Role for T cells in normalization of mural cell behaviors in tumor vessels and injured arteries.

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Abstract

Current understanding regarding the involvement of acquired immune cells in the homeostatic maintenance of vascular structures and functions is limited. Recently, an unexpected role for T cells in normalization of tumor vessels was reported: type 1 T helper cells (Th1) cells contribute to tumor vessel normalization by promoting pericyte coverage. Although there remains a controversy over the origin and the fate of pericytes, appropriate control of mural cell behaviors is an important matter for the homeostatic maintenance of vessels. In our previous study, we accidentally found that smooth muscle cells (SMCs) in endothelia-removed arteries massively escaped from tunica media to extravascular spaces under T cell-deficient conditions, suggesting that T cells are involved not only in organizing pericyte anchoring to capillaries but also in controlling SMC trafficking in injured arteries. In this review, we discuss the roles for T cells in controlling mural cell behaviors to maintain vessel structures in healthy states.

Keywords

T cells, SCID mice, nude mice, wire injury, smooth muscle cells, RGS5, $S1P_1$

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1. Introduction/background

The two major cellular components of vessels, including capillaries and large vessels, are endothelial cells (ECs) and mural cells (MCs). The mode of EC-MC interaction is a key to whether vessels stay in a healthy state or undergo pathological states. As we previously reported, healthy human ECs suppress the proliferation of smooth muscle cells (SMCs), thus keeping injured arteries from undergoing stenosis, whereas degenerative human ECs enhance the proliferation of SMCs, thus exacerbating stenosis (Nishio M et al., 2015a; Nishio M et al., 2015b). During embryogenesis, defective EC-MC interactions due to lack of N-cadherin localization at intercellular junctions result in lethal hemorrhage as shown in Sphingosine 1-phosphate receptor 1 ($S1P_1$) knockout mice (Paik JH et al., 2004). We found that Regulator of G protein signaling 5 (RGS5), which is a master gene responsible for aging- and oxidative stress-dependent degeneration of healthy anti-proliferative/anti-stenotic ECs into pathogenic pro-proliferative/pro-stenotic ECs (Nishio et al., 2015a), disturbs $S1P_1$-dependent signaling and hampers N-cadherin localization at intercellular junctions (Nakahara et al., 2015). Those findings suggest that RGS5/$S1P_1$ signaling axis plays important roles to maintain EC-MC interactions in healthy states.

In tumor vessels, EC-MC interactions are disordered. Deformed networks of capillaries with inadequate pericyte coverage cause insufficient supply of oxygen and anti-tumor drugs, thus exaggerating hypoxic and drug resistant states within tumor tissues. Currently, there are limited options to normalize tumor vessels. However, valuable information that illuminates the mechanism for tumor vessel normalization has been accumulating. In RGS5 knockout mice, tumor vessel normalization along with the recovered maturation of pericytes was observed (Hamzah J et al., 2008). A recent finding further illustrated an unexpected involvement of type 1 T helper (Th1) cells in normalization of tumor vessels by promoting pericytes coverage (Tian L at al., 2017). Since $S1P_1$-dependent signaling reportedly promotes the egress of T cells from lymph nodes (Chiba K et al., 2006), Th1 recruitment from lymph nodes to tumor vessels might be enhanced

in RGS5 knockout mice to promote tumor vessel normalization via eliminated RGS5- mediated inhibitory signals on S1P/$S1P_1$-dependent lymphocyte egress.

In both physiological and pathological contexts, the mechanism for the regulation of EC-MC interactions is an important subject to be elucidated. At the same time, an understanding of the origin and fate of pericytes is a significant theme. A recent study has shown that a population of pericytes in the central nervous system is derived from CD206-positive M2 macrophages that were generated in primitive hematopoiesis in the yolk sac (Yamamoto S et al., 2017). Regarding the fate of pericytes, however, there is a controversy. A previous report showed that pericytes give rise to mesenchymal stem cells (MSC) in various human tissues (Crisan M et al., 2008), whereas a recent finding indicates that pericytes do not behave as MSC in vivo (Guimarães-Camboa N et al., 2017).

