diabetes mellitus, Vogt-Koyanagi-Harada disease, and autoimmune hepatitis have also been associated with HLA–DRB1*0405 in the Japanese population (12–14). HLA–DRB1*0405 may contribute to the pathophysiology of several autoimmune diseases.

In addition, this study revealed that the frequency of HLA–DRB1*0101 was higher in patients with anti–MDA-5 antibody–positive DM than in patients with anti-aaRS antibody–positive PM/DM or patients with PM/DM without anti-aaRS antibody or ILD, although the number of enrolled patients was small. Previously, the HLA–DRB1*01 and *04 alleles were shown to play roles in the susceptibility to and progression of RA (15). Specifically, these alleles are associated with anti–citrullinated protein antibody (ACPA)–positive RA. Residues 70–74 of the DRβ chain (QRRAA) in both HLA–DRB1*0101 and DRB1*0405 constitute an important region for antigen presentation. QRRAA may indirectly influence outcome via ACPA production (15). Among PM/DM patients in the Japanese population, HLA–DRB1*0101 or *0405 can also be associated with the production of autoantibodies against MDA-5 or aaRS. QRRAA may be a critical sequence in the pathophysiology of anti–MDA-5 antibody–positive DM and anti-aaRS antibody–positive PM/DM. These antibodies are strongly associated with the development of ILD in PM/DM.

The HLA class II haplotypes are more important than individual alleles. DQB1 and DPB1 should be investigated in all of the patients and healthy donors included in this study. However, DQB1 and DPB1 alleles were not sufficiently investigated in all samples. This was a limitation of the present study. We plan to analyze the HLA class II haplotypes in patients with anti–MDA-5 antibody–positive DM in a future study.

In conclusion, HLA–DRB1*0101/*0405 is associated with susceptibility to anti–MDA-5 antibody–positive DM in the Japanese population. These alleles were also associated with ILD in patients with PM/DM.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Kawaguchi had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Review

Proangiogenic hematopoietic cells of monocytic origin: roles in vascular regeneration and pathogenic processes of systemic sclerosis

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Summary. New blood vessel formation is critical, not only for organ development and tissue regeneration, but also for various pathologic processes, such as tumor development and vasculopathy. The maintenance of the postnatal vascular system requires constant remodeling, which occurs through angiogenesis, vasculogenesis, and arteriogenesis. Vasculogenesis is mediated by the *de novo* differentiation of mature endothelial cells from endothelial progenitor cells (EPCs). Early studies provided evidence that bone marrow-derived $CD14^+$ monocytes can serve as a subset of EPCs because of their expression of endothelial markers and ability to promote neovascularization *in vitro* and *in vivo*. However, the current consensus is that monocytic cells do not give rise to endothelial cells *in vivo*, but function as support cells, by promoting vascular formation and repair through their immediate recruitment to the site of vascular injury, secretion of proangiogenic factors, and differentiation into mural cells. These monocytes that function in a supporting role in vascular repair are now termed monocytic pro-angiogenic hematopoietic cells (PHCs). Systemic sclerosis (SSc) is a multisystem connective tissue disease characterized by excessive fibrosis and microvasculopathy, along with poor vascular formation and repair. We recently showed that in patients with SSc, circulating monocytic PHCs increase dramatically and have enhanced angiogenic potency. These effects may be induced in response to defective vascular repair machinery. Since $CD14^+$ monocytes can also differentiate into fibroblast-like cells that produce extracellular matrix proteins, here we propose a new hypothesis that aberrant monocytic PHCs, once mobilized into circulation, may also contribute to the fibrotic process of SSc.

Key words: Angiogenesis, Endothelial progenitor cells, Monocytes, Scleroderma, Vasculogenesis

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Introduction

Postnatal blood vessel formation is important for tissue repair and regeneration, but the regulation of this critical process is not fully understood. Maintenance of the postnatal vascular system requires constant remodeling in response to injury and senescence. This may occur by synergic effects of three distinct processes: (i) angiogenesis, which refers to the formation of new blood vessels that sprout from preexisting vessels by a process involving the proliferation and migration of mature endothelial cells (ECs); (ii) vasculogenesis, which refers to the *de novo* differentiation of mature ECs through the recruitment and differentiation of endothelial progenitor cells (EPCs); and (iii) arteriogenesis, which refers to the remodeling of nascent vessels via the recruitment of mesenchymal cells, such as pericytes and smooth muscle cells (Fisher et al., 2006). Since the first description of EPCs as circulating primitive cells that contribute to postnatal vasculogenesis (Asahara et al., 1997), numerous *in vitro* and *in vivo* studies have been carried out to clarify the mechanisms of postnatal vascular formation and repair, as well as the contribution of EPCs to the pathogenesis of vascular diseases, and to develop potential therapeutic strategies that promote tissue regeneration or attenuate pathologic neovascularization. However, a great deal of controversy about EPCs and their roles in postnatal vascular

formation has arisen because of discrepancies in how EPCs are defined (Watt et al., 2010).

The major problem in defining EPCs derives from the lack of specific markers. In the landmark paper by Asahara et al, EPCs were characterized using EC marker-positive cells, which were selected as a cell fraction from peripheral blood mononuclear cells that was enriched in cells expressing CD34 or vascular endothelial growth factor (VEGF) receptor type 2 (VEGFR-2). These cells contributed to the revascularization and salvage of ischemic hind limbs in animal models (Asahara et al., 1997). Currently, it is widely accepted that there are at least two types of EPCs that can be discriminated based on their surface antigen expression, proliferation potential, and time of emergence in the cell culture system (Prater et al., 2007). The first subset is endothelial colony-forming cells (ECFCs) or late-outgrowth EPCs, which are regarded as "true EPCs," based on their potential for clonogenic expansion *in vitro* and their ability to form vessels *in vitro* and *in vivo* (Prater et al., 2007). Circulating precursors of ECFCs have not been identified yet, but they are known to express CD34 and CD31, and to lack the expression of CD133, CD45, and CD14 (Estes et al., 2010).

The cells originally identified as EPCs in various assays are in fact hematopoietic lineage cells that display pro-angiogenic properties, and are now termed pro-angiogenic hematopoietic cells (PHCs). PHCs include several different circulating cell types that are identified in the literature as circulating angiogenic cells (CACs), circulating endothelial precursors, monocytic EPCs, early-outgrowth EPCs, and colony-forming unit (CFU)-ECs. They are hematopoietic progenitors derived from the bone marrow (BM) that fall into at least two distinct major subsets: $CD14^+$ monocytic PHCs (the dominant population) and $CD14^-$ non-monocytic PHCs, which are primitive cells positive for CD34, CD133, and VEGFR-2 (Peichev et al., 2000). Currently, it is generally accepted that PHCs do not give rise to ECs, but rather work as pro-angiogenic support cells (Richardson and Yoder, 2011).

In this review, we focus on the vascular regenerative functions of PHCs originating from the monocytic lineage and their potential roles in the pathogenesis of systemic sclerosis (SSc), a multisystem connective tissue disease characterized by excessive fibrosis and widespread microvasculopathy.

Pro-angiogenic capacity of $CD14^+$ monocytes

EC-like features of $CD14^+$ monocyte-derived cells have been reported ever since Asahara et al.'s 1997 paper was published. Fernandez et al. described a subset of $CD14^+$ monocytes that become adherent within 24 hours of the culture and change their morphology to that of EC-like cells with Weibel-Palade bodies (Fernandez et al., 2000). When cultured with multiple pro-angiogenic growth factors, these $CD14^+$ monocytes gradually lose their expression of hematopoietic markers, such as CD14 and CD45, and display an up-regulated expression of EC markers, including von Willebrand factor (vWF), CD144, CD105, CD34, CD36, acetylated low-density lipoprotein-receptor, endothelial nitric oxide synthase, VEGF receptor type 1 (VEGFR-1), and VEGFR-2 (Fernandez et al., 2000; Schmeisser et al., 2001). In these reports, the cultured EC-like cells formed tubular structures in three-dimensional gel cultures that consisted of short sprouts from the EC-like colonies.

Subsequently, the *in vivo* functional capacity of monocytes was evaluated using animal models for neovascularization. In a study by Urbich et al, peripheral blood-derived $CD14^+$ monocytes were incubated on a fibronectin-coated plate under pro-angiogenic conditions for 4 days, and the recovered adherent monocytes were transplanted into the hind-limb ischemia mouse model (Urbich et al., 2003). The transplanted monocyte-derived cells were incorporated into the vascular structure and promoted neovascularization. In another study, peripheral blood- or BM-derived $CD34^-CD14^+$ monocyte lineage cells accelerated re-endothelialization in a monocyte chemoattractant protein 1 (MCP-1)-dependent manner in a rat model for balloon-injured artery (Fujiyama et al., 2003). These findings together suggest that a subset of $CD14^+$ monocytes can differentiate into the endothelial lineage and contribute to *in vivo* neovascularization and vascular repair (Urbich and Dimmeler, 2004).

A specific marker for this unique monocyte subset has not been identified, but the expression of VEGFR-2 in circulating $CD14^+$ monocytes is essential for their capacity to differentiate into the EC lineage (Elsheikh et al., 2005). Upon vascular injury, a subset of $CD14^+$ monocytes is mobilized into the circulation, adheres to the injured endothelium, and differentiates into EC-like cells, although whether or not monocyte-derived EC-like cells are integrated properly into the endothelium and serve as fully functional ECs has not been confirmed.

