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Lymphatic Vessel Abnormalities Arising from Disorders of Ras Signal Transduction

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Abstract

A number of genetic diseases in man have been described in which abnormalities in the development and function of the lymphatic vascular (LV) system are prominent features. The genes that are mutated in these diseases are varied and include genes that encode lymphatic endothelial cell (LEC) growth factor receptors and their ligands and transcription factors that control LEC fate and function. In addition, an increasing number of genes have been identified that encode components of the Ras signal transduction pathway that conveys signals from cell surface receptors to regulate cell growth, proliferation and differentiation. Gene targeting studies performed in mice have confirmed that the LV system is particularly susceptible to perturbations in the Ras pathway.

Introduction

A major function of the lymphatic vascular (LV) system is to return extravasated fluid from tissues to the peripheral blood circulation (Oliver and Alitalo 2005). Disruption of this function of the LV system results in the accumulation of extracellular fluid and painful swelling known as lymphedema (Alitalo 2011; Radhakrishnan and Rockson 2008). In addition, other pathologies can result from defective LV circulatory function including leakage of lymphatic fluid into body cavities such as the pleural space (chylothorax) or peritoneum (chylous ascites). Disorders of the LV system may be inherited or acquired. Genes responsible for the development of several different inherited LV disorders have now been identified. Examples are *FLT4* that encodes vascular endothelial growth factor receptor 3 (VEGFR-3) in hereditary lymphedema 1A (Milroy's disease), and transcription factor genes *SOX18* and *FOXC2* in hypotrichosis-lymphedema-telangiectasia syndrome and lymphedema distichiasis syndrome respectively. Of other heritable diseases in which disorders of LV function have been reported, several have in common that the causative genes encode components of the ubiquitous Ras signal transduction pathway. This pathway acts downstream of numerous cell surface receptors in most cell types to regulate diverse responses including growth, proliferation, survival and differentiation. Studies of mice with targeted mutations in the Ras pathway have further highlighted its role in the control of LV function and have indicated that the LV system may be particularly sensitive to alterations in the strength or duration of Ras signaling.

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Ras Signal Transduction

Ras family molecules are small guanine nucleotide binding proteins attached to the inner leaflet of cell membranes as a result of lipid modification (e.g. farnesylation) at their carboxy-terminal end (Wennerberg et al. 2005). There are multiple Ras isoforms that differ in their patterns of tissue expression and location within cells. H-, N- and K-Ras are the most commonly studied isoforms. Ras proteins act as molecular switches that convert between inactive GDP-bound and active GTP-bound forms. In response to ligand recognition, cell surface receptors promote the recruitment of guanine nucleotide exchange factors (RasGEFs) to membranes that eject GDP from the Ras guanine nucleotide-binding pocket, thereby allowing Ras to bind GTP that is present at higher concentrations than GDP in the cytoplasm (Bos et al. 2007). Ras-GTP then triggers the activation of several different downstream pathways that include the mitogen activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) signaling pathways (Buday and Downward 2008) (Fig. 1). These pathways drive cellular responses in part through the activation of transcription factors. Inactivation of Ras is mediated by Ras GTPase-activating proteins (RasGAPs) that through physical interaction with Ras increase its ability to hydrolyze bound GTP to GDP many fold (King et al. 2013). Given the central role of Ras in cellular signal transduction it is not surprising that perturbations in this pathway result in disorders of tissue homeostasis. Thus, somatic activating mutations in Ras that render it refractory to inactivation by RasGAPs are found in 30% of all human cancers (Prior et al. 2012). Furthermore, in humans and mice with germline mutations in genes of the Ras signaling pathway there are multiple anomalies of tissue homeostasis. One physiological system that is commonly affected in these disorders is the LV system that is the subject of this review. Following is a discussion of how mutations in genes that encode Ras regulators, Ras itself and Ras effector molecules impact upon LV function in both species.

