Chapter 2
Cuff-Induced Neointimal Formation in Mouse Models

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Abstract Ischemic heart failure caused by atherosclerosis is a major cause of death worldwide. Although remarkable technological advances have been made in the treatment of coronary heart disease, there is as yet no treatment that can sufficiently suppress the progression of atherosclerosis, including neointimal thickening. Therefore, a precise understanding of the mechanism of neointimal hyperplasia will provide the development of new technologies. Both ApoE-KO and LDLR-KO mice have been employed to generate other relevant mouse models of cardiovascular disease through breeding strategies. Although these mice are effective tools for the investigation of atherosclerosis, development of a progressive atherosclerotic lesion takes a long time, resulting in increase of both the costs and the space needed for the research. Thus, it is necessary to develop simpler tools that would allow easy evaluation of atherosclerosis in mouse models. In this review, we discuss our experience in generating mouse models of cuff-induced injury of the femoral artery and attempt to provide a better understanding of cuff-induced neointimal formation.

Keywords Cuff placement • Mice • Polyethylene tube

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2.1 Introduction

Epidemiological studies reveal that coronary heart diseases caused by atherosclerosis, including myocardial infarction and other forms of ischemic heart disease, are major causes of death worldwide [1]. If coronary blood flow is impaired by the development of atherosclerosis, interventions such as balloon angioplasty and endovascular stent placement are employed to overcome the vascular occlusion. These interventions can produce mechanical damage to the vasculature, including endothelial cells, smooth muscle cells (SMCs), and the adventitia [2–4]. Destruction of the endothelial cell layer is observed in the early phase after these interventions, with the formation of a thin thrombus layer covering the vascular surface [5]. Within several weeks, the vascular endothelial cells completely cover the neointima. Endothelial injury causes recruitment and adherence of circulating leucocytes, which results in the progression of neointimal formation [6]. The extent of neointimal formation has been reported to be correlated with the number of macrophages in the neointima [7]. Macrophages and neutrophils enhance the inflammatory response through the release of growth factors such as fibroblast growth factor (FGF), transforming growth factor-beta (TGF-β), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) [5]. Human studies have shown the existence of a correlation between chronic inflammation after stent placement and intimal thickening [7]. The release of the aforementioned growth factors and cytokines by the injured endothelium and infiltrating inflammatory cells leads to SMC migration and proliferation, which is preceded by transition of the SMCs from a contractile to a synthetic phenotype with excessive extracellular matrix (ECM) deposition in the intima [8, 9]. Although remarkable technological advances have been made in the treatment of coronary heart disease, none of the available treatments up to date can sufficiently suppress atherosclerosis or entirely prevent restenosis after angioplasty [10–12]. Although in-stent restenosis can be alleviated by the use of drug-eluting stents, a number of cases treated with drug-eluting stents are still reported to develop restenosis [13, 14]. There also remains the question of the safety of drug-eluting stents, including in relation to the higher frequency of occurrence of thrombotic events observed with the use of drug-eluting stents as compared to bare-metal stents [15, 16]. Moreover, cases with neointimal hyperplasia occurring after bypass surgery or allograft cardiac transplantation cannot be treated with drug-eluting stents [17, 18]. Thus, a more precise understanding of the mechanism of neointimal hyperplasia will provide the development of new technologies. In this review, we discuss our experience in generating mouse models of cuff-induced injury of the femoral artery and attempt to provide a better understanding of cuff-induced neointimal formation.

2.2 Rabbit Models of Cuff-Induced Injury

Many previous studies have reported rabbit models of cuff-induced injury. Two types of materials have been used for the generation of these rabbit models, a polyethylene tube and a silastic tube. In 1969, Mizukawa et al. have reported neointimal formation induced by the insertion of a polyethylene tube in the rabbit carotid artery [19]. Histological analysis revealed that the neointima was composed of SMCs, but had no other characteristics of atherosclerosis, e.g., foam cells [20–22]. Importantly, the polyethylene tube produced only mild, not severe, injury of the endothelial cells. Hirosumi et al. carried out an in-depth investigation of the morphometric changes induced in an artery by the insertion of a polyethylene tube by scanning electron microscopy and light microscopy (1.5 cm long PE-280; inner diameter, 2.15 mm; outer diameter, 3.25 mm; Becton, Dickinson and Company) [23]. At 30 min after cuff placement, small endothelial defects and adherence of a small number of platelets were observed in the subendothelium. At 2 h, a number of leukocytes were recruited to this cuff-injured area. The endothelial defects increased for 24 h, and leukocytic infiltration of the internal elastic lamina and endothelial cells was observed. After the 3rd day, regeneration of spindle-shaped endothelial cells occurred and covered the exposed subendothelium. However, the leukocytic infiltration persisted. The internal elastic laminae were irregular, swollen, and wavy. The SMCs under these laminae were markedly deformed and edematous. Although the endothelial cells became flat after 1 week, gaps were often observed at the cell junctions. Light microscopy revealed regression of the edematous media and SMC-like intimal cell proliferation. After 2 weeks, the endothelial cells completely covered the subendothelium, and the gaps between the cells acquired normal tightness. Leukocytes were no longer seen at the endothelial surface. On the other hand, Booth et al. were the first to use a biologically inert soft and flexible Silastic cuff, 2.1 cm length with an internal volume of 0.3 cm^3 in the carotid artery of a New Zealand White male rabbit [24]. Neointimal formation consisted largely of SMC-like cells, similar to the case in the polyethylene cuff-induced injury. No foam cells were detected in any of the sections obtained from the carotid arteries of the New Zealand White rabbits at 7 days after the placement of the Silastic cuff. Moreover, an intact endothelial layer was observed after 7 days. The endothelial cells remained in contact with each other, and no denudation or exposure of the subendothelium was seen at 24 h after placement of the silastic cuff [25]. Kockx et al. revealed that silicone cuff-induced neointimal formation can be distinguished into three phases [26]. The first phase begins within 2 h, with polymorphonuclear leukocyte (PMN) infiltration from the luminal surface toward the intima and the inner media. In the second phase, which starts within 12 h, the replication rate of the SMCs in the media increases by about 20-fold as compared with that in unmanipulated arteries. The third phase, beginning from day 3, is characterized by the appearance of subendothelial SMCs that are immunoreactive for α-SM actin (α-SMA). Although the neointima formed in response to both injury caused by a polyethylene and that caused by a silastic cuff consists of SMCs,

