Biochemical and Biophysical Research Communications **286,** 195–199 (2001)
doi:10.1006/bbrc.2001.5355, available online at http://www.idealibrary.com on IDEAL®

# A Critical Role for Syk in Endothelial Cell Proliferation and Migration

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Received July 13, 2001

**Syk is a protein-tyrosine kinase that is widely expressed in haematopoietic cells and involved in coupling activated immunoreceptors to downstream signaling. On the other hand, Syk-deficient mice showed severe petechiae *in utero* and died shortly after birth. Recently we have shown the expression of Syk in endothelial cells and morphological defects of these cells in embryonic Syk-deficient mice. Here we report that both proliferation and migration of human umbilical vein endothelial cells were severely impaired by adenovirus-mediated expression of Syk dominant negative mutants. Furthermore, a close relationship between Syk kinase activity and extracellular signal-regulated kinase activation was suggested. Our results indicate that Syk plays a critical role in endothelial cell functions, including morphogenesis, cell growth, migration, and survival, and contributes to maintaining vascular integrity *in vivo*.** 

*Key Words:* **Syk; protein-tyrosine kinase; endothelial cells; migration; proliferation; adenovirus; HUVECs.**

Syk protein-tyrosine kinase contains a C-terminal kinase domain and tandem N-terminal SH2 domains that bind phosphorylated immunoreceptor tyrosine-based activation motif and play critical roles in signaling through immune receptors (1, 2). Syk is expressed by all haematopoietic cells; it is essential for lymphocyte development and signal transduction via immune receptors in non-lymphoid cells (3, 4). On the other hand, Syk-deficient mice show a lethal phenotype associated with severe petechiae, diffuse haemorrhage and chylous ascites (5, 6). This latter phenotype is suggestive of loss of vascular integrity although Syk expression has not been examined in vascular endothelial cells. Remarkably, the bleeding phenotype is transferable when Syk-deficient bone marrow is transplanted into wild-type mice (7, 8). Classical tests of platelet function, such as bleeding time, are normal in radiation chimeras reconstituted with Syk-deficient platelets (7, 9). Thus the mechanism of this bleeding is currently unclear. In our previous study, we have shown the expression of Syk in endothelial cells and morphological defects of these cells in embryonic Syk-deficient mice (10). In this study, we have examined the effect of kinase negative Syk expression on the cell proliferation and migration of cultured human umbilical vein endothelial cells (HUVECs) was examined using adenovirus-mediated expression system. Here we report that Syk is required for HUVECs proliferation and migration. Implications of these phenomena in signal transduction are briefly discussed.

Abbreviations used: HUVECs, human umbilical vein endothelial cells; FCS, fetal calf serum; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; ERK, extracellular signal-regulated kinase.

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## MATERIALS AND METHODS

*Cell culture.* HUVECs were purchased from Cell Systems (Seattle, WA). HUVECs were passaged in medium 199 (GIBCO-BRL) containing 10% FCS, 4 mM L-glutamine, 1 nM bFGF, and 1 nM vascular endothelial growth factor (VEGF) on 60-mm diameter dishes coated with collagen type IV (Wako, Japan). HUVECs were not used after the sixth passage. For experimental purposes, HUVECs were plated on collagen-coated dishes and allowed to form a monolayer.

*Immunoblotting and in vitro tyrosine kinase assay.* Cell and tissue extracts (10 $\mu$g of protein) were subjected to SDS–PAGE using a 10% gel followed by transfer to polyvinylidene difluoride membranes (Immobilon P, Millipore) and immunoblotting with anti-Syk antibody. For *in vitro* tyrosine kinase assay, lysates (1 mg protein) from HUVECs were immunoprecipitated with anti-Syk antibody. The immunoprecipitates were incubated in a reaction mixture containing 50 mM Hepes/NaOH pH 8.0, 10 $\mu$M $Na_3VO_4$, 50 mM $MgCl_2$, 5 mM $MnCl_2$ with or without 10 $\mu$M ATP for 10 min at 30°C. The reactions were terminated by boiling for 3 min with SDS–PAGE sample buffer and analyzed by immunoblotting using anti-phosphotyrosine antibody (4G10) or anti-Syk antibody as described previously (11).

