Cell cycle and apoptosis genes in atherosclerosis

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Chapter 3

Macrophage Rb and atherosclerosis

Macrophage Retinoblastoma deficiency leads to enhanced atherosclerosis development in ApoE-deficient mice

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ABSTRACT

The cellular composition of an atherosclerotic lesion is determined by cell infiltration, proliferation and apoptosis.The tumor suppressor gene retinoblastoma (Rb) has been shown to regulate both cell proliferation and cell death in many cell types.

To study the role of macrophage Rb in the development of atherosclerosis, we used apoE-deficient mice with a macrophage-restricted deletion of Rb (Rb^{del} mice) and control littermates (Rb^{fl} mice). After 12 weeks feeding a cholesterol-rich diet, the Rb^{del} mice showed a 51% increase in atherosclerotic lesion area with a 39% increase in the relative number of advanced lesions.Atherosclerotic lesions showed a 13% decrease in relative macrophage area and a 46% increase in relative smooth muscle cell area, reflecting the more advanced state of the lesions.The increase in atherosclerosis was independent of *in vitro* macrophage modified lipoprotein uptake or cytokine production.Whereas macrophage-restricted Rb deletion did not affect lesional macrophage apoptosis, a clear 2.6-fold increase in lesional macrophage proliferation was observed.

These studies demonstrate that macrophage Rb is a suppressing factor in the progression of atherosclerosis by reducing macrophage proliferation.

Cardiovascular disease (CVD) has long been the leading cause of mortality and disability in developed countries, and it is rapidly becoming the number one killer in developing countries.[1] The primary cause of CVD is atherosclerosis, which is a multi-factorial complex disease that starts in childhood and progresses throughout life. Atherosclerosis is initiated by subendothelial accumulation of cholesterol-engorged macrophages. Gradually, these lesions develop towards more advanced lesions characterized by increased deposition of extracellular lipid cores, fibrous material, and necrosis covered by a smooth muscle cell (SMC)-rich cap.[2,3] The cellular composition of an atherosclerotic lesion is an important determinant of its stability. In general, lesions rich in cholesterol-loaded macrophages and extracellular lipid deposits are prone to rupture. On the other hand, lipid-poor lesions with a prominent presence of fibroblasts, SMCs and collagen are relatively stable and resistant to rupture.[4] Cell proliferation and cell death are important processes in regulating macrophage and SMC numbers in the atherosclerotic lesion and may thereby directly influence lesion stability.[5]

Indeed, recent mouse studies demonstrated that genes involved in regulating cell proliferation and cell death play an important role in progression of the atherosclerotic lesion coinciding with changes in the cellular composition. Deletion of the tumor suppressor gene p53, an essential molecule in both cell proliferation and apoptosis, strongly exacerbated atherosclerosis in apoE-deficient (apoE$^{-/-}$),[6] LDL receptor deficient (LDLR$^{-/-}$)[7] and APOE*3-Leiden[8] mice. Recently Merched et al. showed that the p53 downstream target p21, an inhibitor of cell cycle progression via inactivation of cyclin-CDK complexes during the G1 phase of the cell cycle, has strong pro-atherogenic functions.[9] In addition, mouse studies showed that (hematopoietic) inactivation of p27, another cyclin-CDK regulating cell cycle inhibitor, exacerbated atherosclerosis on an apoE$^{-/-}$ background.[10,11] Taken together, these studies indicate an important role for p53, p21, and p27 in controlling atherogenesis.

Retinoblastoma (Rb), the first tumor suppressor gene identified molecularly, also plays a pivotal role in regulating cell proliferation and apoptosis. Rb is a nuclear phosphoprotein that arrests cells during the G1-phase of the cell cycle by forming complexes with the members of the E2F transcription factor family. The E2F family of transcription factors has binding sites in the promoters of many of the genes that are involved in cell-cycle progression.[12] In addition, loss of Rb function can trigger a p53-dependent apoptotic pathway, which may serve as an intrinsic protective mechanism to eliminate cells in which the Rb pathway is deregulated.[13] This is supported by the finding that Rb-deficient mice die in mid-gestation with widespread apoptosis.[14-16]

Although Rb is known to be a major factor in cell cycle progression and cell death, to date the exact role of Rb in atherosclerosis has not been elucidated. To investigate the role of macrophage-Rb in the development of atherosclerosis we crossed mice with a macrophage specific Rb deficiency[17,18] onto an apoE$^{-/-}$ background. Effects of macrophage Rb deletion on atherosclerosis development were evaluated using morphometric analysis of atherosclerotic lesion area and classification of lesion severity. In addition, detailed immunohistochemical analyses were performed to analyze lesion composition and the contribution of cell proliferation and cell death to atherosclerosis development upon macrophage-restricted Rb deletion.

