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Adhesion of B Cell Lines to Endothelial Cells from Human Lymphoid Tissue Modulates Tyrosine Phosphorylation and Endothelial Cell Activation

Lilian I. Reyes,* Paula Escobar,* María R. Bono,* and Mario Rosemblatt2*†

Through the production of cytokines and growth factors the endothelium of secondary lymphoid organs plays a crucial role in controlling lymphocyte migration to the lymphoid microenvironment, an essential step in the initiation of the immune response. Here we demonstrate that direct contact of B cell lines with tonsil-derived human endothelial cells resulted in changes in the phosphorylation state of endothelial cells, causing their functional activation. We found a rapid (<15-s) and transient dephosphorylation, followed by a rapid rephosphorylation of tyrosine residues of the focal adhesion kinase, paxillin, and ERK2. Maximal rephosphorylation occurred after 15–30 min of B cell contact. Preincubation of lymphoid B cells with an adhesion-blocking Ab directed against \( \alpha_\beta \) integrin abrogated adhesion-mediated changes of endothelial cell tyrosine phosphorylation, suggesting that cell contact was essential. Similar patterns of tyrosine phosphorylation, but with slightly different kinetics were induced after cross-linking of \( \beta_\gamma \) integrin or CD40 on endothelial cells. Functional activation of endothelial cells by B cell adhesion was confirmed by the production of IL-6, IL-8, monocyte chemoattractant protein-1, M-CSF, and macrophage inflammatory protein-1β mRNA. However, direct cross-linking of \( \beta_\gamma \) integrin and CD40 failed to accomplish the same functional activation. These data indicate that direct contact of lymphoid B cells with the endothelium from lymphoid tissue induce endothelial cell signaling, resulting in chemokine and cytokine production. This phenomenon may provide a mechanism for the remodeling of the endothelium from lymphoid tissues, thus contributing to the free migration of lymphocytes and other cells into the lymphoid organs. The Journal of Immunology, 2002, 169: 5881–5888.

The vascular endothelium represents an important barrier to lymphocyte traffic, preventing circulating lymphocytes from entering peripheral tissues. However, during inflammation altered endothelium secretes numerous cytokines and chemokines that promote lymphocyte adhesion and extravasation into inflamed tissue. The process of lymphocyte extravasation during inflammation has been extensively studied and involves the participation of a variety of adhesion receptors and their respective ligands (1, 2). On the other hand, the endothelium of secondary lymphoid organs is specially adapted to permit, under normal physiological conditions, the free crossing of lymphocytes, a step crucial to the development of a normal adaptive immune response. This migration employs some of the same adhesion receptors that are activated during the inflammatory process (3). Despite the fact that the functional relevance of this phenomenon has been well recognized, there are few studies examining the mechanisms that regulate the unique structural and functional adaptation of the endothelium from lymphoid tissue.

A few studies have focused on the interaction of B cells with cells of nonhemopoietic origin. In a recent study performed with purified tonsil stromal cells of nonendothelial origin it was demonstrated that tonsil B cells adhere to TNF-activated stromal cells, producing maximal proliferation of B cells (4). A different preparation of tonsil stromal cells characterized as follicular dendritic cells was also used to study the effect of B cell interaction, which resulted in enhanced proliferation of B cells and increased production of Ig and IL-6 (5). Both preparations of tonsil stromal cells produced IL-6 constitutively, suggesting that tonsil stroma may play a crucial role in the growth and development of B cells.

LeBien and co-workers (6) reported that the interaction of B cells with bone marrow-derived stromal cells initiated a signaling cascade on the stromal cells. This resulted in changes in the phosphorylation state of stromal focal adhesion kinase (pp125FAK) (4), paxillin, and ERK2 as well as IL-6 production. Although they did not identify the particular type of cell interacting with the B cells, they demonstrated that adhesion was independent of the \( \alpha_\beta \) integrin/VCAM-1 interaction (7).

Although integrins show no intrinsic protein kinase activity, their engagement activates signaling cascades commonly associated with growth factor stimulation, cell matrix interactions, and cell shape. Several studies have demonstrated that the ligation of the integrin \( \alpha_\beta \) on T lymphocytes induces tyrosine phosphorylation in proteins such as the pp125FAK (8–10), phospholipase A2 (11), phospholipase C\( \gamma \), p59\( \gamma \)/p56\( \kappa \)k, paxillin, and mitogen-activated protein kinase (MAPK) (9, 12). Also, pp125FAK co-localizes with integrins at the site of cell attachment to the extracellular matrix proteins, it binds several adapter and signaling molecules, and its kinase activity and autophosphorylation are mediated by cell adhesion through integrins (13). These as well as other studies

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have demonstrated the importance of β1 integrin in the activation and differentiation of T and B cells (10, 14) as well as in other cell types, such as NK cells (15, 16) and monocytes (17). Additionally, studies performed on regular endothelium show that some of these signals induce endothelial cell activation, which, coupled to cyto- kine and chemokine secretion, affects cytoskeletal reorganization, inducing changes in cell shape (18, 19) that ultimately may affect lymphocyte transmigration.