Circulating $CD14^+$ monocytes as a primary source of PHCs

The cultivation of circulating mononuclear cells in medium favoring endothelial differentiation has been used to identify EPCs and to expand circulating EPCs. In these cultures, it is difficult to determine which precursor cells give rise to the EPCs, because the starting cell population is heterogeneous, and cellular phenotypes change over time in culture. In the original protocol by Asahara et al. peripheral blood mononuclear cells were cultured on fibronectin for 7 days (Asahara et al., 1997). Currently, CACs are described as the cell type of origin for these cultured cells (Hirschi et al., 2008). Typically, these cells do not form colonies in culture, but they have EC features, including the ability to bind Ulex lectin Europeus Agglutinin-1, to take up acetylated low-density liproprotein, and to express CD31, CD105,

VEGFR-2, and vWF. The vast majority of the cells recovered in these cultures express both CD45 and CD14, indicating their monocytic origin.

In contrast, Hill et al. developed a semi-solid clonogenic assay, in which peripheral blood mononuclear cells that did not adhere to fibronectin within 48 hours were reseeded on fibronectin, and formed cell clusters (Hill et al., 2003). These cells are termed CFU-ECs or CFU-Hill, and express EC markers, including CD31, CD105, CD146, VEGFR-2, CD144, and vWF (Hill et al., 2003). However, unlike the CAC-derived cells, nearly all the cells within the CFU-EC clusters express the hematopoietic marker CD45, but only a tiny fraction express CD34 (Rohde et al., 2006). In addition, the depletion of $CD14^+$ monocytes from the mononuclear cells before seeding effectively prevents colony formation. CACs and CFU-ECs are primarily derived from $CD14^+$ monocytes, and thus are now categorized together as PHCs or early-outgrowth EPCs (Prater et al., 2007). Most importantly, PHCs cannot proliferate or form tubular structures *in vitro* without a co-culture with mature ECs. Several studies reported that PHCs can integrate into tubular structures and differentiate into EC-like cells *in vivo* (Elsheikh et al., 2005; Kuwana et al., 2006), but it is uncertain whether the EC-like cells can exert the full range of endothelial functioning.

PHCs are distinct from ECFCs or late-outgrowth EPCs, which appear 10-21 days after circulating mononuclear cells are plated in medium favoring endothelial differentiation (Ingram et al., 2004; Yoder et al., 2007). These cultured cells display a cobblestone morphology and express EC markers but not hematopoietic markers. Circulating precursor cells that give rise to ECFCs display clonal proliferative potential, self-renewal, and the ability to form vessels *in vivo*, compatible with features of traditional EPCs. A recent genome-wide transcriptional profiling of early- and late-outgrowth EPCs revealed strikingly different gene expression signatures between these cell populations, which provided evidence that the early-outgrowth EPCs are hematopoietic cells with a molecular phenotype linked to monocytes, whereas late-outgrowth EPCs exhibit commitment to the endothelial lineage (Medina et al., 2010). Based on these findings, it has been proposed that the term EPCs should be reserved for ECFCs (Prater et al., 2007; Watt et al., 2010; Richardson and Yoder, 2011). Whether rare ECFCs are derived from hemangioblasts in the BM or from endothelial stem cells that reside in the endothelium remains undetermined (Yoder, 2010).

Roles of monocytic PHCs in neovascularization

PHCs, whether in the monocytic or non-monocytic lineage, are no longer defined as "true EPCs," although they clearly participate in blood vessel formation and vascular repair, and thereby contribute to the maintenance of vascular homeostasis. A function in vascular regeneration was suggested for monocytic PHCs in a vascular injury model, in which green fluorescent-labeled $CD14^+$ monocytes integrated into the endothelium and improved the re-endothelialization (Elsheikh et al., 2005). Indeed, monocytic PHCs are widely accepted to function in a supporting role in vascular repair, and several different mechanisms for their involvement have been described.

First, monocytic PHCs can release a variety of potent, soluble pro-angiogenic growth factors, including VEGF, hepatocyte growth factor (HGF), granulocyte colony-stimulating factor (G-CSF), and stromal cell-derived factor-1 (SDF-1) (Rehman et al., 2003; Urbich et al., 2005). When secreted locally, these factors induce increased vascular permeability, the enhanced proliferation and migration of mature ECs, and the recruitment of progenitor and inflammatory cells from the BM.

Second, immunohistochemical studies in mouse have revealed that monocytic cells attach to the injured vascular lumen immediately after injury and change their morphology to EC-like cells; some of these cells then behave like ECs (Fujiyama et al., 2003; Elsheikh et al., 2005), although it is still unclear if monocytic PHCs are truly integrated into the vascular structures or simply localize there because of their adhesive characteristics. These EC-like cells may supplement the function of impaired ECs at the site of vascular injury, until they are replaced by mature ECs differentiated from ECFCs.

Finally, several lines of evidence have shown that monocytic cells contribute to arteriogenesis (Heil and Schaper, 2004). Mural cells, including pericytes and smooth muscle cells (SMCs), are essential for vessel maturation and stability, but their origin is not fully understood. In a chimeric mouse model for neovascularization, most BM-derived peri-endothelial cells were positive for CD45, CD11b (a monocyte marker), and NG2 proteoglycan (a pericyte marker) (Rajantie et al., 2004), indicating that the pericyte and monocyte lineages have a common origin. Pericyte precursors can differentiate into various mesenchymal cells, including SMCs, fibroblasts, and myofibroblasts (Diaz-Flores et al., 2009), an ability shared by circulating monocytes, which are now considered oligopotent progenitor cells (Seta and Kuwana, 2010). These findings together suggest that monocytic PHCs differentiate into EC-like cells as well as other elements of the vasculature, such as pericytes and SMCs, during the vascular repair process. In addition, monocytic PHCs comprise approximately 0.1% to 2% of peripheral blood mononuclear cells (Dimmeler et al., 2001; Elsheikh et al., 2005; Prater et al., 2007), although the frequency of monocytic PHCs varies depending on the method used to define them. Regardless, monocytic PHCs clearly predominate over non-monocytic PHCs and ECFCs in their absolute numbers in circulation (Prater et al., 2007). The potential mechanisms by which monocytic PHCs provide supportive functions in the neovascular microenvironment are summarized in Fig. 1. During this

process, the monocytic PHCs work in concert with platelets, residential ECs, non-monocytic PHCs, and ECFCs to form new blood vessels (Semenza, 2007).

Monocytic PHCs as oligopotent progenitors

Circulating $CD14^+$ monocytes exhibit heterogeneity in terms of their surface markers, phagocytic activity, and differentiation potential. They are committed precursors in transit from the BM to their ultimate sites of activity. Until recently, monocytes were believed to differentiate only into phagocytic and/or antigen-presenting cells, such as macrophages, dendritic cells, and osteoclasts. However, accumulating evidence indicates that circulating monocytes may differentiate into a variety of other cell types as well, including mesenchymal or endothelial lineage cells (Seta and Kuwana, 2010). Specifically, we described a primitive cell population termed monocyte-derived multipotential cells (MOMCs), which have a fibroblast-like morphology and a unique molecular phenotype positive for CD14, CD45, CD34, and type I collagen in culture (Kuwana et al., 2003). MOMCs include progenitors that differentiate into a variety of non-phagocytes, including bone, cartilage, fat, skeletal and cardiac muscle, neurons, and endothelium (Kuwana et al., 2003, 2006; Kodama et al., 2005, 2006).

At present, several distinct human cell populations derived from circulating $CD14^+$ monocytes have been reported to differentiate into non-phagocytes. Zhao and colleagues demonstrated that pluripotent stem cells generated from circulating monocytes by repeated stimulation with a high concentration of macrophage-colony stimulating factor and phorbol myristate acetate differentiate along several distinct cell lineages, including macrophages, T cells, epithelial cells, endothelial cells, neuronal cells, and hepatocytes (Zhao et al., 2003). Monocytic EPCs also differentiate into cardiomyocytes (Badorff et al., 2003), and monocytic EPCs residing within the circulating $CD14^+CD34^{low}$ cell population differentiate not only into endothelial cells, but also into osteoblasts, adipocytes, or neurons (Romagnani et al., 2005). Finally, fibrocytes are identified as circulating BM-derived cells, which home to sites of tissue injury, differentiate into fibroblasts, and contribute to tissue repair and fibrosis (Bucala et al., 1994). The origin of the fibrocytes is a subpopulation of circulating $CD14^+$ monocytes (Abe et al., 2001).

A variety of $CD14^+$ monocyte-derived cultured cell populations with distinct phenotypes and differentiation potentials have been reported in the literature, but their circulating precursors among the $CD14^+$ monocytes have not been identified to date. All of these cell populations can be enriched by the short-term culturing of circulating monocytes in medium containing different soluble factors and on plates coated with specific matrix proteins. Circulating fibrocytes express the chemokine receptors CCR3, CCR5, CCR7, and CXCR4 (Strieter et al., 2007), but the monocytic precursors of MOMCs are in the $CD14^+CXCR4^{high}$ population. A recent report showed that fibrocytes generated in the absence or presence of fetal calf serum exhibit different morphologies and gene expression profiles (Curnow et al., 2010).