the injury caused by the polyethylene cuff was associated with injury of the endothelial cells, whereas that caused by the silastic cuff was devoid of injury to the endothelium.

2.3 Mouse Models of Cuff-Induced Injury

Mouse models have been used popularly as experimental animal models due to their well-defined genetic background and the possibility of manipulating the mouse genome. Mouse models are useful for evaluating the roles of specific molecules in vascular remodeling, such as in the case of post-percutaneous coronary intervention (PCI) restenosis. Apolipoprotein (Apo) E-knockout (KO) mice have been used extensively because they develop spontaneous atherosclerosis [27, 28]. Furthermore, the atherosclerotic lesions in this mouse model closely resemble the atherosclerotic lesions, both stable and unstable, in humans. Low-density lipoprotein (LDL) R-KO mice also serve as good models to evaluate human atherosclerotic lesions [29]. However, unlike the ApoE-KO mice, the LDLR-KO mice do not develop spontaneous atherosclerosis [28]. Both ApoE-KO and LDLR-KO mice have also been utilized to generate other relevant mouse models of cardiovascular disease through a variety of breeding strategies. Although these mice are effective tools for the investigation of atherosclerosis, development of a progressive atherosclerotic lesion takes a long time, resulting in an increase of both the costs and the space for the research. Mouse arteries, unlike the arteries of larger animals, are too small for transluminal injury to be induced with a balloon. Thus, it is necessary to develop tools for easier evaluation of atherosclerosis in mouse models.

Three methods for the induction of neointimal formation in mice have been mainly reported [10]. In the arterial ligation model, blood flow through the common carotid artery is disrupted by ligation near the distal bifurcation [30]. The decrease in the blood flow resulting from the dramatic reduction of the vessel diameter results in the formation of an extensive SMC-rich neointima within 2–4 weeks. This model has the advantage of reproducibility due to the ease of use of the method for inducing neointimal hyperplasia. Lindner and colleagues were the first to report mechanically induced endothelial denudation model, which involves passage of a flexible guidewire three times in the carotid artery [31]. This procedure completely removes the endothelial cell layer, which results in the recruitment of platelets to the denuded surface and activation of the medial SMCs. Neointimal hyperplasia is typically observed within 2 weeks after the injury. This procedure is the most similar to angioplasty. However, this procedure is a relatively challenging procedure, which leads to difficulty in achieving reproducible results. Subsequently, Sata et al. modified the wire injury method to develop a more convenient and reproducible results [32]. Since the wire injury model shows complete denudation of the endothelial cell layer, it may not be suitable for investigation of the involvement of endothelial cell-specific molecules in the development of atherosclerosis. Use of a

polyethylene cuff also causes damage to the endothelial cell layer as described above, but it produces milder injury as compared to wire injury. The endothelium itself is not directly injured by a polyethylene cuff. In fact, endothelial cells have reported to remain relatively intact after perivascular cuff placement [33]. Moroi et al. first adapted an external vascular cuff model used in rabbits to mice deleted with endothelial cell-specific eNOS gene [34]. Since the virus-mediated vector and drug can be injected easily between the cuff and the vessel, this cuff-induced injury model is also excellent for the observation of changes in the adventitia associated with arteriosclerosis [35]. Thus, the use of mouse models of cuff-induced injury has increased in recent years.

