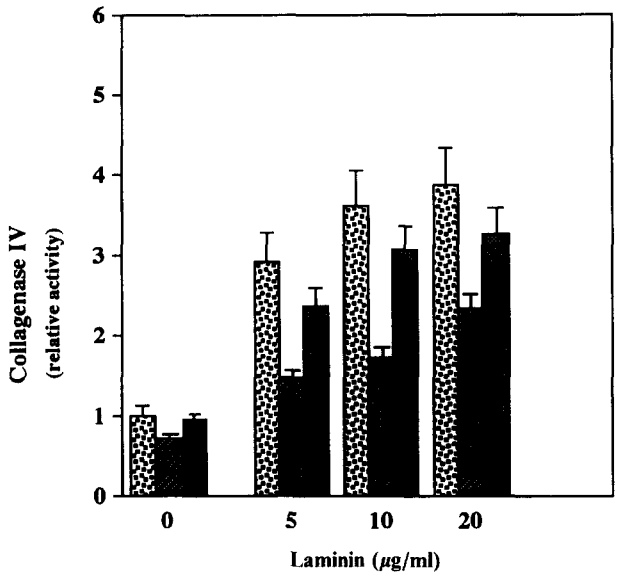

Figure 3. Effect of 1-butanol and 2-butanol on laminin induced MMP-2. HT1080 cells were exposed to 1-, and 2-butanol for 6 h. Collagenase IV activity is presented relative to unstimulated levels. Experiments were run in duplicate and repeated at least twice. ▨, control; ▩, 1-butanol; ■, 2-butanol.

Physiological activation of PLD results in increased intracellular concentrations of phosphatidic acid [28–34], hence, we examined whether phosphatidic acid itself is capable of inducing MMP-2 in tumor cells. Liposomal phosphatidic acid increased the collagenolytic activity of the various tumor cells used in this study in a dose-dependent manner, as shown here for HT1080 cells (Figure 4A). Increased accumulation of phosphatidic acid in the treated cells can also be accomplished by exposure of the cells to a bacterial PLD. This treatment resulted in a time- and dose-dependent increase in MMP-2 production (Figure 4B). Recently, Imamura *et al.* [46] raised the possibility that the lyso-phosphatidic acid rather than phosphatidic acid is responsible for the intracellular signal propagation. In our hands, lyso-phosphatidic acid failed to induce collagenolytic activity in the tumor cells (data not shown). Exposure of normal (non-metastatic) NIH 3T3 fibroblasts to exogenous phosphatidic acid or to bacterial PLD failed to induce MMP-2; in contrast, these agents increased production of MMP-2 in cells that were rendered metastatic by transformation with H-ras (Table 1).

Discussion

The present results show, for the first time, that induction of collagenase IV by laminin is preceded by activation of PLD. The existence of a causal relationship between these two events is further indicated by the following findings: (i) Attenuation of PLD-catalyzed production of phosphatidic acid by 1-butanol, an alternative substrate of PLD, attenuated induction of MMP-2 by laminin. (ii) Elevation of cellular phosphatidic acid, either by addition of exogenous phosphatidic acid or by treatment with a bacterial PLD, mimicked laminin-induced production of MMP-2. Furthermore, similar to laminin, these effects of elevated phosphatidic acid could only be observed in metastatic cell lines or in cells that were made metastatic by transformation with H-ras. Therefore, these findings strongly suggest that both activation of PLD and the subsequent generation of phosphatidic acid participate in the cascade leading to induction of MMP-2 by laminin and strengthen the link between this cellular response and the metastatic process.

The present results raise several interesting and important questions regarding the mechanisms of PLD activation by laminin. Rapid activation of PLD follows receptor ligation by a variety of substances such as hormones, growth factors and neurotransmitters as demonstrated in numerous systems [17–20], but