Since circulating $CD14^+$ monocytes change their morphology, gene expression profiles, and function over

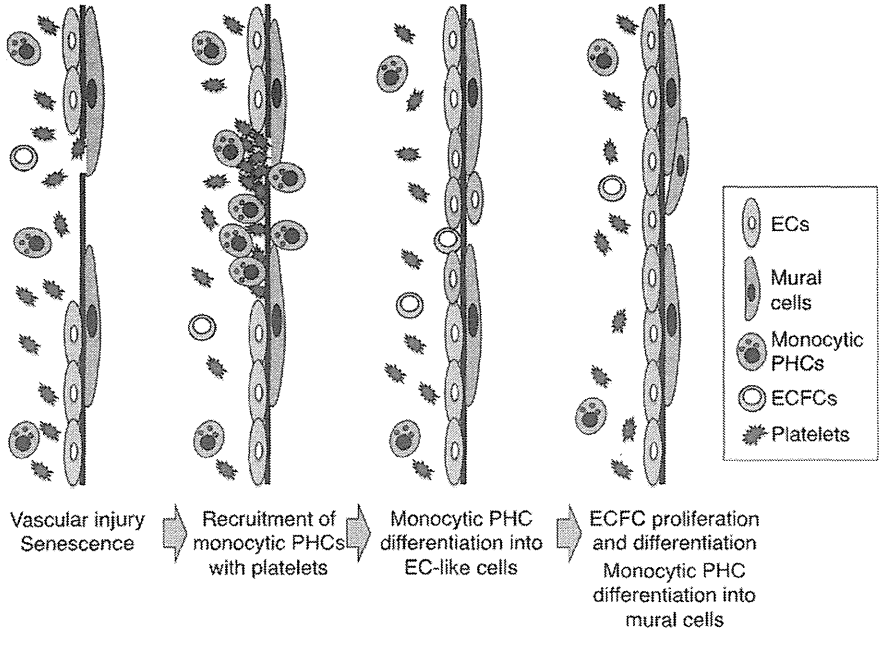

Fig. 1. Potential roles of monocytic PHCs in neovascularization. Monocytic PHCs are recruited to the site of vascular injury, differentiate into EC-like cells, and function as ECs by being incorporated into the vascular structure until ECFCs differentiate into mature ECs. Monocytic PHCs also provide supportive functions by releasing angiogenic factors, chemokines, and proteases to enhance the proliferation, migration, and maturation of the cells required for vascular regeneration. In the late phase of vascular recovery, monocytic PHCs differentiate into mural cells.

time *in vitro* (Seta and Kuwana, 2010), the heterogeneity among culture-enriched monocytic cells does not necessarily indicate that they originate from different circulating precursors. In other words, it is possible that culturing the same $CD14^+$ monocyte precursors in different conditions can generate cell progenies with different characteristics. In fact, monocyte-derived oligopotent cells have common characteristics, including a spindle shape, the expression of CD34 when cultured on fibronectin or type I collagen, and a low proliferative capacity (Seta and Kuwana, 2007). These characteristics are shared by monocytic PHCs, which are oligopotent for differentiation into mesenchymal cells other than EC-like cells. It is possible that monocytic PHCs and other monocyte-derived primitive cells, such as MOMCs and fibrocytes, are all derived from circulating $CD14^+$ precursors.

Roles of monocytic PHCs in neovascular responses in SSc

Given the critical role of monocytic PHCs in postnatal vascular formation and repair, alterations in their numbers and/or functions may contribute to the pathogenic processes of various vascular diseases. In this regard, we focused on SSc, which is characterized by excessive fibrosis and microvascular abnormalities. SSc vasculopathy mainly affects small arteries and causes reduced blood flow and tissue ischemia, leading to clinical manifestations such as digital ulcers and pulmonary arterial hypertension (LeRoy, 1996). Two types of vascular pathology are progressive intimal proliferation and fibrosis, and the loss of capillaries. The mechanism of SSc vasculopathy is not fully understood, but increasing evidence indicates that an endothelial injury is a primary event in the pathogenesis of scleroderma (Guiducci et al., 2007). The persistent increase in pro-angiogenic factors, such as VEGF, platelet-derived growth factor, and SDF-1 observed in SSc patients indicates a strong pro-angiogenic response to vascular damage (Liakouli et al., 2011). Nailfold capillaroscopic findings reveal giant capillaries in the early phase of the disease, and the loss of capillaries and vascular disorganization in the late phase (Herrick and Cutolo, 2010). Severe capillary loss may result from vascular damage, but there is almost no evidence of vascular recovery. In addition, the formation of abnormal blood vessels like giant and bushy capillaries indicates an inadequate vascular repair process. These findings together suggest that, in patients with SSc, the vascular repair machinery does not work properly, and the disease progresses toward irreversible structural changes, despite the strong neovascular push. Thus, impaired angiogenesis and vasculogenesis were proposed in an intriguing hypothesis to explain the pathogenesis of SSc vasculopathy (Manetti et al., 2010).

To test this hypothesis, several studies have been conducted to quantify the circulating $CD14^-CD34^+$ $CD133VEGFR^+$ EPCs, which are now regarded as a non-monocytic subset of PHCs, in patients with SSc. We first reported that there is a reduced number of non-monocytic PHCs in SSc patients (Kuwana et al., 2004). In subsequent analyses by other groups, some confirmed our finding (Zhu et al., 2008; Mok et al., 2010), but

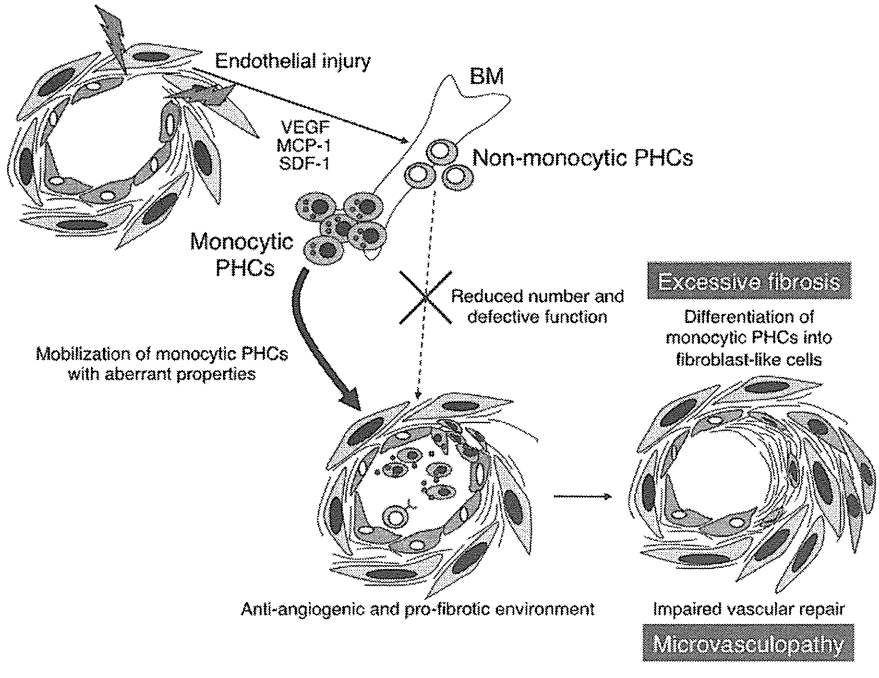

Fig. 2. Potential roles of monocytic PHCs in the pathogenesis of SSc. Growth factors and chemokines produced at the site of endothelial injury mobilize a variety of progenitor cells, including monocytic PHCs. The strong anti-angiogenic environment at the affected site prevents adequate vascular repair, leading to microvasculopathy. In the pro-fibrotic environment, accumulated monocytic PHCs differentiate into fibroblast-like cells and promote excessive fibrosis.

others showed an increase in non-monocytic PHCs in SSc patients (Del Papa et al., 2006; Avouac et al., 2008). Thus, the effect of SSc on the number of circulating non-monocytic PHCs remains a matter of debate (Kuwana and Okazaki, 2012). On the other hand, there is little information on the roles of monocytic PHCs in SSc vasculopathy.

We recently evaluated the number of monocytic PHCs in SSc patients using a culture system previously developed to enrich for MOMCs (Yamaguchi et al., 2010). The MOMCs enriched in this culture can differentiate into EC-like cells and promote blood-vessel formation *in vitro* and *in vivo* (Kuwana et al., 2006), and thus correspond to monocytic PHCs. Unexpectedly, we observed a paradoxical increase in monocytic PHCs in SSc patients compared with healthy controls. Intriguingly, the monocytic PHCs derived from SSc patients showed enhanced *in vitro* tubular structure formation compared with those from healthy controls. Furthermore, in a murine tumor neovascularization model, the transplantation of SSc-derived monocytic PHCs dramatically promoted tumor growth and tumor vessel formation *in vivo*, indicating that monocytic PHCs have enhanced angiogenic activity in SSc patients, an effect that has also been observed in a chick embryo chorioallantoic membrane assay (Ribatti et al., 1998) and in the SCID mouse skin xenograft model (Liu et al., 2005), in which the normal tissue surrounding an SSc skin graft showed a prominent increase in new blood vessel formation. The increased number and enhanced angiogenic potency of the monocytic PHCs are likely to be compensatory responses to damaged vessels.