Figure 1 summarizes the current understanding of the mechanism of tumor vessel normalization as well as the molecular insight into EC-MC interactions and the quality control of ECs (i.e. anti-stenotic *versus* pro-stenotic). Although the precise evaluation of the differentiation capacities of pericytes requires future investigations, an elucidation of trafficking and dynamicity of MCs across diverse tissues is still an important subject for an advanced understanding of vascular biology.

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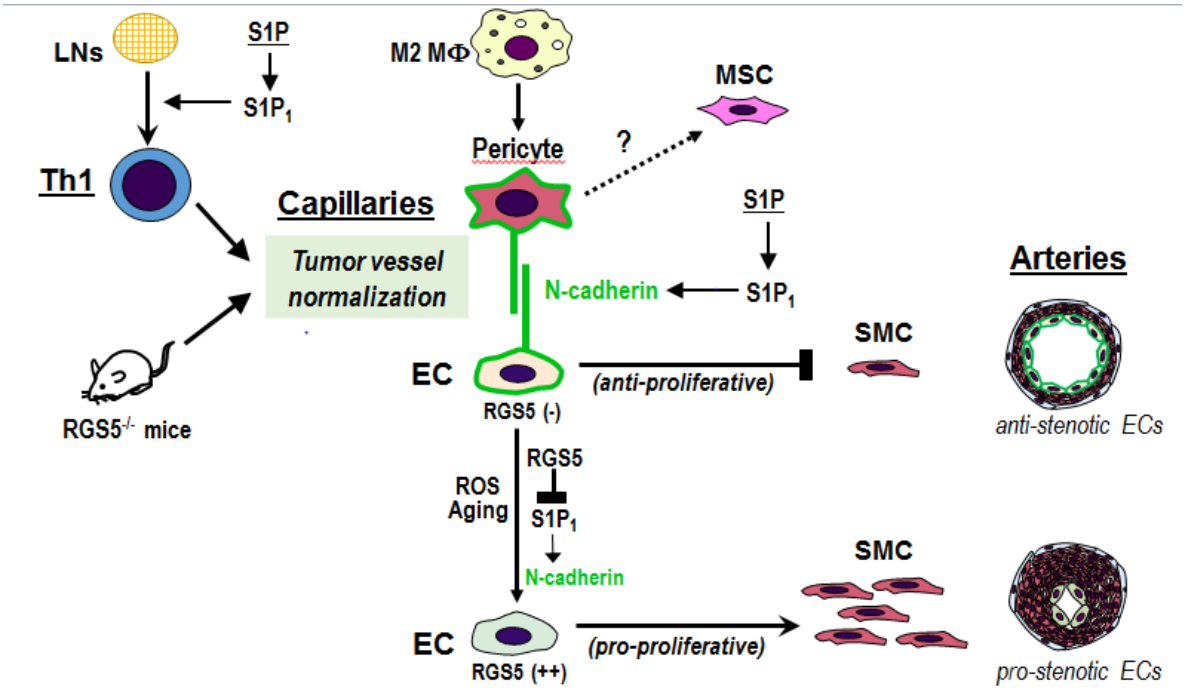

Figure 1. Regulation of mural cell behaviors in capillaries and larger arteries

RGS5 deficiency (Hamzah J et al., 2008) and the presence of Th1 cells (Tian L at al., 2017) induce tumor vessel normalization by promoting pericyte coverage on capillaries. RGS5, which converts healthy anti-stenotic ECs into generative pro-stenotic ECs (Nishio et al., 2015b), hinders S1P1-dependent signals and disturbs localization of N-cadherin (Nakahara et al., 2015). During embryogenesis, S1P1-dependent signals play indispensable roles in forming EC-pericyte interactions via N-cadherin (Paik JH et al., 2004). S1P1-dependent signals reportedly induce T cell egress from lymph nodes (Chiba K et al., 2006). Some pericytes in the brain are derived from M2 macrophages (Yamamoto S et al., 2017). There is a controversy regarding the relationship between pericytes and mesenchymal stem cells (MSCs) (Crisan M et al., 2008; Guimarães-Camboa N et al., 2017)

