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Control of atherosclerotic plaque vulnerability: Insights from transgenic mice

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1. ABSTRACT

Atherosclerosis is a complex, progressive disease of the large systemic arteries. This multi-factorial disease is characterized by accumulation of lipids, cells and extracellular matrix in the vessel wall. The quest to unravel the molecular mechanisms leading to progression of human atherosclerotic plaques has lead to the development of a variety of animal models. Mice are easily amendable to transgenesis and multiple mutant and inbred strains have been generated in which potential regulators are manipulated and subsequently studied for effects on the development and progression of atherosclerosis. The scope of this review is to discuss the relevance, advantages and disadvantages of genetically-engineered mice to investigate mechanisms of plaque vulnerability. Features of human vulnerable lesions, such as large lipid-rich necrotic cores, active inflammation, matrix remodeling and signs of intraplaque hemorrhage are represented in mouse lesions. Here, we will discuss how atherosclerosis is modified by manipulations in apoptosis, lesional lipid metabolism, inflammatory pathways, matrix remodeling and thrombotic pathways in genetically-engineered mice, emphasizing the insights that have been gained from these studies for the control of plaque vulnerability.

2. DEFINING PLAQUE VULNERABILITY

The majority of clinical cardiac manifestations (~70%) such as acute myocardial infarction (MI), unstable angina and sudden cardiac death result from plaque rupture (1-3). Virmani *et al* defined plaque rupture as a fibroatheroma with a disruption in the cap where the overlying thrombus is continuous with the lipid core (1). It was originally thought that all acute coronary events were the result of plaque rupture. However, an occlusive thrombus can also be caused by plaque erosion (4), in which plaques do show an overlying thrombus and absent endothelium at the site of erosion, but no evidence of rupture (1). A less common, but documented cause of atherothrombosis is the calcified nodule, which erupts through the fibrous cap into the lumen resulting in the formation of a thrombus (1) .

Because the etiology of coronary thrombi is diverse, it is important to characterize vulnerable, rupture-prone plaques. Plaques prone to rupture were defined as vulnerable, when these plaques displayed thin fibrous caps, large lipid cores, and macrophage accumulation (5). In a comprehensive classification scheme for atherosclerotic lesions, Virmani *et al* referred to lesions that are most likely to rupture as thin fibrous cap atheromas (1). Such plaques are characterized by a thin fibrous cap, a large lipid core, and an inflammatory infiltrate in the shoulder of the plaque. A recent study showed that acute clinical events are preceded by periods of plaque vulnerability in which successive thrombi develop, but later heal, suggesting that plaque disruption in itself is a first step towards occlusive thrombus formation and clinical events (6).

3. USE OF TRANSGENIC MICE TO STUDY PLAQUE VULNERABILITY

3.1. Models

The most widely used mouse models to study mechanisms of atherosclerosis are Apolipoprotein E-deficient (ApoE-/-) mice and LDL-receptor deficient (LDL-R-/-) mice. In the ApoE-/- mice, targeted deletion of the ApoE gene results in hypercholesterolemia, and atherosclerosis will develop spontaneously even on a normal chow diet (7). In the LDL-R-/- model, deletion of the LDL-R results in impaired LDL clearance and modestly elevated plasma cholesterol levels. Development of atherosclerosis is slow on a normal chow diet. On a high-fat-cholesterol diet, rapid development of atherosclerosis is seen (8). In both mouse models, lesions develop in a time-dependent manner starting at the proximal aorta, progressing in time towards the distal aorta, especially at bifurcations where blood flow is disturbed. The lesions progress over time from intial macrophage-foam cell-rich lesions to advanced lesions with a necrotic, lipid core, active inflammation, fibrous deposits and calcification.

Although lesion-development is comparable in ApoE-/- and the LDL-R-/- mice, there are differences between the two models. Due to the absence of the ApoE gene in the ApoE-/- mouse, plasma cholesterol levels are very high (~8-10 mmol/l on chow diet and even up to 80 mmol/l on a high fat diet) and the majority of the plasma cholesterol is carried by VLDL particles and not LDL particles as in humans. The LDL-R-/- mouse has a more human-like lipid profile and most of the cholesterol is carried by LDL particles. Another difference between these models is the base-line inflammatory status. ApoE itself inhibits the proliferation of monocytes and T-cells (9) and ApoE-/- mice have an elevated humoral immune response and an impaired cell-mediated immunity (10). In addition, ApoE modulates clearance of apoptotic bodies and this results in a systemic pro-inflammatory state in ApoE-/- mice (11). These immuno-modulatory properties are certainly relevant in interpreting results of gene manipulations in this model.

Other models are the ApoE3 Leiden model, ApoE2 knock-in model, and the ApoE-/-//LDL-R-/- double knock-out. For the LDL-R-/- mouse, the susceptibility for atherosclerosis and lipid profiles were modified by inbreeding with apobec-1-/-, resulting in increased levels of cholesterol contained in LDL particles and severe atherosclerosis (12). In another approach, LDL-R-/- mice were generated that synthesized ApoB100 exclusively, resulting in elevated cholesterol levels contained in LDL particles and enhanced atherosclerosis (13). All the models are summarized in Table 1. Of these models, the ApoE-/- and the LDL-R-/- model have been used most frequently, also as a starting point for the study of the role of other genes in the process of atherosclerosis. This has been accomplished by inbreeding, overexpression of a gene or using bone-marrow transplantation in the ApoE-/- or LDL-R-/- background.

3.2. Methodological considerations

3.2.1. Bone marrow reconstitution

A method frequently used in atherosclerosis research to study the role of certain genes is bone marrow reconstitution of ApoE-/- or LDL-R-/- mice with bone marrow from transgenic donors. In this procedure, the hematopoietic system of the recipients is replaced by cells of the donor of choice, establishing a new hematopoietic system, from which cells of lymphoid and myeloid origin such as T and B cells, platelets, monocytes and macrophages, can differentiate. An advantage of this model is the timing, the gene of choice can be studied without backcrossing to the ApoE-/- or LDL-R-/- background. For this procedure, mice receive a lethal whole body γ-irradiation and are reconstituted with either transgenic or the appropriate wild-type bone marrow. The majority of studies that have employed this method use LDL-R-/- mice as recipients (14). Bone marrow transplantation in ApoE-/- is not advisable with a transgene on an ApoE+/+ (wildtype) background, as reconstitution with the ApoE+/+ bone marrow will normalize plasma cholesterol and decrease atherogenesis (15). Although in all cases the experimental mice are compared to control mice that undergo the same procedure, the lethal whole body irradiation and replacement of the bone marrow is not without side-effects. Schiller *et al* showed that whole body irradiation affected plaque development site-specifically, accelerating aortic root lesions and reducing thoracic aorta lesions. In addition, the lesions of the aortic root were densely collagenous in

Table 1. Mouse models of atherosclerosis

Mouse	Advantages	Shortcomings	Ref
ApoE-/-	- develops atherosclerotic lesions throughout arterial tree on normal chow diet - lesions resemble human lesions (contain foam cells, necrotic core, fibrous cap, inflammation, calcification)	- lipoprotein profile differs from that in humans (plasma cholesterol carried in VLDL rather than LDL as in humans) - baseline: more pro-inflammatory status	7
LDL-R-/-	- develops atherosclerotic lesions throughout arterial tree - plasma lipid profile similar to human hyperlipidemia - excellent host for bone marrow transplantation experiments	- must be fed high fat diet to develop advanced lesions	8
ApoE3-Leiden	- exhibits hyperlipidemic phenotype similar to that in humans - excellent response to drugs and interventions (particularly lipid lowering drugs)	- must be fed diet containing cholate in order to develop atherosclerosis, however cholate induces inflammation and alters cholesterol levels and fatty acids in liver	178
ApoE-/-//LDL-R-/-	- plasma cholesterol and lipoprotein profile similar to ApoE-/- - develops extensive lesions - aorta shows massive vasovasorum neovascularization (179)	- plasma cholesterol mainly carried in the VLDL fraction	180
ApoE2 knock-in	- has 2-3 fold elevation of plasma cholesterol - develop lesions on a normal chow diet and fat diet (cholate diet is not needed)	- plasma cholesterol mainly carried in the VLDL fraction	181
LDL-R-/- Apobec-1-/-	- lesions are widely distributed - plasma cholesterol mainly carried in the LDL fraction on a chow and in both VLDL and LDL on a high-fat diet - compatible with bone marrow transplantation experiments	- for backcrossing to another transgene or knock-out extra breeding-time is needed	12
LDL-r-/-// $Apob^{100/100}$	- more extensive lesions on a chow diet compared to ApoE-/- and LDL-R-/- - plasma cholesterol mainly carried in the LDL fraction - compatible with bone marrow transplantation experiments	- for backcrossing to another transgene or knock-out extra breeding-time is needed	13
Wild-type C57BL6	- no transgenesis needed	- lesion consist mainly of foam-cell macrophages, not human-like, develop only on a high-fat diet - lesions remain small, mainly in the descending aorta	182

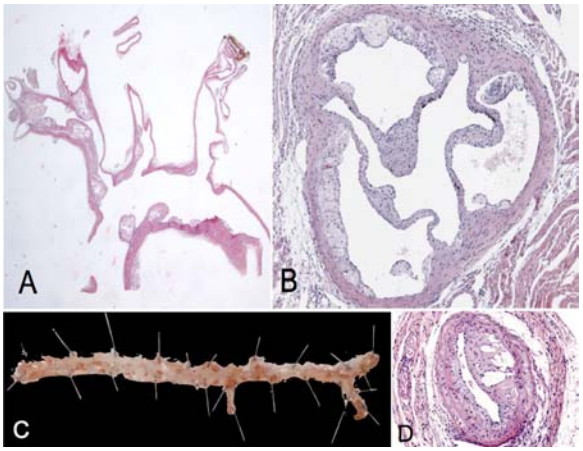

Figure 1. Assessment of atherosclerotic lesion development. Examples of typical sites. Panel A: longitudinal section of the aortic arch (hematoxylin-eosin staining) . Panel B: cross-section of the aortic sinus (hematoxylin-eosin staining). Panel C: 'en face ' preparation of the descending aorta, stained with Sudan IV. Panel D: cross-section of carotid artery with collar-induced atherosclerotic lesion (hematoxylin-eosin staining; method described in (50)).

the non-irradiated animals, while the lesions of the animals that had undergone bone marrow transplantation contained lipid cores and minimal collagen (16). This effect of γ-irradiation does not solely depend on systemic alterations as localized irradiation of the carotid arteries of ApoE-/- mice also induced accelerated atherosclerosis with lesions that were macrophage rich, with a remarkable influx of inflammatory cells, predominantly granulocytes. In addition, intraplaque hemorrhage and erythrocyte-containing macrophages were seen only in lesions of irradiated arteries (17). Thus, it is important to realize that γ-irradiation itself affects the development of atherosclerosis and lesion composition. A second point to consider in bone marrow transplantation studies is that bone-marrow derived cells have the potential to differentiate beyond the myeloid and lymphoid lineages. In an elegant study by Sata *et al*, it was shown that bone-marrow cells can give rise to the smooth muscle cells (SMCs) that contribute to the process of atherosclerosis (18). The effect of certain gene-deficiency in bone marrow transplantation studies can therefore not solely be explained by actions of the lymphoid and myeloid cell lineages in the lesions. Other limitations of bone marrow transplantation often overlooked are -1- potential differences in genetic background of donor and recipients, -2- possible effects of the transgene on bone marrow differentiation, stromal release and homing and -3- incomplete repletion of tissue leukocytes.