Despite the robust pro-angiogenic responses, appropriate blood vessel formation does not occur in patients with SSc. The neovascular process consists of a sequence of highly regulated events, including angiogenesis, vasculogenesis, and arteriogenesis, which are tightly controlled by pro- and anti-angiogenic signals (Semenza, 2007). In this regard, the SSc-affected tissues, such as skin and lungs, exhibit dysregulated endothelial features. In microvascular ECs isolated from the skin of SSc patients, metalloproteinase (MMP)-12 is over-expressed and cleaves urokinase-type plasminogen activator receptor, causing inhibition of the invasion/migration capacities of ECs (D'Alessio et al., 2004; Margheri et al., 2006). Furthermore, the reduction of tissue kallikreins 9, 11, and 12, which exert a mitogenic effect on ECs, and the up-regulation of anti-angiogenic kallikrein 3 were reported in SSc skin (Giusti et al., 2005). In addition, in SSc lesions, ECs lose their expression of VE-cadherin, which is required for vascular tube formation (Fleming et al., 2008). Finally, selective up-regulation of the anti-angiogenic VEGF b isoform was observed in the circulation and skin of SSc patients, indicating a switch from the pro-angiogenic to the anti-angiogenic VEGF isoform in these patients (Manetti et al., 2011). These dysregulated endothelial features at the site of SSc organ involvement are responsible for the disease-related defects in angiogenesis and prevent vascular repair. Together, these data suggest that the balance between pro- and anti-angiogenic responses favors anti-angiogenesis in SSc patients.

Pathogenic roles of monocytic PHCs in SSc

Current data on the functions of monocytic PHCs provide strong hints about their roles in the pathogenesis of SSc. Circulating monocytic PHCs are mobilized from the BM and recruited to SSc-induced lesions in response to chemokines such as MCP-1 and SDF-1, which are up-regulated in the affected skin of SSc patients (Distler et al., 2001; Cipriani et al., 2006). In addition, the hypoxic condition of the affected tissues of SSc patients appears to potentiate the in situ differentiation of circulating monocytic cells into EC-like cells (Bellik et al., 2008). Thus, functionally altered monocytic PHCs accumulate at SSc lesions.

Since monocytic PHCs are oligopotent in terms of their capacity to differentiate into mesenchymal lineage cells (Badorff et al., 2003; Kuwana et al., 2003; Kodama et al., 2005; Romagnani et al., 2005), they may differentiate into fibroblast-like cells, produce collagens and other extracellular matrix proteins, and participate in the fibrotic process. In this regard, recent lines of evidence indicate that $CD14^+$ monocytes are involved in fibrogenesis. For instance, fibrocytes derived from $CD14^+$ monocytes home to the site of tissue injury and contribute to tissue repair and fibrosis by differentiating into myofibroblasts that express αSMA (Abe et al., 2001). In addition, $CD14^+$ circulating monocytes acquire the ability to produce extracellular matrix components, such as type I collagen, in an MCP-1/CCR2-dependent amplification loop (Sakai et al., 2006). Furthermore, an enhanced profibrotic phenotype of circulating $CD14^+$ monocytes was reported in SSc patients with interstitial lung disease (Mathai et al., 2010). Another report described a correlation between fibrotic clinical features and the increased proportion of $CXCR4^+$ circulating cells with monocytic and endothelial markers in SSc patients (Campioni et al., 2008). Therefore, monocytic PHCs may acquire pro-fibrotic characteristics and contribute to the promotion of fibrosis at sites affected by SSc that have a strong anti-angiogenic and pro-fibrotic environment (Fig. 2).

Conclusions

In summary, monocytic PHCs contribute to postnatal blood vessel formation and vascular repair, mainly through their immediate recruitment to the site of vascular injury, their secretion of a variety of pro-angiogenic factors, and their differentiation into mural cells. These cells are also oligopotent; that is, they can differentiate into various cell types in the mesenchymal lineage. This unique feature raises the intriguing hypothesis that monocytic PHCs are involved in the pathogenesis of SSc by participating in two major

pathological features, microvasculopathy and excessive fibrosis. Understanding the roles of monocytic PHCs in the progression of SSc may be key to dissecting its pathogenesis and to developing novel therapeutic strategies for this intractable condition.

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Paradigm shift of childhood thrombotic thrombocytopenic purpura with severe ADAMTS13 deficiency

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Summary

Thrombotic thrombocytopenic purpura (TTP) is a life-threatening generalized disease with pathological conditions termed thrombotic microangiopathy (TMA). TTP is thought to predominantly affect adults and to rarely occur in children. Currently, TTP is defined by a severe deficiency in the activity of ADAMTS13, a metalloprotease that specifically cleaves unusually large von Willebrand factor multimers under high shear stress. Genetic mutations in and acquired autoantibodies to ADAMTS13 cause congenital TTP (termed Upshaw-Schulman syndrome [USS]) and acquired TTP, respectively. Because of very few overt clinical signs for TTP, USS is often misdiagnosed as chronic idiopathic thrombocytopenic purpura or overlooked during childhood. However, in women with USS, pregnancy can induce thrombocytopenia followed by the development of TTP. Furthermore, early childhood cases of acquired idiopathic TTP have not been characterized. From 1998 to 2008, our institution at Nara Medical University functioned as a TMA referral center in Japan and collected a large dataset on 919 TMA patients (Intern Med 2010;49:7–15). This registry contains 324 patients with a severe deficiency in ADAMTS13 activity, including 41 patients with USS and 283 patients with acquired TTP. Of note, the latter population contains 17 patients who were enrolled as children (≤ 15 years old), including 14 children with idiopathic TTP and three with connective tissue disease-associated TTP. Of the 14 patients with idiopathic TTP, five were very young children (under 2 years old). This study focused on these 58 patients (41 USS and 17 acquired TTP) who were diagnosed with a severe deficiency in ADAMTS13 activity during childhood, causing a paradigm shift in our concept of TTP.

Thrombotic microangiopathies (TMAs) are pathological conditions that are characterized by organ dysfunction due to platelet thrombi in the microvasculature, consumptive thrombocytopenia, and microangiopathic hemolytic anemia (MAHA). Two of the typical TMA phenotypes are life-threatening generalized diseases, termed thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome (HUS) [1–4].

A disintegrin-like and metalloproteinase with thrombospondin type 1 motifs 13 (ADAMTS13) is a metalloprotease that specifically cleaves the Tyr1605–Met1606 bond in the von Willebrand factor (VWF)-A2 domain [5]. In the absence of ADAMTS13 activity (ADAMTS13:AC), unusually large VWF multimers (UL-VWFMs) are released from vascular endothelial cells (ECs) and improperly cleaved, causing them to accumulate in the circulation and induce the formation of platelet thrombi in the microvasculature under conditions of high shear stress. Currently, a severe deficiency in ADAMTS13:AC, which results either from genetic mutations in the *ADAMTS13* gene or acquired autoantibodies to ADAMTS13, is thought to be a specific feature of TTP but not HUS [6,7].

TTP was first described in 1924 by Moschcowitz [8], who documented a 16-year-old female who died of multiorgan failure after a clinical disease course of 1 week. An autopsy revealed hyaline membrane thrombi in the small arteries of multiple organs, except for the lung. In 1966, Amorosi and Ultmann [9] examined 16 new patients and reviewed 255 previously documented patients in order to establish a clinical 'pentad', consisting of MAHA, thrombocytopenia, renal failure, fluctuating neurological signs, and fever. Since then, TTP has been considered a life-threatening but rare disease that occurs mainly in adults and presents with predominant neurotropic clinical signs. Because of this classification, the estimated frequency of TTP was low (3.7 per million) [10] before the discovery of ADAMTS13.

On the other hand, in 1955 Gasser et al. [11] described five children who died of acute renal insufficiency, and their autopsies showed prominent necrosis of the renal cortex. This study established the clinical 'triad' for HUS, which consisted of MAHA, thrombocytopenia, and renal insufficiency. In addition, after it was determined that there was a close relationship between HUS and enterohemorrhagic *Escherichia coli* infection, particularly strain O157:H7 that produces a Shiga-like toxin, studies showed that HUS typically affects children with prominent nephrotropic clinical signs [12].

From 1998 to 2008, our institution at Nara Medical University has functioned as a TMA referral center in Japan and collected a large dataset of 919 patients who have TMA but not disseminated intravascular coagulation (DIC) [13]. This registry contains 324 patients with a severe ADAMTS13:AC deficiency (less than 3% of normal), including 41 patients with congenital TTP (Upshaw-Schulman syndrome [USS]) with variable clinical symptoms and 283 patients with acquired TTP. Notably, the latter population includes 17 patients who were diagnosed with TTP as children ($\leq$ 15 years old), including 14 with idiopathic TTP and three with connective tissue disease (CTD)-associated TTP. Surprisingly, the 14 patients with idiopathic TTP included five patients who were very young infants (under 2 years old), which significantly differed from the previous concept of TTP. Therefore, the aim of this study was to characterize these 58 patients (41 USS and 17 childhood TTP) in order to examine the paradigm shift in our understanding of TTP.