2. Atypical behaviors of mural cells in T cell-deficient mice

2.1 Establishment of a method for transplanting human ECs onto the surface of murine arteries

In our previous study, we investigated whether and how ECs contributed to preventing the development of stenosis in injured arteries. The major pathological features of vascular stenosis are migration and subsequent hyper-proliferation of SMCs in the tunica intima, which leads to the formation of neointima. Vascular stenosis generally occurs under conditions where ECs are injured or removed as observed in the case of restenosis after stent therapy. Therefore, it has been suggested that healthy ECs play protective roles in the maintenance of vascular structures by suppressing the proliferation of SMCs, thus preventing neointima formation after vascular injuries. However, it took a long time to prove this hypothesis because commercially available primary cultured human ECs, including human umbilical vein endothelial cells (HUVEC), exclusively enhanced the proliferation of SMCs in *in vitro* co-culture experiments (Shinoda E et al.,1999; Nishio M et al., 2015a). The hypothesis was finally proven by a series of experiments that were performed using ECs generated from human pluripotent stem cells: freshly prepared ECs suppress the proliferation SMCs whereas degenerated or aged ECs enhance SMC proliferation as in the case of HUVEC (Nishio M et al., 2015a).

To evaluate *in vivo* functions of human pluripotent stem-derived ECs, we established a system for an effective transplantation of human ECs onto the luminal surface of murine arteries. First, endothelial layers of murine femoral arteries were mechanically removed by WI operation, where a 0.014-inch diameter guidewire wire inserted into a femoral artery was moved back and forth and rotated several times to thoroughly remove endothelial cells. Immediately after WI operation, human ECs, which had been premixed with BD Matrigel™ Basement Membrane Matrix, were transplanted into the subcutaneous space around the operated artery. We found that transplanted human ECs migrated up to the middle portion of tunica media *via* vasa vasorum and subsequently moved to tunica intima to thoroughly cover the

luminal surfaces of endothelia-removed arteries. We termed this new technique for human EC transplantation as “pervasa vasorum transplantation (PVVT)” (Nishio et al., 2015b). Although we used immunodeficient SCID mice for PVVT in our initial trials, it turned out later that there was no need to use immunodeficient mice as recipients. In immunocompetent ICR mice, transplanted human ECs stayed on the luminal surface at least for one week. Although they were rejected and replaced by host ECs thereafter, one-week-existence of human ECs on the luminal surface of WI-operated arteries effectively prevented the development of stenosis. By applying this system, we elucidated that transplantation of anti-proliferative human ECs prevented the development of stenosis whereas transplantation of pro-proliferative human ECs exacerbated stenosis in injured arteries (Nishio et al., 2015b).

When we performed PVVT using SCID mice, however, we observed a bizarre phenomenon, where SMCs in the tunica media massively escaped to extravascular spaces. A similar phenomenon with a more aggressive phenotype was observed in nude mice. In the following section, we will show the detailed findings.

2.2 WI operations in SCID and nude mice

SCID mice, which lack functionally mature T and B cells, are commonly used as recipients in the transplantation experiments using human cells (Nakahara et al., 2009a; Nakahara et al., 2009b). Therefore, we initially performed WI operations using SCID mice to determine the optimal condition for the transplantation of human ECs. Unexpectedly, tunica media of WI-operated arteries turned into an acellular substance after one week from the operation (Figure 2, right panels). In addition, extravascular spaces were filled with abundant spindle-shaped cells (Figure 2, an asterisk in the right lower panel).