Ras Regulators

SHP-2, SOS1 and Noonan syndrome

Noonan syndrome (NS) is a developmental autosomal dominant disorder characterized by short stature, cardiac and skeletal abnormalities, facial dysmorphism and cognitive impairment (Tartaglia et al. 2010). It is genetically variable and is caused by missense mutations in genes of the Ras-MAPK pathway. Costello syndrome (CS), cardiofaciocutaneous syndrome (CFCS) and LEOPARD syndrome are clinically similar to NS and are also caused by mutations in this pathway (Tidyman and Rauen 2009). Collectively, these syndromes are referred to as RASopathies. In NS, the two most common affected genes are *PTPN11* and *SOS1* that account for 50% and 13% of cases respectively (Tartaglia et al. 2010). *PTPN11* encodes the SHP-2 protein tyrosine phosphatase that is necessary for growth factor receptor induced activation of Ras through an uncertain mechanism that may involve negative regulation of RasGAPs (Dance et al. 2008). The *SOS1* protein, by contrast, functions as a RasGEF (Bos et al. 2007). Mutations of *PTPN11* and *SOS1* in NS result in an impairment of autoinhibitory mechanisms of both proteins, leading to increased activity and hyper-activation of the Ras pathway. LV abnormalities that include prenatal, postnatal, childhood and adult lymphedema, lymphangiectasia (LV dilation) and chylous effusions have long been recognized in NS, although in most instances of LV abnormality, which specific Ras-MAPK gene was affected was not reported (Tartaglia et al. 2010). However, in one recent study it was determined that lymphedema was present in 49% of NS patients with *PTPN11* mutations and 63% of NS patients with *SOS1* mutations (Smpokou et al. 2012) (Table 1).

RASA1 and Capillary malformation-arteriovenous malformation

Capillary malformation-arteriovenous malformation (CM-AVM) is an autosomal dominant vascular disorder the pathognomonic feature of which is the presence of one or more cutaneous CM, together with fast flow blood vascular (BV) lesions in one third of patients (Eerola et al. 2003; Revencu et al. 2008). The affected gene in CM-AVM is *RASA1* that encodes p120 RasGAP. Nonsense, missense, deletion, insertion and splice site substitutions in the *RASA1* gene have all been reported in CM-AVM and all are considered inactivating (Revencu et al. 2008). Disease is thought to result from acquisition of somatic second hit mutations in the inherited wild type *RASA1* allele, which would be consistent with the focal nature of lesions and variable expression of the disease. LV abnormalities have also been observed in CM-AVM including lymphedema, chylous ascites and chylothorax, although their prevalence remains to be determined (de Wijn et al. 2012; Revencu et al. 2008). Recently, near infrared fluorescence lymphatic imaging was used to demonstrate a highly abnormal dermal LV network in a CM-AVM patient that involved localized lymphangiectasia, hyperplasia and abnormal LV pumping function with efflux of lymph from lower extremities into the abdomen and lymphocele-like vesicles in the groin (Burrows et al. 2013).

Consistent with the LV abnormalities observed in CM-AVM, conditional *RASA1* deficient mice that are induced to lose expression of *RASA1* as adults, either in all tissues or specifically within LV endothelial cells (LEC), develop massive LV hyperplasia and chylothorax that results in death several months after *Rasa1* gene deletion (Lapinski et al. 2012) (Table 2). No other spontaneous pathologies are evident in these mice including in the BV system, which is normal. It should be noted, however that non-conditional *RASA1*-deficient mice and EC-specific *RASA1* deficient mice both die during embryonic development as a result of abnormal BV patterning, which implies that *RASA1* must be lost in BECs during embryogenesis in order for the appearance of BV lesions in CM-AVM (Henkemeyer et al. 1995; Lapinski et al. 2012). LEC from induced *RASA1* deficient mice show prolonged MAPK and PI3K signaling induced in response to several different lymphangiogenic growth factors, in particular, VEGF-C, the ligand for VEGFR-3 (Lapinski et al. 2012). This suggests a model in which steady state concentrations of VEGF-C in induced *RASA1* deficient mice drive LV hyperplasia. In agreement with this model, antibody-mediated blockade of VEGFR-3 signaling prevents the development of LV hyperplasia in this model (Lapinski et al. 2012).