*Northern blot analysis.* RNA isolations and hybridizations were performed as described previously (12). Total RNA (20 $\mu$g) was separated using 1.0% formaldehyde gel, transferred to Hybond-$N^+$ membrane and probed with $^{32}$P-labeled human Syk or human $\beta$-actin



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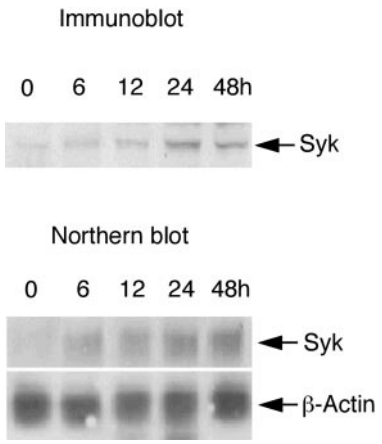


**FIG. 1.** Syk expression is upregulated in response to proliferative stimulation. HUVECs were serum-starved for 24 h in medium 199 containing 1% FCS and 1% bovine serum albumin (resting state) and then stimulated with 10% FCS containing 1 nM bFGF and 1 nM VEGF. The levels of Syk protein and mRNA were determined at indicated time by immuno and Northern blot analyses.

cDNA as a control. After washing, membrane was exposed to X-Omat AR film at −70°C using an intensifying screen.

*Preparation of recombinant adenoviruses.* An adenovirus vector encoding porcine Syk was constructed with the use of an adenovirus expression kit (Takara, Tokyo, Japan). A 2.7-kilobase cDNA fragment containing the entire coding sequence of wild-type or kinase-negative porcine Syk (K395R) was ligated into the pAxCAwt cosmid vector, which contains the modified chicken α-actin promoter with cytomegalovirus-IE enhancer (CAG promoter). Then the recombinant adenovirus pAxCA-Syk(wt) or adenovirus pAxCA-Syk(k−) was prepared by homologous recombination of the expression cosmid cassette and parental viral genome, and amplified to achieve a stock with a titer of approximately $10^9$ plaque-forming units/ml (13). An adenovirus vector encoding LacZ (as a control) was prepared as same method.

## RESULTS

*Upregulation of Syk expression in response to proliferative stimulation.* Most recently we have shown that Syk is expressed in endothelial cells and morphological defects of endothelial cells are observed in embryonic Syk-deficient mice (10). In a preliminary experiment we found that Syk expression in HUVECs was higher in growing cells than in resting cells. This observation prompted us to examine the effect of proliferative stimulation on Syk expression in HUVECs. HUVECs were serum-starved for 24 h in medium 199 containing 1% FCS and 1% bovine serum albumin (resting state) and then stimulated with 10% FCS containing 1 nM bFGF and 1 nM VEGF. As shown in Fig. 1, the levels of Syk protein and mRNA began to increase after 6 h of incubation with 10% FCS containing bFGF and VEGF and reached a maximal level after about 24–48 h. In the basal condition these cells did not express sufficient mRNA to be detectable by classical Northern blot technique. However, after proliferative stimulation, the Syk mRNA was easily detectable in HUVECs. This result suggested the possible involvement of Syk in the growth-signaling pathway of endothelial cells.

*Syk is required for cell growth, migration, and survival of HUVECs.* Although our previous work showed an abnormal morphogenesis and decreased number of endothelial cells in Syk-deficient mice (10), it is uncertain whether this abnormality occurred as the primary effect of Syk deficiency in endothelial cells or as a secondary effect of changes in extracellular milieu such as growth factors, cytokines, and chemokines which affect the growth and maturation of endothelial cell *in vivo.* To address this issue, the effect of kinase negative Syk expression on the cell proliferation and migration of cultured HUVECs was examined using adenovirus-mediated expression system. Syk kinase negative construct contained the ATP binding site mutant of Syk (K395R). Both Syk wild-type and LacZ-expressing adenoviral vectors were used as controls. HUVECs were infected with 20 multiplicity of infection of these vectors. Quantitative and qualitative expression of Syk wild-type and Syk kinase negative mutants in HUVECs were confirmed by immunoblot analysis and *in vitro* tyrosine kinase assays (Figs. 2a and 2b). As shown in Fig. 2c, the proliferation and survival of Syk kinase negative mutants were markedly suppressed compared with Syk wild-type and LacZ-expressing adenovirus infected HUVECs. Similarly, the migration ability of Syk kinase negative mutants were also impaired compared with Syk wild-type and LacZ-expressing adenovirus infected HUVECs (Fig. 2d). These findings suggested that Syk was required for HUVECs proliferation and migration. Therefore, it is believed that the vascular endothelial cell disorder observed in the Syk deficient mice was caused by a decrease in the proliferation and migration activities of endothelial cells during embryogenesis.

*Overexpression of Syk (wt) and Syk (k−) mutant on extracellular signal-regulated kinase (ERK) activation.* To investigate the Syk-mediated signaling mechanism, the effect of overexpression of Syk wild-type and Syk dominant negative mutants in HUVECs on ERK activation was examined. As shown in Fig 3, adenovirus-mediated overexpression of Syk wild-type, but not LacZ control, induced ERK activation. Oppositely, adenovirus-mediated expression of Syk dominant negative mutants (k−) suppressed ERK activation. These results suggest that Syk plays an important role in signaling steps leading to ERK activation in HUVECs.