3.2.2. Assessment of lesion development

Assessment of atherosclerotic lesion development has been performed by various methods. A common method is cross-sectional analysis of the histology of the lesions in the aortic sinus (or root) (19), cross-sectional analysis of the brachiocephalic artery (20) or longitudinal analysis of the whole aortic arch (21) (see Figure 1 for typical examples and Table 2 for an overview). These histological analysis allow detailed description of the lesion phenotype. Remarkably, the aortic sinus is often the preferred site of analysis. However, in humans the aortic sinus is not a typical site and by exception affected by atherosclerotic disease. This is thought to be related to much more profound disturbances in flow in this region in mice (with a heart rate of ~550 beats per minute) compared to humans (~70 beats per minute) (22). Another method is the quantification of the extent of lesion involvement in the entire descending aorta by measuring stained en face

Table 2. Sites of analysis commonly used in mouse models

Site	Method/ analysis	Advantages	Shortcomings
Aortic Sinus	- cross-sectional analysis, showing 3 leaflets with lesions in 1 plane	- easily accessible, tissue slides can be prepared without extensive experience - rapid formation of lesions on a high fat diet - allows analysis of lesion composition	- not a typical site for lesion development in humans, may not be representative for human atherosclerotic lesion development
Aortic Arch	- longitudinal analysis, showing the 3 bifurcations with lesions in 1 plane	- allows analysis of several lesions at the same time - allows analysis of lesion composition	- experience is needed for dissecting and cutting of the arch in the longitudinal plane
Brachiocephalic artery	- cross-sectional analysis, showing a single lesion in this site	- allows analysis of lesion composition - exhibits a highly consistent rate of lesion progression and develops a narrowed vessel characterized by atrophic media and perivascular inflammation - site most often cited as 'rupture-prone' (in older animals)	- experience is needed for dissection of this small artery
Carotid artery – collar-induced	- peri-vascular collar induces accelerated lesion formation on a high fat diet (183) - cross-sectional analysis, showing a single lesion in this site	- lesion formation is accelerated and specifically localized proximal of the collar - allows analysis of lesion composition - allows local therapy (e.g. luminal transfection, or by applying perivascular gel)	- experience needed for placement of the collar
Descending Aorta	- en-face analyses, lipid deposits in the whole descending aorta are stained with Sudan IV or oil red O	- fast	- does not allow analysis of lesion composition - may be biased toward lesions with high lipid content (early lesions) compared to more fibrotic lesions (advanced lesions)
Coronary arteries	- cross-sectional analysis of heart, showing coronary arteries *in situ*	- allows analysis of lesion composition - relevant site (acute coronary events)	- very laborious, difficult to cut the tissue in the right plane, arteries are very small - not yet standardized as a site of analysis

dissected aortas (23). This method does not allow assessment of lesion phenotype and features of plaque stability or vulnerability. It has become clear that multiple measurements on different anatomical locations or using different methods do not always give similar results. Site-specificity in particular has been reported frequently (22), and stresses the importance of examining multiple sites of lesion formation, especially when new mechanisms are studied.

3.2.3. Genetic background

Another source of variation is the genetic background which can selectively influence atherosclerosis. Reardon *et al* showed that the response of brachiocephalic artery atherosclerosis development was sensitive to the purity of the genetic background. Two RAG1-/-//LDL-R -/- strains were studied, one that was ~93% C57BL6 (6 generations backcrossed to C57BL6) and one that was ~99% C57BL6 (10 generations backcrossed to C57BL6). RAG1-/- mice are immuno-deficient due to a lack of mature B and T cells. Two locations were studied, the brachiocephalic artery and the aortic sinus. It was shown that in the strain on a ~93% C56BL6 background, the absence of B and T cells led to a significant reduction in both the aortic sinus and the brachiocephalic artery. In the strain on a ~99% C56BL6 background, effects were site-specific, with a reduction in atherosclerosis in the aortic sinus but not in the brachiocephalic artery (24, 25). This study clearly showed that the comparison of studies with similar (genetic) interventions, can be hampered by differences in strain background or co-segregation of susceptible genes. As recently reviewed by Lusis *et al*, this could be due to 'passenger genes'. Passenger genes flanking the selected locus, may travel with the selected locus and remain present after 10 generations of backcrossing. It was calculated that if the passenger gene is 10 cM from the selected locus, the chance that it will be retained after 10 generations is 39%. A 10 cM region can contain ~200 genes and perturbation of the function of one or more of these genes could lead to unknown and unwanted side-effects. The authors strongly suggested to perform genome-wide scans to test for passenger genes both linked and unlinked to the gene of interest before using transgenic strains (26).

3.2.4. Mouse models and human end stage atherosclerosis

Mouse models have been instrumental in studying atherogenesis, but where they fall short, is the lack of resemblance to human end-stage atherosclerosis, that is plaque rupture and subsequent thrombosis. Actual plaque rupture and clinical events such as myocardial infarction are rare in mice and only seen in extreme situations or in very old mice (20, 27). Two recent papers suggested alternative approaches to induce plaque rupture in ApoE-/- mice. Sasaki *et al* combined ligation and subsequent non-constrictive cuffing of the carotid artery of an ApoE-/- mouse and showed a disruption of the ligation-induced macrophage-rich neo-intima, intra-lesional hemorrhage and thrombosis. Although this model is fast and efficient, the basis is a macrophage-rich intima, rather then a typical ApoE-/- atherosclerotic lesion, located in artery without flow (28, 29). An alternative approach was presented by Johnson *et al*, in which ApoE-/- mice were fed a high fat, cholesterol-enriched diet for up to 9 weeks. The brachiocephalic artery showed small disruptions of the

endothelial layer and thrombotic material was present in the lumen. In addition, the lesions contained more 'buried' fibrous caps, a feature that the authors associated with plaque rupture. Although there was an increased mortality rate in these animals, endothelial disruptions were small and the actual cause of death could not be established (30).

Both studies were recently discussed in a review series that reflect the discussion in the field whether or not a 'true' mouse model for plaque rupture exists (31, 32). On the one hand, Jackson and colleagues have described that the brachiocephalic artery of hypercholesterolemic ApoE-/- mice shows the presence of atherosclerotic lesions with histological features that are suggestive of plaque vulnerability and occasional rupture. Furthermore, it is advocated that mice are not humans and new considerations are recommended to define plaque rupture in mice that are reflective of human plaque rupture (32, 33). On the other hand, Schwartz and colleagues advocate that some of the histopathological criteria to define plaque rupture in mice are misleading and question the relevance of the 'buried fibrous caps' as manifestations of plaque rupture. The authors emphasize that only specific features of plaque vulnerability can be reproduced in mouse models and that it is yet impossible to reproducibly study the entire process of human plaque rupture which should also include thrombotic features, in mice (31, 34).

In the following sections we will discuss aspects of atherogenesis as determined by specific end-points (34), namely changes in apoptosis, lesional lipid metabolism (related to size of the lipid core), inflammatory activity, matrix remodeling (formation of a fibrous cap/protease activation), and thrombotic pathways. Some of these end-points, such as inflammatory activity and thrombotic pathways are also key-features of human vulnerable lesions, as described by Virmani *et al* (1). Changes in apoptosis, lesional lipid metababolism and matrix remodeling are thought to be mechanistically involved in other end-points such as a large necrotic core and a thin fibrous cap. Given the aforementioned limitations (31, 35), studies in transgenic mice have given insight in the impact of deletion or overexpression of certain genes in these specific end-points and lead to useful knowledge on the mechanisms of plaque development and progression and in some cases plaque rupture/vulnerability.

4. LESSONS LEARNED FROM GENETICALLY ENGINEERED MICE

In the following sections, we will focus on (recent) studies that used transgenic mice to study mechanisms of atherosclerotic lesion development and progression. Several excellent reviews are cited for further reading.

4.1. Manipulation of apoptosis

Apoptosis of lesional cells, including macrophages, SMCs and possibly endothelial cells is thought to be important in atherogenesis. This is supported by the observation that apoptotic rates of macrophage and SMCs are increased during lesion progression. Macrophages compose approximately 50% of all apoptotic cells in advanced lesions (36) and this is thought to lead to enhanced lipid-rich necrotic core development. The overall effect of apoptotis on plaque stability or vulnerability has been the subject of intense investigations for which several transgenic mouse models have been used.

4.1.1. Apoptosis in initial lesions

p53 is a tumor suppressor gene that encodes a transcription factor that activates genes regulating growth arrest and apoptosis. p53 is considered a pro-apoptotic factor (37), but the cellular response depends on the level of p53 expression, the presence of other pro- or anti-apoptotic factors, the cell type and importantly the stage of lesion development. Thus, p53 -/- bone marrow transplantation in ApoE3 Leiden mice (38) and LDL-R-/- mice (39, 40) increased early lesion size, suggesting that p53-induced apoptosis limited lesion cellularity and progression. Although it should be noted that p53 is pleotropic in nature and may affect also other processes relevant to atherosclerosis such as cellular metabolism and regulation of cell senescence (41). Manipulations in other apoptotic pathways also showed the impact of apoptosis for lesion development. Like p53, Bax is a pro-apoptotic protein and bone marrow transplantation of Bax-/- bone marrow in LDL-R-/- recipients increased early lesion area and decreased the number of apoptotic macrophages (42). It is of interest that these studies all used bone marrow transplantation to study the effect of genetic deficiency of lesion development, suggesting that p53- and Bax- induced apoptosis of hematopoetic lineage-derived cells (i.e. macrophages) plays an important role in lesion development. However, the data on proliferation and apoptosis of specific cell-types that were affected by the genetic manipulation in these apoptotic pathways was controversial. Increased rates of cell proliferation were shown in the p53-/- mice, while apoptosis was unaffected (39, 40) while in the other study apoptosis was reduced, but cell proliferation was unaffected (38). Thus the role of p53 in proliferation and apoptosis of different lesion cell types is not clear yet.

Direct intervention in macrophage apoptosis pathways in other studies did suggest that macrophage apoptosis is important in atherosclerosis, at least in early lesion development. Arai *et al* studied the role of Aim (apoptosis-inhibitor expressed by macrophages, Spα/Api6), a macrophage survival protein in atherosclerosis development in AIM-/-//LDL-R-/- mice. Of interest, prior genomic analysis was performed to ensure that all mice that were included in this study were homozygous for B6 alleles and genetically identical. In the AIM-/-//LDL-R-/- mice, lesion development was markedly decreased in the aortic root region and along the aorta using en face analysis. The amount of macrophages in the lesions and total number of apoptotic cells were also significantly reduced in the AIM-/-//LDL-R-/- mice, suggesting that loss of AIM increased macrophage apoptosis and decreased early lesion development (43). Using an elegant design in which a novel transgenic ablation strategy was used, Stoneman *et al* also investigated the impact of macrophage apoptosis on lesion development. The mouse strain used expresses the human diphtheria toxin receptor under control of the

monocyte/macrophage-specific CD11b promoter. Treatment with diphtheria toxin conditionally ablates monocytes and macrophages. When mice were treated during early plaque development, plaque area was reduced by 50% and plaque composition was altered, with reduced collagen content and necrotic core formation (44). The number of macrophages in the lesions was not affected, thus it is not known whether treatment with diphtheria toxin was effective (enough) in ablating lesional macrophages. In comparison, mice with a natural ablation of macrophage-colony stimulating factor (M-CSF), also called osteopetrotic (op) mice, displayed a reduced number of blood monocytes and tissue macrophages and on a ApoE-/- background the number and size of atherosclerotic lesions was markedly reduced (~35 fold) (45). Thus, complete ablation of macrophages in the op/op//ApoE-/- mice was more effective than partial ablation of macrophages by apoptosis in reducing atherogenesis. Nevertheless, the studies by Arai *et al* and Stoneman *et al* showed that deficiency of a macrophage survival factor and direct induction of macrophage apoptosis affected initiation of atherosclerotic lesions.

