

Adrenomedullin Haploinsufficiency Predisposes to Secondary Lymphedema

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Secondary lymphedema is a debilitating condition, and genetic factors predisposing to its development remain largely unknown. Adrenomedullin (AM) is peptide encoded, together with proadrenomedullin N-terminal peptide (PAMP), by the *Adm* gene (adrenomedullin gene). AM and its putative receptor calcitonin receptor-like receptor (CLR) are implicated in angiogenesis and lymphangiogenesis during embryogenesis and wound healing, suggesting their possible involvement in secondary lymphedema. To investigate whether AM deficiency predisposes to secondary lymphedema, we used heterozygous adult mice with *Adm* gene-knockin stop mutation, which selectively abrogated AM, but preserved PAMP, expression (*Adm*^{AM+/Δ} animals). After hind limb skin incision, *Adm* messenger RNA expression was upregulated in wounded tissue of both *Adm*^{AM+/+} and *Adm*^{AM+/Δ} mice. However, only *Adm*^{AM+/Δ} animals developed limb swelling and histopathological lymphedematous changes, including epidermal thickening, elevated collagen fiber density, and increased microvessel diameter. Secondary lymphedema was prevented when circulating AM levels in *Adm*^{AM+/Δ} mice were restored by systemic peptide delivery. In human skin, CLR was expressed in tissue components affected by lymphedema, including epidermis, lymphatics, and blood vessels. Our study identified a previously unrecognized role for endogenous AM as a key factor in secondary lymphedema pathogenesis and provided experimental *in vivo* evidence of an underlying germ-line genetic predisposition to developing this disorder.

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INTRODUCTION

Lymphedema is a debilitating condition and a major cause of morbidity following radiotherapy, cancer, surgery, and other injuries (Rockson, 2008a; Tammela and Alitalo, 2010). Lymphedema develops as a result of localized fluid retention and subsequent swelling (edema). This is owing to insufficiency of the lymphatic system, which occurs as a consequence of abnormal structure and/or compromised function of lymphatic vessels (Rockson, 2008a; Tammela

and Alitalo, 2010). It was proposed that the first abnormality during the development of breast cancer-related lymphedema is not lymphatic obstruction, but high fluid filtration that overwhelms vulnerable lymphatics (Stanton *et al.*, 2009a). Blood vessels are therefore also implicated in the pathogenesis of lymphedema, and the edema arising from venous insufficiency, when untreated, can progress into a combined venous/lymphatic disorder (Rockson, 2008a).

Lymphedema often affects the skin. The structural and functional abnormalities in lymphedematous skin reflect a multicellular response to impaired extracellular fluid mobilization and can be distinguished from other mechanisms that lead to interstitial edema (Rockson, 2008a). The presence of epidermal thickening is pathognomonic of lymphedema (Wilson *et al.*, 2004; Tabibiazar *et al.*, 2006; Rockson, 2008a). Lymphedema predisposes to collagen and lipid deposition, cutaneous hypercellularity, progressive fibrosis, and susceptibility to infections (Rockson, 2001; Rutkowski *et al.*, 2010; Wu *et al.*, 2011).

Lymphedema is a heterogeneous condition that is either hereditary or acquired (primary or secondary disorder, respectively) (Rockson, 2008a). Although many genes having a role in lymphatic development have been identified, they are mostly associated with primary lymphedema pathogenesis. For example, mutations in *VEGFR3*, *GATA2*, or *FOXC2* are the underlying causes of either congenital (Milroy disease or Emberger syndrome) or late-onset (distichiasis syndrome)

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Abbreviations: ADM, adrenomedullin gene; AM, adrenomedullin; CALCLRL, gene encoding CLR; CLR, calcitonin receptor-like receptor; EC, endothelial cell; PAMP, proadrenomedullin N-terminal peptide; RAMP, receptor activity-modifying protein

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lymphedema (Tammela and Alitalo, 2010; Wang and Oliver, 2010; Ostergaard *et al.*, 2011). In contrast, little is known about the genetic susceptibility to the risk of developing secondary lymphedema following trauma, malignancy, radiation, or other tissue insults (Rockson, 2008a, b). It remains unknown why lymphedema develops in some individuals following sentinel node removal, yet not in others after axillary clearance for breast cancer (Stanton *et al.*, 2009b). Genetic variation could confer relative risk for or, conversely, protection from the development of secondary lymphedema (Stanton *et al.*, 2009a). Recent association studies support the hypothesis that genetic susceptibility could be an important risk factor for developing secondary lymphedema (Finegold *et al.*, 2008, 2012). However, there is no experimental evidence demonstrating an *in vivo* role for any gene in the pathogenesis of this disorder, and the underlying mechanisms remain to be delineated. In particular, the molecular and physiological relationships between lymphatic and blood vessels and the surrounding tissue microenvironment, which underlie this complex disorder, remain largely uncharacterized (Rockson, 2009). Therefore, therapeutic strategies to prevent or treat lymphedema are limited (Rockson, 2008a; Tammela and Alitalo, 2010).

Adrenomedullin (AM) is a 52-amino-acid vasoactive peptide (Kitamura *et al.*, 1993a; Nikitenko *et al.*, 2002; Brain and Grant, 2004). Together with proadrenomedullin N-terminal peptide (PAMP), AM is encoded by the *ADM* gene (adrenomedullin gene) (Kitamura *et al.*, 1993b). *In vitro*, AM acts through the calcitonin receptor-like receptor (CLR), which is encoded by the *CALCRL* (gene encoding CLR) gene (Poyner *et al.*, 2002). CLR forms heterodimeric receptors with one of the receptor activity-modifying proteins (receptor activity-modifying proteins (RAMPs) 1, 2, or 3). Co-expression of RAMP2 or RAMP3 with CLR in cultured cells leads to AM receptor formation (Poyner *et al.*, 2002).