METHODS

Mice and diet

The experimental animals were obtained by combining mice carrying the floxed Rb gene[18] with LysMCre mice,[17] and apoE-deficient mice[19] resulting in mice homozygously deficient for macrophage-Rb and apoE (LysMCre^{+} Rb$^{loxP/loxP}$apoE$^{-/-}$ or Rb^{del}) and control (LysMcre-negative) littermates that only lack apoE (Rb$^{loxP/loxP}$apoE$^{-/-}$ or Rb^{fl}). Mice were genotyped by polymerase chain reaction (PCR) for LysMCre,[17] Rb$^{loxP/loxP}$[18] and apoE[19] status. For experiments, 8 weeks old male Rb^{del} (n=17) and littermate control Rb^{fl} (n=13) were used. Mice were fed a semi-synthetic cholesterol-rich diet composed essentially according to Nishina et al.[20] supplemented with cocoa butter (15%, by weight) and cholesterol (0.25%, by weight), without cholate (Hope Farms, Woerden, The Netherlands). Mice were given food and water ad libitum. All animal work was approved by institutional regulatory authority and carried out in compliance with guidelines issued by the Dutch government.

Quantification of macrophage Rb deletion by Southern blotting

Southern blotting for the quantification of the deletion of Rb was performed as described before.[21] Mice were intraperitoneally injected with 1 ml thioglycollate broth (3% wt/vol.). After 4 days, DNA was isolated from PBS flushed peritoneal macrophages and digested with Pst1. Southern blots were hybridized with a 450-bp Pst1-PvuII probe, detecting a 5.0 kb wild type allele and floxed allele and a 4.5 kb deleted allele.

Blood sampling and analysis

Blood samples were collected in EDTA-coated vials (Sarstedt, Nümbrecht, Germany) by bleeding from the tail vein. Plasma cholesterol and triglyceride levels were measured enzymatically using commercially available kits (Roche Diagnostics GmbH, Mannheim, Germany). Total blood leukocyte (CD45^{+}), T-cell (CD3^{+}), B-cell (CD19^{+}) and monocyte/granulocyte (CD11b^{+}) numbers were determined by FACS analysis (FACSCalibur, BD Biosciences, California, USA) following standard protocol (TruCOUNT, BD Biosciences, California, USA), as described before.[22] Qualitative analysis of peripheral blood was performed on May-Grunwald-Giemsa (MGG) stained blood smears, according to standard procedures. The acute phase inflammatory markers Serum Amyloid A (SAA, BioSource International, Inc, Camarillo, CA) and fibrinogen[23] were analyzed by ELISA on plasma samples according to standard protocols.

Atherosclerosis analysis

After 12 weeks on a cholesterol-rich diet, mice were sacrificed. Heart and aorta were perfused with PBS, formalin fixed (pH 7.4) overnight and embedded in paraffin. From the entire aortic root area of the heart, four 5 μm cross-sections with an interval of 40 μm were used for quantification of atherosclerotic lesion area.[24] Sections of the aortic root area were routinely stained with hematoxylin-phloxine-saffran (HPS) for morphometric analysis, and characterization of the lesion and with Sirius red for collagen. Areas were determined using Leica Qwin image software (EIS, Asbury, NJ).

Lipid core area was defined by the presence of cholesterol clefts and extracellular lipids. In addition, necrosis was defined by the presence of pyknosis, karyorrhexis, or complete absence of nuclei.[25] Lipid core area and necrosis area were measured using morphometric analysis, as described above.

Atherosclerotic lesions were classified on severity (i.e. early lesions or advanced lesions) as described before.[22] The number observed in each category is expressed as a percentage of the total number of lesions observed within one group of mice (Rb^{del} or control Rb^{fl} group). All analyses were performed double blindly without prior knowledge of the genotype.