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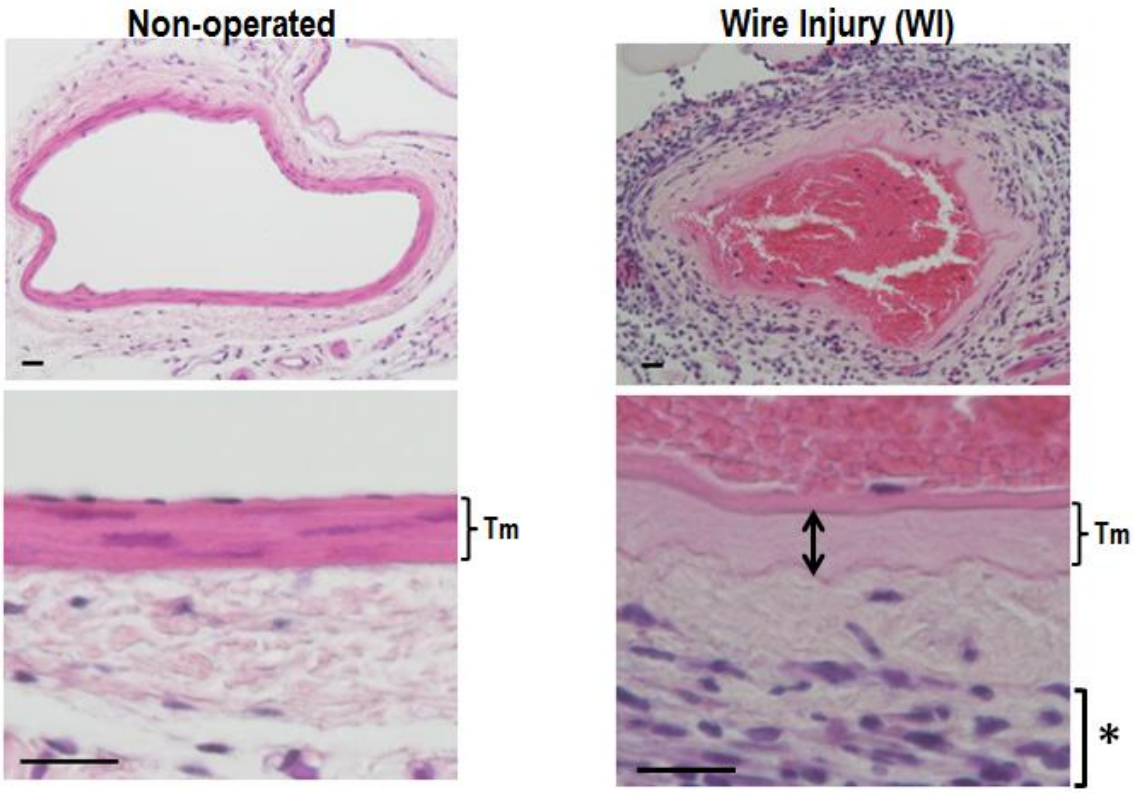

Figure 2. Wire injury operation induces disappearance of cellular components in tunica media in SCID mice.

SCID mice, which lack functional T cells and B cells, were subjected to wire injury (WI) operation in the femoral artery. After one week, mice were sacrificed and histology of the femoral artery was examined by hematoxylin and eosin (HE) staining. WI operation induced disappearance of cellular components in tunica media (Tm), which showed amorphous structures as indicated by an arrow in the lower right panel. Around the WI-operated arteries, spindle-shaped cells were massively infiltrated as indicated by an asterisk in the lower right panel. Scale = 20 mm

Although there were no signs of necrotic tissue destructions, we thought that the spindle-shaped cells might be certain kinds of immune cells. However, they were negative for CD56, a NK marker, and negative for F4/80, a macrophage marker (data not shown). Surprisingly, they were exclusively positive for smooth muscle actin, a SMC marker (Figure 3), indicating that SMCs had massively

escaped from the tunica media toward extravascular spaces. We termed this bizarre phenomenon as "massive escape of mural cells toward extravascular spaces (MEMTES)", which seems to be the cause of acellular appearance of the tunica media of WI-operated arteries of SCID mice.