## DISCUSSION

Syk-deficient mice showed severe petechiae *in utero* and died shortly after birth, however to date, the mechanism of this bleeding has been unidentified. Two other mutations, phosphlipase C-γ2 and SLP-76-deficient

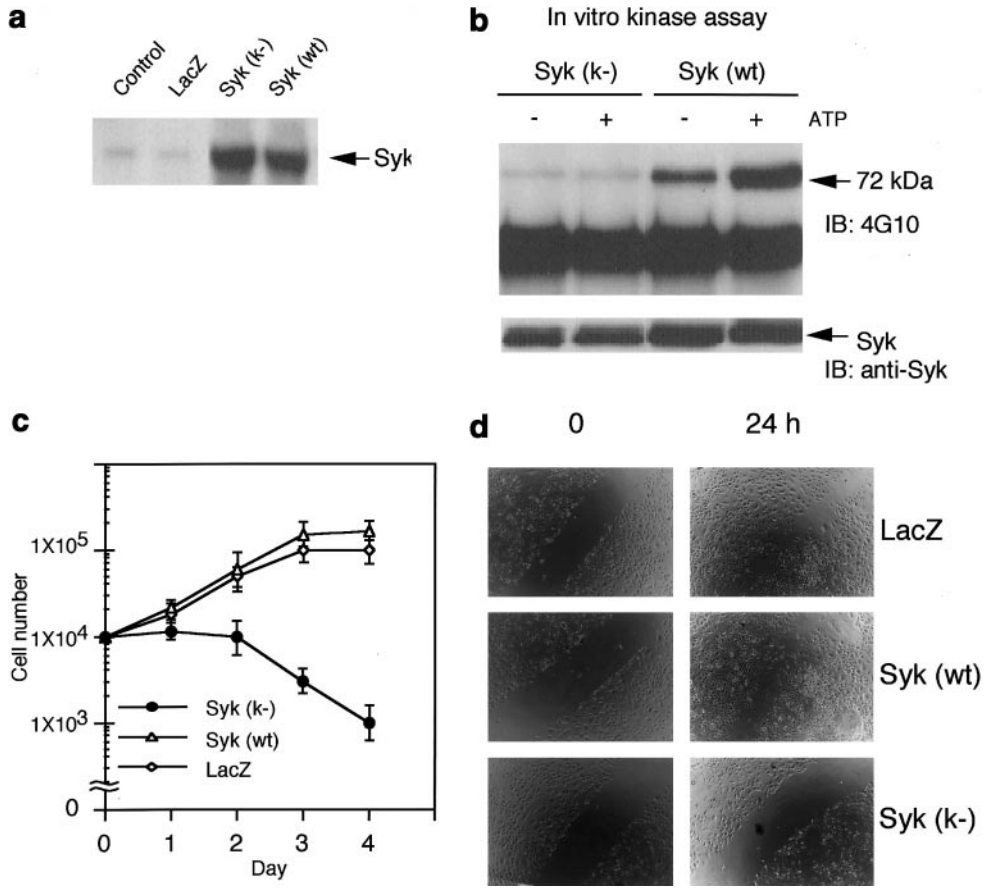


**FIG. 2.** Effect of adenovirus-mediated expression of Syk dominant negative mutants on cell proliferation and migration of cultured HUVECs. (a) Adenovirus-mediated expression of Syk wild-type (wt) and Syk kinase negative mutants (k−) in HUVECs. (b) *In vitro* tyrosine kinase assay of Syk wild-type and Syk kinase negative HUVECs. (c) Proliferation of HUVECs 2 days after adenoviral infection was determined by cell counting. Results are the means ± standard deviations of three independent experiments. (d) Effect of Syk dominant negative mutants on migration of cultured HUVECs. Migration into the scratch wounded area of a HUVEC monolayer was impaired in Syk dominant negative mutants.

