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Mutant Tie2 causing venous malformation signals through Shc

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Abstract

Tie2 is a receptor tyrosine kinase expressed predominantly in endothelial cells. A missense mutation in the intracellular domain of Tie2 resulting in an arginine to tryptophan substitution causes an inherited form of vascular dysmorphogenesis, venous malformation (VM). The signalling pathways activated by mutant Tie2 and responsible for formation and maintenance of the abnormal vessels in VM are not known. In this study, we have sought to define these pathways by identifying phosphoproteins interacting with mutant Tie2 expressed in endothelial cells. We find R849W Tie2 is constitutively active in endothelium and recruits and phosphorylates a 52 kDa protein. This protein is identified as p52 ShcA. We show endothelial cells expressing VM-mutant Tie2 are protected from cell death and expression of dominant-negative ShcA inhibits the anti-apoptotic activity of the mutant receptor. Suppression of this pro-survival signalling could be a therapeutic option for inducing regression of lesional vessels.

Keywords: Endothelial; Receptor tyrosine kinase; Adaptor protein

Inherited diseases of the vasculature can provide insights into the processes of blood vessel development and maintenance, as well as indicating key molecules regulating these processes. Venous malformations (VM) are common vascular anomalies consisting of vascular masses containing highly dilated vessels with very few supporting smooth muscle cells [1]. VM can arise sporadically or be inherited. An autosomal dominantly inherited form of VM (OMIM number 600195) has been shown to segregate with a C to T transition at position 2545 in the receptor tyrosine kinase Tie2 in three unrelated families [2,3]. This transition is the single most common mutation reported in inherited VM and results in a substitution of an arginine for a tryptophan at position 849 (R849W) in the intracellular domain of the receptor [2].

Tie2 is a receptor tyrosine kinase expressed predominantly in endothelial cells where it is required for vessel maturation and maintenance [4]. In vivo Tie2 is involved in microvessel sprouting, integrity, and survival [5,6]. Activation of Tie2 in cultured cells inhibits apoptosis and promotes monolayer integrity [7–9]. The ligands for Tie2 have been identified as the angiopoietin family of proteins and the best characterized of these are angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2) [10]. Ang1 is an agonist for Tie2 and is expressed in mesenchymal and smooth muscle cells [11], whereas Ang2 can act as an antagonist suppressing the effects of Ang1 [12].

The mechanism by which mutant Tie2 gives rise to the aberrant vascular structures in VM is not known. Expression studies in insect cells as well as mammalian cells demonstrate that R849W Tie2 has constitutively elevated tyrosine kinase activity compared with wild-type receptor [2,3,13]. Furthermore, mutant Tie2 inhibits apoptosis in cultured endothelial cells and this may be important in allowing VM vessels to survive and expand without the normal support provided by smooth muscle cells [13]. In addition to the receptor, the downstream signalling pathways activated by R849W Tie2 provide potential therapeutic targets for inducing regression of VM. The signalling intermediates

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recruited to R849W Tie2 and mediating its functional effects have not yet been identified. In this study, we have sought to identify proteins interacting with VM-mutant Tie2 and determine their functional significance.

Materials and methods

Materials. Human umbilical vein endothelial cells (HUVEC) were isolated as described previously [14]. Wild-type and R849W-mutant Tie2 were obtained from the American Tissue Culture collection and subcloned into the expression vector pCR3 (Invitrogen Life Technologies, Paisley UK). Dominant negative Y317F p52 ShcA was a kind gift from Dr. A.R. Frackleton Jr. [15]. Endothelial cells were cultured as described previously [14]. All other reagents were as previously detailed [13].