AM and its receptors are implicated in angiogenesis and lymphangiogenesis during embryonic development, wound healing, and cancer. The role for *Adm*, *Calcrl*, and *Ramp2* during embryogenesis has been defined in knockout mice studies. These revealed close correlation among phenotypes for these genes, supporting the view that *in vivo* AM effects are mediated through CLR/RAMP heterodimers (Brain and Grant, 2004; Nikitenko *et al.*, 2006c). The complete loss of *Adm* expression (that affects both AM and PAMP) in homozygous knockout mice leads to embryonic death at E13.5–14.5 owing to generalized edema or hemorrhage (Caron and Smithies, 2001; Shindo *et al.*, 2001). This is phenocopied in homozygous *Calcrl*- and *Ramp2*-knockout mice with embryonic lethality and edema occurring at E12.5–13.5 (Fritz-Six *et al.*, 2008; Ichikawa-Shindo *et al.*, 2008). The development of edema in these embryos is attributed to blood vessel fragility and leakage, or to jugular lymphatic sac hypoplasia accompanied by structurally unaffected regional lymphatic vessels (Kahn, 2008). AM also has a role in tumor neovascularization (Nikitenko *et al.*, 2006c). Its exogenous administration in wild-type mice ameliorates severe injury-induced swelling (Jin *et al.*, 2008), stimulates wound healing

of pressure ulcers (Harada *et al.*, 2011), and induces angiogenesis and lymphangiogenesis. In human endometrial tissue, CLR localizes to blood and lymphatic microvessels (Maybin *et al.*, 2011). In cultured microvascular endothelial cells (ECs), CLR interacts with AM (Nikitenko *et al.*, 2006a). Primary human blood and lymphatic ECs express *CALCRL* and *RAMPs* messenger RNAs (mRNAs), supplemented AM promotes their proliferation *in vitro* and reduces endothelium monolayer hyperpermeability induced by inflammatory mediators (Hippenstiel *et al.*, 2002; Vart *et al.*, 2007; Fritz-Six *et al.*, 2008). These findings suggest that in developing embryos and reproductive tissues endogenous AM signaling through CLR might be essential in both blood vessels and lymphatics. However, whether it is relevant to mature animals and human pathology, including secondary lymphedema, remains unclear (Kahn, 2008).

Here, we tested the hypothesis that endogenously produced AM affects the development of secondary lymphedema. To our knowledge, the present study identified a previously unrecognized role for *Adm* in the pathogenesis of this disorder and provided previously unreported experimental *in vivo* evidence of a genetic susceptibility or predisposition to the risk of developing secondary lymphedema.

RESULTS

We used mice with *Adm*-knockin stop mutation that selectively abrogates AM, but not PAMP, expression (*Adm*^{AM}-knockin animals) (Figure 1a; (Shimosawa *et al.*, 2002)). Although the homozygous mutation (*Adm*^{AM^Δ/Δ}) in mice was lethal, heterozygous (*Adm*^{AM+/Δ}) fetuses were viable and adult mice (also termed here as *Adm*^{AM} haploinsufficient animals) were fertile, with their plasma AM levels approximately half (~53%) of those of wild-type animals, whereas PAMP levels remained unaffected (Supplementary Table S1 online).

Primary congenital or adult-onset lymphedema is absent in adult *Adm*^{AM} haploinsufficient mice

Under physiological, i.e., unchallenged, conditions, adult *Adm*^{AM+/+} and *Adm*^{AM+/Δ} mice showed no apparent abnormality in the skin, dermal microvessels, and function of lymphatic vessels (Supplementary Material online, Additional Results and Supplementary Figure S1 online). On the basis of these findings, we concluded that *Adm*^{AM} haploinsufficiency by itself did not significantly affect (lymph)angiogenesis during embryonic development and caused no symptoms or signs of congenital or adulthood-onset primary lymphedema.

Secondary lymphedema is a phenotypic feature of adult *Adm*^{AM} haploinsufficient mice, and it is reversible upon exogenous AM supplementation

To evaluate a role for endogenously produced AM in secondary lymphedema development, we used an established experimental model of hind limb skin incision surgery (Kanter *et al.*, 1990). *Adm* mRNA expression in the skin was increased after surgery in both *Adm*^{AM+/+} and *Adm*^{AM+/Δ}