SPREDs

Sprouty-related Ena/VASP homology 1 domain-containing proteins (SPREDs) are intracellular membrane-binding proteins of which there are three different isoforms in mammals (Bundschu et al. 2007). Sprouty proteins inhibit Ras-MAPK signaling. The mechanism of inhibition is controversial, although recently it has been shown that SPRED-1 targets the neurofibromin-1 (NF1) RasGAP to membranes, thereby allowing NF1 to inactivate Ras (Stowe et al. 2012). This would explain the phenotypic similarity between Legius syndrome and neurofibromatosis in man that are caused by inactivating mutations of SPRED-1 and NF1 respectively (McClatchey 2007; Messiaen et al. 2009). LV abnormalities have not been reported in Legius syndrome or neurofibromatosis or in mice that lack individual SPRED isoforms or NF1. Notably, however, mice that lack both SPRED-1 and SPRED-2 develop a hyperplastic network of dilated blood-filled LV associated with subcutaneous hemorrhage, edema and mid-gestation lethality (Taniguchi et al. 2007). LEC from these mice show increased activation of MAPK in response to VEGF-C stimulation, which supports the view that increased activation of Ras downstream of VEGFR-3 is responsible for the LV phenotype. It is conceivable that SPRED proteins engage other RasGAPs, in addition to NF1, and that loss of membrane targeting of these other RasGAPs

underlies the increased MAPK activation in SPRED1/2 deficient LEC, although this remains to be determined.

Ras Isoforms

K-Ras and Noonan syndrome

NS may also be caused by activating mutations in *KRAS*, in less than 3% of cases (Carta et al. 2006; Schubbert et al. 2007; Schubbert et al. 2006). Most *KRAS* mutations in NS are missense that, similar to the somatic Ras mutations that occur in cancer, result in impaired Ras hydrolysis of GTP. In cancer, Ras mutations result in a complete loss of intrinsic Ras GTPase activity and resistance of Ras to the action of RasGAPs. By contrast, *KRAS* mutations in NS are generally less activating. One example is the T58I mutation that results in a reduced intrinsic GTPase activity and partial resistance to RASA1 and NF1. In an aborted fetus with NS that was caused by a T58I *KRAS* mutation, increased nuchal translucency and edema were noted that are both indicators of LV system dysfunction (de Mooij et al. 2011). Consistent with this, distended jugular lymphatic sacs were observed in the same fetus.

K-Ras and Cardiofaciocutaneous syndrome

CFCS, which like NS is also genetically variable, may also be caused by germline mutations in *KRAS* or genes that encode components of the MAPK cascade (Roberts et al. 2006). One *KRAS* mutation that has been identified in CFCS is P34R that shows normal intrinsic GTPase activity but is insensitive to RASA1 and NF1. Notably, a CFCS patient with a P34R *KRAS* mutation developed both lymphedema and chylothorax (Schubbert et al. 2006).

H-Ras and Costello syndrome

CS is caused by missense activating mutations of *HRAS* (Aoki et al. 2005). The majority of *HRAS* mutations are at G12 that is required for proper orientation of Q61 involved in GAP-promoted Ras hydrolysis of GTP (King et al. 2013). In CS, missense mutations at G12 are generally less activating than the G12V mutation, which is the most common *HRAS* mutation detected in human cancers (Seeburg et al. 1984). CS patients with *HRAS* G12E and G12D mutations have been reported to develop chylous ascites and chylothorax respectively (Kerr et al. 2006; Lo et al. 2008).

Ras isoforms in mice

Studies of Ras knockout and Ras over-expressing mice have confirmed its function as an essential regulator of LV development and function (Ichise et al. 2010). Approximately 50% of mice that lack expression of H- and N-Ras develop chylous ascites associated with intestinal LV hypoplasia. By contrast, essentially all mice that lack expression of H- and N-Ras and are heterozygous for a null allele of *Kras* show this phenotype. Conversely, mice that over-express H-Ras in EC show LV hyperplasia, edema and chylothorax. Most striking in these different mouse models is that altered Ras expression does not affect BV development or function.