In the present study we investigated the effect that B cell adhesion may have on the induction of signaling events and the functional activation of endothelial cells from lymphoid tissue. For this we cocultured human B cell lines with human tonsil high endothelial cells (HUTEC). We demonstrate that direct adhesion of these cells induced early tyrosine dephosphorylation events on proteins identified as pp125FAK, paxillin, and ERK2, followed by rephosphorylation of these same proteins. Since HUTEC show high expression of β1 integrin and CD40, we studied signaling events produced after cross-linking of these molecules and demonstrated similar patterns of tyrosine phosphorylation, although with slightly different kinetics. Moreover, we demonstrated that B cell contact induces functional endothelial activation, leading to the production of IL-6, IL-8, macrophage chemotactic protein-1 (MCP-1), M-CSF, and monocyte inflammatory protein-1β (MIP-1β). Hence, contact of lymphocytes with endothelial cells from lymphoid tissue activates signaling cascades and produces factors that may ultimately be involved in the unique adaptation of the endothelium of lymphoid tissue to lymphocyte traffic.

Materials and Methods

Reagents, cells, and Abs

Mouse anti-human β1 integrin (Lia1/2; IgG1) (20) and anti-human αv integrin (HP2/1; IgG1) (21) were provided by Dr. Sánchez-Madrid (Servicio de Immunología, Hospital de la Princesa, Madrid, Spain). Mouse anti-human β1 integrin (TS2/16.2.1; IgG1) and anti-human CD40 (G28-5; IgG1) were obtained from American Type Culture Collection (Manassas, VA). Rabbit anti-paxillin (H114), anti-pp125FAK (A17), and anti-ERK2 (C14) Ab were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-ERK1 (MK12) mAb was purchased from Transduction Laboratories (Lexington, KY). Anti-phosphoysotyrosine Ab (anti-pTyr Ab; 4G10) was furnished by Dr. S. Fischer-Carpe (Compepe, Evry, France). Anti-Fy5 (clone 2C3), an mAb to Duffy Ag receptor for chemokines (DARC), a high endothelial cell marker (22), was supplied by Dr. J.-P. Cartron (Institut National de la Santé et de la Recherche Médicale, Unité 76, Institut National de Transfusion Sanguine, Paris, France). HRP-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were obtained from Biosys (Compiegne, France). HRP-conjugated goat anti-mouse IgG were obtained from Dako (Glostrup, Denmark). Other Abs used are as indicated in Ref. 23. All other reagents were purchased from Sigma (St. Louis, MO) unless noted otherwise.

HUTEC were obtained from human tonsils and characterized as previously described (see below) (23). In brief, human tonsils obtained after routine tonsillectomy were sequentially rinsed in sterile PBS, 70% ethanol, and sterile PBS, and the covering epithelium was removed. After cutting in small pieces, the tonsils were digested with 400 μg/ml collagenase A and 100 μg/ml DNase type I for 1 h at room temperature. The mixture was sedimented at 1 × g for 2 min, and the supernatant was centrifuged at 250 × g for 10 min. The cells were washed twice in RPMI 1640 (Life Technologies, Grand Island, NY) and plated at a density of 2 × 10⁴ cells/cm² in RPMI 1640 supplemented with 2 mM l-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% FBS (Life Technologies). FACScan studies of these cells showed a homogeneous population of cells expressing several endothelial cell markers (23), such as intracellular expression of von Willebrand factor, vimentin, and lymphocyte vascular adhesion protein-2, a marker displayed by endothelial cells of lymphoid organs (24). They express CD40 and integrin subunits β1, αv, α5, and α6. Although 50% of early passage cells express ICAM-1, and 20% express VCAM-1 constitutively, later passages show that the proportion of cells expressing ICAM-1 remains unchanged, while the fraction of cells expressing VCAM-1 decreased to <1% (data not shown). The cells are negative for markers characteristic of follicular dendritic cells (R4/23, XI M4, and CD11b), interdigitating dendritic cells (CD11c), and monocyte/macrophage (CD11b, CD14, and β2 integrin chain) (for details, see Ref. 23). More recently, we have determined that these cells are positive for DARC, a cell surface marker specific for human high endothelial venules (HEVs; data not shown) (22).