Immunohistochemistry

Serial sections were stained for macrophages and SMCs using a rabbit antibody to mouse macrophages (AIA-312040, 1/1500, Accurate Chemical and Scientific) and a monoclonal alpha-smooth muscle cell actin antibody (1/1600, DAKO A/S, Denmark), respectively. AIA-312040-positive and alpha-smooth muscle cell actin-positive areas were quantified using threshold values that discriminated between antibody-positive and antibody negative lesion areas, as described before.[8] In addition, nuclear counting in AIA-312040-positive and alpha-smooth muscle cell actin-postive areas was performed for quantification of macrophage and SMC numbers. Analysis on individual lesions was performed on lesions ranging from 0-50x10^3 μm^2 (n=16 individual lesions for both Rb^{del} and Rb^{fl} mice) and 50-150x10^3 μm^2 (n=24 and n=16 individual lesions for Rb^{del} and Rb^{fl} mice, respectively). To label proliferating cells, sections were stained using a monoclonal rat anti-mouse Ki-67 antibody (DAKO A/S Denmark) and for apoptosis using the Terminal Deoxynucleotidyl Transferase End-Labeling (TUNEL) technique (In situ cell detection kit POD, Roche Diagnostics GmbH, Mannheim, Germany).[8,26] Macrophages and SMCs positive for either Ki-67 or TUNEL were expressed as a percentage of the total number of macrophages and SMCs present.[22]

To analyze monocyte differentiation, spleen cryo-sections of Rb^{del} and Rb^{fl} mice were stained for macrophage markers using the antibodies: FA-11 (a kind gift from S. Gordon, Oxford University, UK), Mac1 (a kind gift from G. Kraal, VUMC, The Netherlands), ERTR9 (a kind gift from G. Kraal, VUMC, The Netherlands), and F4/80 (a kind gift from W. Buurman, UM, The Netherlands).

Uptake of modified lipoproteins and cytokine measurements in *in vitro* cultured bone marrow derived macrophages

Bone marrow-derived macrophages (BMM) were obtained according to standard procedures. Culturing, analyses and modified lipoprotein uptake experiments were performed as described by Kanters et al.[25] TNFα and interleukin-10 production were quantified at 0, 3, 6, and 24 hours after LPS (O111:B4, Sigma-Aldrich) stimulation (100 ng/ml) by ELISA (Biosource, Etten-Leur, The Netherlands).[25]

Statistical analysis

Data were analyzed using the non-parametric Mann-Whitney rank sum test (Graphpad Software, San Diego California USA). Data are expressed as mean±SD. Frequency data for lesion classification were compared by means of the Fisher's exact test.

Correlation analysis was performed using the Spearman's rank order correlation. *P*-value < 0.05 was regarded as significant.

RESULTS

General characteristics of apoE-deficient Rb^{del} mice

Male Rb^{del} (n = 17) and control Rb^{fl} (n = 13) littermates were fed a cholesterol-rich diet from 8 weeks of age on. During the study, the mice appeared healthy and displayed no signs of abnormalities. As shown in Table 1, after 12 weeks of a cholesterol-rich diet challenge, mean body weight was not different between Rb^{del} and Rb^{fl} mice. Plasma cholesterol and triglyceride levels (Table 1) and lipoprotein profiles (data not shown) were not different between Rb^{del} and Rb^{fl} mice. Moreover, absence of macrophage Rb did not affect hematocrite, $CD3^+$, $CD19^+$, and $CD11b^+$ leukocyte concentrations (Table 1). Subdivision of the $CD11b^+$ population did not reveal an effect of LysMCre-induced Rb deletion on either circulating $CD11b^+$ monocyte or circulating $CD11b^+$ granulocyte numbers. Additional, detailed pathological analysis of May-Grunwald Giemsa (MGG) stained blood smears also did not yield any abnormalities in the peripheral blood of Rb^{del} mice.

The degree of Rb deletion is dependent on the effectiveness of the Cre-recombinase in deleting the loxP-flanked Rb allele. Yields of thioglycollate-elicited peritoneal macrophages isolated from Rb^{fl} and Rb^{del} mice, for quantification of Rb deletion by Southern blot analysis, were similar (data not shown). Southern blot analysis revealed that in Cre-recombinase expressing mice the deletion of the floxed allele was almost complete in the heterozygous state ($LysMCre^+Rb^{loxP/WT}$;Figure 1A, lane 2) and complete in the homozygous state ($LysMCre^+Rb^{loxP/loxP}$; Figure 1A, lane 3) confirming effective deletion of Rb in macrophages.

Table 1. General characteristics of male Rb^{del} and Rb^{fl} mice after 12 weeks of feeding a cholesterol-rich diet.