4.1.2. Apoptosis in advanced lesions

The impact of macrophage apoptosis on advanced lesions is less clear. In the study of Stoneman *et al*, macrophage apoptosis by diphtheria toxin treatment was also induced in mice with established lesions. Diphtheria toxin treatment did induce macrophage apoptosis and reduced macrophage content, but did not affect plaque area or composition (44). This was unexpected as macrophage death in advanced lesions was predicted to enlarge the necrotic core, especially as advanced human lesions were shown to have defective clearance of apoptotic bodies (46, 47). It is possible that in this model sufficient phagocytic cells were present to clear apoptotic bodies and necrotic debris. This could be the remaining macrophages (still ~50%) or SMCs which were found to be highly effective phagocytes in a study by the same laboratory (48). In this study by Clarke *et al*, the impact of SMC apoptosis was investigated using the same diphtheria toxin model, but now the human diphtheria toxin receptor (hDTR) was under control of a minimal Tagln promoter, also known as SM22α. In normal arteries, diphtheria toxin induced 50-70% loss of SMCs, but did not cause inflammation, reactive proliferation, remodeling or aneurysm formation. In contrast, SMC apoptosis in established atherosclerotic lesions of SM22α-hDTR ApoE-/- mice induced marked fibrous cap thinning, loss of collagen, accumulation of cell debris and intense inflammation (48). These studies show that selective SMC apoptosis in established lesions has great impact on plaque composition and induced some of the key features present in human vulnerable lesions (active inflammation, thin cap, large necrotic core), while selective macrophage apoptosis has only limited effects on advanced murine lesions.

Finally, p53-induced apoptosis was also further investigated in established lesions by Mercer *et al.* p53-/-//ApoE-/- mice showed increased descending aorta plaque formation and increased plaque area in the brachiocephalic artery after 14 weeks of high fat diet. Cell proliferation rates were increased, while apoptotic rates were decreased in the brachiocephalic artery. Apoptotic cells were both macrophages and SMCs. To examine the separate contributions of p53-induced macrophage and SMC cell-turnover in the brachiocephalic artery, p53+/+ bone marrow was reconstituted in p53-/- mice. This renders a transgenic mouse with 'normal' wildtype hematopoetic cell lineages but p53-deficient SMC and stromal cells. Surprisingly, these mice showed reduced aortic plaque formation, but no change in brachiocephalic artery plaque area (which is another example of site-specificity). Furthermore, both cell proliferation and apoptosis were reduced in the brachiocephalic artery. Further *in vitro* studies showed that endogenous p53 protected SMCs and stromal cells against apoptosis, but promoted apoptosis in macrophages. This study showed that the pro-apoptotic effect of p53 was indeed dependent on the cell-type. Thus, endogenous p53 protected lesional SMCs against apoptosis and reduced atherosclerosis (49). A puzzling observation in this study is that using a p53 deficiency model it was concluded that endogenous p53 decreased apoptosis, while overexpression of p53 in a model of collar-induced atherosclerosis resulted in a marked decrease in the cap-to-intima ratio of the lesion due to apoptosis and induced features of plaque vulnerability and even rupture (50). The same phenomenon was found for Fas-ligand. Fas is a apoptosis-mediating receptor, and upon binding of Fas, apoptosis is initiated. Endothelial overexpression of Fas-ligand in ApoE-/- mice decreased lesion formation at several different anatomical locations (51). However, adenoviral-mediated Fas-ligand gene transfer in advanced carotid lesions reduced the number of cap cells and induced intraplaque hemorrhage, buried caps and iron deposits two weeks after transfection, albeit in a minority of the animals (52).

In conclusion, depending on the model, cell type, type of intervention and stage of atherosclerosis development, apoptosis has been shown to contribute to plaque growth (see Table 3 for overview). In the mouse models, macrophage apoptosis seems less important in plaque progression. It should be noted that in the studies using bone marrow transplantation, intervention in apoptotic pathways could have influenced bone marrow differentiation, homing and number of circulating cells. This could have affected the development of atherosclerosis, independent of plaque apoptosis. As for plaque vulnerability, induction of apoptosis at the right time and place was shown to induce plaque rupture in collar-induced atherosclerosis.

4.2. Manipulation of lesional lipid uptake and efflux

During atherogenesis, cholesterol and modified lipoproteins accumulate in the vessel wall in macrophage foam cells. Normal control of macrophage lipid metabolism is a highly regulated system with a balanced uptake and export. In the atherosclerotic vessel wall, equilibrium between cholesterol export and uptake in macrophage foam cells is no longer maintained. Using mouse models, the effect of interventions in lipid uptake into and efflux out of the lesions and the effect on atherogenesis have been studied extensively. Here, we will focus on several key-

Table 3. Apoptosis

			Features of plaque vulnerability							
Transgene	**Intervention**	**Lesion size**	**Apoptosis**	**Necrotic core**	**Inflammation**	**Matrix content**	**Intraplaque hemorrhage**	**Site investigated**	**Gender-specific?**	**Ref**
p53-/-	BMT ApoE3-Leiden	Early +	-	Necr index +	Mp +	ND	ND	Aortic sinus	ND	38
p53-/-	BMT LDL-R-/-	Early +	=	ND	ND	ND	ND	Desc. aorta (en face)	ND	40
p53-/-	BMT LDL-R-/-	Early +	=	ND	ND	ND	ND	Aortic sinus; desc. aorta (en face)	ND	39
Bax-/-	BMT	Early +	Mp -	ND	ND	ND	ND	Aortic sinus	ND	42
Aim	Aim-/-//LDL-R-/-	Early -	-	ND	Mp -	SMC - Collagen -	ND	Aortic sinus; desc. aorta (en face)	ND	43
CD11b hDTR	BMT CD11b hDTR in ApoE-/-	Early – Adv =	+ +	Size – Size =	Mp – Mp -	Collagen - Collagen =	Not present Not present	Brachioc. art ; desc. aorta (en face)	ND	44
SM22α-hDTR	SM22α-hDTR//ApoE-/-	Adv =	SMC +	Size +	+	Collagen -	Not present, Cap thickn -	Aortic sinus; brachioc. art	ND	48
p53-/-	p53-/-//ApoE-/-	Adv + and =	SMC -	ND	Mp =	Collagen = SMC =	=	Desc. aorta (en face) ; brachioc. art	ND	49
p53-/-	p53+/+ BMT to p53-/-//ApoE-/-	Adv - and =	SMC -	ND	Mp =	SMC =	=	Desc. aorta (en face) ; brachioc. art	ND	49
p53-/-	p53-/- local overexpresion	Adv =	+	=	Mp =	=	Cap thickn. - Rupture	Carotid (collar-induced)	ND	50
FasL	FasLigand EC overexpression in ApoE-/-	-	=	ND	Mp -, CD4 =, CD8 -	ND	ND	Desc. aorta (en face); Aortic Arch, ThAo, AbAo	ND	51
FasL	FasLigand local overexpression	Adv =	+ cap cells	=	=	=	+ (infrequent) Iron deposits	Carotid (collar-induced)	ND	52

Symbols and abbreviations: '+' means increase, '–' means decrease, '=' means no difference, AbAo = addominal aorta, adv = advanced lesion, BMT = bone marrow transplantation, brachioc. art = barchiocephalic artery, desc. aorta = descending aorta, EC = endothelial cell, early = early lesions, hDTR = human diphtheria toxin receptor, Mp = macrophage content, ND= not determined/measured, SMC = smooth muscle cell content, thickn = thickness, ThAo = thoracic aorta.

players such as scavenger receptor and ATP binding cassette transporters and compare the results of studies that have used genetically-engineered mice (see Table 4).

4.2.1. Scavenger receptors

Cholesterol uptake is mediated via several scavenger receptors (SR). SRs most studied for their involvement in atherosclerosis are the class A scavenger receptor (SR) AI and AII, the class B SRs CD36, BI and BII and the class E SR LOX-1 (53). For SR-A, the results of studies using transgenic mice are not consistent. Although deficiency of SR-A reduced lesion area in hyperlipidemic LDL-R-/- mice and ApoE-/- mice (54 and reviewed in (59)), overexpression of SR-AI/II in LDL-R-/- or ApoE-/- mice either did not affect atherosclerosis (55, 56) or reduced atherosclerosis in the aortic arch area (57). Furthermore, a recent study in SR-AI/II-/-//ApoE-/- mice (back-bred 7 generations on C57BL6) showed increased plaque areas in the aortic sinus of male mice, however plaque area of female mice was not affected. Further *in vitro* analysis showed a reduction in macrophage foam cell formation of male SR-AI/II-/-//ApoE-/- peritoneal macrophages but not in female macrophages (58). Thus, the actual role of SRAI/AII in the development of atherosclerosis remains to be clarified. As reviewed by Linton *et al* (59), SR-A deficiency appeared to have more pronounced effects on atherosclerosis in ApoE-/- mice than in LDL-R-/-, which could be due to the anti-inflammatory and anti-oxidant properties of ApoE itself. In ApoE-/- mice, the absence of ApoE could aggravate the pro-atherogenic effects of SR-AI. Thus, it is likely that the outcome of studies that have investigated the impact of SR-AI deficiency or overexpression have been influenced by the choice of the model, genetic background and extent of hyperlipidemia. Also, it is important to realize that this receptor is involved in induction of macrophage foam cell apoptosis and the clearance of apoptotic cells (60). As mentioned earlier, ApoE itself is also involved in the clearance of apoptotic cells (11), thus in a setting in which both SR-BI and ApoE are lacking, synergistic effects on apoptotic cell clearance are expected. Finally, there are no studies yet that have described the impact of either deficiency or overexpression of the SR-AI/AII receptor on established lesions.