The human B cell lines Ramos and Daudi and the T cell line Jurkat as well as the mouse B cell line A20 were obtained from American Type Culture Collection. The human T cell line JM was a gift from Dr. M. Fellous (Institut Pasteur, Paris, France). TBCL-10 is a cell line obtained in our laboratory from human tonsil B cells transformed with EBV. All these cells were maintained in RPMI 1640 supplemented with 10% FBS, 2 mM l-glutamine, 100 IU/ml of penicillin, and 100 μg/ml of streptomycin.

Cell lysates, Western blot, and immunoprecipitation studies

To assess the effect of cell adhesion on protein phosphorylation, tonsil endothelial cells were grown to confluence in 35-mm plates (0.8 × 10⁶ cells) for Western blots or in 60-mm plates (1.5 × 10⁶ cells) for immunoprecipitation and coincubated at 4°C over ice with lymphoid cell lines (test cells) for 30 min. After this time, the cocultures were brought to 37°C for varying amounts of time. Test cells were removed by washing with 2 ml ice-cold PBS containing phosphatase inhibitors (10 mM NaF, 5 mM EDTA, 0.4 mM Na3VO4, and 10 mM sodium pyrophosphate) and were reserved for analysis. Endothelial cells were washed an additional three or four times with ice-cold PBS until the test cells were removed by scraping off the culture plate by light microscopy. For Western blot studies the endothelial cells were lysed directly in the plate with freshly prepared Laemmli sample buffer containing 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM sodium vanadate, 0.2% aprotinin, and 1 mM PMSF at 100°C. Lysed cells were scraped from the plate, collected in microfuge tubes, and boiled for 5 min. Finally, the lysates were passed several times through a 26-gauge syringe needle and centrifuged for 15 min at 12,000 × g. Proteins were separated by PAGE on 10% gels and transferred to nitrocellulose sheets. Blots were blocked for 1 h at room temperature, incubated with the anti-pTyr Ab overnight at 4°C, and developed using an ECL kit (Amersham, Arlington Heights, IL). To confirm the effect of adhesion on phosphorylation, Ramos cells were preincubated with an anti-β1 integrin-blocking mAb at 4°C for 30 min and washed to remove unbound Ab, and the experiment was continued as described above. To determine the effect of protein cross-linking on protein phosphorylation, endothelial cells were preincubated for 30 min at 4°C with 10 μg/ml anti-β1 integrin or anti-CD40 Abs before incubation for different times (15 s to 30 min) at 37°C with the cross-linking Ab (10 μg/ml). The medium containing unbound Abs was aspirated and discarded, and the adherent endothelial cells were washed twice with ice-cold PBS containing phosphatase inhibitors and treated for analysis as described above.

For the immunoprecipitation studies supernatants of cell lysates were precleared with 50 μl protein G-Sepharose coated with primary Ab overnight at 4°C, washed four times with cold immunoprecipitation buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM Na3VO4, 0.2 mM PMSF, and 0.5% Nonidet P-40 (pH 7.4) (1)). Lysates incubated for 5 min with sample buffer (40 mM Hepes, pH 7.4, 1% NP-40, 250 mM NaCl) and precleared by SDS-PAGE, immunoblotted with an anti-pTyr mAb, and detected using ECL. Where indicated, immunoblots were stripped by incubation in 0.1 M glycine/HCl, pH 2.3–3, at room temperature for 15 min, followed by incubation with 1 M NaCl in PBS for 2 min, and were washed twice with 0.1% TBS/Tween 20 for 10 min each time, blocked again as described above, and reimmunostained with the appropriate Ab as indicated.

For quantitation, blots were examined by scanning densitometry using a ScanMaker E3 (Microtek, Arredondo Beach, CA), and data were normalized with NIH Image 1.6 software. Phosphorylated levels of each protein were corrected for protein loading based on the scanning values obtained after reprobing the membranes with the corresponding Ab and were expressed as the level relative to the untreated control value.