		Rb^{fl}	Rb^{del}
Weight (g)		26.0±0.9	26.0±3.0
Plasma lipid levels (mmol/L)	Cholesterol	31.7±9.5	28.6±8.3
	Triglycerides	1.8±1.0	1.8±1.3
Hematocrite		0.49±0.02	0.49±0.03
Blood leukocytes (10^6 cells/mL)	$CD3^+$ cells	3.5±1.3	3.1±0.9
	$CD19^+$ cells	8.1±2.4	7.2±2.8
	$CD11b^+$ cells	4.0±0.6	4.4±1.2
	$CD11b^+$ monocytes	1.0±0.3	0.8±0.2
	$CD11b^+$ granulocytes	3.2±0.6	3.6±1.1
Inflammation parameters (µg/ml)	SAA	41.7±10.1	140.4±186.7
	Fibrinogen	2.5±0.5	2.2±0.2

Analysis of atherosclerotic lesion area

Comparable body weight, blood composition, plasma lipid levels, and complete deletion of the Rb-floxed allele in $LysMCre^+Rb^{loxP/loxP}$ mice, allowed us to selectively

dissect the effect of macrophage Rb on atherosclerosis development in these mice. Mice fed the cholesterol-rich diet for 12 weeks were sacrificed for collection of heart, aorta, and other organs. Morphometric analysis of total atherosclerotic lesion area revealed a significant 51% increase in the Rb^{del} mice, as compared with control Rb^{fl} mice (26.8±13.2 vs. 17.7±9.4 x10^4 μm^2, P=0.04, Figure 1B). In addition, lesion classification (early vs. advanced) showed that Rb^{del} mice had a significantly lower incidence of early lesions (21.6% vs. 43.5%, P=0.04) and a significantly increased incidence of advanced lesions (78.4% vs. 56.4%, P=0.04) as compared to Rb^{fl} control mice, indicating an enhanced progression of atherosclerosis in Rb^{del} mice (Figure 1C).

To evaluate whether this enhanced atherosclerosis in Rb^{del} mice is due to an enhanced uptake of modified LDL and/or differences in inducble cytokine production in macrophages, we performed *in vitro* analysis using bone marrow derived macrophages (BMM). BMM from Rb^{del} and Rb^{fl} mice did not differ in endocytosis of either oxidized LDL or acetylated LDL in two different doses (Figure 2A). LPS stimulation of BMM resulted in an increase in both TNFα and Il-10 production in Rb^{del} and Rb^{fl} BMM (Figure 2B and C). However, Rb^{del} BMM did not differ from Rb^{fl} control BMM in LPS-induced TNFα and Il-10 production. These data show that Rb^{del} mice have normal modified LDL uptake and are not affected in the production of either the pro-inflammatory cytokine TNFα or the anti-inflammatory cytokine Il-10.

In addition, plasma levels of the acute phase inflammatory marker Serum Amyloid A (SAA) and fibrinogen were not significantly affected (Table 1), although