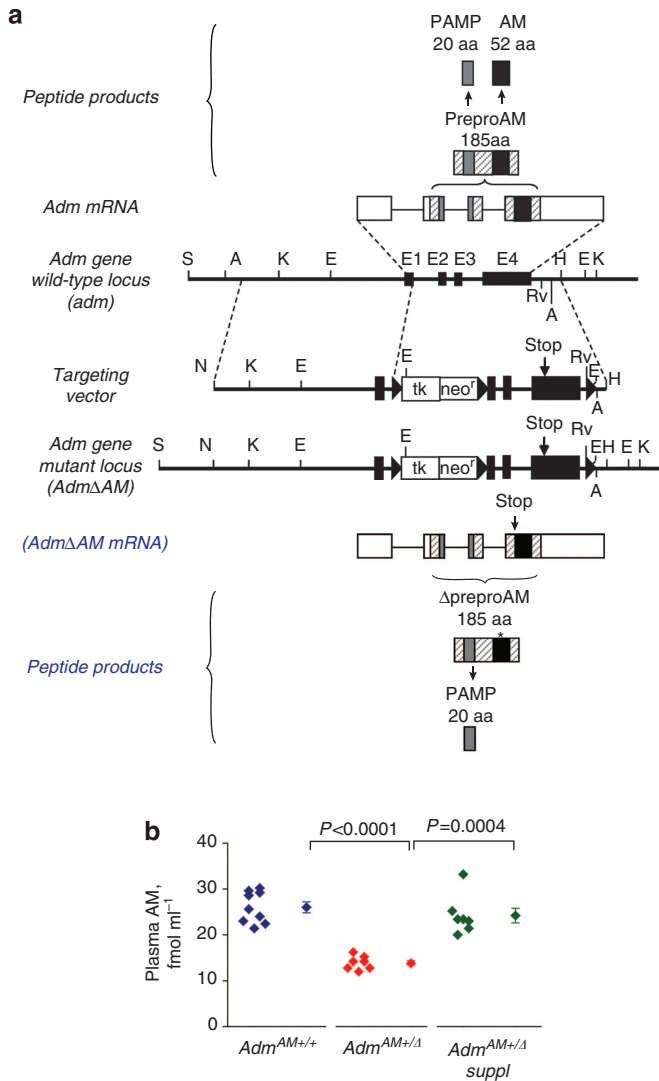


Figure 1. Scheme of targeted knockin stop mutation in *Adm* gene (modified from Shimosawa et al., 2002). (a) Wild-type *Adm* locus, resulting *Adm* messenger RNA (mRNA), preproAM, AM, and PAMP peptide products (top). Targeting vector, mutation-containing locus *Adm Δ AM*, resulting knockin stop mutant *Adm Δ AM* mRNA, preproAM (Δ preproAM), and PAMP peptide products (bottom). Solid boxes, exons; solid triangles, lox-P sequences. Knockin stop mutation (Stop) at the beginning of the AM-coding region (exon 4) preserves PAMP but not AM translation from the Δ *Adm* mRNA. (b) AM was measured (7–9 mice in each experimental group) and compared (Welch two-sample *t*-test) in plasma from wild-type (*Adm*^{AM+/+}) and *Adm*^{AM+/ Δ} haploinsufficient mice without (*Adm*^{AM+/ Δ}) or with exogenous AM supplement (*Adm*^{AM+/ Δ} suppl). aa, amino acid.

mice (Supplementary Figure S2 online), suggesting a functional role for AM during tissue injury. We used osmotic minipump (effective in the range 10⁻⁸ to 10⁻⁶ mol/l) (Shimosawa et al., 2003) to restore circulating AM levels in *Adm*^{AM+/ Δ} mice (Figure 1b).

Following hind limb skin incision, adult *Adm*^{AM+/ Δ} but not *Adm*^{AM+/+} mice developed symptoms and functional changes associated with lymphedema. First, *Adm*^{AM+/ Δ} mice showed impaired ability to move, swelling of the limb, and pitting (or non-pitting in severe cases) edema (Figure 2a and b;

Supplementary Figure S3a and b online and Supplementary Table S2 online). These changes in the *Adm*^{AM+/ Δ} mice were accompanied by decreased accumulation of Evans blue in inguinal lymph nodes after intradermal injection of the dye into the hindlimb footpads (Figure 2c and d), suggesting that edema was accompanied by aberrant lymph flow and fluid uptake by regional lymphatics. All acquired changes were partially, but significantly, prevented upon AM supplementation (Figure 2).

Second, histopathological features characteristic of lymphedema (thickening and hyperplasia of the epidermis, dermal hypercellularity, and increased collagen fiber density) were found in the hind limb skin of *Adm*^{AM+/ Δ} mice after injury (Figure 3a and b; Supplementary Figure S3c and d online). After injury, we also detected an increased deposition of fibrin, used as a marker of plasma filtration and vascular leakage (Chen et al., 2005) in *Adm*^{AM+/+} and *Adm*^{AM+/ Δ} mice (Supplementary Figure S4a online), compared with that before surgery (Supplementary Figure S1h online). Fibrin accumulation was more pronounced in the skin of *Adm*^{AM+/+} animals compared with *Adm*^{AM+/ Δ} animals (Supplementary Figure S4a online). Furthermore, Evans blue accumulation in the dermis after its intravenous injection was elevated in *Adm*^{AM+/+} compared with *Adm*^{AM+/ Δ} mice (Supplementary Figure S4b online). These findings were in accordance with our observation of changes in skin collagen density in *Adm*^{AM+/ Δ} animals (Figure 3a and c). All histopathological alterations in the skin of the adult *Adm*^{AM+/ Δ} mice were partially, but significantly, prevented by AM supplementation (Figure 3; Supplementary Figures S3 and S4 online).