RNase protection assay (RPA)

Endothelial cells were grown in P-60 petri dishes to confluence, washed with PBS three times, and seeded in RPMI, and test cells were added at 4°C for 30 min. Medium was then aspirated, and test cells or mAbs to the β1 integrin or CD40. After this, cells were incubated with the cross-linking Ab (10 μg/ml) for 1, 3, or 6 h at 37°C. Total RNA was isolated using TRIzol as directed by the manufacturer (Invitrogen, Carlsbad, CA) and was analyzed using the RibonQuant kit (BD PharMingen, San Diego, CA) as directed by the manufacturer. Probes were synthesized using [32P]UTP with the hCK-4 and hCK-5 template sets from BD Pharmingen. Hybridization was performed using 5 μg of RNA from each
sample. The protected probes were purified and resolved in 5% SDS-PAGE. Dried gels were exposed to a phosphorimaging screen, and protected fragments were visualized on a Molecular Imager FX (Bio-Rad, Hercules, CA). The identity of each protected fragment was established by analyzing its migration using a standard curve as indicated by the manufacturer. Samples were normalized to the housekeeping genes L32 and GAPDH, and protected bands were quantitated by densitometry analysis with the QuantityOne software (Bio-Rad).

Cytokine measurements

For the determination of cytokine production, endothelial cells were grown in 24-well plates to confluence. Before the assay, the cells were washed three times with RPMI medium and maintained in medium with 2% FBS during the assay. Supernatants were collected 24 h after stimulation. Assays were performed in triplicate. The amount of IL-8 present in the culture supernatants was measured by a capture ELISA kit (BD Pharmingen) according to the manufacturer’s instruction.

TNF bioassay

TNF was quantified by evaluating the cytotoxicity of culture supernatants over actinomycin D-treated murine fibroblast L929. For this, L929 cells were plated in a 96-well microtiter plate at 2 × 10⁴ cells/well and incubated overnight at 37°C in 10% CO₂. The next day the medium was removed, and 50 μl of a stock actinomycin D solution (8 μg/ml) was added to the wells, and incubation was continued overnight at 37°C in 10% CO₂. After this the cells were washed and stained with 50 μl of 0.05% Crystal Violet in 20% ethanol for 10 min at room temperature, washed with water, and allowed to dry. To evaluate cell staining, 100 μl methanol was added to each well, and the OD was read on a microtiter plate reader at 595 nm (25). Each assay was performed in triplicate.

Results

Adhesion of B cell lines induces changes in protein tyrosine phosphorylation on endothelial cells

Recently LeBien and co-workers (6, 7) presented data indicating that upon contact, the lymphoblastoid cell line Ramos is capable of inducing changes in the state of phosphorylation of several proteins in human bone marrow stromal cells. Also, we have previously shown that B cell lines as well as tonsil-derived lymphocytes bind to HUTEC via the α₅β₃ integrin (23). Based on these data and to obtain further understanding on the signaling pathways triggered by B cell adhesion on endothelial cells from lymphoid organs, we investigated whether B cell binding can alter the pattern of Tyr phosphorylation of HUTEC. In these experiments endothelial cells stimulated by lymphoid B cell adhesion consistently showed changes in tyrosine phosphorylation of three main bands of 125, 70, and 45 kDa (see below).

To assess whether the observed changes in protein phosphorylation corresponded to modifications in proteins originating from endothelial cells, lymphoid B cells were independently recovered after adhesion and submitted to the same analysis as endothelial cells. None of the changes in tyrosine phosphorylation found in endothelial cells were detected on lysates of the lymphoid B cells that had been in contact with HUTEC (not shown).

Next we investigated whether the proteins affected in tyrosine phosphorylation corresponded to previously described pp125FAK (125 kDa), paxillin (70 kDa), and ERK1/2 (45 kDa) (7). Tyrosine phosphorylation was analyzed by immunoblot with an anti-pTyr mAb after immunoprecipitation with the corresponding Ab. The same blots were stripped and reprobed with Abs against pp125FAK, paxillin, and ERK2 as required and were scanned for quantitation (Fig. 1E).

Fig. 1A shows that the anti-pp125FAK Ab immunoprecipitated a protein of 125 kDa. This protein was constitutively phosphorylated on Tyr in unstimulated HUTEC and was rapidly and transiently dephosphorylated (<15 s) after B cell stimulation, followed by rephosphorylation, returning to its basal level after 5 min. Although the degree of dephosphorylation was moderate (50%), it was consistent in multiple experiments.

The cytoskeletal protein paxillin migrates on gel electrophoresis as a 70-kDa band and has usually been coupled with the activation and phosphorylation of pp125FAK (26, 27). Fig. 1B shows that the
mAb against paxillin immunoprecipitates from B cell-stimulated HUTEC a protein with a 70-kDa band on SDS-PAGE. The kinetics of dephosphorylation and rephosphorylation of this protein follow the same pattern as those of the anti-pp125FAK-immunoprecipitated protein.

