Activation of protein kinases A and C increase lymphocyte penetration through endothelial monolayers

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A brief incubation of lymphocytes with either PMA, stimulating protein kinase C, or with dibutyryl-cAMP, leading to protein kinase A activation, led to increased lymphocyte penetration through intact endothelial monolayers in vitro The PMA-induced penetration could be dose-dependently down-regulated with a protein kinase C inhibitor, H7. Similarly HA 1004, being mainly a protein kinase A inhibitor, decreased the dibutyryl-cAMP induced penetration. Treatment of lymphocytes with PMA and cAMP did not alter the expression of CD44 homing receptors on lymphocytes Stimulation of lymphocytes with dibutyryl-cGMP or calcium ionophore had no effect on lymphocyte penetration These results suggest that activation of both protein kinases A and C is important in the lymphocyte binding to endothelium.

Protein kinase A, Protein kinase C; Endothelial cell

1. INTRODUCTION

Recirculating lymphocytes exit the blood circulation via specialized high endothelial venules (HEV) lined by a typical high endothelium (HE) [1]. Lymphocytes have homing receptors on their surface. In mice MEL-14 defines a receptor responsible for lymphocyte homing into peripheral lymph nodes and LPAM-1 a receptor responsible for homing into Peyer's patches [2,3]. On the endothelial side specific vascular addressins recognize these homing receptors [4]. Besides these organ- and/or tissue-specific receptor-ligand pairs also non-specific mechanisms exist. Lymphocyte function antigens (LFA-1), which belong to the integrin family, are the counterpart for ICAM-1 and ICAM-2 on endothelial cells [5,6]. Besides strengthening the adhesion, the interaction between LFA-1 and ICAM-1 provides an additional intracellular signal [7,8].

Activation of in vitro cultured endothelial cells by several cytokines and lipid mediators leads to increased binding of lymphocytes to endothelial monolayers [9–13]. For instance, IL-1α operates via cAMP in the induction of binding to endothelial monolayers [14]. Similarly all these cytokines also increase the penetration on lymphocytes through the endothelium [15,16]. Our recent results indicate that the signal transduction pathway during IL-1 stimulation in the binding phase is similar to the penetration phase [16].

Much less is known about the activation status of lymphocytes in correlation to their binding to and penetration through endothelial monolayers. It is demonstrated in this paper that treatment of lymphocytes with a protein kinase C pathway stimulator, PMA, and protein kinase A stimulator, dibutyryl-cAMP, lead to rapid increase in the number of lymphocytes penetrating through endothelial layer. This penetration event was dose-dependent, and it was possible to inhibit it by relevant PKC or PKA inhibitors. The rapid activation of protein kinases and induction of lymphocyte penetration did not correlate to increased expression of lymphocyte homing receptor Hermes-3.

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2. MATERIALS AND METHODS

2.1. *Cells*

Human umbilical vein endothelial cell-line EA.hy 926 was obtained from Dr C.-J.S. Edgell (University of North Carolina, NC, USA) [17]. Peripheral blood leukocytes were isolated with a density gradient centrifugation (Ficoll-Isopaque, Nycomed, Norway) in a routine manner. Adherent monocytes were depleted by two consecutive 1 h incubations in plastic Petri dishes and the remaining loose cells were used in the lymphocyte penetration assay.

2.2. *Penetration assay*

0.4×10^6 endothelial cells were seeded on a Millipore filter (Sartorius, Vangard Int., NJ) placed under a glass O-ring (diameter 15 mm) in a 24-macrowell tissue culture plate (Nunclon). After an overnight incubation, the EC were growing as a tight monolayer. The cultures were washed with warm media and 2×10^6 lymphocytes were placed on top of the EC layers into the glass O-rings. After a 3 h incubation at 37°C, the media on top of the filter were removed. The filters were then washed, fixed in 70% ethanol overnight and stained with Mayer's hemalun. From these preparations it was possible to determine separately the leukocytes remaining on top of the EC-monolayer, and the number of cells which were under the monolayer and had penetrated through the endothelial monolayer into the filter. The number of penetrated lymphocytes was calculated from at least

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20 individual high power fields and figures are given as number of cells penetrated/1 mm^2 of endothelial monolayer. The endothelial monolayer was verified by ordinary light microscopy and cross-section preparations in the electron microscopy.

2.3. *Reagents*

Monoclonal Hermes-3 antibody detecting CD44 was obtained as a generous gift from Sirpa Jalkanen (University of Turku, Finland). Stimulators of intracellular signal transduction: *N*6-2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate (cAMP), *N*2,2′-*O*-dibutyrylguanosine 3′,5′-cyclic monophosphate (cGMP), calcium ionophore A23187 and phorbol 12-myristate 13-acetate (PMA) were all purchased from Sigma In some experiments the lymphocytes were treated with second messenger stimulators in combination with protein kinase C inhibitor 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H7), with a cyclic nucleotide protein kinase (A) inhibitor HA1004 Both enzyme inhibitors were purchased from Seikagaku Kogyo (Tokyo, Japan).