The class B SR CD36 accounts for ~60-70% of the accumulation of (mildly) oxidized LDL (oxLDL) in macrophage foam cells (61). Studies with genetically-engineered mice in general pointed to a pro-atherogenic role of CD36 (58, 62-64). There are however, some discrepancies. In one study, CD36 deficiency in ApoE-/- mice decreased plaque aortic area (measured en face), and decreased plaque area in the aortic sinus of male but not of female mice (64). Moore *et al* also showed a decrease in aortic plaque area in female mice by en face analysis, however aortic sinus plaque area was increased. Male mice

Table 4. Lesional lipid metabolism

			Features of plaque vulnerability							
Transgene	**Intervention**	**Lesion size**	**Apoptosis**	**Necrotic core**	**Inflammation**	**Matrix content**	**Intraplaque hemorrhage**	**Site investigated**	**Gender-specific?**	**Ref**
SR-A-/-	SR-A-/- (BL6) SR-A-/- BMT in LDL-R-/-	- -	ND ND	ND ND	ND ND	ND ND	ND ND	Aortic sinus Aortic sinus; desc. aorta (en face)	No No	54
SR-A tg overexp	SR-A tg BMT in ApoE-/-	=	ND	ND	ND	ND	ND	Aortic sinus	ND	56
SR-A Tg overexp	SR-A tg BMT in ApoE-/-	=	ND	ND	ND	ND	ND	Aortic sinus; desc. aorta (en face)	ND	55
SR-A Tg overexp	SR-A Tg BMT in LDL-R-/-	-	ND	ND	ND	ND	ND	Aortic sinus; desc. aorta (en face)	ND	57
SR-A-/-	Sr-A-/-//ApoE-/-	+/=	ND	=	Mp =	SMC =	ND	Aortic sinus; desc. aorta (en face)	Yes (m +, f =)	58
CD36-/-	CD36-/- BMT in ApoE-/-	-	ND	ND	Mp =	ND	ND	Aortic sinus; desc. aorta (en face)	No	63
CD36-/-	CD36-/-//ApoE-/-	Adv – sev. timepoint	ND	ND	ND	No Calc. in CD36-/-//ApoE-/-	ND	Aortic sinus; desc. aorta (en face)	ND	62
CD36-/-	CD36-/-//ApoE-/-	-	ND	ND	ND	ND	ND	Aortic sinus; desc. aorta (en face)	No	64
CD36-/-	CD36-/-//ApoE-/-	=/+	ND	=	Mp – in f	SMC =	ND	Aortic sinus; desc. aorta (en face)	Yes (m =, f +)	58
SR-BI-/-	SR-BI-/-//ApoE-/-	Complete occlusion	ND	+ but ND quantitative	ND	ND	Fibrin+	Coronary artery	ND	67
SR-BITg overexp	SR-BI Tg inj. in LDL-R-/-	-	ND	ND	ND	ND	ND	Aortic arch (en face)	ND	72
SR-BI-/-	SR-BI-/- BMT in LDL-R-/-	+	ND	ND	ND	ND	ND	Desc. aorta and aortic arch (both en face)	ND	71
SR-BI-/-	SR-BI-/- BMT in LDL-R-/-	+	ND	ND	ND	ND	ND	Aortic sinus; desc. aorta (en face)	ND	74
SR-BI-/-	SR-BI-/- BMT in LDL-R-/-	- early + adv	ND	+ in adv but but ND quantitative	ND	ND	ND	Aortic sinus	ND	75
Lox1-/-	Lox-/-//LDL-R-/-	-	ND	ND	ND	ND	ND	Desc. aorta (en face), aortic arch, AbAo, ThAo	ND	76
ABCA1 Tg	ABCA1 Tg (BL6) ABCA1 Tg/ApoE-/-	- +	ND	ND	ND	ND	ND	Aortic sinus	No	78
ABCA1 Tg	ABCA1Tg/LDL-R-/-	-	ND	ND	ND	ND	ND	Aortic sinus	No	80
ABCA1 Tg	ABCA1Tg/ApoE-/-	-	ND	ND	ND	ND	ND	Aortic sinus	ND	79
ABCA1 Tg	ABCA1Tg BMT in LDL-R-/-	- sev. timepoints	-	Size -	Mp -	Collagen -	ND	Aortic sinus	ND	81
ABCA1-/-	ABCA1-/- BMT in LDL-R-/-	+	ND	ND	Mp -	Collagen +	ND	Aortic sinus	ND	83
ABCG1-/-	ABCG1-/- BMT in LDL-R-/-	-	ND	ND	Mp =	SMC =	ND	Aortic sinus	ND	84
ABCG1-/-	ABCG1-/- BMT in LDL-R-/-	+	ND	ND	ND	ND	ND	Aortic sinus	ND	86
ABCG1-/-	ABCG1-/- BMT in LDL-R-/- and ApoE-/-	- -	+ ND	ND ND	ND ND	ND ND	ND ND	Aortic sinus; desc. aorta (en face)	ND	85
ABCG1-/-	ABCG1-/- (BL6 background)	+	ND	ND	ND	ND	ND	Aortic sinus	ND	88
ABCG5/G8 Tg	ABCG5/G8 Tg/LDL-R-/-	-	ND	ND	ND	ND	ND	Aortic sinus; coronary artery	ND	89
ABCB4-/-	ABCB4-/- BMT in LDL-R-/-	+	ND	+	Mp =	ND	ND	Aortic sinus	ND	90
NPC+/-	NPC+/-//ApoE-/-	= (two separate exp)	+	+	ND	ND	ND	Aortic sinus	ND	92

Symbols and abbreviations: '+' means increase, '–' means decrease, '=' means no difference, AbAo = abdominal aorta, adv = advanced lesion, BL6 = C57BL6 background, BMT = bone marrow transplantation, Calc = calcification, desc aorta = descending aorta, early = early lesion, inj = injection, f = female, m = male, Mp = macrophage content, ND= not determined/measured, sev. = several, SMC = smooth muscle cell content, thickn = thickness, ThAo = thoracic aorta.

showed no differences (58). These authors ascribed the different effects on aortic sinus plaque area to the background of the mice. In their study mice were back-crossed 7 generations to C57BL6, while in the other study this was 4 times (64). These two studies were discussed in an accompanying editorial by Witztum who also stressed the impact of background on susceptibility to atherosclerosis. Also, it was noted that the two studies not only were different in the anatomical site that was analyzed but also in the time point of analysis. While Moore *et al* studied 1 time-point (after 8 weeks of high fat diet), other studies investigated later time points (9-12 weeks of high fat diet) (65). The group of Febbraio recently studied the long-term effects of CD36 deficiency and showed that lesion size continued to be smaller in CD36-/-//ApoE-/- mice fed a western type diet for 35 weeks (62). Comparison of these studies (see also Table 4) emphasizes that to allow comparison between studies, one needs to investigate the impact of the intervention at both early and late time points, at different anatomical sites and in both sexes.

SR-BI has a high homology to CD36, but a different function in lipid metabolism. SR-BI mediates selective cholesterol uptake from and transfer to HDL, and

is important in reverse cholesterol transport (66). Mice deficient in SR-BI and ApoE develop severe dyslipidemia and exhibit several features of human coronary disease, such as occlusive coronary atherosclerosis, myocardial infarction and cardiac hypertrophy. Of note, most reports discussed thus far that intervened in lipid homeostasis focused on plaque size and in general lacked evaluation of features of plaque stability or vulnerability (see also Table 4). The study by Braun *et al* is an exception as plaque phenotype was thoroughly investigated. The coronary arteries of SR-BI-/-//ApoE-/- mice showed features of plaque vulnerability, such as lipid-rich cores, inflammation and contained fibrin deposits. If these were due to actual ruptures was not shown. Also, the extent of atherosclerosis at other locations was not reported on. Unfortunately, these mice die prematurely at 6 weeks of age, which limits their use as a model of plaque vulnerability (67).

Two subsequent studies investigated whether the phenotype of the SR-BI-/-//ApoE-/- can be rescued. Yu *et al* showed that ApoE+/+ bone marrow transplantation in the SR-BI-/-//ApoE-/- mice rescued the lethal phenotype by improving dyslipidemia and reducing lesion development (68). The same group also showed that the severely altered cholesterol homeostasis in the SR-BI-/-//ApoE-/- macrophages was partly due to the high plasma lipid levels and defective intracellular cholesterol trafficking, including intracellular free cholesterol sequestration, lysosomal lipid engorgement and inefficient cholesterol efflux to HDL and ApoA1 (69). In the second study, the premature death was overcome by the generation of a new inbred strain, the SR-BI-/- hypomorphic ApER61 mouse, that is SR-BI deficient and has <5% expression of hepatic ApoE expression. These mice had no evidence of coronary disease unless challenged with a high fat diet, which caused 50% mortality ~32 days after onset of the diet. In this study, aortic root lesions were 25 times larger compared to SR-BI-/- mice on a high fat diet, but phenotypic features were not specifically reported. The phenotype of the coronary lesions was similar to that reported in the SR-BI-/-//ApoE-/- mice (70). This transgenic mouse represents an interesting model of coronary heart disease, although it is not clear yet whether the fibrin deposits in the coronary lesions are caused by true plaque ruptures.

Other mouse studies that investigated the role of SR-BI in atherosclerosis in general confirm the protective role of SR-BI in the development of atherosclerosis (71-73). This was partly attributed to macrophage expression of SR-BI as bone marrow transplantation studies with SR-BI-/- promoted atherosclerosis (71, 74). However, this effect may also be time-dependent. Van Eck *et al* showed that macrophage SR-BI deficiency increased the development of advanced lesion after 9-12 weeks of high fat diet. However, after 4 weeks of high fat diet, macrophage SR-BI deficiency reduced fatty streak formation (75). Thus, depending on the stage of lesion development SR-BI is either pro- or anti-atherogenic, and this is likely to involve interaction with other SR-BI dependent pathways.

Finally, the Class E SR, Lox-1 is a receptor for oxLDL in endothelial cells and recently the role of this SR was studied using transgenic mice. Mice deficient in Lox-1 developed less atherosclerosis both on the wild-type and LDL-R-/- background after a high fat diet for 18 weeks (76).

4.2.2. ATP-binding cassette transporter family

The effects of genes and proteins involved in lipid efflux has also been studied using transgenic mice. Members of the ATP-binding cassette (ABC) transporter family modulate circulating and tissue cholesterol levels (reviewed in (77)) and several members have been linked to cardiovascular disease. ABCA1 was one of the first ABC transporters studied in transgenic mice. Overall overexpression of ABCA1 reduced aortic lesion development in C57BL6 on a high fat diet (78), ApoE-/- and LDL-R-/- mice, albeit not in all studies (78-80) (see Table 4). Macrophage-specific overexpression was shown to inhibit lesion progression in LDL-R-/- mice. Interestingly, lesions were collagen-rich, with smaller necrotic cores and less apoptotic macrophages (81). The latter observation fits with the reported role of ABCA1 in the engulfment of apoptotic cells (82). Macrophage deficiency by ABCA1-/- bone marrow transplantation in LDL-R-/- mice resulted in larger and more advanced lesions (83). Overall, these studies point to athero-protective effects of ABCA1. Part of the beneficial effects may be explained by the role of this transporter in the clearance of apoptotic cells.

While ABCA1 exports cholesterol and phospholipids to lipid-free apolipoproteins, ABCG1 promotes cholesterol efflux from cells to HDL and other lipoprotein particles but not to lipid-free ApoAI (77). ABCG1 is a key regulator of macrophage lipid metabolism and its deficiency would be expected to deteriorate lesion development. However, three studies all using bone marrow transplantation in LDL-R-/- to identify the role of macrophage ABCG1 deficiency on atherogenesis did not come to the same conclusion (84-86). While Out *et al* reported that macrophage ABCG1 deficiency moderately increased atherosclerosis in LDL-R-/- mice, Baldan *et al* and Ranalletta *et al* observed an atheroprotective effect. These studies were compared in an editorial by Curtiss ((87) and Table 4). The methodological differences were significant and most likely responsible for the variation in outcome. Again the degree of hyperlipidemia, genetic background and most importantly standard assessment of disease initiation rate and progression at multiple anatomical sites were mentioned as potential confounders. As rightfully expressed by Curtiss, the standard assessment of disease rate will be a technical challenge for all studies using genetically-engineered mice. In a follow-up study, Out *et al* further investigated the role of ABCG1 in which hyperlipidemic ABCG1-/- mice on a C57BL6 background were used. Again a moderate increase in lesion size was reported. The authors proposed that the effect of ABCG1 deficiency is determined by the level of serum cholesterol. Above a critical level of 900 mg/dL, induction of other efflux mechanisms may dominate or ABCG1 deficiency affects other processes that enhance atherogenesis at high cholesterol levels (88).