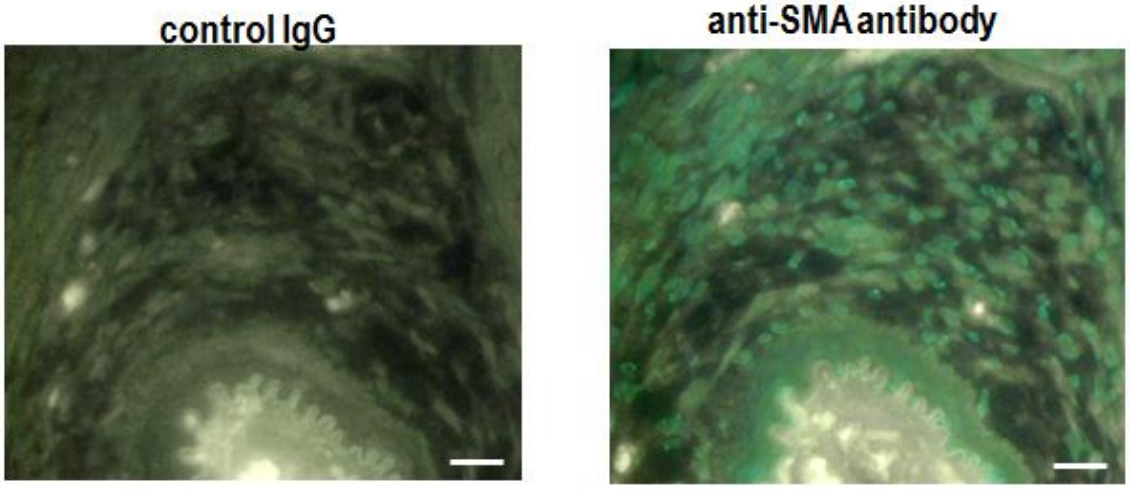

Figure 3. Wire injury operation induces massive escape of smooth muscle cells to extravascular spaces in SCID mice.

Femoral arteries of SCID mice that received WI operation one week before was subjected to immunostaining studies using an anti-smooth muscle actin (SMA) antibody. The cells distributed in the extravascular space of the operated artery were positively stained by anti-SMA antibody. Scale = 20 mm

We next performed WI operation using nude mice, which lack T cells. MEMTES was also observed in nude mice but the phenotype was significantly severer than SCID mice (Figure 4). The areas of MEMTES were not limited to extravascular spaces but extended to the regions within skeletal muscle (Figure 4, arrow head in the right panel) and adipose tissues. Cells within skeletal muscle and adipose tissues were positive for SMA but negative for CD56, F4/80, CD19, a B cell marker, Pax7, a satellite cell marker, and Myf5, a myoblast marker (data not shown), indicating that they were indeed SMCs but not immune cells or myoblasts. The reason why nude mice showed a severer MENTES remains elusive. The

presence of B cells, which are absent in SCID mice, might possibly have influenced the severity of MEMTES. Alternatively, it might be possible that the genetic background of nude mice modified the phenotype of MENTES. In any event, there is a correlation between deficiency of T cells and incidence of MENTES.

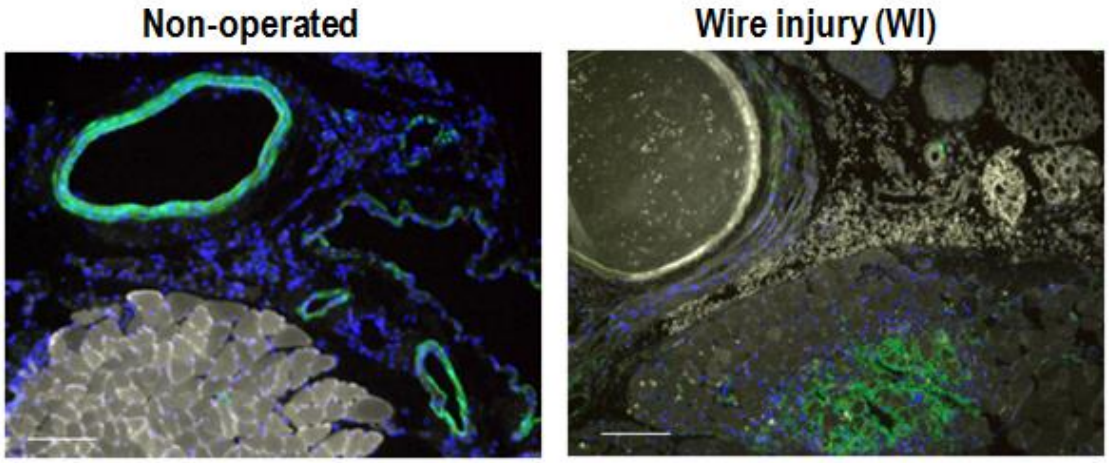

Figure 4. Wire injury operation induces disappearance of smooth muscle cells from tunica media in nude mice.