Finally, we investigated the possibility that the 45-kDa protein could correspond to ERK1 or ERK2. Immunoprecipitates with anti-ERK1 or anti-ERK2 Abs of endothelial cell lysates obtained after activation for varying times with Daudi B cells showed tyrosine phosphorylation only of ERK2 (Fig. 1C) and not of ERK1 (data not shown). Changes in the state of phosphorylation of ERK2 differ from those of pp125FAK and paxillin on two aspects. Firstly, ERK2 remains dephosphorylated for a longer period of time, and secondly, its rephosphorylation surpasses the initial basal level with a maximum of Tyr phosphorylation at 30 min returning quickly to its basal degree of phosphorylation (not shown).

Finally, to confirm that the observed changes in phosphorylation were produced by Ramos cells, adhesion to the endothelial cells was blocked by preincubating Ramos cells with the anti-α1 integrin mAb HP2/1 (23, 28). This Ab blocks adhesion of Ramos and Daudi cell to HUTEC by >90% (data not shown) (23). Fig. 1D reveals that no change in the phosphorylation of endothelial cells was detected when Ramos cell adhesion was blocked (only the 15 min point is shown). Quantitation of these experiments by scanning densitometry (Fig. 1E) confirmed these results. Thus, the observed changes in protein phosphorylation of pp125FAK, paxillin, and ERK2 clearly correspond to changes produced on endothelial cells upon B cell binding.

Cross-linking of β1 integrin and CD40 lead to changes in protein tyrosine phosphorylation on endothelial cells

It is known that there is information transfer between regular vascular endothelium and lymphocytes, and the involvement of integrins and other adhesion molecules in this process has been demonstrated. Although integrins show no intrinsic protein kinase activity, their engagement induces tyrosine phosphorylation commonly associated with changes in cell shape, secretion of growth factors, and lymphocyte transmigration (13). Furthermore, recent reports have shown that cross-linking of the β1 integrin present on NK cells induces changes in tyrosine phosphorylation of pp125FAK and paxillin (15). Since our preparation of tonsil endothelial cells exhibits high expression of the β1 integrin chain, we evaluated integrin-mediated signaling events in tonsil endothelial cells by cross-linking β1 integrin.

Fig. 2 shows that cross-linking β1 integrin on endothelial cells produced changes in the phosphorylation state on the same proteins of 125, 70, and 45 kDa seen during B cell adhesion. However, some differences should be underlined. Firstly, on β1-activated HUTEC, dephosphorylation is more modest than on B cell-induced activation. Second, rephosphorylation of pp125FAK and paxillin reached a higher level than the basal state (30 min), persisting for ~1 h before returning to unstimulated levels (not shown). Another important difference is seen on the kinetics of ERK2 rephosphorylation, since after β1 integrin cross-linking this protein starts its rephosphorylation earlier (5 min) compared with B cell adhesion (30 min).

These changes in protein tyrosine phosphorylation generated after β1 integrin cross-linking were not detected in control cultures treated with an isotype-matched Ab or with the second Ab alone or in experiments in which the anti-β1 mAb was not submitted to cross-linking with a second Ab (not shown). Furthermore, to insure that the changes observed in HUTEC phosphorylation were not due to some nonspecific effect of membrane protein cross-linking, we examined the effect of cross-linking of surface MHC class I Ags, a molecule highly expressed in HUTEC. Under this conditions no changes in the state of phosphorylation of the endothelial cells were observed (data not shown).

It has been reported that proinflammatory cytokines can increase CD40 expression, while ligation of CD40 by CD154 (CD40L) on endothelial cells induces the up-regulation of several intercellular adhesion molecules as well as chemokine secretion, thus contributing to the pattern of leukocyte migration and extravasation in inflammation and immunity (29). Since our previous results showed that tonsil-derived endothelial cells express the CD40 molecule in culture (23), we next evaluated the effect of CD40 cross-linking on protein tyrosine phosphorylation of HUTEC by treating these cells with an anti-CD40 mAb (G28-5) for different times. Cross-linking of CD40 also produced time-dependent changes in tyrosine phosphorylation of pp125FAK, paxillin, and ERK2 (Fig. 3). An initial dephosphorylation (<15 s) was followed by a rephosphorylation at longer times. The kinetics of tyrosine phosphorylation of the 125-, 70-, and 45-kDa proteins were similar to

![Figure 2](http://www.jimmunol.org/DownloadedFrom/bcell/125938)

