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HLA class I signal transduction is dependent on Rho GTPase and ROK

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Abstract

Chronic rejection is the major limitation to long-term allograft survival. HLA class I signaling pathways have been implicated in this process because ligation of class I molecules by anti-HLA antibodies (Ab) initiates intracellular signals in smooth muscle cells (SMC) and endothelial cells (EC) that synergize with growth factor receptors to elicit cell survival and proliferation. Anti-HLA Ab mediate cell proliferation and survival through a focal adhesion kinase dependent pathway that requires the integrity of the actin cytoskeleton. In this study, we investigated the role of Rho and Rho-kinase (ROK) in class I signal transduction. We show that class I ligation results in activation of Rho and increased stress fiber formation. In addition, inhibitors of Rho GTPase and ROK block HLA class I-mediated tyrosyl phosphorylation of paxillin and FAK, central elements of the focal adhesion signaling complex. These results suggest that HLA class I-induced signaling in EC is dependent on Rho GTPase and ROK.

Keywords: MHC; HLA; HLA class I; Rho; Rho-kinase; Actin; Endothelium

Chronic rejection is the leading cause of late renal, heart, and lung allograft loss and is estimated to affect more than 40% of recipients within the first five years following transplantation. The hallmark of chronic rejection is transplant arteriosclerosis (TA), which is characterized by a diffuse, concentric intimal thickening resulting in occlusion of the vessels of the graft and deterioration of organ function. The histologic appearance of TA shows marked proliferation and hyperplasia of intimal SMC, macrophages, and EC. Although the etiology of TA is not well understood, there is a strong association between TA and production of Ab to donor HLA antigens following transplantation [1,2].

Studies by our group and others have shown that ligation of HLA class I molecules with human and murine anti-HLA Ab transduces activation signals in EC and initiates cell proliferation in a model relevant to the development of transplantation associated vasculopathies [3–10]. Engagement of HLA class I molecules by anti-HLA Ab stimulated tyrosine phosphorylation of intracellular proteins, increased fibroblast growth factor receptor (FGFR) expression, and enhanced proliferative responses to basic fibroblast growth factor (bFGF) [4–6]. More recently, we provided evidence that anti-MHC class I Ab triggers a pro-survival signaling cascade resulting in phosphorylation of PI3K and Akt and up-regulation of the anti-apoptotic proteins Bcl-2 and Bcl-xL in EC [9]. Consistent with the in vitro experiments, biopsies from heart allograft recipients with evidence of Ab-mediated rejection showed increased Bcl-2

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expression on the vascular endothelium of the graft [9]. These results have led us to conclude that anti-HLA Ab contribute to the process of TA by transducing signals promoting cell survival and proliferation.

A central step in HLA class I signal transduction pathway is phosphorylation of two elements of the focal adhesion signaling complex, focal adhesion kinase (FAK) and paxillin [8]. HLA class I-mediated phosphorylation of the focal adhesion proteins was inhibited by cytochalasin D and latrunculin A, suggesting a role for the actin cytoskeleton in the signaling process [8]. The association between HLA class I clustering and actin cytoskeleton rearrangement was also described by Ash et al. [11] and Huet et al. [12]. They showed that crosslinking of HLA class I on the surface of human fibroblasts leads to the reorganization of HLA molecules in linear arrays directly super-imposed over stress fibers positioned underneath the membrane. There is mounting evidence that the Rho GTPase family of molecules, Rho, Rac, and Cdc42, plays a central role in actin cytoskeleton rearrangement [13]. The Rho family of proteins, RhoA, RhoB, and RhoC, has been shown to contribute to contractility and formation of stress fibers and focal adhesions. The Rac proteins including Rac1, Rac2, Rac3, and RhoG all stimulate the formation of lamellipodia and membrane ruffles, whereas, the Cdc42 GTPases stimulate the formation of filopodia [13]. Stress fibers are filaments of actin or F-actin, which are connected to the focal adhesion complex. Two Rho effectors, Rho-kinase (ROK) and the mammalian homolog of diaphanous (mDia), promote the assembly of stress fibers and focal adhesions.

The results presented here demonstrate that crosslinking class I molecules in human endothelial cells with anti-HLA Ab induces a rapid and striking increase in Rho activation, as judged by the increase in the level of Rho-GTP, and in the assembly of stress fibers in these cells. Our results also show that inhibition of RhoGTPase or ROK using the selective inhibitor of ROK Y-27632 [14] and the Rho-family inhibitor *Clostridium difficile* toxin B (TcdB) [15] prevented class I-mediated phosphorylation of FAK and paxillin. These findings are consistent with a model in which anti-HLA Ab-mediated clustering of class I molecules activates Rho and ROK which promote the formation of stress fibers and lead to the enhanced tyrosine phosphorylation of FAK and paxillin.