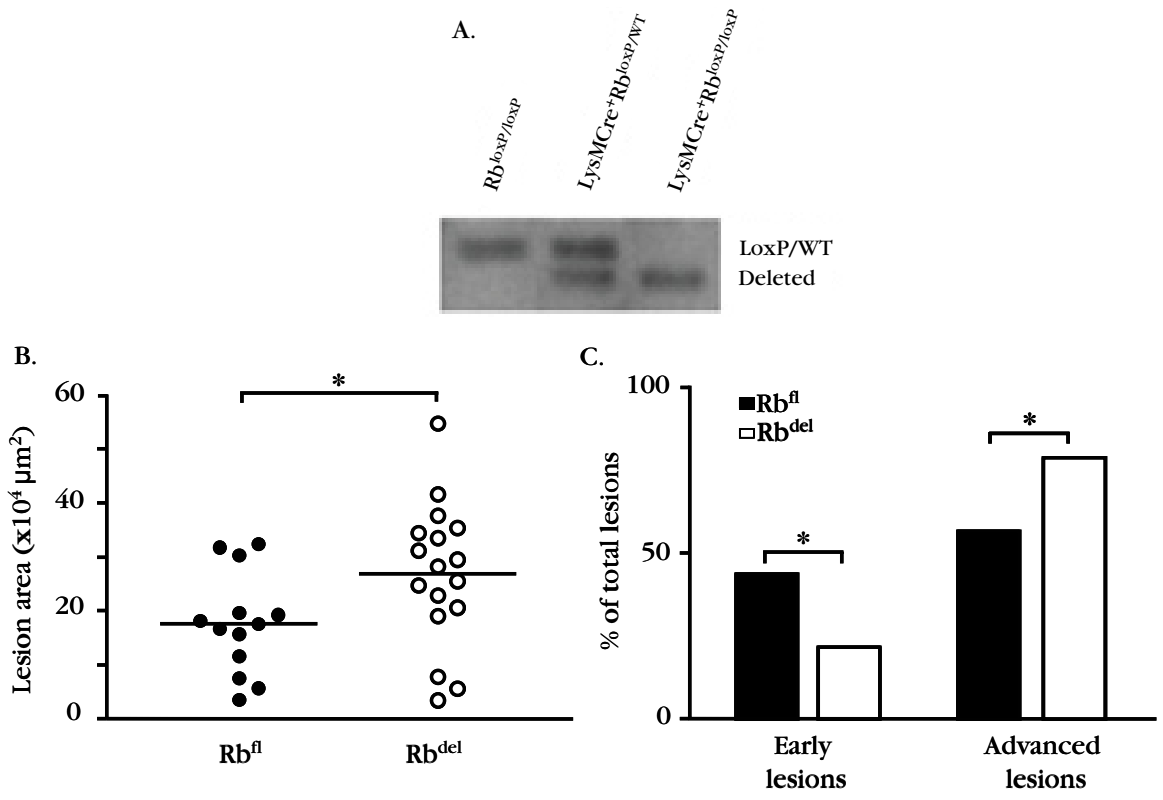

Figure 1. (A.) Southern blot analysis of thioglycollate-elicited peritoneal macrophages. (B.) Aortic atherosclerotic lesion area in Rb^{fl} (closed circles, n = 13) and Rb^{del} (open circles, n = 17) mice. Line represents mean area for each group. (C.) Lesion classification of Rb^{fl} (black bars) and Rb^{del} (white bars) mice. *P<0.05.

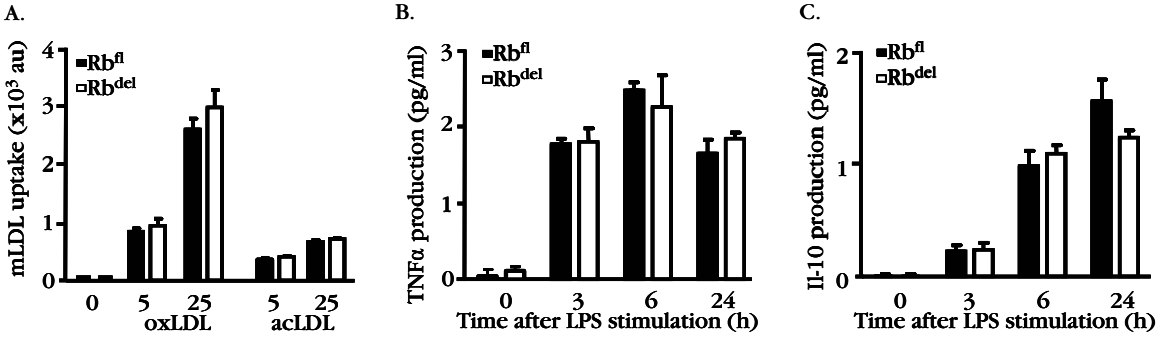

Figure 2. (A.) Uptake of oxidized LDL and acetylated LDL in different doses (μg/mL) by BMM from Rb^{fl} (black bars) and Rb^{del} (white bars) mice. (B, C) Rb^{fl} and Rb^{del} macrophages were stimulated with LPS. TNFα (B.) and Il-10 (C.) production were measured in the supernatant by ELISA.

SAA showed a tendency towards an increase. To evaluate a possible relationship between inflammation and atherosclerotic lesion area a Spearman's rank order correlation analysis was performed. No correlations were found for either Rb^{del} or Rb^{fl} mice between SAA levels and atherosclerotic lesion areas (Correlation coefficient of 0.06 for Rb^{del} (P = 0.83) and 0.33 for Rb^{fl} (P = 0.35) mice). In line with this, no correlations were found either between fibrinogen levels and atherosclerotic lesion areas (Correlation coefficient of -0.04 for Rb^{del} (P = 0.89) and -0.30 for Rb^{fl} (P = 0.32) mice). These data indicate that inflammation, as measured by plasma SAA and fibrinogen levels, does not contribute to the observed difference in atherosclerotic area between Rb^{del} and Rb^{fl} mice.

Moreover, Rb plays a critical role in monocytic differentiation.[27] To evaluate whether the enhanced atherosclerosis in Rb^{del} mice is due to a defect in monocyte differentiation into macrophages, we immunohistochemically stained spleen sections of Rb^{del} and Rb^{fl} mice. No differences were found on the differentiation markers FA-11, Mac1, ERTR9, and F4/80 between Rb^{del} and Rb^{fl} mice (data not shown) indicating that macrophage specific deletion of Rb does not affect the differentiation of monocytes into macrophages.