mice, show a similar phenotype of *in utero* bleeding (14, 15). Both of them are substrates for Syk/ZAP-70 in the haematopoietic system, though their expression in endothelial cells has not been reported. It was recently reported that collagen-induced platelet aggregation, granule release and tyrosine phosphorylation of phosphlipase C-γ2 were markedly impaired in SLP-76-deficient platelets (16). These results suggest that the haemorrhagic diathesis could be a consequence of platelet dysfunction. In contrast, classical tests of platelet function, such as bleeding time, are normal in radiation chimeras reconstituted with Syk-deficient platelets (7, 9). In this study, we suggest that Syk is required for cell growth, migration and survival in HUVECs. In our previous study, electron microscopic analysis of Syk-deficient mice revealed an abnormal morphogenesis of vascular endothelial cells during embryogenesis. Our results strongly suggested that the bleeding is caused by a dysfunction in Syk-deficient vascular endothelial cells during embryogenesis. Remarkably, the bleeding phenotype is transferable when Syk-deficient bone marrow is transplanted into wild-type mice (7, 8). We suppose that the mechanism of bleeding disorder in day 16.5 fetuses of Syk-deficient mice might be same as that in radiation chimeras. Because recent study suggested that vascular endothelial cell is differentiated from haematopoietic stem cells and endothelial progenitor cells circulate in blood vessel (17), and vascular endothelial cell progenitors incorporated into sites of active angiogenesis and these cells differentiated into vascular endothelial cells. Therefore, in radiation chimeras reconstituted with Syk−/− fetal liver, it can be considered that Syk-deficient endothelial cells gradually substitute for normal endothelial and cause bleeding. If this is true, Syk appears deeply implicated in cellular reorganization of vascular endothelial cells during angiogenesis. In this point, Syk may be a potential target for angiogenesis inhibition in tumor growth. Oppositely, Coopman *et al.* proposed that Syk was a potent modulator of epithelial cell growth and a potential tumor suppressor in human breast carcinomas (18). Thus, Syk may represent an

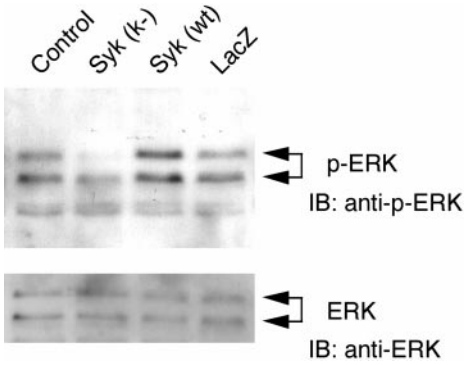


**FIG. 3.** Effect of expression of Syk (wt) and Syk (k−) mutant on ERK activation. HUVECs were equally infected with 20 multiplicity of infection of LacZ, Syk wild-type (wt), and Syk kinase negative mutants (k−), and 2 days later, ERK activation were examined by immunoblot analysis probed with anti-phospho ERK antibody and anti-ERK antibody, respectively.

approach to develop new cancer therapy strategies by controlling its kinase activity.

In this study, we showed that adenovirus-mediated overexpression of Syk dominant negative mutants suppressed ERK activation in HUVECs. In haematopoietic cells, a close relationship between Syk kinase activity and ERK activation was documented (19–21). For example, Costello *et al.* reported the critical role for the tyrosine kinase Syk in signaling step leading to ERK and c-Jun N-terminal kinase activation through high affinity IgE receptor of mast cells (19). Sada *et al.* also reported that Syk kinase activity was essential for ERK activation in mast cells (21). Similarly, in B cells, Jiang *et al.* reported that B cell antigen receptor cross-linking-mediated activation of ERK2, although maintained in Lyn-deficient cells, was abolished in Syk-deficient cells (20). Recent works have shown that Syk exhibits a more widespread expression pattern and multifunction in non-haematopoietic cells such as epithelial cells, hepatocytes, fibroblasts, neuronal cells and breast tissues (12, 18, 22, 23). In fibroblasts, we recently reported that Syk was expressed in human nasal fibroblasts and Syk expression affected lipopolysaccharides-induced RANTES production in fibroblasts of nasal polyps (23). In this report, Syk was found to be required for lipopolysaccharides-induced c-Jun N-terminal kinase activation which affects RANTES production. In hepatocytes, our previous work suggested that Syk plays an important role in signaling steps leading to MAP kinase activation by G-protein-coupled receptors (12). In neuronal cells, we found that adenovirus-mediated overexpression of Syk induced supernumerary neurite formation and ERK activation in P19 cells (22). Thus, although Syk has been believed to be specifically involved in immunoreceptor signaling, Syk appears to play a general physiological function in various tissues.

In summary, our results suggest that Syk plays a critical role in physiological regulation of endothelial cells and contributes to maintaining vascular integrity *in vivo.* Further investigations are needed in order to clarify the exact role and physiological target(s) of Syk in the growth-signaling pathway of vascular endothelial cells.

## ACKNOWLEDGMENTS

This work was supported in part by the Uehara Foundation of Japan, Fellowships of the Japan Society for the Promotion of Science for Young Scientists (to R.I.), Grant-in-Aid for the Research for the Future Program from the Japan Society for the Promotion of Science, Grant-in-Aids for Scientific Research (B), Scientific Research on Priority Areas (A) from Ministry of Education, Culture, Sport, Science, and Technology, Japan.

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