Materials and methods

Antibodies and reagents. The anti-HLA class I monoclonal Ab (mAb)W6/32 (IgG2a isotype) directed against a monomorphic epitope on class I antigens was produced from hybridoma HB-95 supplied by the American Type Culture Collection (Manassas, VA). The mouse IgG isotype control was supplied from Sigma (St. Louis, MO). The anti-phosphotyrosine mAb 4G10 was obtained from Upstate Biotechnology (Lake Placid, NY). Anti-FAK (C-20) Ab and protein A/G plus-agarose were obtained from Santa Cruz Biotechnology (CA). Anti-paxillin Ab was obtained from Transduction Laboratories (Lexington, KY). The Rho GTPase inhibitor *C. difficile* cytotoxin was purchased from List Biological Laboratories (Campbell, CA). The ROK inhibitor Y27632 was purchased from Calbiochem Biochemical (San Diego, CA). Reagents used to determine Rho GTPase activity were purchased from Pierce (IL).

Cell culture. EC from single donors were obtained from Clonetics (San Diego, CA). Cell culture reagents including endothelial cell basal medium (EBM), fetal calf serum (FCS), epidermal growth factor (EGF), bovine brain extract, hydrocortisone, and gentamicin were obtained from Clonetics (San Diego, CA). Cells were grown to a confluence of 80–90% and incubated for 16 h in serum free EBM media before treatment, unless stated differently.

Rho GTPase "pull down" assay. Active Rho GTPase was affinity-purified and measured as previously described [16]. After treatment with anti-class I Ab, cells were washed once with PBS (4 °C) before lysis in the presence of proteinase inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM PMSF). Lysates were incubated for 1 h at 4 °C with an immobilized glutathione disc and 400 μg of GST-Rhotekin-RBD for Rho or 20 μg of GST-Pak-PBD for Rac1 and Cdc42 activity assays. "Pull down" and lysate samples were analyzed by Western blot using an anti-RhoA,B,C, anti-Rac or, anti-Cdc42 Ab (Pierce, IL) and signals were generated using Super Signal West Pico Chemiluminescence Substrate (Pierce, IL). Bands were scanned using the GS-710 Calibrated Imaging Densitometer (Bio-Rad) and the data were quantified using the Quantity One software program (Bio-Rad).

F-actin staining. EC grown to 80–90% were starved for 2 h in basal medium containing gentamicin and 1% BSA followed by treatment with mAb W6/32 (10 μg/ml) or isotype control mouse IgG for different times. Where indicated, EC were pretreated with 10 μM Y27632 for 1 h at 37 °C prior to stimulation with mAb W6/32. Treated EC were fixed with 2% paraformaldehyde and permeabilized with 0.075% saponin. The presence of F-actin was visualized by direct staining with Texas Red–phalloidin (Molecular Probes). Cells were analyzed with Zeiss Axioplan 2 microscope with the Zeiss FluoArc 100 watt Mercury light source.

FAK and paxillin phosphorylation. Levels of FAK and paxillin phosphorylation were determined as previously described [8]. Briefly, FAK or paxillin proteins were immunoprecipitated and the level of phosphorylation was determined by Western blot using the anti-phosphotyrosine Ab 4G10. Where indicated, EC were pretreated with 5 or 10 μM of the ROK inhibitor Y27632 for 1 h or with 2.5, 5 or 10 nM of the Rho GTPase inhibitor TcdB overnight at 37 °C prior to stimulation with mAb W6/32.

Results

Ligation of HLA class I molecules by anti-HLA Ab induces Rho activation in EC

The capacity of anti-HLA class I Ab to activate Rho in EC was determined using "pull down" assays. As shown in Fig. 1, ligation of HLA class I molecules with the anti-HLA class I mAb W6/32 on the surface of EC induced a rapid and transient increase in Rho activity with a peak at 1 min. Densitometric scanning showed anti-class I Abs induced an 18-fold and 7-fold increase in the level of Rho activity at 1 and 2 min, respectively, when compared to untreated EC (Fig. 1). Rho activity remained unchanged when cells were treated with mouse IgG (data not shown). Confirmation that similar