Third, these lymphedematous changes were accompanied by a significant increase in average cross-sectional areas of both lymphatic and blood dermal microvessels in *Adm*^{AM+/ Δ} mice (Figure 4). The number of blood, but not lymphatic, microvessels was decreased in *Adm*^{AM+/+}, but not in *Adm*^{AM+/ Δ} , mice. Upon AM supplementation, the observed changes were partially prevented in the blood and to a lesser extent in lymphatic microvessels (Figure 4). Epidermal thickening and hyperplasia in lymphedema are frequently accompanied by lymphatic vessel dilation, and this frequently occurs as a result of lymphatic endothelium hyperproliferation, especially when infection is an underlying cause or when inflammatory tissue response is involved (Baluk et al., 2005; Tabibiazar et al., 2006; Kajiya et al., 2009). Dilation of the microlymphatics in *Adm*^{AM+/ Δ} mice after surgery was not accompanied by alterations in their numbers (Figure 4e), their hyperplasia, and increased proliferation or number of lymphatic ECs (Supplementary Figure S5 online), suggesting that lymphangiogenesis was grossly unaffected. Incision wounding-onset lymphedema in *Adm*^{AM+/ Δ} mice was associated with elevated dermal blood/lymphatic microvessel number ratio, whereas in *Adm*^{AM+/+} mice this parameter was markedly decreased, when compared with animals before the surgery (Supplementary Figure S6 online).

A summary of the impact of endogenous and exogenous/supplemented AM on acute secondary lymphedema development (3 days after surgery) is presented in Supplementary Table S2 online and Supplementary Figure S7 online.

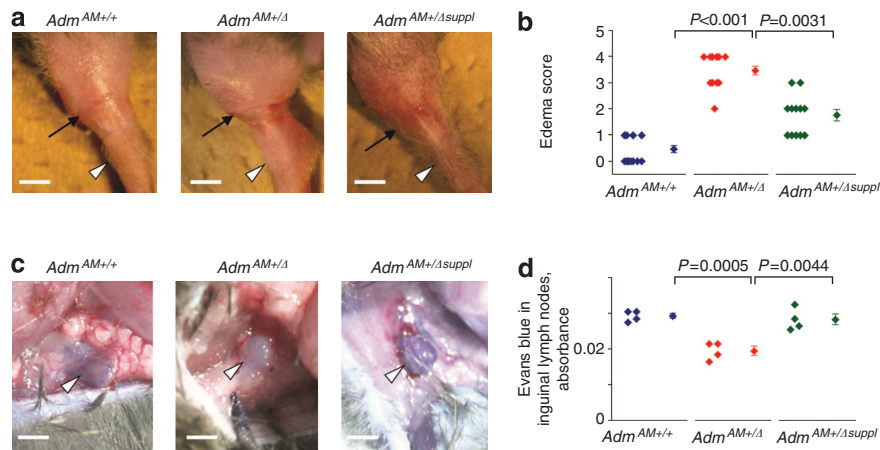


Figure 2. Edema and aberrant fluid uptake in the skin of the adult *Adm^{AM}* haploinsufficient mice after hind limb skin incision wounding surgery. Wild-type (*Adm^{AM+/+}*) and *Adm^{AM}* haploinsufficient mice without (*Adm^{AM+/-}*) or with exogenous AM supplement (*Adm^{AM+/-suppl}*) were used for surgery. (a) The incision site (black arrow) and the ankle (white arrowhead) are indicated (bar = 5 mm). For comparison, the images of the sham-operated limbs are presented in Supplementary Figure S3a online. (b) Hind limb swelling degree was assessed and compared (Wilcoxon rank test) between groups. (c, d) Evans blue uptake in inguinal lymph nodes was assessed (four mice in each experimental group) and compared (*t*-test). Representative images of the inguinal lymph nodes (white arrowheads) after dye uptake are shown (bar = 1.5 mm).

CLR is expressed in lymphatic and blood vessels in human skin tissue

Finally, to investigate the potential targets of endogenously produced AM in the skin, we analyzed *CALCRL/CLR* expression in tissue samples and primary cells by using immunostaining, GeneChip expression array analysis, immunoblotting, and deglycosylation (Figure 5; Supplementary Figures S8–S10 online). In human skin, CLR was expressed in microvascular lymphatic and blood vessel ECs (MVLEC and MVBEC, respectively), as well as in the epidermis and vascular smooth muscle cells (Figure 5a; Supplementary Figure S8a online). In other human tissues, e.g., myometrium, CLR was also localized in both lymphatic and blood endothelium (Supplementary Figure S8b online). Primary cultured human dermal MVLECs and MVBECs both expressed *CALCRL* and *RAMPs 2 and 3* mRNAs (Figure 5b; Supplementary Figure S9 online), which is in accordance with previous reports on the predominant expression of *CALCRL/CLR* in ECs (Nikitenko et al., 2003, 2006a). In cultured MVLECs, CLR generated functional AM receptors (Supplementary Material online; Additional Results and Supplementary Figure S10 online).

DISCUSSION

Secondary lymphedema occurs after surgery or trauma, but risk factors and molecular mechanisms underlying resistance or predisposition to its development remain largely unknown. Therefore, pharmacological strategies to prevent or treat this disorder are limited. Here, we show that endogenously produced AM prevents, and that its deficiency predisposes to, the development of secondary lymphedema in adult mice. Importantly, such genetic predisposition could be corrected through AM supplement therapy. Therefore, to our knowledge, this study provides previously unreported experimental *in vivo* evidence of a genetic susceptibility to the risk of developing secondary lymphedema.