Other ABC transporters that have been studied less intensively include ABCG5/G8 and ABCB4. For ABCG5/G8, overexpression in LDL-R-/- mice attenuated the development of atherosclerosis. Plasma cholesterol levels were also significantly decreased, so it remains to be determined whether this could totally explain the decrease in lesion area or whether ABCG5/G8 overexpression on lesional cells was also involved (89). Recently, Pennings *et al* reported on the role of ABCB4 in lesion development. The role of ABCB4 in cholesterol metabolism is not fully elucidated yet, however macrophage deficiency of ABCB4 in LDL-R-/- mice increased lesion area and increased the size of the necrotic core, despite lower serum cholesterol levels (90).

For human lesions, it is hypothesized that the loss of equilibrium in lipid metabolism during early lesion development can induce macrophage apoptosis, leading to smaller lesions with less lipid/necrotic core, rendering an anti-atherogenic pathway. At late stage lesion development however, deficient lipid loading or efflux may lead to loss of macrophage function and phagocytotic potential and subsequent macrophage apoptosis. This may lead to enhanced lipid/necrotic core formation and is considered a pro-atherogenic pathway (47, 91). However, the effect of interventions in macrophage lipid uptake or efflux and macrophage apoptosis (see previous paragraph) have been studied as separate entities in most studies. Few experimental studies yet have linked interventions in macrophage lipid metabolism to apoptosis, size of the lipid/necrotic core, progression of atherosclerosis or plaque vulnerability (see Table 4). Feng *et al* did associate intracellular cholesterol trafficking and lesion development, using Niemann-Pick C heterozygous mice. NPC1+/- macrophages have a defect in trafficking of lipoprotein cholesterol to the endoplasmic reticulum which inhibits free-cholesterol apoptosis despite increased cellular stores of free cholesterol. Analysis of advanced lesions of NPC+/-//ApoE-/- mice showed a marked decrease in late lesional necrosis and macrophage apoptosis. Lesion area was unchanged. Thus, this study shows that intact intracellular cholesterol trafficking is important for the regulation of macrophage apoptosis and necrotic core formation (92).

In conclusion, transgenic mice have been central in studies designed to delineate the role of key players such as SR and ABC transporter family members in atherogenesis. Although this has given a wealth of data, results have not always been consistent and this was attributed to differences in strain background, analysis method, time-points and anatomical sites investigated and extent of hyperlipidemia. For the control of plaque vulnerability, Table 4 shows that in many of the discussed studies, characterization of the lesions was limited to plaque area measurements and the majority of studies did not assess size of the necrotic core or other features of plaque vulnerability. Thus firm conclusions on whether or not scavenger receptors and ABC transporters are involved in plaque vulnerability cannot be made. The studies with SR-BI//ApoE-/- mice are an exception in displaying acute coronary events, similar to those in humans and these mice can thus be considered as a model for vulnerable lesions. Although it will be a challenge to standardize the analysis of the coronary lesions, this model will be very valuable for future intervention studies.

4.3. Manipulation of inflammatory activity

Numerous cytokine and cytokine receptors are expressed in atherosclerotic lesions. It is well known that inflammation is an important mechanism in both the initiation and progression of atherosclerosis (93, 94). There is an extensive amount of papers using transgenic mice to study inflammatory mechanisms during atherogenesis and clearly these studies have been instrumental in our current understanding. Several excellent reviews on the role of cytokines and chemokines have been published recently (95-97). To avoid overlap, we will focus on novel insights in the field, coming from papers published in 2007. These include new studies on the role of the CC-chemokine receptor 5 (CCR5) and tumor necrosis factor receptors (TNF-R), the effect of blockade of interferon (IFN)-γ and the impact of other (subsets of) immuno-regulatory cells.

4.3.1. CCR5

CCR5 is a chemokine receptor expressed on monocytes/macrophages and T-lymphocytes. Earlier studies have shown that total body deficiency of CCR5 in ApoE-/- mice did not reduce early plaque development (98). CCR5-/- bone marrow transplantation in LDL-R-/- mice also did not affect lesion size, but did induce features of improved plaque stability as judged by a decreased inflammatory cell content and an increased collagen content (99). The effect of CCR5 deficiency on diet-induced, more advanced atherosclerosis was investigated by two laboratories in a joined paper, showing that CCR5 deficiency reduced plaque area at two anatomical locations (aortic root and distal aorta), in two independent strains of CCR5-/- mice and at two different time-points. Interestingly, the same studies were performed in CCR1-deficient mice, but in these mice development of diet-induced atherosclerosis was increased, pointing to an athero-protective role of CCR1. Further analysis of the plaque phenotype in the CCR5-/-//ApoE-/- mice showed features of plaque stability, such as reduced numbers of lesional macrophages, increased SMC content and a reduced Th1-type pro-inflammatory response (100). In a second study, Quinones *et al* specifically studied the effect of CCR5 deficiency on late-stage atherosclerosis. Here it was confirmed that CCR5 deficiency did not influence early atherosclerosis, but did protect against advanced lesion development at different time-points, both on a normal chow and a high-fat diet. Interestingly, these differences were found in the aortic sinus but also in the coronary arteries, an anatomical site rarely studied in mice models. In addition, adoptive transfer studies showed that the atheroprotective effect of CCR5 deficiency resided in the hematopoetic cell lineage as bone marrow transplantation resulted in a similar phenotype. In contrast, transfer of purified T cells in thymectomized CCR5-/-//ApoE-/- mice did not affect plaque size (101). Both Braunersreuther *et al* and Quinones *et al* used a multi-faceted approach and showed a consistent pro-atherogenic effect of this chemokine receptor.

4.3.2. TNF

The pro-inflammatory cytokine TNF exerts its effects by activating membrane receptors TNF receptor-I (TNF-R1 or p55) and TNF-RII (or p75). Mice deficient in TNF and ApoE-/- have reduced development of atherosclerosis and this was largely ascribed to TNF produced by hematopoetic cell-lineages (102). Lesion progression and plaque composition were not altered in older p55-/-//ApoE-/- mice (assessed in brachiocephalic artery) (103), although an earlier report demonstrated less diet-induced atherosclerosis in the aortic sinus of p55-/- wild-type mice (104). Chandrasekharan *et al* recently showed that the p75 receptor is required for TNF-induced leukocyte-endothelial interaction *in vivo* (105) and the same authors also showed reduced lesion areas in 16 wk old chow-fed p75-/-//ApoE-/- mice (105). For further investigation of the p55 receptor, Zhang *et al* used an alternative approach to delineate the role of production of TNF by leukocytes or resident vascular wall cells in the development of atherosclerosis. To this end, carotid artery-to-carotid artery interposition grafting was performed with p55-/- and wildtype mice as donors and ApoE-/- mice as recipients. Wildtype carotid grafts demonstrated more atherosclerosis than p55-/- grafts suggesting that arterial wall TNF receptor p55 expression contributed to the development of this graft-induced atherosclerosis. En face analysis showed that atherosclerosis in the thoracic aorta was also decreased slightly in 60 week old female p55-/-//ApoE-/- (106). The magnitude of reduction in the thoracic aorta was significantly less compared to the carotid grafts and different compared to earlier studies (103), which could be explained by either differences in method of analysis or site-specific effects. These studies suggest that depending on the anatomical location, both TNF-receptors are involved in initial and advanced atherosclerosis.

4.3.3. INF-γ

INF-γ is considered a pro-inflammatory cytokine produced by T cells and natural killer (NK) cells, and promotes Th1 immune responses (96). Earlier studies in LDL-R-/- and ApoE-/- mice have shown that deficiency of INF-γ or its receptors reduced atherosclerosis at several anatomical sites, although there appeared to be a gender-specific effect (107, 108). Koga *et al* recently explored the role of IFN-γ in progression of atherosclerotic plaques and used postnatal blockage of IFN-γ by repeated gene transfers of soluble mutant of IFN-γ receptor (sIFN-γR) in ApoE-/- mice. sIFN-γR treatment was started at 12 weeks of age and prevented plaque progression and decreased accumulation of intra-plaque macrophages and lipid and increased SMC content, which were interpreted as features of plaque stability (109). Lesions were only investigated in the aortic sinus area and this site is not susceptible to atherosclerosis in humans. Therefore it would have been interesting to include mice at an older age, to study the effects of sIFN-γR treatment on more advanced lesions and at other anatomical locations. This would strengthen the conclusion that (therapeutic) intervention in a pro-inflammatory pathway after lesion initiation was effective in reducing lesion progression. This effect of IFN-γ blockade was not specific for advanced lesions as second paper from the same authors showed that this treatment was also effective in reducing early plaque formation (110).

4.3.4. Macrophage subsets

Inflammatory cells are involved in every stage of atherogenesis and the focus has evolved from macrophages to T cells and recently to subsets of macrophages and T cells and other inflammatory cell types such as natural killer T (NKT) cells, dendritic cells and mast cells. The role of different subsets of T cells has been recently reviewed (96, 111). In 2007, attention also turned to macrophage subsets. In two back-to-back papers, the role Ly-6^{hi} and Ly-6^{lo} monocytes was studied in atherosclerosis. Swirsky *et al* used ApoE-/- mice on a high fat diet and showed that the hypercholesterolemia was associated with a dramatic increase in the Ly-6^{hi} monocyte subset. The Ly-6^{hi} cells adhered to activated endothelium, infiltrated in the lesion and differentiated in lesional macrophages (112). In the second study, Tacke *et al* employed a sophisticated labeling technique and showed that both Ly-6^{lo} and Ly-6^{hi} monocytes entered the lesions, although the entry of Ly-6^{hi} monocytes was 20 times higher. The Ly-6^{lo} monocytes were more prone to develop into plaque cells expressing the dendritic cell marker CD11c. In addition, these monocyte subsets used different ways of entry, while Ly-6^{lo} monocytes used a CCR5-dependent pathway, the infiltration of the Ly-6^{hi} subset unexpectedly appeared to be mediated not only by CCR2 but also by CX3CR1 and CCR5. It was also speculated that CX3CR1 may be a selective therapeutic target to modulate monocyte entry into atherosclerotic lesions (113, 114).

4.3.5. NKT and dendritic cells

Other inflammatory cell types recently examined for their role in atherogenesis were NKT cells and dendritic cells. VanderLaan *et al* used an adoptive transfer model to selectively reconstitute or deplete NKT cells in immune-deficient, functional T and B cell-lacking RAG1-/-//LDL-R-/- mice. Transfer of mature peripheral lymphocyte populations of the spleen of Vα14Jα18 TCR transgenic mice rendered NKT cell enriched mice, and transfer of CD1d-/- mice rendered NKT cell deficient mice. NKT cell enrichment resulted in an increased plaque area in the aortic root, but not in the aortic arch. Although the results were site-specific, this study demonstrated that NKT cells are pro-atherogenic even in the absence of an exogenous stimulus (115, 116). Dendritic cells have also gained attention for a potential role in atherosclerosis. Shaposhnik *et al* used GM-CSF-/- mice to determine the effect on dendritic cell content of atherosclerotic lesions on a LDL-R-/- background. GM-CSF is known to promote dendritic cell formation *in vitro* and aortic sections of GM-CSF-/-//LDL-R-/- showed a dramatic decrease (60%) in CD11c+ dendritic cell content, but no change in overall content of monocytic cells. Plaque area was also significantly decreased, albeit in female mice only and this was ascribed to the decrease in dendritic cell content (117).