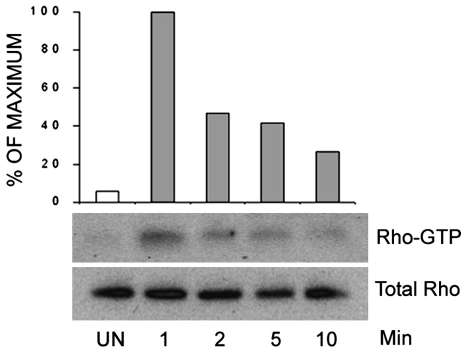

Fig. 1. Ligation of HLA class I molecules on EC results in a rapid and transient activation of Rho. EC were treated with 10 μg/ml of mAb W6/32 for 1, 2, 5 or 10 min or untreated (UN). For each experimental condition, active Rho (Rho-GTP) was affinity-purified from cell lysates and analyzed by Western blot. Total Rho protein present in cell lysates was determined by Western blot analysis (lower panel). Quantification of Rho protein was performed by scanning densitometry. Histogram values are expressed as the percentage of the maximal Rho activation. Results of three independent experiments show a maximum increase of Rho after 1 min stimulation.

amounts of Rho were recovered from cell lysates was obtained by immunoblotting with anti-Rho Ab (Fig. 1, lower panel). No significant changes in Rac1 and Cdc42 activity were observed when EC were treated with 10 μg/ml W6/32 for up to 15 min (data not shown).

Ligation of HLA class I molecules on EC increases stress fiber formation

To investigate if HLA class I-induced Rho activation results in stress fiber formation, EC were stained with fluorochrome-conjugated phalloidin. EC treated with W6/32 for 1 min showed a marked increase in stress fiber staining compared to isotype IgG control treated or untreated cells (Fig. 2). Pretreatment of EC with the ROK inhibitor Y27632, prior to stimulation with anti-class I Ab, completely inhibited class I-induced stress fiber formation in EC. These results are consistent with the hypothesis that ROK plays a role in class I-mediated reorganization of the actin cytoskeleton and formation of stress fibers.

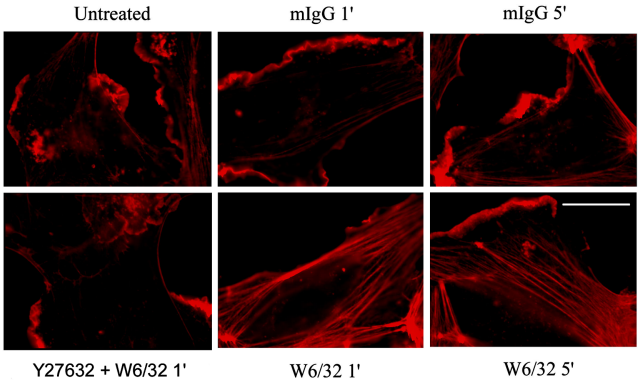

Fig. 2. Ligation of HLA class I molecules on EC induces stress fiber formation. EC were serum starved for 2 h in basal medium containing 1% BSA before treatment. EC were treated for 1 or 5 min with 10 μg/ml mIgG, 10 μg/ml W6/32 or untreated. Alternatively, EC were pretreated with 10 μM Y27632 for 1 h followed by treatment for 1 min with 10 μg/ml W6/32. Cells were stained with Texas-Red–phalloidin and analyzed by fluorescence microscopy (600× magnification). The results are representative of three independent experiments.

Rho GTPase and ROK inhibitors prevent HLA class I-mediated phosphorylation of paxillin and FAK

To study the effect of Rho GTPases on HLA class I-mediated activation of focal adhesion proteins, we tested the effect of the Rho GTPase inhibitor TcdB [15,17] and a selective inhibitor of ROK, Y-27632 [14] on class I-induced phosphorylation of FAK and paxillin. EC were pretreated with increasing concentrations of TcdB before stimulation with 10 μg/ml W6/32 for 10 min, conditions known to stimulate maximum tyrosine phosphorylation of FAK and paxillin [8]. Cell lysates were immunoprecipitated with anti-FAK Ab and the level of protein tyrosine