Glossary

ADAMTS13	**a disintegrin-like and metalloproteinase with thrombospondin type 1 motifs 13**
ADAMTS13:AC	**ADAMTS13 activity**
ADAMTS13:INH	**ADAMTS13 inhibitor**
ai-TTP	**acquired idiopathic TTP**
BU	**Bethesda unit**
CR-TTP	**chronic relapsing TTP**
CTD	**connective tissue disease**
DIC	**disseminated intravascular coagulation**
EC	**endothelial cell**
FFP	**fresh frozen plasma**
HPS	**hemophagocytic syndrome**
HUS	**hemolytic uremic syndrome**
ITP	**idiopathic thrombocytopenic purpura**
MAHA	**microangiopathic hemolytic anemia**
PE	**plasma exchange**
PNH	**paroxysmal nocturnal hemoglobinuria**
SNP	**single nucleotide polymorphism**
TMA	**thrombotic microangiopathy**
TTP	**thrombotic thrombocytopenic purpura**
UL-VWFM	**unusually large VWF multimer**
USS	**Upshaw-Schulman syndrome**
VWF	**von Willebrand factor**
VWF-CP	**VWF-cleaving protease**
WPBs	**Weibel-Palade bodies**

Diagnostic criteria for thrombotic microangiopathy and thrombotic thrombocytopenic purpura

As previously described [13], patients were considered to have TMA if they met all of the following criteria:

- MAHA (hemoglobin [Hb] $\leq$ 12 g/dL), Coombs test negative, undetectable serum haptoglobin (< 10 mg/dL), more than two fragmented red blood cells (RBC) (schistocytes) in a microscopic field with a magnification of 100, and concurrent increased serum lactate dehydrogenase (LDH) above the institutional baseline;
- thrombocytopenia (platelet count $\leq 100 \times 10^9$/L);
- a variable degree of organ dysfunction (renal or neurological involvement) without DIC [14,15].

It is difficult to differentially diagnose HUS and TTP based on routine laboratory data. Therefore, as a rule, the plasma levels of ADAMTS13:AC were determined for all patients who were suspected to have TMA, and patients with a severe ADAMTS13:AC deficiency were classified as having TTP regardless of the clinical signs. This protocol was important because our registry included patients with congenital TTP or an ADAMTS13:AC deficiency (USS), which generally have fewer clinical signs, often isolated thrombocytopenia, than patients with acquired TTP.

Within the large dataset of 324 patients with a severe ADAMTS13:AC deficiency who were enrolled in our registry between 1998–2008 [10], 58 patients were diagnosed with a severe ADAMTS13:AC deficiency during childhood, of which 41 had congenital TTP (USS) and 17 were diagnosed with acquired TTP, including 14 with idiopathic TTP and three with CTD-associated TTP.

Assays for plasma ADMTS13:AC and ADAMTS13:INH

Until March 2005, ADAMTS13:AC was determined with a classic VWFM assay in the presence of 1.5 mol/L urea using purified plasma-derived VWF as a substrate according to the method described by Furlan et al. [16]. In our laboratory, the detection limit of this assay was 3% of the normal control [17].

In 2005, our laboratory developed a novel chromogenic ADAMTS13-act-ELISA using both an N- and C-terminal tagged recombinant VWF substrate (termed GST-VWF73-His). This assay was highly sensitive, and the detection limit was 0.5% of the normal control [18]. Since 2005, the classic VWFM assay was completely replaced with this novel chromogenic act-ELISA. Both assays show a high correlation between the plasma ADAMTS13:AC levels ($R^2 = 0.72$, $P < 0.01$) with similar means ± SD in healthy individuals (102.4 ± 23.0% vs. 99.1 ± 21.5%), as was shown previously [18]. Thus, the results obtained using the chromogenic act-ELISA were used in this study. In addition, we have categorized plasma ADAMTS13:AC levels of < 3%, 3~ < 25%, and 25~50% of the normal control as a severe, moderate, and mild deficiency, respectively.

Since 2005, ADAMTS13:INH has also been evaluated with the chromogenic act-ELISA by means of the Bethesda method [19]. Prior to this inhibition assay, the tested samples were heat-treated at 56 °C for 60 min to eliminate endogenous enzymatic activity. The ADAMTS13:INH assay consists of two steps. In the 1st step, the test or control plasma is heat-inactivated, mixed with an equal volume of intact normal pooled plasma, and incubated for 2 hours at 37 °C. After the incubation, the residual enzyme activity is measured. One Bethesda unit is defined as the amount of inhibitor that reduces the enzymatic activity by 50% of the control value, and values greater than 0.5 U/mL are considered significant.

Pathogenesis of thrombotic thrombocytopenic purpura

ADAMTS13-producing cells

ADAMTS13 is a metalloproteinase that consists of 1427 amino acids and a multi-domain structure, including a signal peptide, short propeptide, metalloproteinase domain, disintegrin-like domain, thrombospondin-1 (TSP1) domain, cysteine-rich domain, spacer domain, seven additional TSP1 repeats, and two CUB domains [20]. The *ADAMTS13* gene is located on chromosome 9q34, and initial northern blotting studies indicated that ADAMTS13 mRNA is exclusively expressed in the liver [20]. Subsequent immunological studies with *in situ* hybridization analyses indicated that ADAMTS13 is unambiguously produced in hepatic stellate cells (Itoh cells) [21]. However, ADAMTS13 was also identified in platelets [22], vascular ECs [23], and kidney podocytes [24]. Therefore, an outstanding and important question is which organ is most responsible for maintaining the plasma levels of ADAMTS13:AC. In this regard, we have two observations that suggest that the liver is the major ADAMTS13-producing organ. Childhood patients with advanced billiary cirrhosis due to bile duct atresia often showed pathological features of TMA with low plasma levels of ADAMTS13:AC (20–30%), but these clinical signs disappeared and plasma ADAMTS13:AC rapidly recovered to normal levels after a successful liver transplantation [25]. Adulthood patients with cirrhosis that was largely related to hepatitis C infection tended to have lower plasma ADAMTS13:AC levels that correlated with their clinical severity, and the lowest values were approximately 20–30% of the normal levels [26].

Cleavage of unusually large von Willebrand factor multimer

Although the mechanism by which TTP develops in the absence of ADAMTS13:AC has not been fully elucidated, accumulating evidence has provided a hypothesis as illustrated in *figure 1* [27]. In this proposed model, UL-VWFMs are produced exclusively in vascular ECs and stored in an intracellular organelle termed Weibel-Palade bodies (WPBs) and then released into the circulation upon stimulation. Under physiological conditions, epinephrine acts as an endogenous stimulus but other stimuli are largely unknown. In contrast, under non-physiological conditions, DDAVP (1-deamino-8-D-arginine vasopressin), hypoxia, and several cytokines such as interleukin (IL)-2, IL-6, IL-8, and tissue necrotizing factor (TNF)-α act as stimuli that up-regulate VWF release. Once ECs are stimulated, UL-VWFMs and P-selectin, both stored in WPBs, move to the membrane surface of ECs, where P-selectin anchors UL-VWFMs on the EC surface [28]. Under these circumstances, high shear stress generated in the microvasculature induces a change in the UL-VWFM molecule that alters its conformation from a

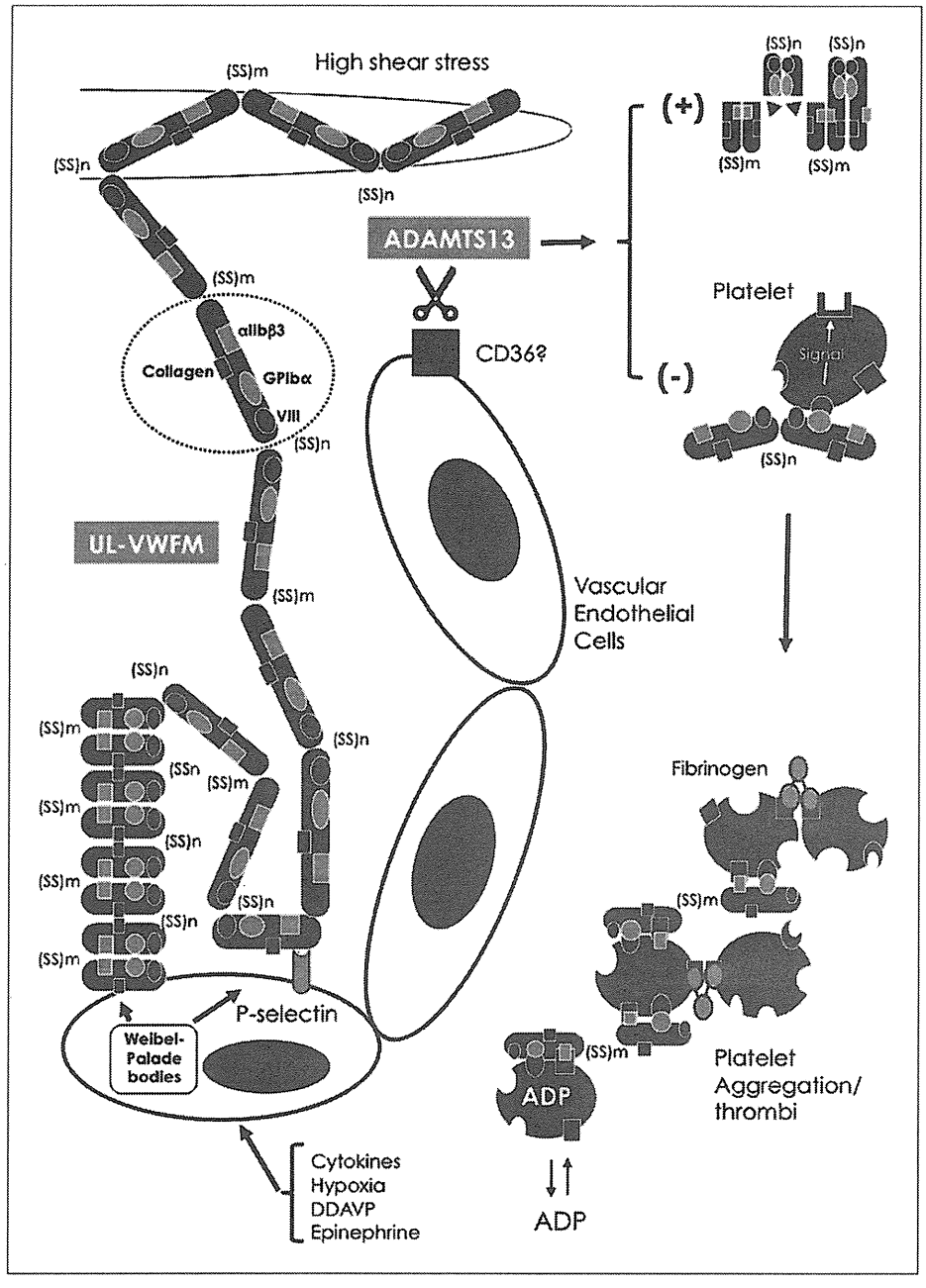

FIGURE 1

Proposed mechanism of platelet thrombi under high shear stress in the absence of ADAMTS13:AC