4.3.6. Mast cells

The involvement of mast cells in atherogenesis was investigated in two recent papers. Mast cells have several functions that may affect plaque progression. Mast cells release histamine that affect (micro)vascular permeability, and produce cytokines such as TNF-α. Mast cell-derived chymase and tryptase can activate matrix

Table 5. Inflammation - summary of studies published in 2007

			Features of plaque vulnerability							
Transgene	**Intervention**	**Lesion size**	**Apoptosis**	**Necrotic core**	**Inflammation**	**Matrix content**	**Intraplaque hemorrhage**	**Site investigated**	**Gender-specific?**	**Ref**
CCR5-/- CCR1-/-	CCR5-/-//ApoE-/- CCR1-/-//ApoE-/-	- + and =	= ND	ND ND	Mp -. T cell – T cell +	SMC – SMC =	ND ND	Aortic sinus; desc. aorta (en face)	ND ND	100
CCR5-/-	CCR5-/-//ApoE-/- CCR5-/- BMT in ApoE-/-	= early - adv - adv	ND ND	ND ND	ND Mp -	ND ND	ND ND	Aortic sinus ; coronary artery Aortic sinus	No ND	101
p75-/-	p75-/-//ApoE-/-	-	ND	ND	ND	ND	ND	Aortic sinus	ND	105
p55-/-	p55-/- and +/+ grafts in ApoE-/-	-	ND	ND	Mp =	SMC =	ND	Carotid	ND	106
sIFNγR gene transfer	sIFNγR i.m. in ApoE-/-	- adv - early	ND ND	- - but ND quantitative	Mp – - but ND quantitative	SMC +, matrix + - but ND quantitative	ND ND	Aortic sinus; desc. aorta (en face) Aortic sinus; desc. aorta (en face quantitative	ND ND	109, 110
Vα14Jα18 TCR Tg CD1d-/- (NKTcells)	Vα14Jα18 BMT in RAG-/-//LDL-R-/- CD1d-/- BMT in RAG-/-//LDL-R-/-	+ and = = and =	ND ND	ND ND	ND ND	ND ND	ND ND	Aortic sinus ; Aortic arch Aortic sinus ; Aortic arch	ND ND	115
GM-CSF -/- (DC)	GM-CSF-/-//LDL-R-/-	- and =	ND	ND	Mp =, DC – CD4 -	Elastin content – SMC =, collagen =	ND	Aortic sinus; desc. aorta (en face)	Yes (f -, m =)	117
$Kit^{W\text{-}sh/W\text{-}sh}$ (mast cells)	$Kit^{W\text{-}sh/W\text{-}sh}$//LDL-R-/-	-	-	-	Mp -, T-cell -	Collagen +, Cap thickn. +	ND	Aortic arch; desc. aorta (en face)	ND	121

Symbols and abbreviations: '+' means increase, '–' means decrease, '=' means no difference, adv = advanced lesion, BL6 = C57BL6 background, BMT = bone marrow transplantation, DC = dendritic cells, desc. aorta = descending aorta, early = early lesion, f = female, i.m. + intra-muscularly, m = male, Mp = macrophage content, ND= not determined/measured, sev. = several, SMC = smooth muscle cell content, thickn = thickness.

metalloproteinases (MMP) and cathepsins. In addition, mast cell chymase and tryptase degrade HDL and thus affect cholesterol efflux from lesions (118, 119). Bot *et al* used systemic activation of mast cells during plaque progression in ApoE-/- mice which led to a robust plaque expansion. Local activation of perivascular mast cells by applying dinitrophenyl-albumin in a gel around the lesion led to increased incidence of plaque hemorrhage, macrophage apoptosis and vascular leakage, all features of increased plaque vulnerability. All these features could be prevented by mast cell stabilization (by the mast cell stabilizer cromolyn) (120). In a second study, Sun *et al* showed that mast cell-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice on a LDL-R-/- background had decreased lesion size, lipid deposition, T cells and macrophage numbers, and increased collagen content and fibrous cap development, all features suggestive of a more stable plaque phenotype. The mechanism did not involve mast cell-derived TNF-α as adoptive transfer of TNF-α-deficient or wild type mast cells both restored atherogenesis. In contrast, similar plaque phenotypes were found after adoptive transfer of IL-6 and IFN-γ-deficient mast cells, suggesting that part of the mechanism by which mast cells affected atherogenesis involved mast cell-derived IL-6 and IFN-γ . *In vitro* studies confirmed that IL-6 and IFN-γ-deficient mast cells did not induce expression of matrix-degrading proteases from vascular cells. This study suggests a direct participation of mast cells and mast cell-derived IL-6 and IFN-γ in mouse atherogenesis(121).

In conclusion, manipulation of immunomodulatory pathways during atherogenesis has been investigated extensively using transgenic mice. It should be noted that the studies in this field using ApoE-/- mice may have been biased to some extent by the activated immune response due to the deficiency of ApoE (see paragraph 3.1). Recent studies investigating pro-inflammatory mediators (e.g. CCR5) have applied a more multifaceted approach, combining results from independent laboratories, using different strains, analyzing different anatomical sites and time-points (see Table 5). Recent studies also focused on other inflammatory cell types such as NKT cells and dendritic cells. Studies published in 2007 mainly used transgenic mice with interventions in or deficiencies of pro-inflammatory mediators, resulting in decreased lesion size and a more stable plaque phenotype in the majority of studies. Thus in view of the control of plaque vulnerability, inhibition of (these) pro-inflammatory pathways or cell types will stabilize lesions.

4.4. Manipulation of matrix remodeling (protease activation) and calcification

One of the features associated with human plaque rupture is a thin and collagen-poor fibrous cap. The lack of collagen or other extracellular matrix components is the result of impaired synthesis by plaque SMCs and increased matrix breakdown. Increased rates of SMC apoptosis (see 4.1) at late stage atherosclerosis will indirectly result in decreased deposition of extracellular matrix. Increased synthesis and activity of proteases such as MMPs and cathepsins are responsible for increased matrix breakdown, thinning of the fibrous cap and ultimately plaque rupture (2).

4.4.1. Matrix metalloproteinase's

Transgenic mice either deficient or overexpressing MMPs have been used to study plaque progression and events related to plaque rupture (reviewed in (122, 123)). Johnson *et al* compared lesion size and phenotype of MMP3-, MMP7-, MMP9- and MMP12-deficient ApoE-/- mice to single ApoE-/-knock-out mice (124). Lesions in the brachiocephalic artery were significantly larger in the MMP3-/-//ApoE-/- and MMP9-/-//ApoE-/- mice than in controls. In both MMP3 and MMP9 double knock-outs, lesion phenotype showed more features of plaque vulnerability such as reduced SMC content and increased number of macrophages, albeit only in the

MMP9 knock-outs. Lipid core size or T-cell numbers were not quantified. The lesions of the MMP3 and MMP9 double knock-outs also contained more 'buried fibrous caps', This is a feature that these authors have associated with features of plaque vulnerability in other studies (32, 33). MMP7 deficiency did not change lesion area, but did increase the SMC content. MMP12 deficiency reduced plaque area, increased SMC content and decreased macrophage content. It was concluded that MMP3 and MMP9 may protect against atherosclerosis, MMP7 was relatively neutral and MMP12 induced lesion expansion and features of plaque vulnerability (124). Other studies, however, have reported different results. Luttun *et al* showed decreased plaque areas and macrophage content in MMP9-/-//ApoE-/- mice, while MMP12 deficiency did not affect plaque area and macrophage content. Site of analysis in this study however, was the descending aorta, and not the brachiocephalic artery (125). Also, the background of the mice used in both studies was unusual, namely mixed C57BL6 and 129 in (124) and mixed C57BL6, 129SvJ, 129SSJ, CDI and 129SvEvTac in (125), which could also explain the controversial results.

Other studies have shown that depending on the site of expression, MMP9 (over)expression can induce features of plaque rupture. Thus, transient overexpression of MMP9 via luminal adenoviral delivery in a pre-existing collar-induced lesion, induced outward remodeling, cap thinning and increased the incidence of intra-plaque hemorrhage. The latter was associated with the presence of neo-vessels (126). Gough *et al* transplanted macrophages expressing an autoactivating form of MMP9 in ApoE-/- mice and showed enhanced elastin degradation and significant plaque disruption in the brachiocephalic artery. Lesion showed fibrin deposits and intraplaque hemorrhage but no luminal thrombi. In this model, ruptures of advanced lesions could be reproducibly induced, clearly suggesting that mouse lesions can rupture if sufficient amounts of active protease is expressed at the right place and time (127).

Other interventions that can manipulate MMP activity in atherosclerotic lesions are either deficiency or overexpression of the natural inhibitors of MMPs, the tissue inhibitors of matrix metalloproteinase's (TIMPs). For TIMP1, discrepant results were reported in studies that used TIMP1-/-//ApoE-/- mice or overexpressed TIMP1 in ApoE-/- mice. Plaque area was decreased in the TIMP1-/-//ApoE-/- mice (128) and in 1 study adenoviral delivery also decreased plaque area as well ((129), but had no effect in the other study (130). Study set-up however, was different. In the studies in which the effect of overexpression was studied, one study analyzed the aortic sinus (and found decreased plaque areas) and the other study analyzed the brachiocephalic artery (and found no differences). In the knock-out study the aortic root was analyzed. This is again an example in which differences in study set-up led to distinct results. Interestingly, in the same study where TIMP1 overexpression had no effect on plaque size, TIMP2 overexpression resulted in a marked reduction of plaque size, increased SMC and collagen content and reduced macrophage content and reduced frequency of buried fibrous caps. These were interpreted as features of increased plaque stability. This study also presented a methodological aspect: conventional first generation adenoviral delivery can be used to study short term effects (up to 4 weeks), while helper-dependent virus technology can be used to examine the effects of long-term delivery (up to 11 weeks) (130).

4.4.2. Cathepsins

Fewer studies have used transgenic mice to investigate the effect of cysteine proteases such as cathepsins. Cathepsins have collagenolytic and elastolytic properties and are also involved in major histocompatibility complex class II antigen presentation, apoptosis and lipid metabolism (131, 132). Both cathepsin K (CatK) and cathepsin S (CatS) deficiency on a ApoE-/- or LDL-R-/- background reduced plaque area and increased plaque matrix content. The mechanism by which CatS deficiency prevented plaque growth involved reduced degradation of medial elastic laminae and reduced leukocyte transmigration (133, 134). CatK deficiency increased collagen content, but also affected macrophage foam cell formation. Macrophage foam cells displayed increased lipid uptake, which was stored in lysosomes that had increased in size (135). Subsequent microarray and pathway analysis suggested that CatK deficiency altered plaque phenotype not only by decreasing proteolytic activity, but also by stimulating TGF-β signaling, and suggested a role for caveolin-1 and CD36 in the lipogenic phenotype of CatK-deficient atherosclerotic lesions (136). These data suggest that CatK and CatS deficiency may have a protective role in atherosclerosis by increasing fibrosis; however, cathepsin K deficiency also aggravated foam cell formation in mice. Cathepsin L (CatL) was recently investigated using CatL-/-//LDL-R-/- mice. It was shown that CatL deficiency significantly decreased plaque size and lipid core size, reduced collagen levels, T cells, macrophages and SMCs. The reduced plaque collagen content in the deficient mice levels seems contradictionary with the elastinolytic and collagenolytic properties of CatL. However, it was hypothesized that the reduced elastinolysis and better-preserved internal elastica delayed SMC migration, and subsequently caused reduced presence of SMC in the lesion and reduced collagen levels (137).