2.4 Method for Creation of Cuff-Induced Injury in the Femoral Artery

Dr. Moroi kindly taught us the procedure for the cuff placement [34]. Before the operation, the cuff (Intramedic Polyethylene Tubing (PE-50, inner diameter = 0.58 mm), Becton Dickinson) needs to be prepared. Polyethylene tube was cut for each length 2 mm and cut in longitudinal direction. We tied polyethylene tube directly to 8–0 nylon suture with needle (Bear Medic Corporation) in two places on both side and made stringlike. The mice were anesthetized by an intraperitoneal (IP) injection of sodium pentobarbital at the dose of 40–50 mg/kg. Then, the mice were fixed in the supine position and their skin was shaved from the inguinal region to the medial aspect of the thigh above the knee and disinfected with 70 % ethanol. The skin was next lifted with tweezers under stereomicroscopic observation, and an incision about 7–10 mm length was placed in the inguinal region. The subcutaneous connective tissue was carefully removed to expose both the femoral artery and the femoral vein. The femoral artery is separated from the femoral vein by blunt dissection with micro tweezers. A stringlike nylon suture with polyethylene tube is sent under a separating femoral artery. The break of the polyethylene tube is expanded by pulling stringlike nylon suture of polyethylene tube. The polyethylene tube is placed around the femoral artery. Finally, the surrounding tissue is straightened and the skin is sutured (Fig. 2.1). A sham operation is performed without polyethylene tube placement in the other femoral artery, as control. The mice are anesthetized by IP injection of pentobarbital 14 days after the operation and fix in the supine position. After laparotomy, the diaphragm is incised to expose the heart. Ice-cold 4 % paraformaldehyde is injected into the systemic circulation via the cardiac apex at a pressure of 100 cm H_2O, followed by incision of the right auricular appendage. Both the cuffed-femoral artery and the sham-operated artery of the opposite sides are removed and immersed in OCT compound. For the immunohistochemical staining, 5- to 6-μm-thick sections are prepared from each sample. Paraffin sections are more suitable for the measurement of intimal thickness than frozen sections. Femoral artery samples embedded in

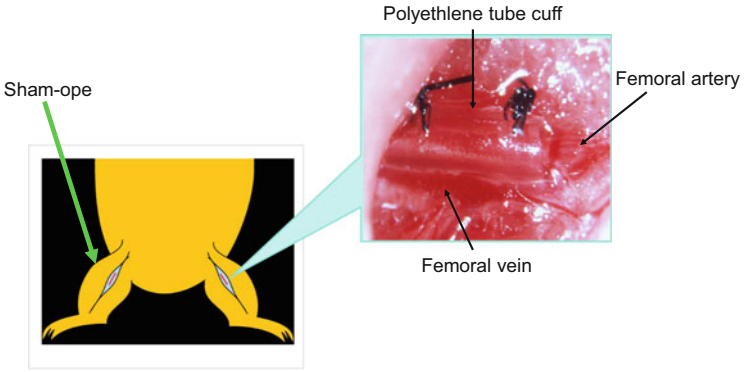

Fig. 2.1 Cuff placement for the femoral artery

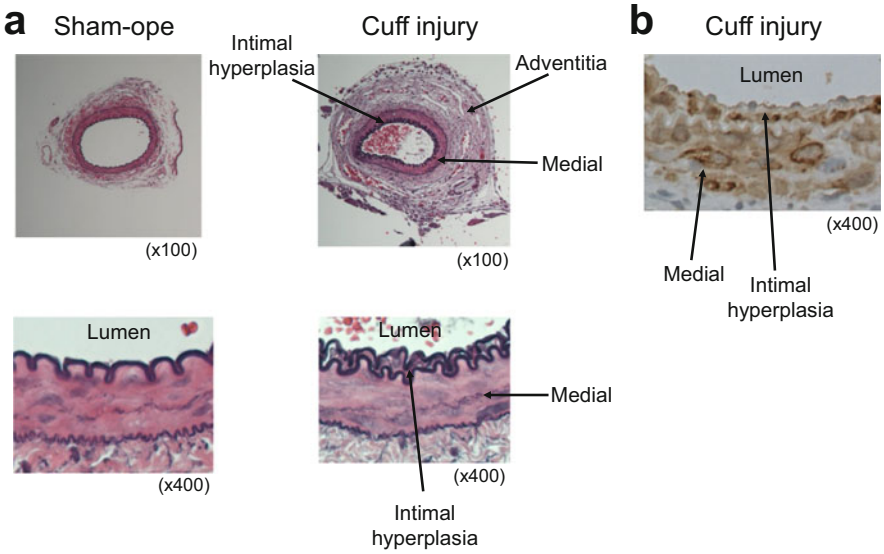

Fig. 2.2 Representative arteries after sham-operated or cuff placement (**a**) and alpha smooth muscle actin staining for cuff-injured artery in C57B6 mice (**b**)

paraffin were cut from one edge to the other edge of the cuffed portion. The areas of the lumen, intima, and media were measured in 10 cross sections using the image analyzer. Images are digitized and captured with a CCD camera connected to a personal computer (Fig. 2.2a). Measurements are performed at a magnification of $\times$200 using the Scion Image analysis software. For each artery, the luminal area, area inside the internal elastic lamina, and the area encircled by the external elastic lamina are measured. The medial area is calculated as the area encircled by the external elastic lamina area inside the internal elastic lamina, and the intimal area is calculated as the area inside the internal elastic lamina-luminal area. To calculate

the medial thickness for each vessel cross section, the linear distance between the internal elastic lamina and external elastic lamina is measured independently in 10 places, separated by 90° each and then averaged. The ratio of the intimal to the medial area and the percent luminal stenosis are calculated based on these measurements [10, 34].