Immunoprecipitation and Western blotting. Briefly, endothelial cells were washed in PBS and lysed at 4 °C by addition of lysis buffer (50 M Tris, pH 7.4, containing 50 mM NaCl, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM EGTA, and Complete protease inhibitor cocktail). Lysates were cleared of particulate matter by centrifugation at 13,000*g* for 10 min. For analysis of cell proteins, samples of lysates were mixed with Laemmli sample buffer containing 100 mM dithiothrietol and boiled. For immunoprecipitation, antibodies recognizing Tie2 were added to lysates and incubated with agitation at 4 °C for at least 1 h. Pre-washed protein-G–agarose was added and incubations continued for at least 1 h. Immunoprecipitates were recovered by centrifugation, washed four times with lysis buffer, and proteins solubilized by boiling in Laemmli sample buffer containing 100 mM dithiothrietol. Proteins were resolved by sodium dodecyl sulfate gel electrophoresis and transferred to nitrocellulose membranes electrophoretically. Following probing with appropriate antibodies, immunoreactive proteins were visualized with peroxidase-conjugated secondary antibody and a chemiluminescent detection system [16].

Cell survival and apoptosis. Analysis of survival of transfected HUVEC was performed as described previously [17]. Briefly, cells were transfected with vector encoding the appropriate Tie2 together with green fluorescent protein (GFP). Twenty-four hours post-transfection, cells were washed and placed in serum-free medium. Cell survival was determined by counting the number of viable transfected (GFP-positive) cells in ten designated areas in gridded tissue culture wells before and after 18 h serum deprivation. In parallel cultures apoptotic index of transfected (GFP positive) cells was determined as described previously following staining with 0.2 μg/ml 4′,6′-diamidino-2-phenylindole [17].

Statistical evaluation. Data are presented as means and standard error of means. Statistical analysis was performed using Student's *t* test.

Results

To gain insight into the signalling pathways activated by R849W Tie2, and the functional consequences for endothelial behaviour, mutant Tie2 was expressed in endothelial cells. Parallel experiments were performed with endothelial cells transfected with wild-type Tie2. As shown in Fig. 1, and consistent with previous data, R849W Tie2 was found to be constitutively tyrosine phosphorylated, in contrast to the phosphorylation state of similar levels of the wild-type receptor. Examination of Tie2 immunoprecipitates revealed a number of proteins showing elevated tyrosine phosphorylation and co-immunoprecipitating with mutant receptor in cells expressing R849W Tie2 (Fig. 1). These were of molecular mass 98, 90, 70, and 52 kDa. Most prominent among these phosphoproteins was one of mass 52 kDa. To identify this protein, blots were probed with antibodies recognizing candidate phosphoproteins with a

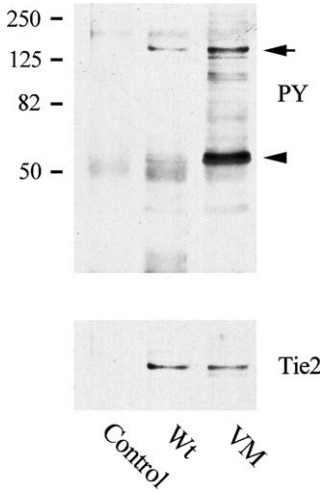

Fig. 1. R849W Tie2 is constitutively phosphorylated and associated with phosphoproteins in endothelial cells. Endothelial cells transfected with empty vector (control), wild-type (Wt) or VM-mutant Tie2 (VM) were lysed and Tie2 immunoprecipitated. Tie2 and associated proteins were resolved on SDS–PAGE, transferred to nitrocellulose membranes, and probed for tyrosine phosphorylated proteins (PY). The position of tyrosine phosphorylated Tie2 is indicated with an arrow. A number of other tyrosine phosphorylated proteins co-immunoprecipitate with VM-mutant Tie2, including a prominent phosphoprotein of approximately 52 kDa (arrowhead). Re-probing with anti-Tie2 indicates the overexpressed receptor. Positions of molecular mass markers are indicated in kilodalton.

similar molecular mass and the 52 kDa protein was identified as the adaptor protein ShcA. Endothelial cell lysates probed with anti-ShcA antibodies reveal three main isoforms expressed, 66, 52, and 44 kDa (Fig. 2). Immunoprecipitation of expressed R849W Tie2 resulted in co-immunoprecpitation of the 52 kDa form of ShcA (Fig. 2). Close examination of blots revealed 52 kDA ShcA was present in two forms, one running slightly above the main 52 kDa band. The two bands corresponding to p52 most likely represent different phosphorylation forms of the protein. Interestingly, the slightly higher molecular mass form of p52 was preferentially recruited to the mutant