The mechanisms of lymphedema development after wound infliction are largely unknown and yet to be delineated in our, as well as in other, *in vivo* models (Tabibiazar et al., 2006; Tammela et al., 2007; Rutkowski et al., 2010). It is proposed that genetic features, which are not accompanied by easily detectable morphological abnormalities, can predispose lymphatic and blood vessels to dysfunction/insufficiency that underlies secondary lymphedema only when coupled with a sufficient initiating stimulus (Rockson, 2008a). However, such genetic factors have not yet been identified. The *Adm* locus has an essential role in regulating vascular integrity and permeability during embryogenesis. Either the deletion of the protein-coding region, resulting in abrogated expression of both AM and PAMP (Caron and Smithies, 2001; Shindo et al., 2001), or the introduction of a knockin stop mutation resulting in functional inactivation of AM expression only (Shimosawa et al., 2002) led to embryonic lethality in homozygous mice, and this was attributed to generalized edema or hemorrhage in the first two models. Previous studies showed that upon exogenous administration AM has a role in lymphatic EC biology *in vitro* and in wound healing *in vivo*. However, the role for endogenous AM in the pathogenesis of secondary lymphedema remained unknown. We found that *Adm^{AM}* haploinsufficiency did not cause primary lymphedema, but that *Adm* expression was upregulated upon skin surgery. We hypothesized that lymphatic and/or blood microvessels in *Adm^{AM+/-}* mice could be affected by interventions that cause secondary lymphedema. After skin incision, we detected macroscopic (tissue swelling), functional (aberrant fluid uptake), and histopathological (epidermal thickening, increased collagen deposition, and dilated blood/lymphatic vessels) features, which are pathognomonic of secondary lymphedema, in *Adm^{AM+/-}* but not in *Adm^{AM+/+}* mice. These findings support the view that both lymphatic and blood vessels are involved in the pathogenesis of

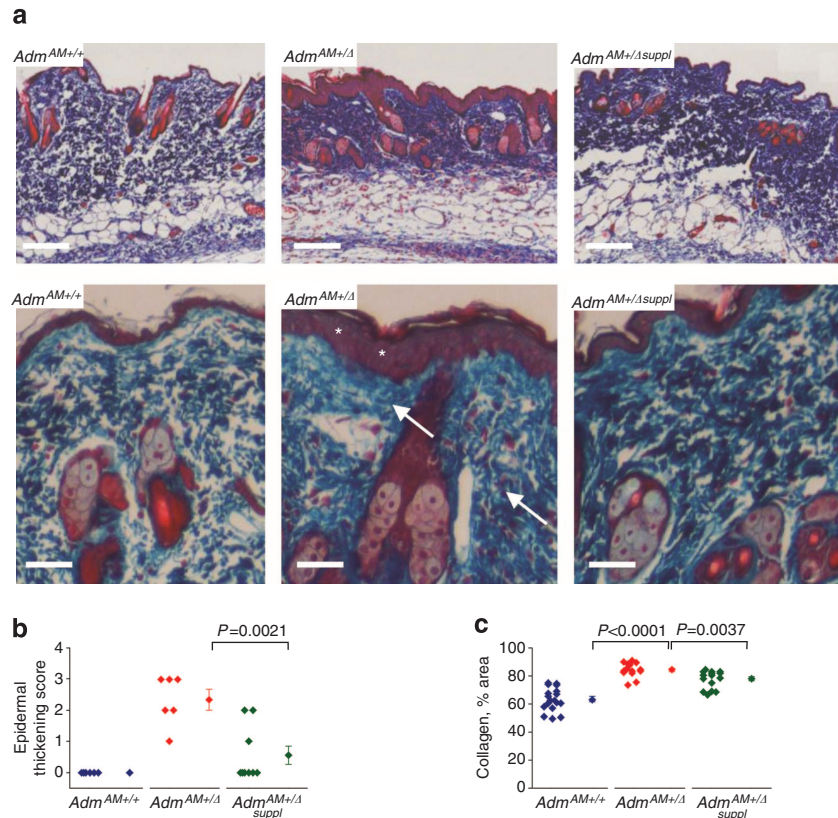


Figure 3. Histopathological features characteristic of lymphedema in the skin of the adult *Adm^{AM}* haploinsufficient mice after hind limb skin incision wounding surgery. (a) Azan staining. The representative images from six to eight mice in each group are shown. Lower panels (bar = 50 μ m) are magnified images from the same group as on the upper panel (bar = 100 μ m). Note thickened epidermis (white asterisks) and increase in collagen fiber (blue color, white arrows) density in *Adm^{AM+/-}* in comparison with wild-type mice. (b) Epidermal thickness was measured (six to eight mice in each group) and compared (Wilcoxon rank test). (c) The areas occupied by collagen fibers are presented as the percentage of “signal/area” ratio and compared (Welch two-sample *t*-test).

secondary lymphedema. It is possible that upon tissue wounding the high fluid filtration overwhelmed fragile blood vessels and/or vulnerable lymphatics in *Adm^{AM+/-}* mice, subsequently leading to impaired fluid uptake and edema, as suggested in breast cancer patients (Stanton *et al.*, 2009a). Such alterations within skin tissue could have ultimately led to lymph stagnation and elevated collagen fiber density, subsequently exerting further effects on grossly morphologically intact but possibly functionally impaired lymphatics and/or blood vessels and contributed to their constitutive dilation/dysfunction. How vascular and lymphatic insufficiencies are coordinated to predispose to secondary lymphedema and whether lymphedematous changes in AM-deficient mice develop in part owing to non-lymphatic vessel-dependent mechanisms remain to be defined. Nevertheless, our data reveal that aberrant expression of a single gene is sufficient to affect both lymphatic and blood vessels and predispose to the development of secondary lymphedema, thus suggesting a crucial, possibly even “gatekeeping”, role for AM in this disorder, at least upon wounding. In addition, our study uncovers a previously unrecognized spectrum of potential AM targets in human skin by demonstrating that CLR is expressed in both lymphatic and blood vessel endothelium, as well as in the epidermis, which are all affected in lymphedematous organs (Wilson *et al.*, 2004;

Tabibiazar *et al.*, 2006; Rockson, 2008a; Nakamura *et al.*, 2009).