Nude mice were subjected to wire injury (WI) operation in the femoral artery. After one week, mice were sacrificed and histology of the femoral artery was examined by immunostaining studies using an anti-smooth muscle actin (SMA) antibody. Similar to the case of SCID mice, smooth muscle cells (SMCs) were lost from tunica media (right) but only a small population of SMA-positive cells were detected in the extravascular spaces (arrows in right panel). Interestingly, a number of SMA-positive cells were accumulated within skeletal muscle tissues (arrowheads in right panel) and adipose tissues (data not shown). SMA-positive cells were negative for F4/80, a macrophage marker, CD56, an NK cell marker, CD19, a B cell marker, Pax7, a satellite cell marker, Myf5, a myoblast marker (data not shown). Autofluorescence signals from hemoglobin in erythrocytes and myoglobin in skeletal muscle cells were decolorized according to the method described previously (Nishio et al., 2015a). Scale = 100 mm

2.3 Model: Immune cells and mural cell behaviors in injured arteries

The findings obtained from SCID mice and nude mice suggest that T cells are involved in preventing MEMTES and the absence of T cells causes the disappearance of SMCs from tunica media without inducing inflammatory reactions in injured arteries, thus altering the tunica media into an "acellular" substance (Figure 5).

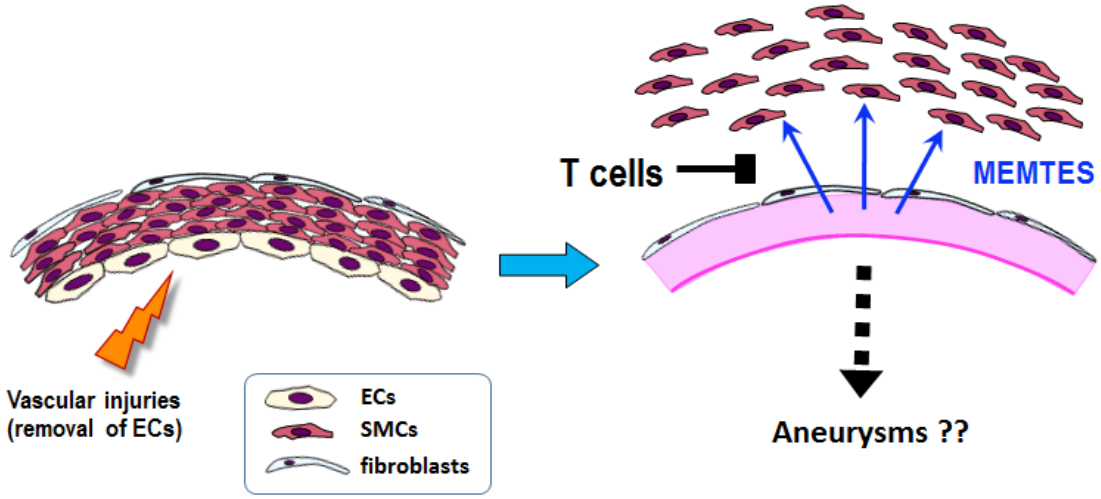

Figure 5. Model for T-cell-mediated prevention of mural cells loss from vascular walls

WI-operated arteries, whose ECs were mechanically removed, undergo a massive escape of mural cells toward extravascular spaces (MEMTES) under T cell-deficient conditions as observed in SCID and nude mice (Nishio et al., 2015b). The histology of those MEMTES arteries resembles the pathology of aneurysms of non-atherosclerotic basis such as aneurysms on the circle of Willis. Whether $CD4^+$ T cells or $CD8^+$ T cells as well as whether a specific clone of T cells or a broad spectrum of T cells are involved in prevention of MEMTES is a matter to be elucidated.