2.5 Etiology of Cuff-Induced Neointimal Formation

Since cuff-induced neointimal formation showed positive staining with an α-SMC antibody (Fig. 2.2b), the neointima appears to be almost entirely composed of SMCs. Neointimal formation induced by cuff placement can be considered as a consequence of migration and/or proliferation of the SMCs induced by some factors such as PDGF-BB or inflammatory cytokines (Fig. 2.3). Although the exact mechanisms underlying the neointimal formation in cuff-induced injury models are still uncertain, I would like to refer to reports from the literature of studies carried out using genetically manipulated mouse models to discuss the mechanisms of neointimal formation (Table 2.1).

2.5.1 Factors Directly Inducing SMC Proliferation and Migration

Cuff replacement around the femoral artery induced neointimal hyperplasia, which was exclusively composed of SMCs, as mentioned above. Platelet-derived growth factor (PDGF)-BB, which is mainly secreted from platelets, is one of the most

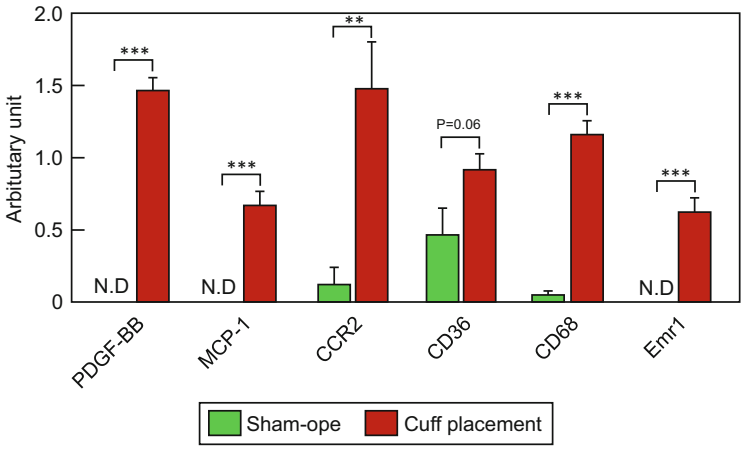

Fig. 2.3 Comparison of the gene profiles between sham-operated artery and cuff-injured artery

Table 2.1 Representative cuff placement in genetically manipulated mouse models

Gene	Location	Neointimal formation	References	
LR11-KO	Carotid artery	Reduced	Arterioscler Thromb Vasc Biol	2007 [38]
Rag-1-KO	Carotid artery	Increased	Arterioscler Thromb Vasc Biol	2002 [45]
CD4-KO	Carotid artery	Reduced	J Am Heart Assoc	2013 [50]
eNOS-KO	Femoral artery	Increased	J Clin Invest	1998 [34]
iNOS-KO	Carotid artery	Reduced	Circ Res	1999 [59]
sEH/ApoE double-KO	Femoral	Reduced	Arterioscler Thromb Vasc Biol	2010 [61]
sEH/ApoE double-KO				
Tlr4-KO	Femoral	Reduced	Circulation	2002 [64]
Tlr2-KO	Femoral	Reduced	Cardiovasc Res	2005 [65]
CatK-KO	Carotid	Reduced	Hypertension	2014 [66]
AT1 receptor-KO	Femoral	Reduced	Circulation	2002 [74]
AT2 receptor-KO	Femoral	Increased	Circulation	2002 [74]
Mas-KO	Femoral	Increased	Hypertension	2014 [78]
Cu/ZnSOD-KO	Femoral	Unchanged	Journal of atherosclerosis and thrombosis	2011 [81]
HAS2Tg (SMC-specific)	Femoral	Increased	PLoS One	2013 [39]
IRS1-KO	Femoral	Increased	Circulation	2003 [85]
IRS2-KO	Femoral	Increased	Circulation	2003 [85]

potent factors inducing migration and proliferation of the SMCs after balloon angioplasty. The expression levels of PDGF-BB mRNA in the cuffed artery were significantly increased as compared to those in the sham-operated artery (Fig. 2.3). An antagonist of the PDGF-BB receptor was shown to suppress the proliferation of vascular SMCs after balloon-induced injury in a rat model [36]. PDGF-BB induces vascular SMC proliferation via, at least in part, regulation of the cell cycle. Schwaiberger et al. demonstrated that local treatment with a cyclin-dependent kinase inhibitor inhibited cuff-induced neointimal formation, accompanied by reduction of the phosphorylation level of signal transducer and activator of

transcription 3 (STAT3), but not of Akt, ERK1/2, or p38MAPK activation [35]. These data suggest that PDGF-BB-STAT3 signaling is involved in cuff-induced neointimal formation.

In addition to PDGF-BB-STAT3 signaling, PDGF-BB has been reported to augment the migration of SMCs through the LR11, a member of the LDL receptor family/urokinase-type plasminogen activator receptor (uPAR) pathway [37]. Ohwaki et al. found that balloon-injured intimal SMCs showed strong expression of LR11 in rat arteries. High-fat diet-fed LR11-KO mice showed a decrease of cuff-induced neointimal formation [38]. In in vitro experiments, the secreted soluble form of LR11 (soILR11) promoted the migration of THP-1 induced by PDGF-BB, an effect that was completely canceled by the anti-uPAR antibody [38]. These data suggest that PDGF-BB induced by cuff-induced injury is one of strongest regulators of neointimal formation.