Ways to prevent or treat lymphedema are limited and mainly palliative (Rockson, 2008a; Tammela and Alitalo, 2010; Wang and Oliver, 2010). The development of an experimental model for the commonest form of lymphedema will lead to further insight into the underlying molecular mechanisms, and potentially effective therapies (Shin and Rockson, 2008). *Adm^{AM}* haploinsufficient mice represent an example of a new, to our knowledge previously unreported, type of an *in vivo* model for studying secondary lymphedema occurring as a result of an underlying germ-line genetic alteration. Certain strategies (e.g., VEGF-C or non-steroidal anti-inflammatory drugs) have been tested to treat postsurgery-onset lymphedema in experimental models (Tammela *et al.*, 2007; Nakamura *et al.*, 2009). Here, we report partial but significant prevention of secondary lymphedema in *Adm^{AM+/-}* mice by postsurgical and systemic AM supplementation, which attained circulating peptide levels comparable to those in *Adm^{AM+/+}* mice. These findings suggest that circulating vasoactive factors could have a role in secondary lymphedema and that vascular treatments could potentially be used to alleviate its development. In our *in vivo* model, *Adm* expression in injured tissue was upregulated, as previously reported for VEGF-C or VEGFR-3

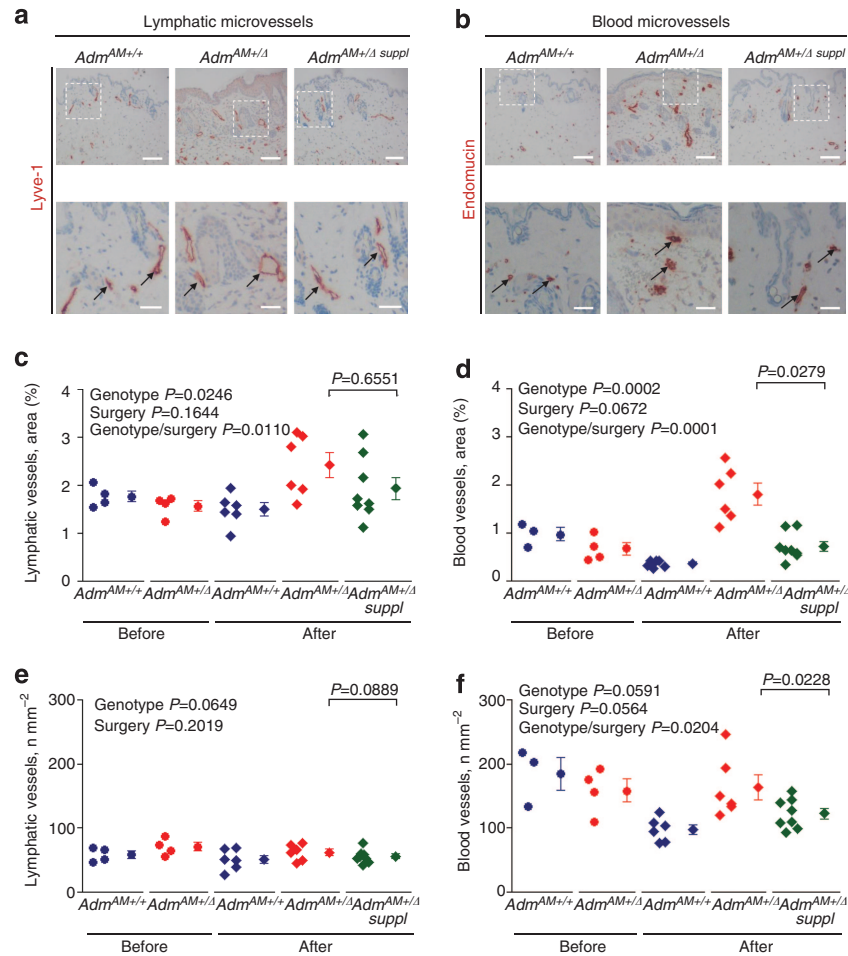


Figure 4. Microvessel morphology and numbers in the skin of the wild-type and adult *Adm*^{AM} haploinsufficient mice after incision wounding.

Immunohistochemistry was performed on sections from wild-type (*Adm*^{AM+/+}) and *Adm*^{AM} haploinsufficient mice without (*Adm*^{AM+/-}) or with exogenous AM supplement (*Adm*^{AM+/- suppl}) (six to eight mice in each group). Lower panels (bar = 50 μm) are magnified boxed areas from the upper panel (bar = 100 μm) of images. (a) Lyve-1 (lymphatic vessel endothelium marker; black arrows) and (b) endomucin (blood vessel endothelium marker; black arrows) immunostaining. Lymphatic and blood microvessel (c, d) average cross-sectional area and (e, f) numbers. The values for the experimental groups before the surgery are shown for direct comparison with post-incision wounding conditions. The comparisons among the first four groups were done using two-way ANOVA testing for effects of genotype (genotype, i.e., *Adm*^{AM+/+} or *Adm*^{AM+/-}) and incision wounding (surgery) independently; where it was suggested by the plots and data, the possible interactions between these factors (i.e., whether the effect of genotype was different after surgery to before; genotype/surgery) were also tested (*P*-values are shown in upper left corner), followed by Welch two-sample *t*-test for the groups 4 and 5 (*Adm*^{AM} haploinsufficient mice with or without AM; *P*-value is shown in the upper right corner).