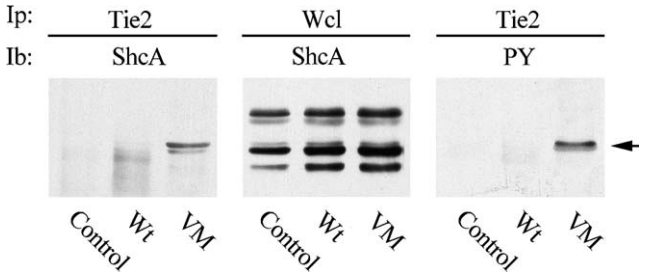

Fig. 2. R849W Tie2 associates with 52 kDa ShcA. Endothelial cells transfected with empty vector (control), wild-type (Wt) or VM-mutant Tie2 (VM) were lysed. Whole cell lysates (Wcl) or Tie2 immunoprecipitates were resolved by SDS–PAGE, immunoblotted, and blots probed with antibodies recognizing ShcA and phosphotyrosine (PY) as indicated. Endothelial cells express 46, 52, and 66 kDa forms of ShcA but only the 52 kDa form is associated with VM-mutant Tie2 and tyrosine phosphorylated (arrow).

form of Tie2, even though this form was present at much lower levels than the lower mass form of p52 (Fig. 2). ShcA p52 co-immunoprecipitating with R849W Tie2 was clearly tyrosine phosphorylated. These data indicate that mutant Tie2 constitutively interacts with tyrosine phosphorylated 52 kDa ShcA.

Previous studies have found that endothelial cells expressing VM-mutant Tie2 show increased survival compared with cells expressing wild-type Tie2, and this increased survival may contribute to lesion formation and/or maintenance [13]. ShcA has been reported to mediate cell survival [18,19]. It was of interest therefore to test whether ShcA could mediate part of the pro-survival activity of R849W Tie2. To do this, endothelial cells were transfected with wild-type and mutant Tie2 together with a plasmid encoding GFP and a dominant-negative form of p52ShcA. This dominant-negative form retains the SH2 and PTB domains of ShcA but has a phenylalanine in place of tyrosine at position 317, thus preventing recruitment of Grb2/SOS and downstream signalling by the adaptor [15]. Following transfection, endothelial cells were subjected to serum-deprivation and the survival of transfected cells was monitored. Cells expressing VM-mutant Tie2 showed significantly increased survival in the absence of serum compared with cells expressing wild-type Tie2 (Fig. 3). Importantly, expression of dominant-negative p52 ShcA resulted in suppression of the pro-survival activity of mutant Tie2 down to levels seen with wild-type receptor (Fig. 3).

The p52 ShcA-dependent increased survival of endothelial cells expressing VM-mutant Tie2 most likely reflects an effect on endothelial apoptosis. To test this, endothelial cells were transfected with wild-type of VM-mutant Tie2 together with plasmid encoding GFP and dominant-negative p52 ShcA. Following transfection, cells were subjected to serum-deprivation and the apoptotic index of transfected cells was assessed at 18 h. Consistent with the effects on cell survival, VM-mutant Tie2 suppressed endothelial apoptosis. Expression of dominant-negative p52 ShcA inhibited the suppression of endothelial apoptosis by R849W Tie2 (Fig. 4).

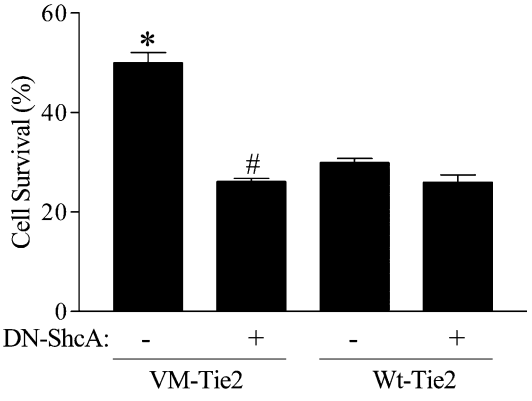

Fig. 3. R849W Tie2 promotes endothelial survival via ShcA. Endothelial cells were transfected with VM-mutant Tie2 or wild-type Tie2 together with control vector or dominant-negative ShcA (DN-ShcA), as indicated. Endothelial survival following 18 h serum-deprivation was determined as described in Materials and methods. Data are shown as mean percentage of endothelial survival and standard error of mean for three independent experiments. *Significant increase in cell survival vs. wild-type Tie2 ($p < 0.001$), #significant inhibition of VM-Tie2 effect by DN-ShcA ($p < 0.02$).