**FIGURE 2.** Cross-linking of the β1 integrin chain modifies the phosphorylation state of pp125FAK, paxillin, and ERK2 in tonsil endothelial cells. Endothelial cells were left untreated or were treated with the anti-β1 integrin mAb TS2/16.2.1 for 0.25, 5, and 30 min, collected at each time point, and lysed. The lysates were immunoprecipitated with anti-pp125FAK (A), anti-paxillin (B), and anti-ERK2 (C) Abs and visualized by immunoblotting with anti-phosphotyrosine Ab (upper panels). The amount of each protein loaded in each lane was examined by reprobing the membrane with the corresponding Ab as indicated (lower panels). The results shown here are representative of three independent experiments. D. Quantitation of pp125FAK, paxillin, and ERK2 tyrosine phosphorylation at each time point in response to contact with Daudi cells.
levels of several cytokines. In these experiments we compared untreated HUTEC with cells pretreated with either the human B cell line Daudi, the tonsillar B cell line TBCL-10, the mouse B cell line A20, and human T cell lines, Jurkat and JM. As a positive control for the assay we used HUTEC treated with TNF. The TNF treatment of HUTEC showed protected RNA fragments for several of the cytokines present in the templates (data not shown), confirming previous data indicating that TNF induces a variety of chemokines on the endothelium. All RPAs were quantitated by comparison with the L-32 or GAPDH housekeeping genes (not shown). Interestingly, untreated HUTEC showed a low constitutive level of MCP-1 mRNA, which increased to a maximum 3 h after a change of culture medium (Fig. 4A). On the other hand, the level of the mRNA for this chemokine was markedly increased by the incubation of HUTEC with human B cell lines Daudi and TBCL-10. Maximum levels were obtained after 3 h of coculture, and this was maintained after 6 h of coincubation. In contrast, compared with the untreated controls at 1, 3, and 6 h, human T cell lines and the mouse B cell line A20 do not affect the level of MCP-1 mRNA over the basal levels. Also, we observed that the level of IL-8 mRNA increased after incubation of HUTEC with human B cell lines (Fig. 4A). As shown in Fig. 4A, endothelial cells treated with TBCL-10, in contrast to other human B cell lines tested, induce messages for other cytokines besides MCP-1 and IL-8. Since the pattern of cytokines induced by this cell line was very similar to that observed for TNF, we investigated whether TBCL-10 cells produce TNF or induce HUTEC to produce it. In fact, we demonstrated that TBCL-10 produces a detectable amount of TNF in culture, while Daudi or Ramos cells do not (data not shown). Considering this last point, we found that after 1 h of coculture Daudi cells induced in HUTEC a low level production of MIP-1β mRNA, which remained unaltered for 3 h, disappearing after 6 h of cell contact (Fig. 4A). We further evaluated whether cross-linking of β1 integrin or CD40 also produced an increase in the mRNA levels of MCP-1 and IL-8. No changes in mRNA levels for these chemokines were observed when HUTEC were cross-linked for different time periods (data not shown) even though this treatment produces patterns of tyrosine phosphorylation similar to those observed after β1 integrin cross-linking (Fig. 3). Thus, cross-linking of β1 integrin or CD40 produced changes in protein phosphorylation similar to those produced by adhesion of B cell lines, although the kinetics were slightly different. In addition to the above-mentioned proteins, cross-linking of CD40 induced after 30 min the strong phosphorylation of an unidentified protein of 90 kDa.

B cell (but not T cell) adhesion induces tonsil endothelial cell activation and the production of mRNA for several cytokines

Numerous studies show that activated endothelium produces a variety of factors, including cytokines and growth factors that promote lymphocyte extravasation or attract leukocytes to inflammatory sites (30–32). To evaluate the effect of cell adhesion on chemokine production by endothelial cells, we coincubated these cells with several cell lines and examined by RPA the mRNA

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**FIGURE 3.** Cross-linking of CD40 modifies the phosphorylation state of pp125FAK, paxillin, and ERK2 in tonsil endothelial cells. Endothelial cells were left untreated or were treated with the anti-CD40 mAb G28-5 for 0.25, 5, and 30 min; collected at each time point, lysed, and immunoprecipitated with anti-pp125FAK (A), anti-paxillin (B), and anti-ERK2 (C) Abs. The immunoprecipitated proteins were visualized by immunoblotting with anti-phosphotyrosine Ab (upper panels). The amount of each protein loaded in each lane was examined by reprobing the membrane with the corresponding Ab as indicated (lower panels), and the protein level was used to calculate the relative changes in phosphorylation. The results shown here are representative of three independent experiments. D. Quantitation of pp125FAK, paxillin, and ERK2 tyrosine phosphorylation at each time point in response to contact with Daudi cells.
at each time point, and RPA was performed using hCK-5 (A) or hCK-4 (B) template sets (BD PharMingen) containing the cytokines indicated.