Unusually large von Willebrand factor multimers (UL-VWFMs) are produced in vascular endothelial cells (ECs) and stored in Weibel-Palade bodies (WPBs). UL-VWFMs are released from WPBs into the circulation upon stimulation by cytokines, hypoxia, DDAVP, and epinephrine. P-selectin that co-migrates from WPBs anchors UL-VWFMs on the vascular EC surface. Under these circumstances, high shear stress changes the molecular conformation of UL-VWFM from a globular to an extended form, allowing ADAMTS13 to access this molecule. In the absence of ADAMTS13:AC, UL-VWFMs are left uncleaved, allowing them to excessively interact with platelet glycoprotein (GP) Ibα and activate platelets via intraplatelet signaling, which results in the formation of platelet thrombi (dotted circle indicates a VWF subunit, which contains a set of binding domains with factor VIII, subendothelial collagen, platelet GPIbα, and integrin αIIbβ3).

globular to an extended form, allowing ADAMTS13 to cleave UL-VWFM. In this context, it has been postulated that multiple exocites within the disintegrin-like/TSP1/cysteine-rich/spacer (DTCS) domains of ADAMTS13 play an important role in interacting with the unfolded VWF-A2 domain [29]. Furthermore, although a direct link to TTP pathogenesis had not been shown, in 1994 Tandon et al. [30] reported that approximately 80% of patients with acquired TTP had autoantibodies to CD36. Recently, Davis et al. [31] showed that recombinant (r) ADAMTS13 specifically binds to rCD36 *in vitro*. Thus, it is possible that ADAMTS13 more efficiently cleaves newly released UL-VWFMs that exist as solid-phase enzymes

anchored to the vascular EC surface by binding to CD36 because CD36 is a receptor for TSP1, which is a repeated domain within the ADAMTS13 molecule.

In 2001, we clearly showed that pre-existing UL-VWFMs in the plasma of USS patients began to disappear within 1 hour and completely disappeared 24 hours after ADAMTS13 was replenished with infusions of fresh frozen plasma (FFP) as shown in *figure 2* [32]. Retrospectively, these results indicated that exogenous ADAMTS13 could efficiently cleave both UL-VWFMs that pre-existed in the circulation and the newly produced molecules at the EC surface. Related to this phenomenon, Zhang et al. [33] recently analyzed the crystal structure of the VWF-A2 domain and found that the ADAMTS13 cleavage site within this domain is not exposed to the outer surface of the molecule, indicating that the enzyme cannot readily access this site. More recently, Zanardelli et al. [34] proposed that the '2-site initial interaction mechanism between VWF and ADAMTS13', in which a binding site in the VWF C-terminal domains (D4CK) is constitutively exposed, allows this domain to interact with the ADAMTS13 C-terminal domains [TSP1(5-8)/CUB]. Under high shear stress, the '2-site initial interaction' may help expose this binding site within the VWF-A2 domain and favor the correct positioning of the ADAMTS13 spacer domain. Once the higher-affinity interaction between the spacer domain and the VWF-A2 domain is achieved, the metalloproteinase domain of ADAMTS13 can access and cleave the Tyr1605–Met1606 bond within the VWF-A2 domain.

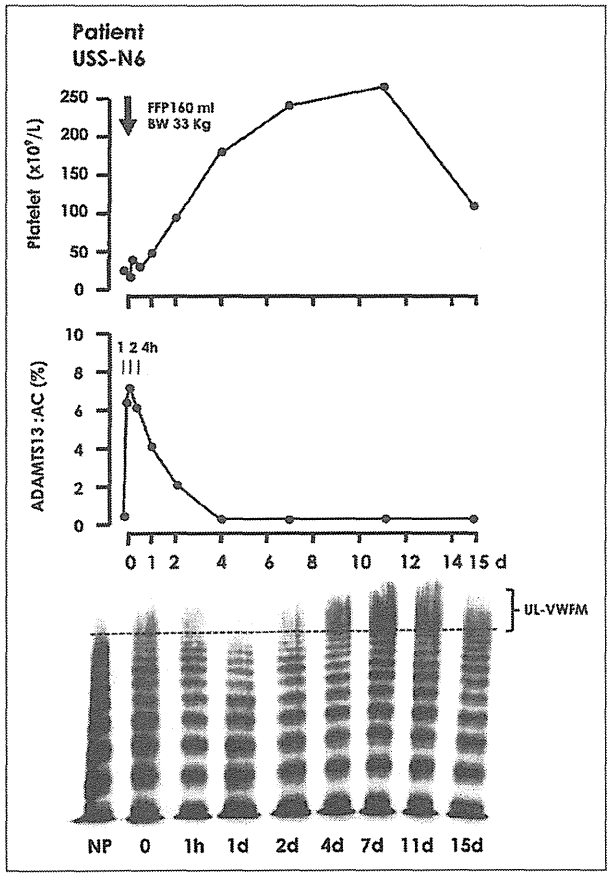

FIGURE 2

Effect of fresh frozen plasma (FFP) infusion on platelet counts, ADAMTS13:AC, and VWFM patterns in patient USS-N6

A total of 160 mL of FFP was transfused into female patient USS-N6 (BW 33 kg). As shown in the top panel, her platelet counts increased from 23 × 10^9/L before the FFP infusion to 251 × 10^9/L at 11 days after the infusion. The middle panel shows the plasma levels of ADAMTS13:AC that were re-examined by the chromogenic act-ELISA using deep-frozen plasma samples. Note that 4 days after the infusion, the plasma ADAMTS13:AC decreased to the pre-infusion level (< 0.5%). In the lower panel, the pre-existing UL-VWFM levels before the FFP infusion rapidly disappeared 24 hours after infusion, and 4 days later, UL-VWFMs re-appeared in the plasma. It should be noted that the platelet count began to decrease concomitantly with the re-appearance of UL-VWFMs (cited from [32] with a slight modification).

Anti-ADAMTS13 autoantibodies

Soejima et al. [35] were the first to report that the cysteine-rich and spacer domains of ADAMTS13 are a major binding site for ADAMTS13 autoantibodies in acquired TTP. Subsequently, Klaus et al. [36] showed that there are multiple antibody binding sites within the ADAMTS13 molecule. Now it is accepted that anti-ADAMTS13 neutralizing autoantibodies target epitopes within the spacer domain [37]. More recently, Pos et al. [38] identified three amino acids, Arg660, Tyr661, and Tyr665, within the spacer domain of ADAMTS13 that are critical for the binding of both the VWF-A2 domain and anti-ADAMTS13 autoantibodies.

Upshaw-Schulman syndrome (congenital TTP/deficiency in ADAMTS13:AC)

Background

The classic hallmarks of USS are repeated childhood episodes of chronic thrombocytopenia and MAHA that are reversed by infusing fresh frozen plasma (FFP). The most striking clinical feature is severe neonatal jaundice with a negative Coombs test that requires exchange blood transfusion therapy. Although USS is now defined as a congenital ADAMTS13:AC deficiency due to genetic mutations, there was a lengthy history that led to this conclusion, as has been described in detail in previous publications [39]. In fact, the term USS had almost been embedded in 1997, when the assay for VWF-cleaving protease (VWF-CP) activity (now ADAMTS13;AC) was established. This is because the pathogenic features that were initially postulated for the disease, such as a defect in 'platelet-stimulating factor', 'decreased plasma fibronectin

level', or 'lack of thrombopoietin', have been entirely excluded by subsequent investigations. Instead, the practical diagnostic term 'chronic relapsing TTP' (CR-TTP) has long been used. This term was coined by Moake et al. [40], who found that UL-VWFMs were present in the plasma of 4 CR-TTP patients during the remission phase, but disappeared during the acute phase. In 1997, Furlan et al. [41] showed that four CR-TTP patients, different from those of Moake et al. [40], lacked VWF-CP activity, but did not address ADAMTS13:INH. Retrospectively, however, each two CR-TTP patients, reported by Moake et al. [40] and Furlan et al. [41], were congenital TTP, and the remaining two each were acquired TTP. Under these circumstances, we re-visited the term USS [17], which included analyzing three Japanese patients with USS, and found that they uniformly had a severe deficiency in VWF-CP activity (determined by VWFM assay in the presence of 1.5 mol/L urea) in the absence of its inhibitors. The parents of these patients were asymptomatic with a moderately decreased activity (17–60% of normal), except for one carrier who had very low VWF-CP activity (5.6% of normal). Later, this carrier was shown to have a unique single nucleotide polymorphism (SNP), a P475S mutation in the *ADAMTS13* gene in one allele, which is very common in Japanese people (9.6% of normal individuals are heterozygous for the P475S mutation) [42]. However, Levy et al. [43] provided solid evidence that linked congenital TTP or USS and *ADAMTS13* gene mutations. Since this discovery, approximately 100 patients have been identified worldwide [44], but the precise incidence is completely unknown because USS is an extremely rare disease.