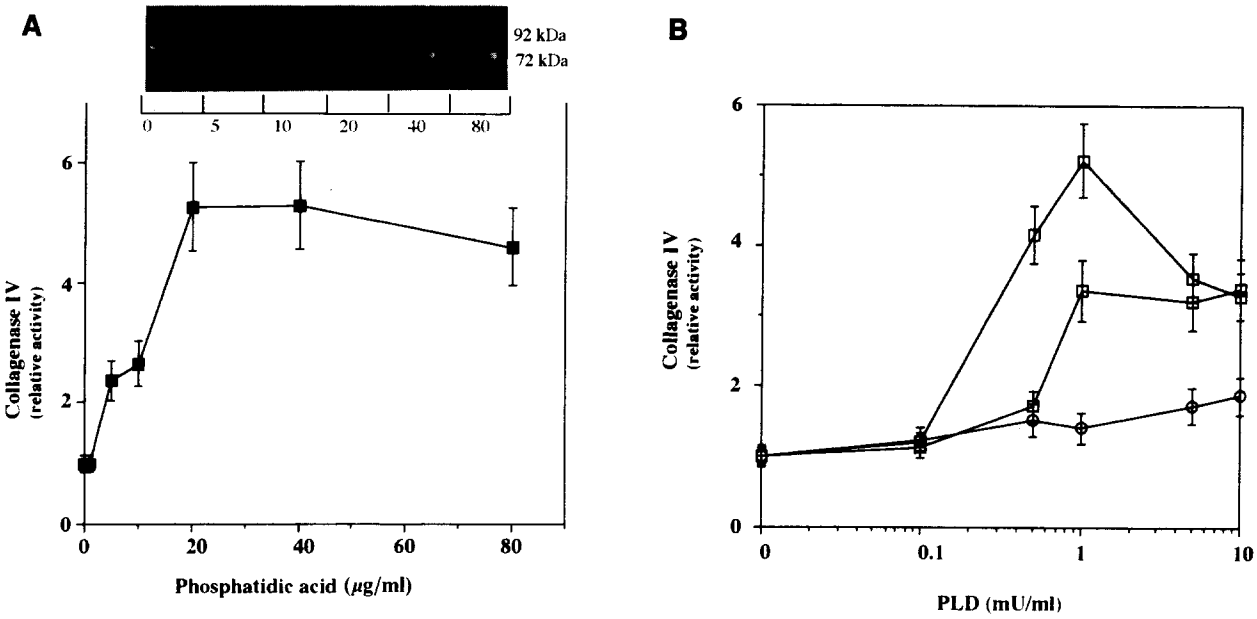

Figure 4. Effect of phosphatidic acid and bacterial phospholipase D on MMP-2 production in HT1080 cells. (A) HT1080 cells were exposed to sonicated dipalmitoyl-phosphatidic acid (Sigma) liposomes at the indicated concentrations for 6 h. Collagenase IV activity is presented relative to unstimulated levels. (B) HT1080 cells were exposed to *Streptomyces chromofuscus* phospholipase D (Sigma) at the indicated concentrations for 2 h (○), 4 h (□) or 6 h (⊡). Collagenase IV activity is presented relative to unstimulated levels. Experiments were run in duplicate and repeated at least twice.

Table 1. Collagenolytic activity in 3T3 and ras-3T3 cells

	Untreated	Laminin (μg)		Phosphatidic acid (μg)		Phospholipase D_b (mU)	
		5	10	10	40	1	5
3T3	1.0	0.88	1.04	0.85	1.15	0.92	0.98
H-ras 3T3	1.0	2.2	3.3	1.8	2.9		nt

nt, not tested.

this is the first report of such activation by a non-collagenous component of the extracellular matrix. The elucidation of receptors for laminin and other extracellular matrix proteins has become an important issue in cell biology because many aspects of cell activity are influenced by cell–matrix interactions. Four groups of membranal/cellular proteins are reported to bind laminin. In the integrin group, at least six different members were reported to bind laminin [35–39]. It is presently unknown which among these receptors are linked to PLD activation and whether this is a direct link. The time-course of phosphatidylpropanol accumulation, utilized here as a measure of PLD activity, clearly shows a lag of at least 15 min. In contrast, PLD activation in hormone-situated cells, e.g. NIH 3T3 cells treated with either platelet-derived growth factor or prostaglandin $F_2\alpha$ [40], or in granulosa cells treated with gonadotropin-releasing hormone [41] is very rapid. This may suggest that activation of PLD by laminin is secondary to another intracellular event. It was recently reported that laminin induces protein dephosphorylation in neural cells [42]. In addition, we have shown that arachidonic acid metabolites from both the cyclo-oxygenase and the lipoxygenase pathways are required for the production of MMP-2 in tumor cells [43, 44]. Since the hydrolysis of phosphatidylcholine by phospholipase A_2 provides an important source of arachidonic acid and because phospholipase A_2 and PLD are often activated in a coordinated fashion [45]

it is reasonable to assume that both enzymes comprise part of a signaling cascade triggered by laminin that culminates in increased production of MMP-2. In this context it may be speculated that the late activation of PLD demonstrated in the present study provides 'second wave' intracellular messengers that regulate delayed and/or sustained cellulr responses of which MMP-2 production may be only one example.

Laminin, both in soluble or surface-bound form, elicits many cell-specific responses, causing secretory cells to become polarized, neural cells to extend axon-like processes, various cells to differentiate and others to migrate [5]. Activation of PLD may conceivably be involved in mediating some or all of these processes, but this remains to be shown. Laminin has a pronounced effect on cell motility, being chemotactic when it is in solution and haptotactic when surface-bound. The possible role of PLD in mediating laminin-induced cell motility is of particular interest because cellular motility is essential for the metastatic process whether it is along a solute gradient (chemotaxis) or along an insoluble one (haptotaxis). Recently, Xu *et al.* [48] have shown that long term ethanol feeding (primary alcohol) impairs binding to laminin. One might speculate that this impairment is due to production of phosphatidyl-ethanol in the cells followed by impairment of PLD mediated signal transduction in the hepatocytes. Since the various receptors of laminin are not yet sufficiently well characterized, the intriguing possibility that in addition to induction of MMP-2, phosphatidic acid might be involved also in the chemotactic response of the metastatic cells, cannot be ruled out.

Acknowledgements

We thank P. Ben-Av and Y. Eli for their valuable help. M.L. is an Yigal Allon Fellow and incumbent of the Shloimo and Michla Tomarin Career Development Chair in Membrane Physiology. This study was supported in part by a grant to R.R. from The Clive and Judy Callman Fund for Cancer Research, UK.

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