Ras effectors

Raf1 and Noonan syndrome

Recognition of Ras-GTP by Raf proteins represents an initial step in activation of the MAPK pathway (Buday and Downward 2008) (Fig. 1). Raf proteins are serine/threonine kinases of which there are three isoforms, named A-Raf, B-Raf and C-Raf (Raf-1). Upon activation, Raf proteins phosphorylate and activate downstream MEK kinases that then

phosphorylate and activate ERK MAP kinases. ERKs phosphorylate and activate different transcription factors such as ternary complex factors (TCFs), and c-fos and c-jun that comprise activator protein 1 (AP-1). Gain of function mutations in Raf-1 have been identified in NS and gain of function mutations in B-Raf and MEKs have been identified in CFCS (Pandit et al. 2007; Razzaque et al. 2007; Roberts et al. 2006). In one case of NS caused by a gain of function Raf-1 mutation, cutaneous microcystic lymphatic malformation and lymphangiectasia were reported as prominent features (Lee et al. 2010).

One mutation hotspot of Raf-1 in NS is centered at S259 (Pandit et al. 2007; Razzaque et al. 2007). In its phosphorylated state, S259 binds 14-3-3 proteins that stabilize Raf-1 in an inactive conformation (Leicht et al. 2007). Kinases that may phosphorylate Raf-1 S259 in EC include PKA, PKC and AKT, the last of which lies downstream of PI3K (Deng and Simons 2013; Ren et al. 2010; Wellbrock et al. 2004) (Fig. 1). Recently, Deng et al generated transgenic mice that express an S259A Raf-1 mutant specifically in EC that results in constitutive activation of the MAPK pathway in the EC lineage (Deng et al. 2013). These mice developed extensive edema and lymphangiectasia in utero. Interestingly, the constitutive ERK activation resulted in increased cardinal vein expression of SOX-18 and downstream PROX1 transcription factors that specify LEC fate (Francois et al. 2008; Wigle and Oliver 1999). Accordingly, increased differentiation of LEC from cardinal vein EC and massively enlarged jugular lymphatic sacs were observed in embryos. This study reveals a critical function for the MAPK pathway in LEC fate specification. Furthermore, findings of the study indicate that altered LEC differentiation is likely to be an important contributor to the development of LV abnormalities in humans and mice caused by mutation in other components of the MAPK pathway.

Ternary complex factors

How MAPK regulate the expression of SOX-18 is uncertain. Recently, however, it has been shown that the early growth response gene 1 (Egr1) transcription factor can activate SOX-18 expression (Petrovic et al. 2010). In turn, expression of Egr1 is regulated by TCFs that bind MAPK and are activated upon MAPK phosphorylation (Wasylyk et al. 1998). Thus, MAPK may link to SOX-18 expression through a TCF-Egr1 route. Net is one member of the TCF family that acts as a repressor rather than activator of Egr1 expression (Ayadi et al. 2001; Criqui-Filipe et al. 1999). In support of the TCF-Egr1 connection, knockin mice that express a form of Net that lacks its DNA-binding domain develop lymphangiectasia and die after birth from chylothorax (Ayadi et al. 2001). Furthermore, these LV abnormalities are associated with increased expression of Egr1.

Class I PI3K

Class I PI3K catalyze the transfer of phosphate to the third position of the inositol ring of phosphatidylinositol 4,5 bisphosphate (PIP₂) resulting in the generation of PI-3,4,5 trisphosphate (PIP₃) (Engelman et al. 2006). This event leads to membrane recruitment and activation of the AKT serine/threonine kinase that phosphorylates several different downstream targets that include tuberous sclerosis proteins (TSCs) and transcription factors such as nuclear factor kappa B (NF κ B) (Fig. 1). Phosphorylation of TSCs results in their inactivation and subsequent triggering of the mechanistic target of rapamycin (mTOR) signaling pathway. Class IA PI3K each comprise of a catalytic subunit (p110 α , or) in association with regulatory subunits (p85 α , p55 α , p50 α , p85 α or p55 α). Binding of regulatory subunits to the cytoplasmic domains of cell surface receptors activates the catalytic activity of class IA PI3K. However, all class IA PI3K catalytic subunits also contain Ras binding domains and direct interaction with Ras-GTP provides an alternative mechanism by which PI3K catalytic activity can be activated. For p110 α , clear-cut evidence of the importance of Ras mediated-PI3K activation for normal LV development was

provided with the generation of a knockin mouse that expressed a form of p110 α that was specifically unable to interact with Ras (Gupta et al. 2007). These mice showed LV hypoplasia and developed chylous ascites, most likely as a consequence of defective LV drainage.