B A

Discussion

Lymphocyte homing to secondary lymphoid tissue, a crucial step for immune surveillance and the development of a normal adaptive immune response (33), is characterized by a multiple stage sequence of interactions between lymphocytes and endothelial cells in HEVs. Numerous reports indicate that some locally produced, HEV-derived cytokines and chemokines, can differentially regulate endothelial cell function and lymphocyte adhesion and transmigration (34). In this regard, normal HEV behaves very much as an inflamed endothelium. There are numerous reports on the participation of adhesion molecules and their ligands on lymphocyte migration through inflamed endothelium (2, 35) as well as through normal HEV (36–38). However, few communications deal with the mechanism leading to the specific structural and functional properties characterizing the endothelium from lymphoid tissue or with the potential signaling events that may arise in high endothelial venules as a consequence of lymphocyte contact (37–39). These signaling events may be specially relevant in view of increasing evidence indicating that cell-cell interactions activate signaling transduction cascades associated with the production and secretion of cytokines and chemokines (30, 40, 41), as well as with cell attachment (42) and changes in cell shape (19), activities clearly related to lymphocyte transmigration.

Although the physiological significance of lymphocyte interaction with high endothelial cells from secondary lymphoid tissue is well recognized most of the data on endothelium-lymphocyte interaction have been obtained with endothelial cells from non-lymphoid tissues. Thus, it appeared important to us to investigate the signaling events induced in endothelium from lymphoid tissue after lymphocyte adhesion. Here we used cultures of HUTEC as a source of endothelial cells. These cells, which exhibit surface expression of DARC, a marker displayed by HEVs and absent from regular HUVEC (22), present several surface molecules involved in adhesive interactions and signal transduction (23). In this report we investigated the early changes in the pattern of protein tyrosine phosphorylation as well as the physiological consequences induced on the endothelial cells as a result of their interaction with human B lymphoblastoid cells. Our results show that B lymphoid cell adhesion on HUTEC induces rapid tyrosine dephosphorylation and rephosphorylation of several proteins; the most prominent were identified by immunoprecipitation experiments as pp125FAK, paxillin, and the ERK2 kinase (Fig. 1). We observed that tyrosine dephosphorylation and rephosphorylation of pp125FAK and paxillin followed similar kinetics, while ERK2 remained tyrosine dephosphorylated for a longer period, followed by increased phosphorylation and a rapid return to the basal level. All these proteins were tyrosine phosphorylated before any treatment, indicating that activation of protein tyrosine phosphatases (PTPs) might be an important early step in endothelium activation. In another work performed with cocultures of bone marrow stromal cells and the B cell line Ramos, it was shown that similar changes occurred in the phosphorylated state of these three proteins (7), except that the early dephosphorylation of pp125FAK and paxillin was not observed. This apparent discrepancy may be explained by the fact that the earliest time points studied in that report were 1 min of coculture, a time at which these proteins may have already undergone rephosphorylation. Additional differences found in the initial state of phosphorylation of ERK2 as well as the kinetics of phosphorylation may be ascribed to the different type of cells used in each case.

Several reports dealing with cell adhesion have reported the phosphorylation of pp125FAK and paxillin (43–46). Although these results were obtained from a variety of models, they suggest that tyrosine phosphorylation of pp125FAK, paxillin, and ERK2 is affected by factors that regulate cell adhesion and migration. On the other hand, the activation of pp125FAK and paxillin may be independent of the activation of MAPKs, as shown in two reports using specific inhibitors of the MAPK pathway (43) or inhibitors of the Ras signaling pathway. In a attempt to understand the mechanisms involved in the B cell-induced tyrosine phosphorylation
and chemokine production by tonsil endothelial cells, we compared the changes generated by B cell adhesion with those induced by β1 integrin or CD40 cross-linking on HUTEC. We found that engagement of β1 integrin or CD40 induced dephosphorylation and rephosphorylation of the same three proteins affected by B cell adhesion (pp125FAK, paxillin, and ERK2). We observed that pp125FAK, paxillin, and ERK2 exhibited similar phosphorylation kinetics upon β1 integrin or CD40 cross-linking (Figs. 2 and 3) and that they remained phosphorylated longer than when the endothelial cells were activated by B cell adhesion (2 h compared with <1 h, respectively; data not shown). Regardless of the stimulus, early signaling events induced on endothelial cells indicated the participation of one or more PTP. Concerning this point, the most striking difference was found on the dephosphorylation of ERK2. After B cell adhesion this protein remained dephosphorylated for almost 30 min before rephosphorylation (Fig. 1C) compared with the 3–5 min required for recovery after β1 or CD40 cross-linking. One possible explanation for this difference may be that B cell adhesion activates different PTPs compared with β1 integrin or CD40 cross-linking. Protein phosphatases PTP1B (47) and PTP-Pro-Glu-Ser-Thr-rich PTP (PTP-PEST) (48) have been implicated in focal adhesion signaling, since both associate with the SH3 domain of p130Cas and paxillin. While PTP1B has been shown to inhibit signaling pathways stimulated by cell adhesion, PTP-PEST was found to be associated directly with paxillin in vitro. These data suggest that these two proteins form a complex containing tyrosine kinase and tyrosine phosphatase activities, thus controlling the phosphotyrosine content of proteins in focal adhesions. Although the phosphatases involved in the dephosphorylation of ERK2 have not been identified, recent results demonstrated that MAPK phosphatase 3 activates ERK2 in a sequential and ordered way, since it exhibits dual specificity. First, it dephosphorylates the phosphotyrosine found in the activation loop of ERK2 and then it dephosphorylates the phosphothreonine residue in the loop (49). These results argue in favor of the fact that surface stimuli through different ligands may activate different sets of PTPs leading to different patterns of phosphorylation. Also, the nature of the tyrosine kinases involved in the rephosphorylation of pp125FAK, paxillin, and ERK2 are unclear. It is likely that pp125FAK autophosphorylates and, in turn, phosphorylates paxillin (13). On the other hand, it has been shown that there is a cooperative interaction between ligation of integrins and growth factors in the activation of MAPKs (19). This may explain our finding that phosphorylation of ERK2 is delayed compared with phosphorylation of pp125FAK and paxillin in B cell adhesion-induced tyrosine phosphorylation.