Histopathological analysis of atherosclerotic lesions

Histopathological analysis of atherosclerotic lesions in the aortic valve area revealed the presence of foam cell rich fatty streaks and fibrous plaques with a lipid core and a cap covering necrotic material, cholesterol clefts and extracellular lipids in both Rb^{del} and Rb^{fl} mice. Figure 3 shows representative photomicrographs of atherosclerotic lesions in Rb^{fl} (A-C) and Rb^{del} (D-F) mice stained with HPS for morphometric analysis (A and D), for macrophages (B and E) and for smooth muscle cells (C and F). Quantification of the lesion area positive for the anti-mouse macrophage polyclonal antibody AIA-312040 in Rb^{del} mice showed a significant 13% decrease in lesion macrophage area as compared to the Rb^{fl} control mice (P=0.03, Table 2). However, analysis of macrophage area on individual lesions within the same size range (0-50x10^3 μm^2 and 50-150x10^3 μm^2, Table 2) showed no difference in macrophage area between Rb^{del} and Rb^{fl} mice. These data indicate that the decrease in relative macrophage area in the lesions of Rb^{del} mice is the result of an enhanced

Table 2. Characteristics of atherosclerotic lesions in Rb^{del} and Rb^{fl} mice.

	Rb^{fl}	Rb^{del}
Lesion macrophage area (% of total lesion area)	62.5±13.5	54.2±12.7*
Individual lesion size		
0-50x10³ µm²	74.7±20.0	75.2±18.4
50-150x10³ µm²	48.6±18.7	46.3±14.6
Macrophage density (per 10³ µm² macrophage area)	3.6±1.0	3.1±1.2
Lesion SMC area (% of total lesion area)	2.8±1.6	4.1±1.8*
Individual lesion size		
0-50x10³ µm²	2.8±1.9	4.6±2.6
50-150x10³ µm²	3.5±2.5	4.7±2.6
SMC density (per 10³ µm² SMC area)	0.9±0.5	1.2±0.9
Collagen area (% of total lesion area)	18.5±10.0	24.9±12.3
Lipid core area (% of total lesion area)	5.3±4.5	6.8±3.2
Necrosis area (% of total lesion area)	2.7±3.2	4.3±4.0