PI3K and CLOVES

Consistent with findings in mice, somatically acquired missense mutations of p110 α (*PI3KCA*) have recently been identified as the cause of CLOVES syndrome in humans (congenital lipomatous asymmetric overgrowth of the trunk with lymphatic, capillary, venous, and combined-type vascular malformations, epidermal nevi, and skeletal anomalies) (Kurek et al. 2012). These mutations, which are frequently observed in cancer, have been identified directly within LV malformations of CLOVES syndrome patients. The mutations result in a constitutively active form of p110 α and activation of AKT. One of these mutations has also been identified in lesional tissue of some patients with Klippel-Trenaunay-Weber syndrome (KTWS) in which LV abnormalities are also commonly present along with other vascular abnormalities and limb overgrowth (Kurek et al. 2012).

AKT

Each of the three isoforms of AKT (AKT1, 2 and 3) are expressed in LEC (Zhou et al. 2010). However, studies of mice that are deficient in each isoform indicate that AKT1 is the dominant isoform required for normal LV development and function (Zhou et al. 2010). In AKT1 deficient mice, LV capillaries are reduced in size and there is an absence of valves in smaller collecting LV. In addition, larger collecting LV are distended and there is reduced pericyte coverage of these vessels. These abnormalities are observed to a much lesser degree in AKT2 or AKT3 deficient mice, whereas compound AKT deficient mice show a similar phenotype to AKT1 deficient mice. By contrast BV development is normal in AKT1 deficient mice (Zhou et al. 2010).

AKT and Proteus syndrome

Like CLOVES syndrome, Proteus syndrome (PS) is a general tissue overgrowth syndrome with a vascular component (Cohen 2005). In PS, vascular malformations are subcutaneous and are predominantly of a lymphatic or lymphovenous type (Hoey et al. 2008). Recently, somatic activating mutations in AKT1 have been identified as the cause of PS in the majority of cases (Lindhurst et al. 2011). This finding is in agreement with the findings in mice showing a predominant role for AKT1 in the regulation of LV.

Conclusions

Mutation of genes that encode components of the Ras signaling pathway commonly result in LV disorders. In several examples, mutations also affect other physiological systems. However, in other examples, effects are limited to BV and LV and in several cases a specific influence upon the LV system is apparent. Why the LV system is particularly susceptible to gene mutations in this pathway is unclear but cannot be explained by restricted expression of gene products to LV, as most have a ubiquitous tissue distribution. In general terms, frequent LV phenotypes may reflect a requirement of the LV system to respond rapidly and robustly to Ras signaling triggered by growth factors during both developmental lymphangiogenesis and lymphangiogenesis in adults, for example, during wound healing. Causative genes of a number of other diseases with a strong or exclusive LV component have yet to be identified. Predictably, some of these genes will encode distinct Ras pathway components. In all instances in which LV disorders are determined to arise from increased Ras induced MAPK and/or PI3K signaling, drug inhibition of either or both effector pathways as appropriate could represent a ready means of therapy.

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Figure 1.

Ras signal transduction. Human diseases with LV abnormalities are indicated with blue text above the respective affected component in the Ras signaling pathway. NS, Noonan syndrome; CFCS, Cardiofaciocutaneous syndrome; CS, Costello syndrome; CM-AVM, Capillary malformation-arteriovenous malformation; CLOVES, Congenital lipomatous asymmetric overgrowth of the trunk with lymphatic, capillary, venous, and combined-type vascular malformations, epidermal nevi, and skeletal anomalies; KTWS, Klippel-Trenaunay-Weber syndrome; PS, Proteus syndrome. Red asterixes indicate those signaling molecules in the Ras pathway where mutation in mice also results in LV abnormalities. The pink dashed line between SPREDs and RasGAPs indicates a potential link between these signaling intermediates as regulators of the LV system in mice. See text and Tables 1 and 2 for details.