ADAMTS13 gene knock-out humans and mice

Although USS patients consistently lack ADAMTS13:AC, they do not always have acute symptoms, and symptoms often become evident only when the patients have infections or become pregnant. In both instances, vascular EC injuries might be involved, and these cases have been indirectly shown to have elevated plasma levels of cytokines or soluble thrombomodulin [45]. However, studies on *ADAMTS13* gene knock-out mice [46,47] showed that UL-VWFMs were detectable in the blood, although the mice did not have acute symptoms. Considering these results, investigators have assumed that an ADAMTS13:AC deficiency is prothrombotic but alone is insufficient to provoke acute symptoms. Therefore, second hits or triggers must exist. However, the lack of symptoms in knock-out mice sharply contrasts the clinical symptoms of USS. For example, USS patients, but not mice, were reported to have acute clinical aggravation soon after receiving infusions of DDAVP [48,49]. However, it is still controversial whether mice have a receptor to DDAVP. Furthermore, there are striking differences between humans and mice during pregnancy. In our studies, nine USS females had a history of pregnancy and all had thrombocytopenia during the 2nd–3rd trimesters. When this thrombocytopenia was not well managed, they developed clinical signs of TTP and the fetus died in many cases [50]. However, this disease course was not found in knock-out mice.

Natural history of 41 Upshaw-Schulman syndrome patients in Japan

USS is inherited in an autosomal recessive fashion, indicating that the female-to-male ratio in the patient population should be one-to-one. However, in our registry of 41 USS patients from 36 families (Table I), the female-to-male ratio was 25-to-16 with an apparent female predominance. Furthermore, all patients had a severe ADAMTS13:AC deficiency (under 3% of normal), except for one USS-GG2 patient (ADAMTS13:AC 2.4–3.4%).

Although severe neonatal jaundice is a typical sign of early-onset bouts of USS, our analysis indicates that such cases represent a relatively small number (16/41, 39%) of patients. Thirty-two patients (32/41, 78%) had repeated episodes of thrombocytopenia during childhood, but many USS patients were primarily misdiagnosed with idiopathic thrombocytopenic purpura (ITP) or Evans syndrome. Therefore, the age at which these patients were diagnosed with CR-TTP or USS was widely distributed from 1 month to 63 years. Sixteen patients (16/41, 39%) were diagnosed with TTP beyond childhood.

Of particular interest, pregnant women with USS inevitably have thrombocytopenia during the 2nd–3rd trimester when the plasma VWF levels rapidly increase with the appearance of UL-VWFM. *Figure 3* presents data for two female patients who were siblings in an USS-L family and were diagnosed based on their precise natural history around pregnancy followed by an examination of ADAMTS13:AC and ADAMTS13:INH [50]. Furthermore, an *ADAMTS13* gene analysis gave a solid diagnosis of USS. As illustrated in these two cases, USS is thought to have two clinical phenotypes, the early-onset and late-onset types. However, generally we cannot find any clear differences in the plasma ADAMTS13:AC levels between these two phenotypes, even when examined by the sensitive act-ELISA.

Regarding severe renal complications in USS patients, we know that two patients thus far have received hemodialysis for chronic renal insufficiency. One patient, USS-C3 (male, born in 1972), was diagnosed with USS at 8 years of age, and then given prophylactic FFP infusions that were continued for the rest of his life. However, his renal function deteriorated yearly and he began to receive hemodialysis when he was 24 years old. During the clinical course of his disease, he experienced chronic heart failure and died of a sudden cardiac attack at 38 years of age. The other patient, USS-JJ3 (male, born in 1980), was diagnosed with USS at 16 years of age, after which he received prophylactic FFP infusions. However, his renal function deteriorated and he has been receiving hemodialysis since he was 26 years old.

TABLE I

Registration of 41 Japanese patients with Upshaw-Schulman syndrome (USS)

No	Patient	Year of birth	Sex	Exchange blood transfusion during newborn period	Thrombocytopenia during childhood	ADAMTS13:AC (%)	Disease-causing ADAMTS13 gene mutations		Age of TTP diagnosis	Prophylactic FFP infusion		Remarks	Ref.
											From when		
1	A4	1999	M	+	+	< 0.5	C-Hetero	p.R268P/ p.C508Y	4 m	+	4 m		[53]
2	B3	1986	F	+	+	< 0.5	Homo	p.Q449X	2 m	+	11 m		[53]
3	C3	1972	M	–	+	< 0.5	Homo	c.414+1G>A	8 y	+	8 y	Dead (chronic heart failure at the age of 36)	[54]
4	D4	1978	F	+	+	< 0.5	C-Hetero	c.414+1G>A/ p.I673F	4 y	+	4 y		[54]
5	E4	1985	M	+	+	< 0.5	C-Hetero	p.I673F/ p.C908Y	5 y	–	–		[54]
6	F3	1993	M	+	+	0.6	C-Hetero	p.R193W/ p.1244+2 T>G	2.5 y	–	–		[54]
7	G3	1987	F	+	+	< 0.5	C-Hetero	c.686+1G>A/ p.R1123C	14 y	–	–		[54]
8	H3	1951	M	–	–	0.6	C-Hetero	p.A250V/ c.330+1G>A	51 y	+	50 y	Dead (renal failure at the age of 51)	[51]
9	I4	1972	M	–	+	< 0.5	C-Hetero	p.H234Q/ p.R1206X	2 y	+	2 y		[55]
10	J3	1977	F	–	+	< 0.5–0.8	C-Hetero	p.R312C/ c.3198del CT	3 y	+	22 y		[56]
11	J4	1979	M	–	+	< 0.5	C-Hetero	p.R312C/ c.3198del CT	5 y	–	–		[56]
12	K3	1976	F	–	+	< 0.5–0.7	C-Hetero	p.Y304C/ p.G525D	27 y	+	27 y		[50]
13	K4	1978	F	+	+	< 0.5	C-Hetero	p.Y304C/ p.G525D	25 y	+	25 y		[50]
14	L2	1967	F	–	–	< 0.5	C-Hetero	p.R125VfsX6/ p.Q1302X	25 y	–	–		[50]
15	L3	1972	F	–	+	< 0.5	C-Hetero	p.R125VfsX6/ p.Q1302X	25 y	–	–		[50]

TABLE I (*Continued*)

No	Patient	Year of birth	Sex	Exchange blood transfusion during newborn period	Thrombocytopenia during childhood	ADAMTS13:AC (%)	Disease-causing ADAMTS13 gene mutations		Age of TTP diagnosis	Prophylactic FFP infusion		Remarks	Ref.
											From when		
16	M3	1969	F	–	–	< 0.5	C-Hetero	p.R193W/ p.R349C	33 y	–	–		[50]
17	M4	1971	F	–	–	< 0.5	C-Hetero	p.R193W/ p.R349C	30 y	–	–		[50]
18	N6	1986	F	+	+	< 0.5	C-Hetero	p.H234R/ c.3220delTACC	4 y	+	4 y		[17]
19	O4	1958	F	–	–	< 0.5	C-Hetero	p.I178T/ p.Q929X	26 y	+	26 y		[50]
20	P3	1971	M	-	+	< 0.5	C-Hetero	p.C908Y/ p.C322G, p.T323R, p.F324L	3 y	+	21 y		[42]
21	Q1	1983	M	+	+	< 0.5–0.7	C-Hetero	p.G227R/ p.C908Y	6 y	+	11 y		[56]
22	Q2	1988	M	+	+	< 0.5	C-Hetero	p.G227R/ p.C908Y	2 y	+	7 y		[56]
23	R5	1982	F	–	+	< 0.5	C-Hetero	p.R193W/ p.A606P	23 y	+	23 y		[50]
24	S3	1982	F	–	+	0.9	Not determined		4 y	+	*		
25	T4	1981	F	+	+	< 0.5	Homo	c.3220delTACC	1 m	+	*		[56]
26	U3	1990	F	+	+	< 0.5	Homo	c.2259delA	4 m	+	*		[56]
27	V3	1983	F	+	+	< 0.5	C-Hetero	p.W1081X/ p.R193W	6 y	+	6 y		[56]
28	W4	1990	F	–	+	< 0.5	C-Hetero	p.G550R/Not determined	15 y	+	15 y		[56]
29	X5	1963	F	–	–	< 0.5	Not determined		40 y	–	–		
30	Y3	1960	F	–	+	< 0.5	C-Hetero	p.G385E/ p.R1206X	45 y	+	45 y		[56]
31	Z3	1971	F	–	+	< 0.5	Homo	p.R193W	25 y	–	–		[50]
32	AA3	1987	F	–	–	< 0.5	Not determined		19 y	–	–		
33	BB3	1947	M	–	–	< 0.5	Homo	p.R193W	55 y	–	–		[56]
34	CC5	2004	M	+	+	< 0.5	C-Hetero	p.Q723K/ p.R398C	2 y	+	2 y		[56]

TABLE I (*Continued*)

No	Patient	Year of birth	Sex	Exchange blood transfusion during newborn period	Thrombocytopenia during childhood	ADAMTS13:AC (%)	Disease-causing ADAMTS13 gene mutations		Age of TTP diagnosis	Prophylactic FFP infusion		Remarks	Ref.
											From when		
35	DD5	2007	F	–	+	< 0.5	C-Hetero	p.R268P/ p.Y304C	1 m	–	–		[56]
36	EE4	2003	M	+	+	< 0.5	Homo	c.2259delA	4 y	–	–		[56]
37	FF3	1991	F	–	+	< 0.5	Homo	p.Q449X	6 y	+	6 y		[56]
38	GG2	1931	M	–	–	2.4–3.4	Homo	p.C1024R	63 y	+	63 y	Dead (stroke at the age of 79)	[56]
39	HH4	2003	F	+	+	< 0.5	C-Hetero	p.Q449X/ c.4119delG	1 y	–	–		[56]
40	II3	1977	F	+	+	< 0.5	Not determined		9 m	+	10 y		
41	JJ3	1980	M	–	+	< 0.5	C-Hetero	c.1885delT/ p.C908Y	12 y	+	25 y	Hemodialysis	[56]

C-Hetero: compound heterozygotes; Homo: homozygotes.