Although several reports indicate that lymphocyte adhesion to endothelial and stromal cells from lymphoid tissue promotes changes in the state of phosphorylation of both cell types of the interacting pair, the physiological outcome of these changes has only been studied in a small number of cases. In one such report it was shown that coculturing tonsilar B cells with IL-6-secreting follicular dendritic cells from tonsil stroma resulted in an increased B cell proliferation and in an augmented secretion of Ig by these B cells (5). Interestingly, although a number of studies have reported the secretion of IL-6 by stromal cells (5, 6, 42), only one of these reports indicates that IL-6 secretion was associated with B cell adhesion (6).

Endothelial cells participate in lymphocyte and monocyte cell extravasation by secreting chemotactic chemokines that facilitate cell adhesion and transmigration (50). For example, the chemokines IL-8 and MCP-1 that promote monocyte and T lymphocyte migration respectively (30) are produced by inflamed endothelium after IL-1 stimulation. Here we report that B cell adhesion induces in tonsilar endothelial cells IL-8 secretion as well as messages for the production of MCP-1, IL-8, IL-6, M-CSF, and MIP-1B. Furthermore, lymphocyte migration to lymphoid organs may be influenced by the presence of other cells, as it was reported that the migration of activated T lymphocytes depended on the production of TNF released by cumulating monocytes (51). Here we show that endothelial cells from human tonsils produce, upon B cell adhesion, mRNA for M-CSF and IL-6, growth factors for monocytes and B cells, respectively (Fig. 4). Furthermore, we demonstrated the production of mRNA for chemokines associated with leukocyte transmigration, such as IL-8, MCP-1, and MIP-1B (Fig. 4). Thus, these results show that cross-talk between B cells and lymphoid endothelium induces the activation of signaling pathways leading to the production of a complex combination of cytokines and chemokines by endothelial cells. These factors may not only affect the adhesive and migratory properties of the adhering B cells, but also influence the behavior of other migratory cells.

Our results further suggest that contact of lymphoid B cell with high endothelium of lymphoid tissue may influence cell migration through changes in cell shape. This is supported by a recent report indicating that IL-8 potentiates changes in endothelial cell shape and transmigration of neutrophils (7). Additionally, other factors induced by B cell contact, such as M-CSF, may initiate monocyte or immature dendritic cell differentiation before entering the lymphoid organ or may act in maintaining the maturation state of migrating lymphocytes.

In summary, we have shown that B cell contact with endothelium from lymphoid tissue induces tyrosine dephosphorylation and rephosphorylation of proteins related to the formation of focal adhesion, such as pp125FAK and paxillin. Additionally, we found that B cell adhesion activates the MAPK protein ERK2 and the secretion of a complex mixture of cytokines. We therefore suggest that B cell interaction may be central to the structural and functional remodeling of lymphoid endothelium and to the chemotaxis and transmigration of lymphocytes and other cells into secondary lymphoid organs. It will be of interest to determine how the described phenomena influence B and T cell emigration and to determine the molecular mechanisms involved.

Acknowledgments

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References