Table 1

Lymphatic vessel abnormalities and disorders of Ras signal transduction in man

Disease	Affected gene	Protein/function	Mutation ¹	Phenotype	References
Noonan syndrome (NS)	<i>PTPN11</i>	SHP-2/Ras	Germline GOF	Lymphedema	(Smpokou et al. 2012)
	<i>SOS1</i>	activator SOS1/RasGEF	Germline GOF	Lymphedema	(Smpokou et al. 2012)
	<i>KRAS</i>	K-Ras	Germline GOF	Lymphedema	(de Mootij et al. 2011)
	<i>RAF1</i>	Raf-1/Ras effector MAPK pathway	Germline GOF	Lymphangiectasia Microcystic lymphatic malformation	(Lee et al. 2010)
Capillary malformation-arteriovenous malformation (CM-AVM)	<i>RASA1</i>	RASA1/Negative regulator of Ras	Germline LOF	Lymphedema Chylous ascites Chylothorax Lymphangiectasia LV hyperplasia	(Burrows et al. 2013; de Wijn et al. 2012; Revencu et al. 2008)
	<i>KRAS</i>	K-Ras	Germline GOF	Lymphedema Chylothorax	(Schubbert et al. 2006)
	<i>HRAS</i>	H-Ras	Germline GOF	Chylous ascites Chylothorax	(Kerr et al. 2006; Lo et al. 2008)
Costello syndrome (CS)					
Congenital lipomatous asymmetric overgrowth of the trunk with lymphatic, capillary, venous, and Klippel-Trenaunay-Weber syndrome (KTWS)	<i>PIK3CA</i>	PI3K p110α Ras effector PI3K pathway	Somatic GOF	LV malformation	(Kurek et al. 2012)
	<i>PI3KCA</i>	PI3K p110α Ras effector PI3K pathway	Somatic GOF	LV malformation	(Kurek et al. 2012)
	<i>AKT1</i>	AKT1/PI3K pathway	Somatic GOF	LV malformation	(Hoey et al. 2008)

¹ GOF, gain of function; LOF, loss of function

Table 2

Lymphatic vessel abnormalities and disorders of Ras signal transduction in mice

Gene	Protein/ function	Mutation ¹	Phenotype	Comments	References
<i>Rasa1</i>	RASA1/Negative regulator of Ras	Induced deletion in adults (LOF)	LV hyperplasia Chylothorax Chylous ascites	Absence of spontaneous BV abnormalities	(Lapinski et al. 2012)
<i>Spred1/2</i>	SPRED-1 and -2/Negative regulators of Ras	Germline deletion (LOF)	LV hyperplasia Lymphedema	Single mutants normal	(Taniguchi et al. 2007)
<i>Ras</i>	H-Ras K-Ras N-Ras	Germline deletion (LOF)	LV hypoplasia Chylous ascites	Normal BV development	(Ichise et al. 2010)
<i>Raf1</i>	H-Ras Raf-1/Ras effector MAPK pathway	EC over-expression EC expression of Raf1 S259A (GOF)	LV hyperplasia Lymphedema Lymphangiectasia	Normal BV development Increased LEC differentiation	(Ichise et al. 2010) (Deng et al. 2013)
<i>Net</i>	Net/Transcription repressor in MAPK pathway	Germline DNA-binding domain deletion (LOF)	Lymphangiectasia Chylothorax	Increased expression of Egr1	(Ayadi et al. 2001)
<i>Pi3kca</i>	PI3K p110α Ras effector PI3K pathway	Germline Ras-binding deficient (LOF)	LV hypoplasia Chylous ascites		(Gupta et al. 2007)
<i>Akt1</i>	AKT1/PI3K pathway	Germline deletion (LOF)	LV capillary hypoplasia Absence of valves in collecting LV	Normal BV development	(Zhou et al. 2010)

¹ GOF, gain of function; LOF, loss of function