4.4.3. Calcification

Vascular calcification refers to the deposition of calcium phosphate minerals, frequently in the form of hydroxyapatite in the vessel wall. Calcification is frequently present in the intimal area of human atherosclerotic lesions. The mechanism by which lesions become calcified is not known but may involve dystrophic calcification, and endochondal or direct ossification (138). Until a decade ago, it was regarded as a passive, degenerative process, however the expression of several bone-related regulatory proteins such as bone morphogenetic proteins (BMPs) and osteoprotegerin (OPG) already at the stage of intimal thickening suggests an active regulated process (139). The significance of lesion calcification for clinical events is still debated in literature. Virmani *et al* described a specific lesion type characterized by a calcified nodule that is prone to induce plaque rupture

Table 6. Protease activation

			Features of plaque vulnerability							
Transgene	**Intervention**	**Lesion size**	**Apoptosis**	**Necrotic core**	**Inflammation**	**Matrix content**	**Intraplaque hemorrhage**	**Site investigated**	**Gender-specific?**	**Ref**
MMP3-/- MMP7-/- MMP9-/- MMP12-/-	MMP3-/-//ApoE-/- MMP7-/-//ApoE-/- MMP9-/-//ApoE-/- MMP12-/-//ApoE-/-	+ = + -	ND ND ND ND	ND ND ND ND	Mp = Mp = Mp + Mp -	SMC = SMC = SMC + SMC –	IH =, Buried FC + IH =, Buried FC = IH =, Buried FC + IH =, Buried FC –	All brachioc. artery	ND ND ND ND	124
MMP9-/- MMP12-/-	MMP9-/-//ApoE-/- MMP12-/-//ApoE-/-	- =	ND ND	ND ND	Mp -	Media ruptures – Media ruptures –	ND ND	aorta (cross-sec); desc. aorta (en face)	ND ND	125
MMP9 Tg	Local Overexpression	=	ND	ND	Mp =	Collagen =	+	Carotid (collar-induced)	ND	126
Active MMP9	Active MMP9 BMT in ApoE-/-	=	ND	ND	Mp =	Collagen =	+, fibrin +, FC breaks +	Brachioc. artery; desc aorta (cross-sec)	ND	127
TIMP1-/-	TIMP1-/-//ApoE-/-	-	ND	+	Mp+	= (tendency to +)	=	Aortic sinus	ND	128
TIMP1 Tg	TIMP1 Tg in ApoE-/-	-	ND	ND	Mp + (not meas.))	=	ND	Aortic sinus	ND	129
TIMP1 Tg TIMP2 Tg	TIMP1 Tg in ApoE-/- TIPM2 Tg in ApoE-/- (2^{nd}generation virus)	= -	= -	= =	= Mp -	= Collagen +, SMC +	= Buried FC -	Brachioc. artery	ND ND	130
CatS-/-	CatS-/-//LDL-R-/-	-	ND	-	Mp -, T cells -	Collagen - , elastin -, FC -, SMC -	ND	Desc. aorta (en face) ; aortic arch	ND	134
CatS-/-	CatS-/-//ApoE-/-	-	ND	=	Mp =	Collagen – (early), elastin -, FC+	Buried FC -	Brachioc. artery	ND	133
CatK-/-	CatK-/-//ApoE-/-	-	ND	=	=	Collagen +	-	Aortic arch	ND	135
CatL-/-	CatL-/-//LDL-R-/-	-	ND	-	Early Mp - Adv Mp =	Collagen -, Early SMC - Adv SMC =	ND	Aortic arch	ND	137

Symbols and abbreviations: '+' means increase, '–' means decrease, '=' means no difference, adv = advanced lesion, BL6 = C57BL6 background, BMT = bone marrow transplantation, Brachioc. artery = brachiocephalic artery, cross-sec = cross-sectional analysis, DC = dendritic cells, early = early lesion, f = female, FC= fibrous cap, IH = intraplaque hemorrhage, m = male, Mp = macrophage content, ND= not determined/measured, not meas. = not measured quantitively, SMC = smooth muscle cell content, thickn = thickness.

(1). Other histological studies by Burke *et al* showed that calcification is a reliable marker for plaque vulnerability. Acutely ruptured lesions were more likely to demonstrate calcification then thin-capped atheromas, and healed ruptures showed the highest calcification score (140). In addition, calcification was found preferentially in lesions with outward remodeling, a feature associated with increased risk of rupture (141). On the other hand, it was also shown by the same group that the correlation between coronary calcification and Framingham risk index in patients with sudden cardiac death was only modest (142). In addition, several intravascular ultrasound studies suggested that ruptured lesions of patients with acute coronary events contained less calcium (143, 144), although it was also shown this decrease in calcium was mainly superficial calcium, while the number of small calcium deposits and especially deep calcium deposits increased (145).

Rattazzi *et al* characterized calcification of advanced atherosclerotic lesions in the brachiocephalic artery of ApoE-/- mice and showed that calcification was initiated in younger mice and became apparent in mice between 45-60 weeks of age. By 75 weeks, lesions were highly calcified with 100% frequency. In contrast to calcification in human lesions, chondrocyte-like cells were responsible for depositing hydroxyapatite, mainly via the process of endochondral ossification. Features such as calcified nodules were not reported. In addition, starting at 60 weeks of age a high frequency of chondrocyte-like cells and calcification was observed in the medial layer (~60% of the lesions) (146). In human arteries, medial artery calcification, also known as Mönckenberg's sclerosis, occurs commonly in aged and diabetic individuals and patients with chronic kidney disease and is a strong predictor of coronary artery disease and future cardiovascular events (138). However, Mönckenberg sclerosis can be present independent from atherosclerosis (147), which was not reported to be the case in the aged ApoE-/- mice.

From these studies it is clear that vascular calcification differs in human and mouse vessels. In mice, medial calcification associated with the lesions is a prominent feature. Also, although plaque ruptures have been reported to occur in the brachiocephalic artery of aged ApoE-/- mice (42-54 weeks of age) (20), Rattazzi *et al* did not report on presence of ruptures or features of plaque vulnerability in these highly calcified lesions (146). Intervention studies in which extent of calcification is manipulated in transgenic mice are limited. A recent study in transgenic mice does suggest that calcification of mice lesions is actively regulated by bone-related regulatory proteins. Bennett *et al* studied OPG deficient ApoE-/- mice and showed accelerated lesion progression and increased areas of intimal and medial calcification, suggesting that OPG inhibits plaque progression and calcification. Features of plaque vulnerability in these aged mice (60 weeks) were not reported (148), again suggesting that extensive calcification in mice is not associated with increased risk for plaque disruption.

In conclusion, although results have not always been consistent, transgenic mice in which protease activity has been manipulated have shown that MMPs have diverse functions in regulating plaque matrix content. As shown in Table 6, these studies were more complete in the analysis of plaque phenotype. As expected, MMP deficiency or

overexpression resulted in changes in matrix content, but also changes in the content of inflammatory cells and in some cases intraplaque hemorrhage or plaque rupture. In particular, overexpression of (active) MMP9, either in the fibrous cap or in macrophages has lead to actual plaque rupture in these mice models. There are less studies yet describing the role of cathepsins in atherogenesis. Studies thus far advocate that cathepsin activity is pro-atherogenic. However, in the case of CatK, deficiency also aggravated macrophage lipid uptake and storage, which is unwanted in terms of plaque stability. Studies in which certain cathepsins are overexpressed are not yet available, but are expected to give novel insights for the role of these proteases in the control of plaque vulnerability.

Finally, mice and human lesions do not have the same pattern for intimal and medial calcification. Although it is still debated whether calcification of human atherosclerotic lesions is associated with higher risk for cardiovascular events, medial calcification was predictive for cardiovascular events in diabetic and chronic kidney disease patients. As no reports were made on increased features of plaque vulnerability in the calcified lesions of aged ApoE-/- mice, there does not appear to be an association between plaque vulnerability and calcification in atherosclerotic mice.

4.5. Manipulation of lesional hemorrhage and coagulation factors

4.5.1. Intraplaque hemorrhage and angiogenesis

Intraplaque hemorrhage may contribute to plaque vulnerability and has been shown to occur in human atherosclerotic lesions. Intraplaque hemorrhage is thought to mediate the progression of asymptomatic into vulnerable plaques, as it results in the rapid accumulation of erythrocytes in the plaque, accompanied by macrophage infiltration. Erythrocyte membranes are a rich source of free cholesterol, which contributes to the enlargement of the necrotic core (149). Recently, it was suggested that a likely source of erythrocytes in lesions are leaky, immature vessels within the plaque (150). The role of neovascularization in plaque progression has recently been reviewed (151, 152) and shown to be increased in lesions of patients with acute coronary syndromes (153). In addition, a recent study followed patients over an 18-month period using magnetic resonance imaging to investigate whether intraplaque hemorrhage in carotid artery lesions stimulates plaque progression. Indeed, it was found that hemorrhage into carotid lesions was also associated with carotid atherosclerotic plaque progression (154). Another important factor that may contribute to plaque vulnerabilty is intraplaque hemorrhage, which has been observed in mouse plaques (tables 3-7). The source of intraplaque hemorrhage in humans is thought to occur via microvessels that are present in advanced lesions. These vessels mediate plaque progression by allowing the rapid accumulation of cholesterol-rich erythocyte membranes inside the plaque that contribute to enlargement of the lipid core (149). Although intraplaque hemorrhage was observed in mouse studies (tables 3-7), the link with plaque neovascularization was either not determined or the number of micro-vessels was too small to draw firm conclusions. Studies by Moulton *et al* (155, 156) in ApoE-/- mice investigated the role of angiogenesis and atherosclerosis by the use of angiogenic inhibitors, which significantly reduced plaque area and progression. However, the occurence of intimal vessels was relatively low and whether they had a direct effect on plaque development was inconclusive. Mouse lesions are relatively small and the question is whether adaptive hypoxia-driven neovascularization in these lesions is required. It was calculated that neovascularization is needed when tissue is >100 μm from a capillary (157). In the studies by Moulton *et al*, even the larger lesions (>250 μm thick) showed occasional presence (in ~30% of larger lesions) of intimal vessels, suggesting that intimal vessels are not expected to be abundantly present in mouse lesions . The studies by Moulton *et al* also showed an enhanced presence of adventitial vaso vasorum at (some) sites of advanced lesions. The significance of these vessels for plaque progression is also less clear and needs further investigations. Administration of vascular endothelial growth factor (VEGF), a major inducer of angiogenesis, was shown to promote atherosclerosis in mice deficient in apoE and apoB100 (158). However, other studies suggest that VEGF may not play an angiogenesis-specific role in plaque development. Antibodies directed against Flk-1, the major receptor for VEGF-mediated angiogenesis, had no effect on plaque development (159) and adenoviral transfer of various VEGF isoforms in LDLR/apoB48 double knockout mice had no effect on plaque growth or neovascularization (160). In a recent study by Lucerna *et al*, VEGF-A was focally overexpressed (by adeno-viral transfection) in advanced collar-induced atherosclerotic plaques in ApoE-/- mice. VEGF-A treatment of pre-existing lesions was seen to promote plaque expansion, with a concomitant increase in macrophage and lipid content, whereas it lowered collagen content. In addition, VEGF-A-treated plaques displayed more features of a vulnerable phenotype, such as intraplaque hemorrhage, cap breaks, and intramural thrombi. However, as shown in other studies, VEGF-A overexpression was not accompanied by increased microvessel development in the neointima, suggesting that VEGF-A may destabilize atherosclerotic plaques through an angiogenesis-independent mechanism (161). Thus, the role of plaque neovascularization in mouse atherosclerosis, intraplaque hemorrhage and plaque vulnerability remains unclear.