The ECM has been reported to be a key player in arterial remodeling after vascular injury [4]. Hyaluronan 2 (HAS2), which is a primary component of the ECM, is expressed in neointimal lesions in humans with atherosclerosis and at sites of wire-induced injury of the arteries in mice. Mice overexpressing the murine HAS2 gene specifically in the vascular SMCs (cHAS2/CreSM22a mice) showed markedly enhanced cuff-induced neointimal formation, with augmentation of SMC migration and proliferation, and production of inflammatory cytokines and ROS [39]. Consistent with these data, in a wire-induced injury model, a HA synthesis inhibitor markedly suppressed neointimal formation. The ECM induced by arterial cuff-induced injury promotes migration of SMCs into the intima, leading to neointimal formation.

2.5.2 Roles of BM-Derived Cells in Cuff-Induced Neointimal Formation

The roles of bone marrow (BM)-derived cells in the pathogenesis of atherosclerosis have been extensively studied [40]. Fluorescence in situ hybridization (FISH) analysis has revealed that female-derived BM cells transplanted into male mice were detected in the neointima of male mice (Fig. 2.4). Following BMT from green fluorescent protein (GFP)-transgenic mice to ApoE-KO mice, GFP-positive cells were confirmed in the vascular neointima (4–10 %) and media (5–32 %) in the latter mice. Following bone marrow transplantation (BMT) from LacZ mice to wild-type (WT) mice, a number of LacZ-positive macrophages were found in the neointima (25.7 %), media (7.3 %), and adventitia (73.7 %) of the arteries of the WT mice at 4 weeks after cuff placement [33]. It was worthy of note that more marked infiltration of the adventitia by macrophages was observed after injury induced by cuff placement than after wire injury or ligation injury.

Xu et al. transplanted the BM of GFP-transgenic mice into LDLR-KO mice to identify the cell lineage in the lesion [41]. Two weeks after cuff placement

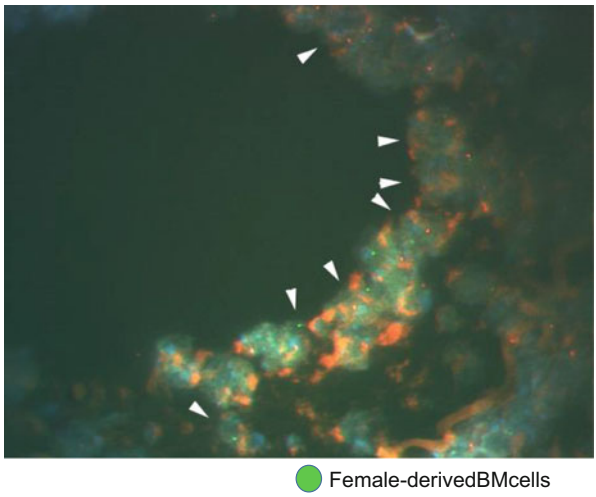

Fig. 2.4 Fluorescence in situ hybridization (FISH) of cuff-injured artery in male mice transplanted female-derived BM cells

following a high-fat diet for 4 weeks, atherosclerotic lesions developing in the intima predominantly consisted of a massive accumulation of foam cells with a number of α-SMA- and GFP-positive cells. In addition to macrophages, adventitial small vessels also showed positive staining for both the endothelium-specific marker CD31 and GFP in mice transplanted with BM obtained from GFP-transgenic mice [41]. Most of the macrophages are GFP-positive, and some of the SMCs and ECs are also GFP-positive. In a cuff-induced vascular injury model, BM-derived cells are recruited to the adventitia and differentiate into macrophages, SMCs, and endothelial cells. Treatment with a blocker of M-CSF, which guides differentiation into macrophages, caused a prominent decrease of macrophages, and inhibition of the PDGF receptor suppressed the recruitment of SMCs to the adventitia after cuff placement [42]. These data suggest that the adventitia plays a pivotal role in neointimal formation induced by cuff placement. Scott et al. demonstrated that the adventitia is important in the first wave of growth after angioplasty following occurrence of neointimal hyperplasia [43].

2.5.3 Roles of T Cells and B Cells in Cuff-Induced Neointimal Formation

The importance of T and B lymphocytes in the process of restenosis after PCI has been pointed out [44]. Rag-1-KO mice, which lack mature B and T cells, have been demonstrated to show increased thickness of cuff-induced neointima in the carotid artery. Reconstitution of the Rag-1-KO mice with B cells from WT mice reduced the neointimal formation. Moreover, both IgG and IgM were detected in the cuff-injured carotid arteries of reconstituted Rag-1-KO mice with B cells [45]. These data suggest that an increase of immunoglobulin by activation of B cells exerts a

protective action against intimal thickening. The neointimal area and intima/media ratio were significantly reduced in mice treated with immunoglobulin administered intraperitoneally for 5 consecutive days starting 1 day prior to cuff placement. On the other hand, immunoglobulin could not suppress cuff-induced neointimal formation, when the treatment was commenced 3 days after cuff placement [46]. When pooled mouse IgG or IgM was given to Rag-1-KO mice by intravenous injection, a significant reduction of intimal thickening was observed as compared with that in the untreated Rag-1-KO mice. Immunoglobulin treatments modify the serum complement C3 profile, and the amount of complement C3 was decreased in the injured arteries. Depletion of complement C3 in the Rag-1-KO mice significantly decreased the degree of intimal thickening [47]. These data suggest that IgG, IgM, and complement C3 are involved in the modulation of the neointimal hyperplasia response to cuff-induced injury.