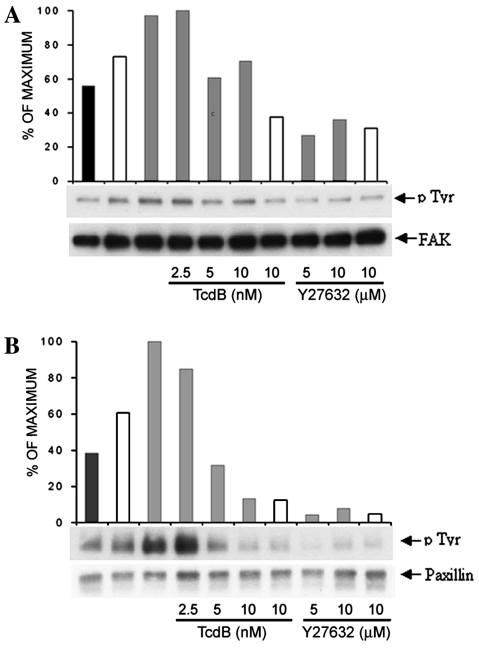

Fig. 3. HLA class I-mediated phosphorylation of FAK and paxillin is dependent on Rho GTPase and ROK. Serum starved EC were treated for 10 min with 10 μg/ml mIgG (black bars), 10 μg/ml W6/32 (grey bars), or untreated (white bar). Starved EC were pretreated with 2.5, 5 or 10 nM TcdB prior to stimulation with W6/32 or left unstimulated. Starved EC were pretreated with 5 or 10 μM Y27632 prior to stimulation with W6/32 or left unstimulated. FAK (A) or paxillin (B) was immunoprecipitated and analyzed by Western blot. Tyrosine phosphorylation (pTyr) was detected with the anti-phosphotyrosine Ab 4G10 (upper panel) and compared to the amount of immunoprecipitated protein (lower panel). Quantification of FAK and paxillin phosphorylation were performed by scanning densitometry. Histogram values are normalized to equal loading results and expressed as the percentage of the maximal increase in tyrosine phosphorylation. The results are representative of three independent experiments.

phosphorylation was determined by Western blot. Ligation of class I molecules by anti-HLA Abs stimulated a 40% increase in phosphorylation of FAK (Fig. 3A). Exposure to TcdB blocked class I-induced phosphorylation of FAK in a dose-dependent manner. Similarly, class I-mediated tyrosine phosphorylation of FAK was inhibited by prior exposure to Y-27632. Immunoblotting with anti-FAK Ab of the FAK immunoprecipitates confirmed that equal amounts of protein were recovered during immunoprecipitation (Fig. 3A, lower panel).

The addition of the Rho GTPase inhibitor TcdB at 5 nM completely prevented the marked increase in class I-mediated tyrosine phosphorylation of paxillin (Fig. 3B). Furthermore, pretreatment of EC with Y-27632 also abrogated the tyrosine phosphorylation of paxillin induced by class I ligation. Immunoblotting the membranes with anti-paxillin Ab verified that similar amounts of paxillin were present in each lane (Fig. 3B, lower panel). These results indicate that Rho and ROK are involved in HLA class I-mediated tyrosine phosphorylation of FAK and paxillin in EC.

Discussion

This study demonstrates that Rho and ROK are important upstream regulators involved in HLA class I signal transduction. We observed a rapid increase in Rho activity upon crosslinking class I molecules with anti-HLA Ab. Rho is principally known for its pivotal role in inducing the assembly of contractile actin and stress fibers [13]. In accordance with this function, we observed marked increases in stress fiber staining upon treatment of EC with anti-HLA class I Ab. This study also shows that inhibition of RhoGTPase or ROK resulted in a striking reduction in class I-mediated phosphorylation of FAK and paxillin. Since both paxillin and FAK are central elements of the focal adhesion complex the data suggest that actin reorganization controlled by Rho GTPase exerts a strong influence on class I-induced assembly of FAK, Src, and paxillin cell matrix adhesions and subsequent phosphorylation of these proteins.

The significance of these findings is underscored by our recent in vitro studies showing that HLA class I-mediated FAK phosphorylation is an early event leading to cell proliferation, activation of the PI3K/Akt signaling pathway, and up-regulation of the anti-apoptotic proteins Bcl-2 and Bcl-xL in EC [9]. Furthermore, in the transplant setting, binding of Ab to the endothelium of the allograft has been shown to lead to induction of protective genes such as Bcl-2 and Bcl-xL which may promote cell survival [9,18,19]. Rho and its target protein ROK regulate cell shape, migration, survival, and proliferation, all of which may be involved in the pathogenesis of TA. For these reasons, inhibitors of these molecules have been evaluated in vivo for their effect on neointima formation. Hattori et al. [20] showed that long-term treatment with fasudil, a ROK inhibitor, suppressed the development of TA in heart allografts in mice. In another study, the ROK inhibitor, Y-27632, was shown to prolong cardiac allograft survival and prevent the intimal thickening of the vasculature of the graft [21].

Together, our previous results [3–6,8,9] and current findings are consistent with a model in which anti-HLA Ab-mediated clustering of class I molecules activates Rho and ROK which promote the formation of stress fibers and phosphorylation and assembly of FAK and paxillin into cell matrix adhesions which act in concert as signaling units. These signaling events activated by Ab ligation of HLA class I molecules may promote cell survival by phosphorylation of FAK and subsequent activation of the PI3K/Akt pathway and up-regulation of Bcl-2 and Bcl-xL. Thus, regulation of the class I signaling cascade by Rho and ROK may play an important role in the development of TA.

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