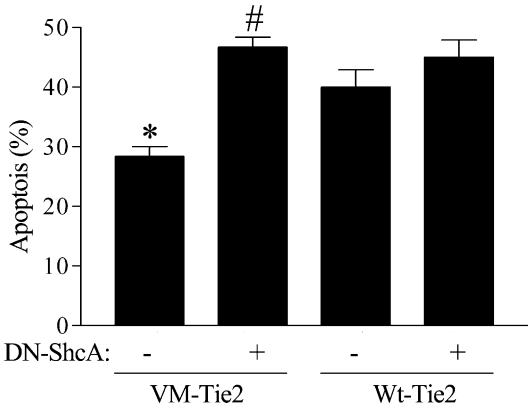

Fig. 4. R849W Tie2 inhibits endothelial apoptosis via ShcA. Endothelial cells were transfected with VM-mutant Tie2 or wild-type Tie2 together with control vector or dominant-negative ShcA (DN-ShcA), as indicated. Endothelial apoptosis following 18 h serum-deprivation was determined as described in Materials and methods. Data are shown as mean percentage of endothelial apoptosis and standard error of mean for three independent experiments. *Significant inhibition of endothelial apoptosis vs. wild-type Tie2 ($p < 0.05$), #significant inhibition of VM-Tie2 effect by DN-ShcA ($p < 0.05$).

Discussion

Here, we show that VM-mutant Tie2 expressed in endothelial cells is constitutively tyrosine phosphorylated and interacts with a number of phosphotyrosine-containing proteins. The major phosphoprotein recruited to VM-mutant Tie2 in the present study was found to be p52 ShcA. VM-mutant Tie2-associated ShcA is, like the mutant receptor, constitutively tyrosine phosphorylated. Furthermore, p52 ShcA was found to be required for the anti-apoptotic/pro-survival effects of mutant Tie2.

ShcA is an adaptor protein widely expressed in mammalian cells and comprises of an amino-terminal PTB domain, central proline-rich sequence, and carboxy-terminal SH2 domain [20]. The protein exists as three isoforms of molecular mass 46, 52, and 66 kDa, which are generated by alternative splicing or alternative translation initiation [20]. All isoforms are able to bind phosphotyrosine residues via their PTB and SH2 domains. The best studied pathway downstream of ShcA is the Ras pathway, although additional signalling pathways can be activated [21]. Following binding of ShcA to an activated receptor tyrosine kinase, such as the insulin receptor, the adaptor protein is phosphorylated at tyrosine-317 and this becomes a recruitment site for Grb2-SOS thereby initiating further downstream signals and effects on cell function [22]. ShcA has been implicated in a range of cellular functions including cell migration, proliferation, and survival [23]. In this study, we show that a

dominant-negative form of ShcA in which tyrosine-317 has been substituted with phenylalanine suppresses the ability of VM-mutant Tie2 from rescuing endothelial cells from apoptosis. These data indicate that the pro-survival activity of VM-mutant Tie2 is mediated by ShcA.

Acquisition of pericytes and smooth muscle cells is important to prevent regression of blood vessels by inhibiting endothelial apoptosis [24]. In VM, the abnormal vascular structures have a relative paucity of vascular support cells [2]. These smooth muscle cell-poor lesional VM vessels are likely to be maintained by the pro-survival activity of the mutant Tie2 [13]. The present study identifies ShcA as a mediator of the anti-apoptotic activity of VM-mutant Tie2 and suggests this adaptor protein, or its associated signalling pathways, as potential targets for therapies aimed at promoting regression of VM vessels in patients with the R849W mutation.

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