Moreover, treatment with immunoglobulin significantly enhanced the secretion of interleukin (IL)-10, suggesting activation of T cells by immunoglobulin [46]. Rag-1-KO mice reconstituted with T cells from WT mice showed a reduction of neointimal formation after cuff placement [48]. Dimayuga et al. carried out a detailed examination of the T-cell fraction. Splenic $CD8^+CD25^+$ T cells and $CD8^+CD28^+$ T cells, but not $CD4^+CD25^+$ and $CD4^+CD28^+$ T cells, were also significantly increased after arterial injury in the WT mice. Rag-1-KO mice given $CD8^+$ T cells showed a significant decrease of neointimal formation as compared to Rag-1-KO mice not given the cells. On the other hand, transfer of $CD4^+$ T cells was not associated with inhibition of the neointimal formation [49]. Neointimal formation induced by cuff placement was significantly reduced in CD4-KO mice as compared with that in the WT mice, because of the higher percentage of $CD8^+$ T cells. Moreover, adoptive transfer of $CD8^+CD28^{hi}$ T cells into recipient Rag-1-KO mice significantly reduced neointimal formation as compared to that of $CD8^+CD28^+$ T cells [50]. Although both $CD8^+$ T cells and $CD4^+$ T cells are activated in response to arterial injury, $CD8^+$ T cells, which constitute at least a fraction of the $CD8^+CD28^{hi}$, are mainly involved in the inhibition of cuff-induced neointimal formation.

2.5.4 Roles of eNOS and iNOS in Cuff-Induced Neointimal Formation

Nitric oxide (NO) is an important vascular regulatory factor that is generated by the enzyme nitric oxide synthase (NOS) [51]. Three different isoforms of NOS are recognized: among these is endothelial NOS (eNOS), which is constitutively expressed mainly in the vascular endothelial cells [52]. eNOS can be an important factor modulating vascular endothelial function and is activated by acetylcholine (ACh) and insulin. eNOS-KO mice exhibit impaired ACh-induced vascular relaxation [53]. The second isoform is inducible NOS (iNOS), which cannot be detected

in normal tissue, but is expressed in several cell types, including macrophages and vascular SMCs, after cytokine stimulation [54], and the third isoform is neuronal NOS (nNOS), which is constitutively expressed mainly in the nervous tissues and skeletal muscle type II.

eNOS-KO mice exhibit increased neointimal formation following cuff placement [34]. Consistent with these data, the neointimal formation induced by ligation is also significantly more pronounced in the eNOS-KO mice than in the WT mice [55]. Since antiplatelet and antihypertensive treatments cannot attenuate the progression of neointimal formation, the neointimal hyperplasia observed in eNOS-KO mice is produced by the direct action of eNOS and not mediated by thrombus formation or high blood pressure [33]. When adenovirus-mediated human endothelial constitutive NOS cDNA (AdCMVceNOS) was transduced into the rat carotid artery after balloon injury, the intima/media ratio decreased significantly because of inhibition of SMC proliferation [56]. These data suggest that NO mediated by eNOS inhibits neointimal hyperplasia induced by vascular injury.

iNOS has been shown to be expressed in the SMCs after cuff-induced vascular injury in rabbits [57, 58], and iNOS-KO mice showed a significant reduction of neointimal thickening induced by cuff placement [59]. Unlike eNOS-deficient mice, iNOS-KO mice showed no reduction of the neointimal hyperplasia associated with mechanically induced endothelial denudation. Both the medial area and medial thickness were increased in the iNOS-KO mice after mechanically induced endothelial denudation [60]. Consistent with these data, Yogo et al. demonstrated that vascular remodeling, but not neointimal hyperplasia, after carotid artery ligation was increased in the iNOS-KO mice [55]. The differences in the procedure used to induce vascular injury may be related to the degree of neointimal hyperplasia and vascular remodeling in the iNOS-KO mice. Cuff placement evokes significant participation of inflammatory cells, including macrophages, in the adventitia as compared to the ligation model, as mentioned above. Increase of inflammation in the adventitia may be associated with increased iNOS expression levels, which may promote neointimal formation induced by cuff placement. Mechanically induced endothelial denudation and ligation may mainly induce migration of the SMCs from the media to the intima, resulting in increased neointimal hyperplasia.

Similar results were also observed in soluble epoxide hydrolase (sEH)/ApoE double-KO mice and inhibition of sEH by 12-(3-adamantan-1-yl-ureido) dodecanoic acid, which suppress metabolism of epoxyeicosatrienoic acids (EETs). sEH/ApoE double-KO mice or mice with inhibition of sEH showed significant reduction of the neointimal formation in the femoral artery cuff model, but not in the carotid artery ligation model. The expressions of proinflammatory genes were significantly reduced in the femoral arteries of the sEH/ApoE double-KO mice [61].