(Nakamura *et al.*, 2009), suggesting that AM levels could be higher locally and that the systemic supplementation was probably not sufficient to completely prevent lymphedema development. Therapeutic approaches using AM have already been developed for the treatment of vascular disease, and this peptide could be delivered topically or systemically to reverse vascular damage associated with pulmonary hypertension or response to tissue injury (Kawai *et al.*, 2004; Nagaya *et al.*, 2004; Tokunaga *et al.*, 2004; Harada *et al.*, 2011). Therefore, similar AM topical application strategies together with systemic delivery could be explored as combinatorial therapies with VEGF-C (Tammela *et al.*, 2007) or other factors (Kajiya *et al.*, 2005), to prevent or treat lymphedema.

Experimental models of acute postsurgical lymphedema (including our model of genetically predisposed secondary lymphedema) are useful in studying mechanisms of human

lymphedema, which represents chronic unremitting condition, because they are associated with lymphatic insufficiency and share characteristic lymphedematous changes in affected tissues/organs (Tabibiazar *et al.*, 2006; Shin and Rockson, 2008). Studies investigating whether *Adm*^{AM} haploinsufficiency also predisposes to long-lasting secondary lymphedema and whether there is a potential for AM treatment in ameliorating or reversing complex changes associated with chronic lymphedema will be necessary in the future. The detection of AM receptor CLR expression in those cell types that are affected in lymphedematous tissues prompts further studies using complex genetic and physiological approaches to dissect underlying mechanisms. For example, animal models using lymphatic/blood vessel EC-specific conditional knockdown technologies (Bazigou *et al.*, 2011) might provide further insight into the lymphatic/vascular insufficiency

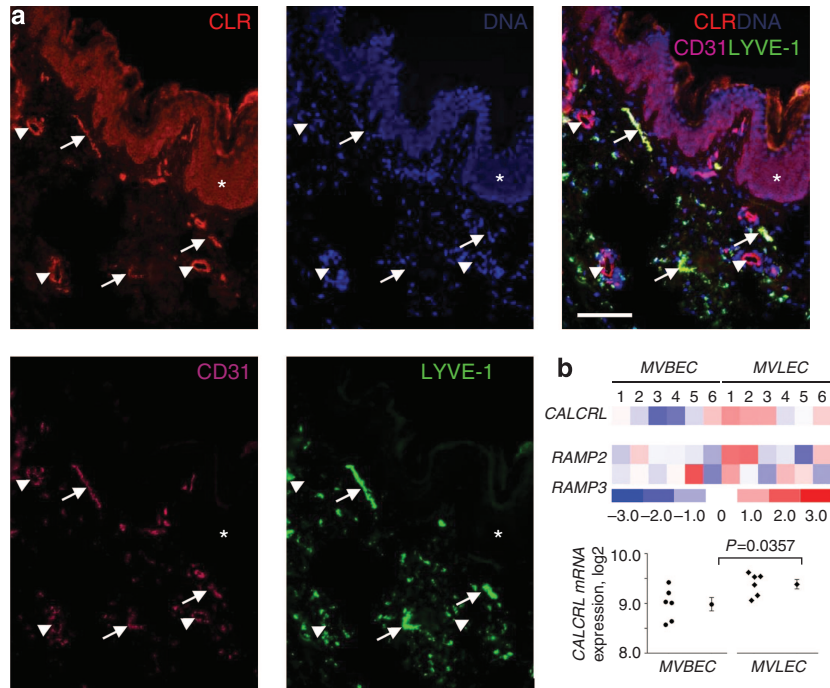


Figure 5. CLR protein and *CALCRL* messenger RNA (mRNA) expression in human skin and primary dermal endothelium. (a) CLR, LYVE-1, and CD31 immunofluorescence and DAPI (DNA) staining (bar = 75 μ m). Lymphatic (CD31-positive/LYVE-1-positive; white arrows) and blood (CD31-positive/LYVE-1-negative; white arrowheads) vessel endothelium, and epidermis (white asterisk). (b) *CALCRL*, *RAMP2*, and *RAMP3* mRNA expression in primary human dermal microvascular blood and lymphatic ECs (MVBE and MVLE) was determined using GeneChip Human Genome array data (E-MEXP-66) from 12 isolates as described in detail in Supplementary Methods online. Heatmap reflects relative changes in gene expression. Color scale—units of SD from the mean values of “rma” log₂ expression units across all samples. Red, gene expression is significantly upregulated; blue, significantly downregulated; white, no significant change. A difference in *CALCRL* expression between MVBE and MVLE is also presented as dot plots (left) alongside the mean and SEM (right; Welch two-sample *t*-test).

underlying secondary lymphedema development upon aberrant AM signaling through CLR.

Apart from previously identified genetic factors, e.g., mutations in *VEGFR3* or *GATA2*, it is likely that additional predisposition mutations contribute to the development of primary and secondary lymphedema (Finegold *et al.*, 2008). Genome-wide data identified a significant association between the *ADM* locus and predisposition to the development of cardiovascular disease (Ehret *et al.*, 2011). Similar studies will be required to address its potential association with secondary lymphedema in humans. Similar to *Adm*^{AM} haploinsufficient mice, individuals with AM deficiency might not have overt symptoms of vascular or lymphatic insufficiency under physiological conditions, and lymphatic or blood vessel abnormalities may therefore remain undetected unless injury occurs. Whether mutations and polymorphisms in *ADM*, *CALCRL*, or *RAMPs* have a role in the pathogenesis of secondary lymphedema in humans remains to be investigated.