3. RESULTS

In the first set of experiments I first isolated lymphocytes from peripheral blood white cells. These lymphocytes were then incubated with various activators of intracellular second messengers prior to adding the lymphocytes to endothelial monolayer-coated Millipore filters. After a 3 h incubation, the loose lymphocytes were washed away, the Millipore filters were fixed with 70% ethanol and stained with Mayer's hemalun. From these preparations it was possible to determine the number of lymphocytes that had penetrated through the endothelial monolayer into the filter.

Previous work had shown that 30 min pretreatment of lymphocytes with PMA, which is a protein kinase C stimulator, increased the number of lymphocytes homoaggregations as well as binding to in vitro cultured endothelial cells [18]. Lymphocytes were incubated for 30 min with PMA (1.0 μM) prior to the penetration assay. PMA pretreatment increased the number of penetrated lymphocytes from 1009 ± 241 to 4865 ± 801 per mm^2 of endothelial monolayer ($P < 0.001$. Fig. 1). Also dibutyryl-cAMP, an analogue of cAMP able to penetrate into the cell, increased lymphocyte penetration up to 2399 ± 614 cells per mm^2 ($P < 0.01$). On the other hand, ditubyryl-cGMP, increasing the intracellular levels of cGMP, as well as the calcium ionophore (A23187), increasing intracellular Ca^{2+} concentrations, had no effect after 30 min preincubation on the number of lymphocytes that had penetrated through endothelial monolayers into the Millipore filter.

Further evidence of the role of activation of protein kinase C in the lymphocyte penetration came from the enzyme inhibition studies. An inhibitor to protein kinase C, H7 had a clear dose-dependent effect on the PMA-induced lymphocyte penetration. A 2 μM dose of H7 had no effect on the PMA-induced penetration; 10 μM H7 already decreased the number of penetrating lymphocyte from 4865 ± 801 to 2710 ± 437 corresponding to a 56% decrease of the ± PMA induced increase.

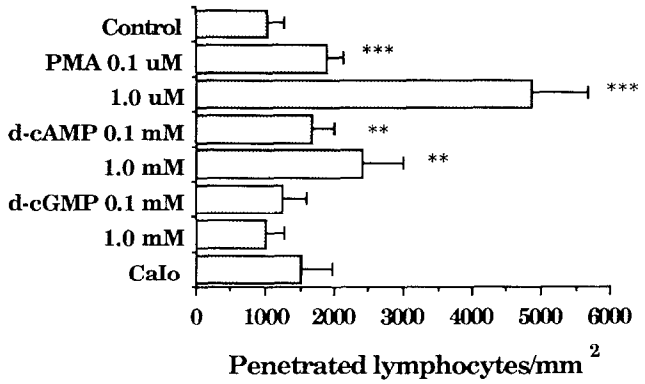

Fig. 1. Regulation of lymphocyte penetration through intact endothelial monolayers Lymphocytes were stimulated with various activators of second messengers for 30 min, washed and placed on the endothelial layer growing on a Millipore filter in a cylinder. After 3 h incubation, the filters were washed, fixed and stained. From these preparations it was possible to determine the number of lymphocytes penetrating through endothelial cells into the filter. The data are expressed as number of penetrated lymphocytes per 1 mm^2 of endothelial layer. Mean ± SD of 7 independent experiments *P*-values are calculated according to paired Student's *t*-test: * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$.

Finally 20 μM of H7 decreased up to 93% of the lymphocyte penetration (Fig. 2).

Similarly HA 1004 is an inhibitor of protein kinase A. HA 1004 could dose-dependently decrease dibutyryl-cAMP induced lymphocyte penetration. The maximal effect was seen at a dose of 20 μM of HA 1004, when 61% of the dibutyryl-cAMP induced increase in lymphocyte penetration was inhibited (Fig. 3).

Hermes-3 (CD44) is an antibody against a lymphocyte homing receptor. In my assay the 30 min incubation with PMA or dibutyryl-cAMP had no effect on the number of Hermes-3-positive lymphocytes or on