2.5.5 *Roles of Inflammatory Cytokines in Cuff-Induced Neointimal Formation*

Inflammation is considered as an important factor in human atherogenesis [8]. An inflammatory response to vascular injury, mediated by proinflammatory cytokines, influences the progression of neointimal formation and development of atherosclerotic lesions.

It has been demonstrated that the expressions of toll-like receptors (Tlrs) 2 and 4 are markedly enhanced in human atherosclerotic plaques and vascular adventitia [62]. Tlr4 serves as the receptor for bacterial lipopolysaccharides (LPS) and also recognizes cellular fibronectin, heat shock protein 60, and endogenous peptides that are produced in response to tissue injury [63]. In the adventitia, not all Tlr4-positive cells are colocalized with macrophages. Although application of LPS between the cuff and artery augmented the neointimal formation induced by cuff-induced injury in the WT mice, no such finding was observed in the Tlr4-KO mice [64]. Application of Pam3Cys-SK4, a synthetic Tlr2 ligand, significantly enhanced the neointimal formation induced by cuff placement in the femoral arteries of the WT mice. No such increase of the neointimal formation was observed in the Tlr2-KO mice. In ApoE-KO mice, application of Pam3Cys-SK4 led to a significant increase in the formation of atherosclerotic plaques [65]. Tlr2 stimulation produced significant induction of inflammatory cytokines in human adventitial fibroblasts in vitro. Treatment with cathepsin K (CatK), which is one of the most potent of mammalian collagenases, increased the mRNA levels of inflammatory cytokines, including Tlr2 and Tlr4. CatK-KO mice showed significantly reduced neointimal formation following cuff placement and ligation, accompanied by a decrease in the expression levels of Tlr2 and Tlr4 mRNA [66]. These findings provide evidence for a link between inflammatory cytokines in the adventitia and intimal lesion formation.

Although Tlr2/4 are expressed on the cell surface, Tlr7/9 are expressed on the endosomes. Tlr7/9 were detected at sites of post-interventional remodeling and accelerated atherosclerosis [67]. In hypercholesterolemic apolipoprotein E*3-Leiden mice, femoral artery cuff placement led to a strong increase of the Tlr7/9 expressions [68]. Blockade of Tlr7/9 with a dual antagonist reduced neointimal thickening and foam cell accumulation; the intima/media ratio was reduced by 64.5 % and luminal stenosis by 62.8 %. Application of the Tlr7/9 dual antagonist also reduced arterial wall inflammation, with reduced macrophage infiltration and altered serum IL-10 levels. Stimulation of cultured macrophages with Tlr7/9 ligands enhanced TNFα expression, which was decreased by coadministration of the Tlr7/9 antagonist. Furthermore, the antagonist abolished the Tlr7/9-enhanced LDL uptake. The antagonist also reduced oxidized LDL-induced foam cell formation, most likely not via decreased influx, but via increased efflux induced by increased IL-10 levels.

2.5.6 Role of RAS in Cuff-Induced Neointimal Formation

The renin-angiotensin-aldosterone system (RAAS) has various physiological actions such as vasoconstriction and is known to be involved in the development of hypertension and atherosclerosis [69, 70]. Specifically, modulation of the local RAAS may play a key role, just like that of the systemic RAS, in the development of cardiovascular diseases. Angiotensin (Ang) II is a peptide that exerts potent vasoconstrictive action via the AT1 receptor-MAPK pathway. Although human renin (hRN)/human angiotensinogen (hANG)-transgenic (Tg) mice showed increased blood pressure and medial thickening even in the absence of cuff placement, the hRN/hANG-Tg mice showed even more pronounced inflammatory vascular remodeling after cuff placement. Treatment with an AT1 blocker inhibited cuff-induced neointimal formation associated with reduced inflammation, but independently of the blood pressure change [71]. When the direct renin inhibitor aliskiren was administered to C57BL/6 mice via an osmotic pump, it inhibited cuff-induced vascular remodeling. The number of adherent leukocytes was increased in the cuff-injured mice not treated with aliskiren, where it was significantly reduced in the aliskiren-treated mice without any change of the blood pressure. Aliskiren decreased the adhesion of THP-1 cells to TNFα-stimulated human umbilical vein endothelial cells [72]. These data indicate that RAS activation augments neointimal hyperplasia induced by cuff placement via increased release of inflammatory cytokines.

Moreover, the ACE expression level increased in a time-dependent manner after cuff placement and was observed in the medial and neointimal layers and the adventitia of the cuffed arteries in FVB/N mice. The intima/media ratio after cuff placement was significantly decreased by ACE inhibitor treatment [73]. Ang II appears to be one of the factors exacerbating cuff-induced neointimal formation. In fact, AT1 receptor-KO mice showed decreased neointimal formation following cuff placement, accompanied by an increase of apoptotic cells among the SMCs [74]. Consistent with these data, the AT1-selective receptor blocker olmesartan suppressed cuff-induced neointimal formation via reducing ERK phosphorylation [75]. On the other hand, neointimal formation induced by cuff placement was increased in AT2 receptor-KO mice. The expressions of bcl-2 and bcl-xL mRNA, which are regulators of apoptosis, were enhanced in the AT2 receptor-KO mice showing enhanced neointimal formation [74].