* P<0.05

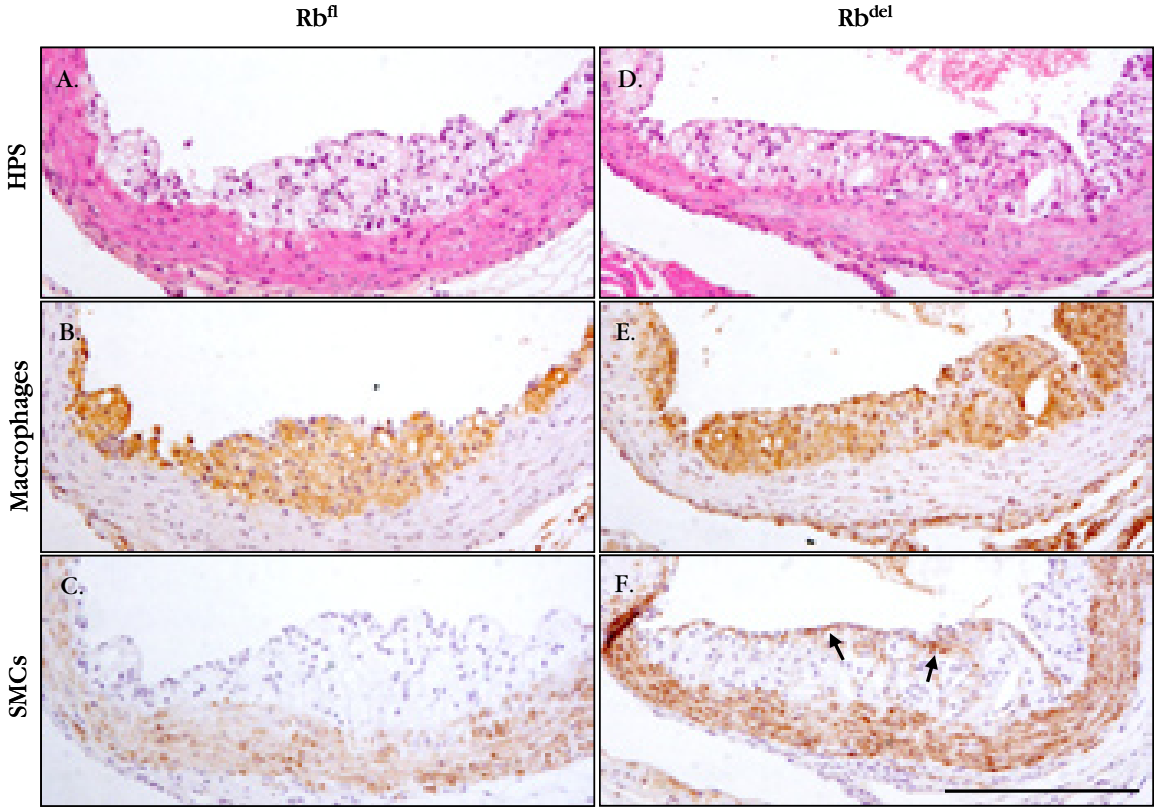

Figure 3. Representative lesions of Rb^{fl} (A-C.) and Rb^{del} (D-F.) mice. Sections were stained with HPS (A and D.), macrophage-specific antibody (B and E.) or alpha-smooth muscle cell actin antibody (C and F). Arrows indicate the presence of a SMC rich cap (F). Magnification 100x, scale bar 100 µm.

progression of atherosclerosis, a primary characteristic of advanced lesion formation,[28] rather than an additional effect of macrophage specific Rb deletion on lesion composition. In the sections stained with the AIA-312040 antibody, it was demonstrated that monocyte adherence to the lesions, as a parameter for endothelial cell activation, was not affected (4.4±2.5 vs. 4.4±2.3 monocytes/lesion for Rb^{del} and Rb^{fl} mice, respectively). Analysis of the SMC area showed a significant 46% increase in lesions in the Rb^{del} mice (P=0.05, Table 2). Analysis of SMC area on individual lesions within the same size range (0-50x10^3 μm^2 and 50-150x10^3 μm^2, Table 2) showed no difference between Rb^{del} and Rb^{fl} mice indicating that the increase in SMC area was also a result of the enhanced progression of atherosclerotic lesions,[28] rather than an additional macrophage-specific Rb effect on lesion composition. The increase in SMC area in the Rb^{del} mice coincided with a non-significant 35% increase in collagen area (P=0.26, Table 2). Both parameters indicate the presence of a thicker fibrous cap in Rb^{del} mice. Nuclear counting revealed no effect of macrophage Rb deficiency on macrophage and SMC numbers (macrophage and SMC density, Table 2) in the atherosclerotic lesions. The lipid core defined by the presence of cholesterol clefts and extracellular lipids was not shown to be affected by macrophage Rb deficiency (P=0.23, Table 2). To complete lesion composition analysis the necrotic core was analyzed. Rb^{del} mice showed a (non-significant) doubling of the necrotic core as compared to Rb^{fl} mice (P=0.13, Table 2).

Cell death and cell proliferation

To investigate whether macrophage-specific deletion of Rb affects cell death in the atherosclerotic lesions, TUNEL-positivity was determined. Lesions of Rb^{del} mice showed an incidence of 1.3±1.0% TUNEL-positive macrophages which did not differ significantly from the incidence of 1.1±1.0% TUNEL-positive macrophages for Rb^{fl} mice (Figure 4A). In addition, the incidence of TUNEL-positive SMCs in Rb^{del} mice (0.5±0.5%) did not differ significantly from the Rb^{fl} control group (0.5±0.5%, Figure 4A), indicating that macrophage Rb deficiency did not affect lesional apoptosis.

To study the effect of macrophage-specific Rb deletion on lesional proliferation nuclear protein Ki-67-positivity was determined for both lesional macrophages and SMCs. As shown in Figure 4B, lesions of Rb^{del} mice showed a significant 2.6-fold increase in the incidence of Ki-67-positive macrophages as compared to lesions of Rb^{fl} mice (3.3±2.4% vs. 1.3±1.2% Ki-67-positive macrophages for Rb^{del} and Rb^{fl} mice, respectively, P=0.02, Figure 4B and C). The incidence of Ki-67-positive SMC nuclei was not affected by the macrophage Rb genotype (0.7±0.6% vs. 0.5±0.4% Ki-67-positive SMCs for Rb^{del} and Rb^{fl} mice, respectively, Figure 4B). Hence, the increased atherosclerosis in Rb^{del} mice coincides with increased proliferation of macrophages in the lesions of these mice.