4.5.2. Coagulation factors

Coagulant proteins such as tissue factor (TF) and factor VII are expressed in human atherosclerotic lesions (162-164). It was shown that macrophages express and secrete TF, particularly at sites of macrophage apoptosis (165), suggesting a link between macrophage viability and thrombotic features. However, a 50% reduction of TF in all cells (TF+/-//ApoE-/-) or a selective reduction in hematopoietic cell-derived TF (TF+/- BMT in LDL-R-/-) did not affect advanced lesion size (34 wks of age). In this study different anatomical sites were investigated using two different methods (en face and cross-sectional analysis of the aortic root) (166). Mice with increased TF activity due to deficiency of TF pathway inhibitor (TFPI+/-//ApoE-/-), showed more atherosclerosis (34 wks of age, two anatomical locations) (167), suggesting that either TF-

Table 7. Coagulation factors

			Features of plaque vulnerability							
Transgene	**Intervention**	**Lesion size**	**Apoptosis**	**Necrotic core**	**Inflammation**	**Matrix content**	**Intraplaque hemorrhage**	**Site investigated**	**Gender-specific?**	**Ref**
TF+/-	TF+/-//ApoE-/- TF+/- BMT in LDL-R-/-	= =	ND ND	ND ND	Mp = ND	SMC = ND	ND ND	Aortic sinus; desc. aorta (en face) ; brachioc.artery ; carotid artery	ND	166
TFPI +/-	TFPI +/-//ApoE-/-	+	ND	ND	ND	ND	ND	Desc. aorta carotids (both en face)	ND	167
Fn-/-	Fn-/-//Apo(a) Tg	Early -	ND	ND	ND	ND	ND	Aortic sinus	Yes (f -, m ? (n too low))	170
Fn-/-	Fn-/-//ApoE-/-	Early -, Adv = (semi-quant)	ND	ND	ND	ND	ND	Aortic sinus	ND	168
Fn Tg	Fn Tg in ApoE3Leiden mice	=	ND	ND	=	=	=	Aortic sinus	No	169
Fn-/-	Fn-/-//apobec1-/-//LDL-r-/-	+ (different time-points)	ND	More adv (semi-quant analysis)	ND	Aortic sinus; desc. aorta (en face)	ND	(171)		
FV Leiden (thromb+)	FV+/+//ApoE-/-	Early =, Adv +	ND	ND	ND	ND	Fibrin + in Adv	Thoracic aorta (en face)	ND	173
FVIII-/-	FVIII-/-//ApoE-/-	Early -, Adv - (less prominent)	ND	ND	Mp -	Early Collagen -	Iron =	Aortic sinus	ND	172

Symbols and abbreviations: '+' means increase, '–' means decrease, '=' means no difference, adv = advanced lesion, BMT = bone marrow transplantation, desc. aorta = decending aorta, early = early lesion, f = female, FC= fibrous cap, Fn = fibrinogen, IH = intraplaque hemorrhage, m = male, Mp = macrophage content, ND= not determined/measured, semi-quant = semi-quantitative analysis, thromb = trombin, SMC = smooth muscle cell content.

independent mechanisms were responsible for the effect of TFPI deficiency, or the differences between these studies were caused by the diversity in study design and analysis.

Other coagulation proteins have also been studied using transgenic mice. Fibrinogen (Fn) deficiency in ApoE-/- only mildly affected development and phenotype of atherosclerotic lesions. Lesions appeared slightly less extensive in 22 wk old Fn-/-//ApoE-/- using a semi-quantitative score, but this was no longer apparent at 26 and 31 wks of age (168). In addition, fibrinogen overexpression did not enhance atherosclerosis in ApoE3-Leiden transgenic mice (169). Fibrinogen deficiency in Apo(a) transgenic mice did reduce lesion size at 16 wks of age after 2 months of high fat diet (170). In contrast, fibrinogen deficiency on a LDL-R-/-//Apobec1-/- background increased lesion size at 24-48 weeks of age and lesions appeared to be more advanced (171). Thus, results for the effect of fibrinogen on atherogenesis are inconclusive, and this is most likely caused by differences in the model used and site of analysis. Deficiency of intrinsic factor VIII (FVIII) in ApoE-/- mice resulted in less early-stage atherosclerosis (8 wks after high-fat diet), despite higher plasma cholesterol levels. However, this effect was less prominent, but still significant in the advanced lesions. Surprisingly, intraplaque hemorrhage or fibrin deposits as a sign of less efficient coagulation were not observed (172). Increased thrombin generation in Factor V Leiden mice resulted in enhanced atherosclerosis but only in advanced lesions (52 wks of age). In addition, lesions contained significantly more fibrin, which was interpreted as a feature of increased plaque vulnerability (173). Another important regulator of thrombosis is the plasminogen activator system. For a discussion on the role of this system in atherogenesis, the reader is referred to a recent review by Fay *et al* (174).

In conclusion, several genetic and mechanical interventions have resulted in the occurrence of intraplaque hemorrhage in mouse lesions, albeit that the mechanisms are still to be elucidated. The link with angiogenesis could not be made in most of the studies, most likely because murine atherosclerotic lesions contain only a small amount of vessels. The new concept of angiogenesis as a contributor to plaque vulnerability has therefore, not been reproduced in mouse models. If intraplaque hemorrhage is viewed as a sign of late stage or very advanced atherosclerosis, its presence does suggest lesion progression up to a certain end-point, albeit that the mechanisms are still to be elucidated. The central location of most massive intraplaque hemorrhages at the border of the media and the intima/lesion may point to a role of penetrating vessels of the vaso vasorum, although this needs to be further investigated. Studies using genetically-engineered mice with interventions in coagulation factors are not abundant yet and have given unexpected results. Reduction of key coagulant factors such as TF did not affect atherogenesis. In contrast, in human atherogenesis, signs of thrombosis are most prominent at late stage disease. In the majority of the discussed mice studies intraplaque hemorrhage was either not determined or not affected. Thus far, only increased thrombin activity (in Factor V Leiden mouse) led to more advanced atherosclerosis and fibrin deposits in the lesions (see Table 7). Coagulation factors such as thrombin have other cellular functions, such as stimulation of SMC proliferation and migration and evoking an inflammatory response of endothelial cells (reviewed in (175)). These functions could contribute to plaque progression. Another point to consider is the differences in mouse and human fibrinolysis, in mice the balance is more towards fast fibrinolysis and thrombi will resolve much faster (176). These differences in fibrinolysis have to be considered in the interpretation of studies describing the effects of coagulation factors in atherosclerosis and thrombosis.

5. CONCLUSIONS

Atherosclerosis is a complex, progressive inflammatory disease of the large systemic arteries and the leading cause of death in the Western World. The quest to better understand the mechanisms leading to progression of human atherosclerotic plaques have lead to the emergence of a variety of mouse models. ApoE-/- and LDL-R-/- mice have been used most frequently. It is important to reflect on a number of methodological issues when interpreting the results of studies using these transgenic mice. First, mouse lesions are not human lesions and certain limitations of the mouse lesion have to be taken into account. Plaque vulnerability is well defined for human lesions, but not yet for mouse lesions. Certain features of 'human' plaque vulnerability such as large necrotic cores and inflammatory activation are present in mouse lesions, but it is yet impossible to reproducibly study the entire process of plaque rupture in mice. When measuring features such as lipid core burden, intraplaque hemorrhage, matrix remodeling, apoptotic rates and inflammatory activity to assess the impact of a gene-intervention in transgenic mice, it is important to be as complete as possible. Lesion size is a poor measure of plaque progression. If the objective is to study lesion development, plaque composition needs to be determined in great detail, next to the measurement of plaque size, and at several stages of the disease.

Furthermore, bone marrow transplantation has proven to be an important tool to study the effect of (gene-) intervention in hematopoetic cell lineages, but lesion composition is likely to be affected by the systemic irradiation. Also, site-specificy has to be considered as a source of discrepant results between studies. The gene of interest may affect atherogenesis differently on diverse anatomical locations. It is important to examine multiple sites of lesion formation, especially when new mechanisms are studied. Although the aortic sinus is a site commonly used for analysis (see Table 2), this site is rarely affected by atherosclerotic disease in humans. Other sites such as the brachiocephalic artery may be more suitable for comparison to human atherosclerotic disease.

Genetic background is another important issue. Certain 'passenger genes' could affect study outcome, and more importantly, this effect is either unknown or unspecified. It was suggested recently to perform genome-wide scans to test for passenger genes both linked and unlinked to the gene of interest before using transgenic strains (26). It is clear that differences in outcome after interventions in the same gene or sets of genes have been caused by these factors. The importance of standardization of analysis, using different anatomical sites, uniform genetic background, and considering the impact of different levels of hyperlipidemia was also emphasized in several editorial comments that accompanied the experimental studies.

In this review, we discussed aspects of atherogenesis as determined by specific end-points, namely changes in apoptosis, lesional lipid metabolism (related to size of the lipid core), inflammatory activity, matrix remodeling (formation of a fibrous cap and protease activation), and thrombotic pathways. A number of interventions in mice resulted in fibrin deposits, intraplaque hemorrhage, or coronary thrombosis, all of which have shown to be associated with rupture of human vulnerable lesions. These interventions were: induction of SMC apoptosis in advanced lesions, SR-BI-/- deficiency in ApoE-/- mice, local overexpression of MMP9 in the luminal endothelium, transplantation of macrophages with an active MMP9 construct, luminal overexpression of VEGF-A and increased activity of thrombin in Factor V Leiden mice. These studies have shown that in the case of MMP9 and SR-BI, mouse lesions can rupture when the intervention is done at the right time and place. The mechanisms for intraplaque hemorrhage in mice lesions are yet to be elucidated but its presence does suggest that mice lesions can progress up to a certain end-point, that occurs in mice as well as in human lesions.

Animal models have been and will be invaluable in elucidating the pathobiology and complex processes of atherosclerosis. An animal model would ideally develop lesions that are histologically reminiscent to human lesions, and their lipid metabolism and fibrinolytic system should also resemble that in humans. Lesions in animal models should also have the same propensity to disrupt as in humans and result in (occlusive) thrombus formation. This may be too much to ask of an animal model. Instead we suggest to use transgenic mice to delineate and validate pathways involved in plaque progression and take into account the unique properties of mouse lesions which may differ from human lesions.

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