Recently, in addition to the Ang II-AT1 receptor pathway, Ang-(1-7), which is synthesized from Ang I and Ang II mainly via ACE2 activity, has been reported to play a crucial role in vascular remodeling via Mas receptor activation [76, 77]. Mas-KO mice showed markedly increased neointimal formation after cuff placement, independently of the AT1 receptor. Treatment with Ang-(1-7) also suppressed neointimal formation, associated with suppression of vascular SMC proliferation, release of inflammatory cytokines and superoxide anion production in the injured artery. On the other hand, these inhibitory effects of Ang-(1-7) were less marked in the Mas-KO [78]. Interestingly, treatment with an

AT1 receptor blocker inhibited neointimal formation induced by cuff placement, accompanied by a decrease in the expression levels of ACE2 and Mas mRNA and an increase in the expression of AT2 receptor mRNA. AT2 receptor-KO mice showed no reduction of the neointimal formation by treatment with Ang-(1-7). These results suggest that in addition to the activities of the ACE2/Ang-(1-7)/Mas axis, blockade of the AT1 receptor could enhance the activities of the ACE2/Ang-(1-7)/AT2 receptor axis and thereby inhibit neointimal formation induced by cuff placement.

2.5.7 Role of ROS in Cuff-Induced Neointimal Formation

Atherosclerosis is associated with increased production of reactive oxygen species (ROS) in the vessel [79]. The superoxide dismutases (SODs) are enzymes that catalyze the dismutation of superoxide anions to hydrogen peroxide; three isoenzymes of the SODs have been identified, namely, manganese SOD (MnSOD), which is localized in the mitochondria; copper/zinc SOD (Cu/ZnSOD), which is localized in the cytosol; and extracellular SOD (EC-SOD). The antioxidant enzyme Cu/ZnSOD metabolizes superoxide anions (O2-) in the vascular endothelial cells [80].

Although there was no difference in the degree of cuff-induced neointimal formation between the Cu/ZnSOD-KO and WT mice, the former showed a significant decrease in the intima/media ratio after cuff placement [81]. This increased medial SMCs in the Cu/ZnSOD-KO mice showed positive staining for SMemb/MHC-B, which is a useful molecular marker of embryonic-type SMCs. Moreover, the expression levels of TNFα, ICAM1, VCAM1, and iNOS in the media were higher in the Cu/ZnSOD-KO mice than in the WT mice, suggesting that Cu/ZnSOD-KO mice showed enhanced inflammation, expression of adhesion molecules, and altered structure of the media post-injury.

When an adenovirus vector expressing EC-SOD (AxCAEC-SOD) was injected between the cuff and the adventitia of the femoral arteries in rat models, neointimal formation was significantly reduced in the AxCAEC-SOD-transfected arteries [82]. Furthermore, proliferation of SMCs in the neointima and media was inhibited by EC-SOD treatment. Augmented iNOS expression, apoptosis, collagen content, and ROS generation in the vascular wall were also reduced by EC-SOD treatment. The amount of generation of ROS may have influence on the degree of medial thickening as well as neointimal formation induced by cuff placement.

2.5.8 *Role of Insulin Signaling in Cuff-Induced Neointimal Formation*

Insulin resistance has been reported to be associated with atherosclerosis; however, the underlying mechanism is still unknown [83]. Insulin resistance is caused by impaired insulin signaling induced by some factors. Insulin activates insulin receptor substrates (IRS) 1 and 2, which are expressed ubiquitously in various tissues, including the endothelial cells and SMCs, through the insulin receptor and the downstream signaling [84]. Both IRS1- and IRS2-KO mice show features of the metabolic syndrome, including insulin resistance, dyslipidemia, and hypertension. Although both genotypes of mice show a similar degree of insulin resistance, the IRS2-KO mice show more pronounced cuff-induced neointimal formation than the IRS1-KO mice, which in turn show more pronounced cuff-induced neointimal formation than the WT mice. IRS2 expression, but not IRS1 expression, is detected in the blood vessels [85]. Insulin resistance in the blood vessels is considered to exacerbate cuff-induced neointimal formation.

On the other hand, mice with phosphatase and tensin homology deleted on chromosome 10 (PTEN), which is an antagonist of phosphatidylinositol 3-kinase (PI3K), exhibited significant reduction of neointimal formation when adenovirus-mediated human PTEN (AdPTEN) type 1 was injected between the cuff and the adventitia. AdPTEN reduced SMC proliferation and release of inflammatory cytokines [86]. Since insulin activates PI3K via the IRS, the overexpression of PTEN in response to cuff-induced injury appears to be a seemingly contradictory finding to that observed in the IRS-KO mice. However, as PI3K has known to be activated by some factors independently of insulin signaling, activated PI3K may enhance neointimal hyperplasia induced by cuff placement under some conditions.

2.6 Conclusion

Cuff placement causes mild endothelial cell damage, SMC proliferation and migration, and inflammation of the adventitia. This is a convenient and reproducible tool and is relatively less expensive and time-consuming. This tool is effective for analyzing the mechanism of vascular remodeling using mice, including genetically engineered mice. Further experiments to identify the precise mechanisms of neointimal hyperplasia by using this tool will provide the development of new technologies in the future.

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