Although these hypotheses should be validated by additional experiments (e.g. WI operations after adoptive transfer of T cells into SCID mice, WI operations using genetically NK-deficient or basophil-deficient mice), our observation has illuminated previously unidentified phenomena that would contribute to an advanced understanding of vascular biology.

3. Future prospects

Our findings regarding unexpected behaviors of mural cells in injured arteries may provide useful ideas in elucidating the etiology of vascular diseases of unknown causes or pathomechanisms. For example, it seems that aneurysms of non-atherosclerotic causes are inclined to develop specific sites in the body including the central nervous system (Figure 6). It was once thought that T cells were absent in the central nervous system (CNS); however, the existence of T cells in the CNS of normal subjects (Togo T et al., 2002) and the presence of lymphatic vessels in CNS (Louveau A et al., 2015) has been proven. Therefore, the possibility cannot be ruled out that the presence of a small number of T cells in the CNS may play roles in preventing the development of aneurysms in the brain arteries and retinal microvessels although the preventing capacity would substantially be small compared to other parts of the body. It is also known that main branches of coronary arteries are the most susceptible sites for the development of aneurysms in Kawasaki disease. A decrement in the number of coronary T cells might possibly occur and thus accelerate the development of aneurysms. Further studies are required to evaluate these hypotheses.

4. Conclusions

The involvement of T cells in regulating mural cells behaviors has been suggested. Currently, there is only limited information regarding the roles for T cells in the homeostatic maintenance of vessels. Detailed investigations will unravel mysteries in vascular biology.

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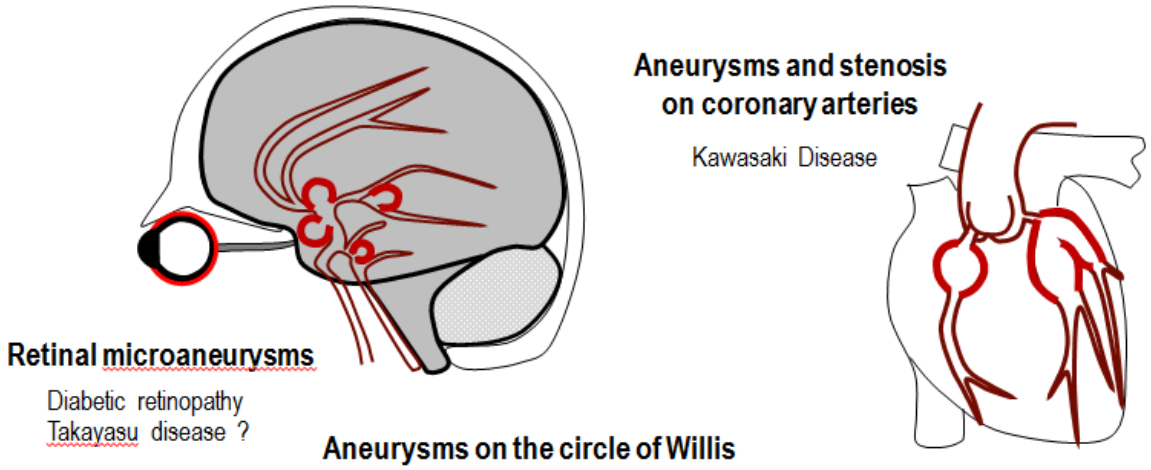

Figure 6. The occurrence of non-atherosclerotic aneurysms and stenosis.

There seems to be a tendency that aneurysms that are not based on atherosclerosis (non-atherosclerotic aneurysms; NAA) occur in specific sites in the body. For example, arteries in the central nervous system (CNS) such as retinal microvessels and arteries on the circle of Willis are the major sites for NAA. Since T cell population in CNS is considerably small, it might be possible that MEMTES could not be sufficiently prevented under vascular injuries. In Kawasaki disease, coronary arteries are affected by NAA by unknown reasons.

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