In summary, we identify AM as a key regulator in the pathogenesis of secondary lymphedema in adult mice. Our data suggest that circulating vasoactive factors, blood, and lymphatic vessels are intimately involved in this disorder. Although it remains to be shown that AM deficiency in humans is associated with this disorder, our experimental

model provides previously unreported *in vivo* evidence that germ-line genetic factors predispose to the development of secondary lymphedema.

MATERIALS AND METHODS

Please see details of the following methods in Supplementary Information online: lymphangiography, Evans blue accumulation, and deglycosylation assays; GeneChip Human Genome array analysis; histology, immunostaining, immunoblotting, and image analysis.

Animals

AM-knockin mice were generated as previously reported (Shimosawa *et al.*, 2002). As the homozygote (*Adm*^{AM/AM}) animals are embryonic lethal, in the present study we used heterozygotes (*Adm*^{AM+/A}), which were back-crossed with C57/B6crj mice for more than 20 generations, and C57/B6crj was used as a wild-type (*Adm*^{AM+/+}) control. Animals were handled in accredited facility in accordance with the institutional animal care policies, and all research protocols have been approved and conformed to the guiding principles for animal experimentation, as outlined by the Ethics Committee on Animal Research of the University of Tokyo, Faculty of Medicine.

AM measurement

AM and PAMP concentrations were measured in the plasma as described previously (Shimosawa *et al.*, 2002).

Skin incision wounding model. Skin incision wounding model (Kanter *et al.*, 1990) was performed in both *Adm*^{AM+/+} and *Adm*^{AM+/-} eight-week-old male mice. Mice were anesthetized by pentobarbital (1 g kg⁻¹ body weight, intraperitoneal), and after shaving the operated area hind limb skin at an achilles tendon was incised. The following modifications were introduced: the Evans blue dye was not injected and inguinal lymph node was not excised. Next, *Adm*^{AM+/-} mice were randomized for treatment with vehicle or synthetic murine AM (Peptid Institute, Osaka, Japan). AM was administered intraperitoneally at a dose of 300 ng kg⁻¹ per hour by ALZET osmotic pump 1002 (Durect, Cupertino, CA) (Shimosawa *et al.*, 2003), which was implanted straight after incision. Three days after operation, animals were anesthetized with pentobarbital and the swelling of the limbs was scored (edema score) in a blind manner from 0 to 5: 0—no edema; 1—edema at cutting edge; 2—edema 1/3 of the hind limb; 3—edema 2/3 of the hind limb; 4—edema in the whole hind limb; and 5—edema above the knee. Skin samples were fixed in phosphate-buffered saline containing 4% paraformaldehyde for 6–12 hours at 4 °C, dehydrated, and embedded in paraffin.

Human skin tissue samples. Human skin tissue samples were obtained from the Royal National Orthopaedic Hospital Musculoskeletal BioBank (Stanmore, UK), snap-frozen, and stored in liquid nitrogen. The Cambridgeshire Research Ethics Committee has approved their use for this study.

Histology, immunostaining, and image analysis

Paraffin-embedded or frozen sections of mouse skin and human tissues were prepared as previously described (Shimosawa *et al.*, 2002; Nikitenko *et al.*, 2006a, b) and stained either with hematoxylin and eosin or Azan or processed for immunostaining. Histopathological analysis was performed by scoring epidermal thickening in the skin. A severity scale from 0 to 4 was used (0, normal; 0.5, focal and rare; 1, focal and mild; 2, diffuse and mild; 3, diffuse and moderate; and 4, diffuse and severe). The tissue area occupied by the collagen fibers, as a measure of fibrotic changes (Chen *et al.*, 2005), was quantified using the Scion Image Software (Scion, Frederick, MD). Immunohistochemistry and immunofluorescence were performed using previously described methods (Nikitenko *et al.*, 2006a, b).

Blood and lymphatic vessel number and density (occupied tissue area) in mouse skin tissue were analyzed as described elsewhere (Kajiya *et al.*, 2009), based on endomucin (blood vessel endothelium marker; (Kuhn *et al.*, 2002)) and Lyve-1 (lymphatic endothelium marker; (Tammela and Alitalo, 2010)) immunostaining and quantified using the ImageJ or Scion Image Software, according to approaches adapted by others (Kajiya *et al.*, 2009). Lymphatic/blood microvessel number ratio was calculated based on obtained numbers for each vessel type.

Statistical analysis

Four independent sets of skin incision wounding experiments were performed in this study, and the phenotype was reproduced. All obtained values and quantitative data for each experimental group were analyzed using R and GraphPad Prism (GraphPad Prism, La Jolla, CA) software programs and presented as dot plots (left) shown alongside means ± SEM (right). The comparisons among the groups were done using either Wilcoxon rank test or two-way ANOVA testing for effects of genotype and surgery independently,

and where it was suggested by the plots and data, we also tested possible interactions between these factors (i.e., whether the effect of genotype was different after surgery to before), followed by Welch two-sample *t*-test for the *Adm*^{AM+/-} mice with or without AM supplement, with a two-tailed significance set at the 0.05 levels and marginal significance set at the 0.1 level. We reported the actual *P*-value for each test.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

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