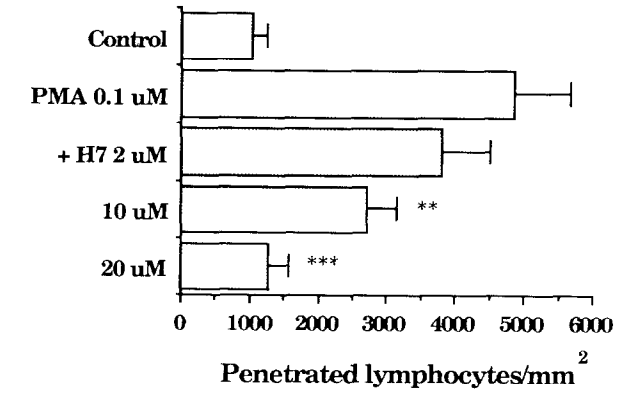

Fig. 2. The role of H7, a protein kinase C inhibitor, on PMA-induced lymphocyte penetration. The lymphocytes were treated with PMA as in Fig. 1. In addition, H7 was added simultaneously with PMA into the indicated samples. H7 had a clear dose-dependent ability to decrease the PMA-induced penetration. Mean ± SD of 7 independent experiments For *P*-values see legend to Fig 1.

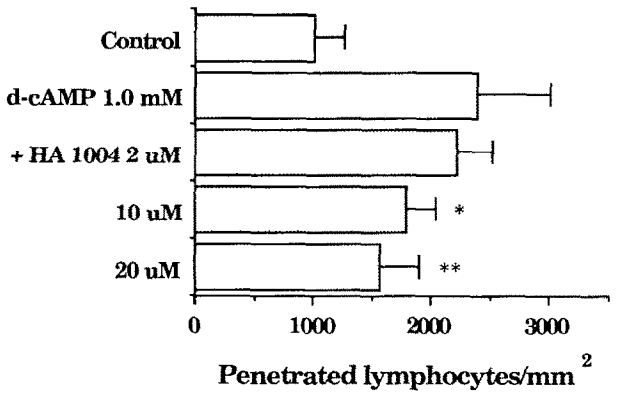

Fig. 3. The role of HA 1004, a protein kinase A inhibitor, on dibutyryl-cAMP-induced lymphocyte penetration. HA 1004 could partially inhibit the induced penetration in a dose-dependent manner. Mean ± SD of 7 independent experiments. For *P*-values see legend to Fig. 1.

the amount of CD 44 on the individual lymphocytes when tested with flow cytometry and compared to non-treated lymphocytes (data not shown).

4. DISCUSSION

The local inflammation process is characterized by an influx of white cells in situ. The regulatory role of endothelial cells has become evident in the initiation of the inflammation process. The white cells bear homing receptors on their surface which recognize their counterpart, vascular addressins on the endothelial side. Only a very small proportion of white cells bound to endothelium will penetrate through it [19]. Therefore the binding to endothelial cells is a necessary but not the only step in white cell invasion from blood circulation to sites of inflammation.

Previous work with PMA had shown that it stimulates protein kinase C in many cell types, like in normal white cells, hematopoietic tumor cells and in endothelial cells [20,21]. Concomitantly PMA induces many cell types to adhere onto plastic surfaces, to each other and to endothelial monolayers [18]. Therefore I asked the question, does increased adherence lead to increased penetration through endothelial monolayers?

We had previously described a reliable technique for analysing the lymphocyte penetration through endothelial monolayers. The endothelial cells were cultured in a cylinder placed upon a Millipore filter and after the endothelial cells had formed a tight monolayer in the cylinder, the tested lymphocytes were placed on top of the endothelial layer in the cylinder [13]. After a 3 h incubation period, the filters were washed, fixed and stained. In this model it was possible to analyse the role of protein kinase A and C in the lymphocyte penetration.

Stimulation of lymphocytes either alone with PMA or with dibutyryl-cAMP increased the number of lymphocytes penetrating through endothelial monolayers. IFN-γ has been shown to activate protein kinase C and calcium fluxes in many cells like endothelial cells. Even though the activation of PKC occurs very rapidly (within 10–15 min) in the endothelial cells after IFN-γ treatment [22], IFN-γ itself requires several hours of incubation before it increases lymphocyte penetration through endothelial cells.

IL-1-induced lymphocyte binding to and penetration through endothelial monolayers has been linked to cAMP as a second messenger. IL-1 stimulation increases within 10 min the intracellular cAMP levels, and forskolin, which is an activator of adenylate cyclase, can mimick IL-1 effects [14]. Dideoxyadenosine, an adenylate cyclase inhibitor, can almost totally inhibit IL-1-induced in vitro lymphocyte homing as well as increasing intracellular cAMP levels, and finally IL-1 leads to activation of protein kinase A in a phosphorylation assay.

IL-1 also requires several hours of incubation with endothelial cells before it increases the lymphocyte binding and penetration. Already a 30 min incubation of lymphocytes with dibutyryl-cAMP led to a significant increase in lymphocyte penetration. The role of the dibutyryl compound to cause this effect can be ruled out due to the fact that dibutyryl-cGMP was totally ineffective in the penetration assay.

Taken together, these results show that stimulation of lymphocyte protein kinase A and C can lead to a rapid increase in the number of lymphocytes penetrating through endothelial monolayers.

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