There have been two fatal USS cases, one is the above-mentioned USS-C3 and the other is patient USS-I4 (male, born in 1972), whose natural history was previously described in detail [51]. Briefly, patient USS-I4 was diagnosed with late-onset USS when he was 50 years old. The next year he received a cholecystectomy and then experienced a bout of TTP, which led to renal insufficiency. He received extensive treatment, including PE and hemodialysis but did not improve, and he died of renal insufficiency at the age of 51 years.

ADAMTS13 gene analysis

The parents of USS patients are usually asymptomatic carriers, and a major population of patients from unrelated parents is a compound heterozygote, while a minor population of patients from related parents is a homozygote [42,43,51–56].

We performed *ADAMTS13* gene analyses in 38 out of 41 USS patients and disease-causing mutations were identified in 37 patients: nine with homozygous and 28 with compound heterozygous *ADAMTS13* gene mutations. Furthermore, five of these 37 patients were siblings. Therefore, within 64 [2 × (37 – 5)] allelic numbers (n) for *ADAMTS13* gene mutations, the three most frequently found mutations were in the following order: p.R193W (n = 8), p.Q449X (n = 5), and p.C908Y (n = 4). All these mutations were unique to Japanese individuals, perhaps to East-Asians, and were totally different from Europeans and white and black Americans. In addition, to date, we have not found an apparent association between specific *ADAMTS13* mutations and clinical phenotypes. However, Camilleri et al. [57] reported that some single nucleotide polymorphisms in the *ADAMTS13* gene could modulate ADAMTS13:AC and its secretion, indicating that further investigations are required.

Patient USS-GG2 (male, born in 1931) suddenly developed a bout of TTP when he was 63 years old. After this incident, he had repeated TTP bouts and required prophylactic FFP infusions under a clinical diagnosis of CR-TTP. Even under these circumstances, he developed a cerebellar infarction at 76 years of age. During the infusion intervals, ADAMTS13:AC was often measured and determined to be 2.4–3.4% of the normal levels but ADAMTS13:INH was not detected. Most recently, the patient was diagnosed with USS with the homozygous missense mutation C1024R based on an *ADAMTS13* gene analysis (unpublished).

Treatment

Except for exchange blood transfusions to treat jaundice in newborns, USS patients usually respond well to small FFP infusions. Therefore, the question arises; what is the best marker for deciding this indication? As suggested above, mild thrombocytopenia seems to occasionally occur in USS patients during childhood, but this condition might be overlooked

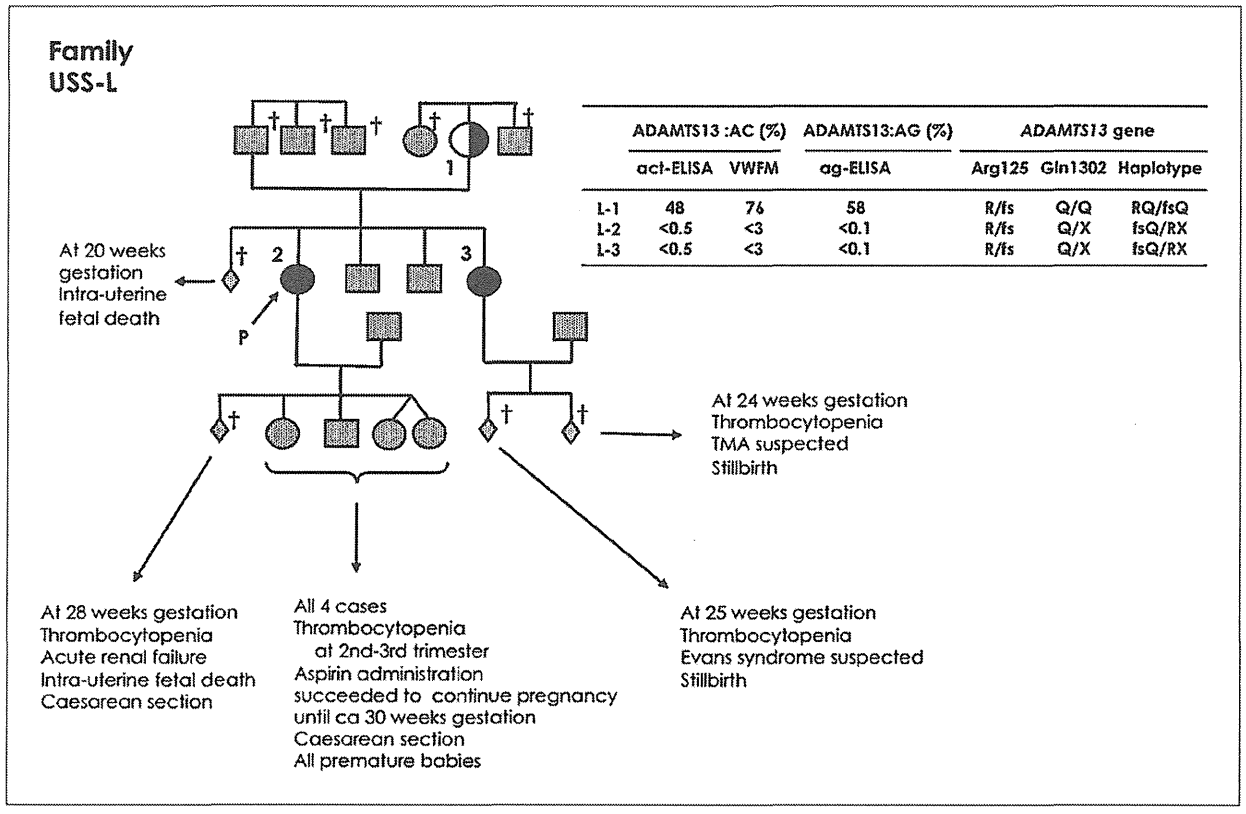

FIGURE 3

Family pedigree and ADAMTS13 analyses in family USS-L

The propositus is L2, and L3 is her younger sister. Both siblings had an abortion along with thrombocytopenia of an unknown etiology. When propositus L2 became pregnant the second time, she had mild thrombocytopenia and her physician recommended low-dose aspirin, which enabled her to maintain the pregnancy until 30 weeks of gestation. However, it was uncertain why and how aspirin worked in this occasion. She delivered a live but premature baby by caesarean section. Then, she successfully bore three more children with the same treatment. All of the babies were premature and alive. During childhood, L2 had no episodes of thrombocytopenia, but L3 was diagnosed with ITP at 3 years of age. At 25 years of age, propositus L2 was diagnosed with USS based on an analysis of ADAMTS13:AC and ADAMTS13:INH. In addition, an *ADAMTS13* gene analysis provided solid evidence that the two siblings are compound heterozygotes for *ADAMTS13* gene mutations (p.R125fsx6/p.Q1302X). Squares and circles indicate males and females, respectively, and an arrow with P indicates the proposita. Closed circles and closed squares represent USS patients. The half-closed circles and squares represent asymptomatic carriers. The cross indicates deceased (cited from [50] with a slight modification).

because of the paucity of clinical signs. Thus, mild thrombocytopenia alone may not be a good marker. However, in clinical practice, some USS patients receive prophylactic FFP infusions (5–10 mL/kg BW) every 2–3 weeks because the half-life of ADAMTS13:AC in the plasma is thought to be 2–3 days, while other patients receive FFP infusions only when acute TTP bouts develop. In our registry, 26 of 41 (63%) USS patients received prophylactic FFP infusions. Currently, USS patients receive FFP infusions based on the physician's observations and the frequency of TTP bouts. However, the efficacy of prophylactic FFP infusions needs to be more precisely evaluated over a long observation period because our two patients who developed renal insufficiency had been receiving FFP infusions since they were clinically diagnosed with CR-TTP.

One serious adverse effect of repeated plasma infusions is that nine out of 41 (22%) USS patients were infected with hepatitis C virus. In this regard, virus-free rADAMTS13 preparations would be a promising product for USS patients.

Acquired thrombotic thrombocytopenic purpura/ADAMTS13:AC deficiency

Figure 4 shows age and gender distribution of acquired TTP. Of 195 patients with acquired TTP and a severe ADAMTS13:AC deficiency due to ADAMTS13:INH, 17 (6%) were childhood patients, including 14 with acquired idiopathic TTP (ai-TTP)