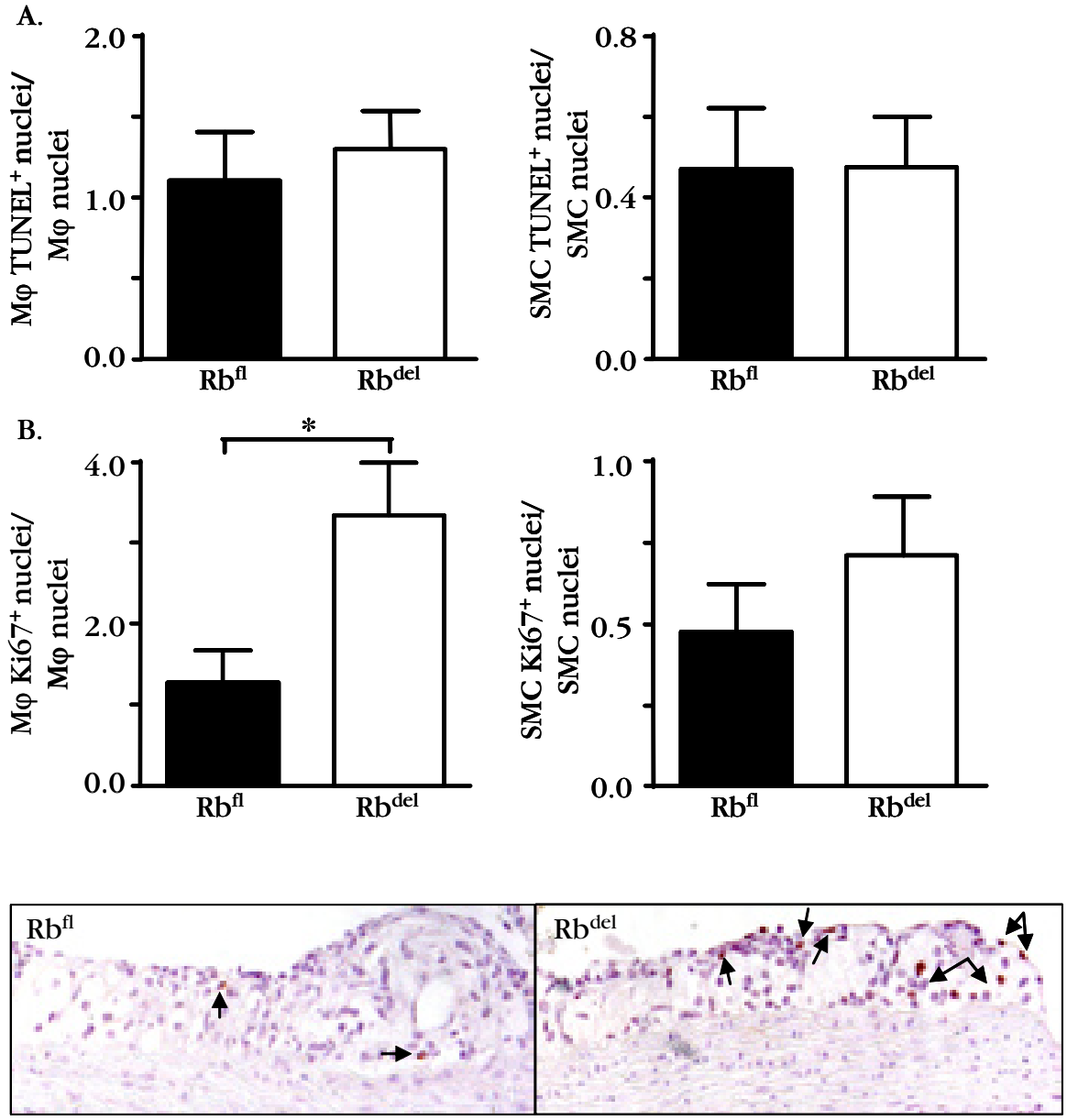

Figure 4. (A.) Apoptosis and (B.) proliferation in macrophages (left panel) and SMCs (right panel) in the atherosclerotic lesions of Rb^{fl} (black bars) and Rb^{del} (white bars) mice. Error bars indicate SEM. *P<0.05. (C.) Representative Ki-67 stained lesions of Rb^{fl} (left panel) and Rb^{del} (right panel) mice. Arrows indicate Ki-67 positive cells. Magnification 150x.

DISCUSSION

In the present study, we investigated the role of macrophage Rb in the pathogenesis of atherosclerosis. ApoE-deficient mice lacking macrophage Rb displayed accelerated atherosclerosis. This was characterized by the presence of more advanced lesions that were rich in smooth muscle cells and collagen and poor in macrophages (Figure 5). *In vitro* analysis showed that the enhanced atherosclerosis in macrophage Rb deficient mice was independent of modified lipoprotein uptake or cytokine production. Whereas macrophage specific Rb deletion did not affect

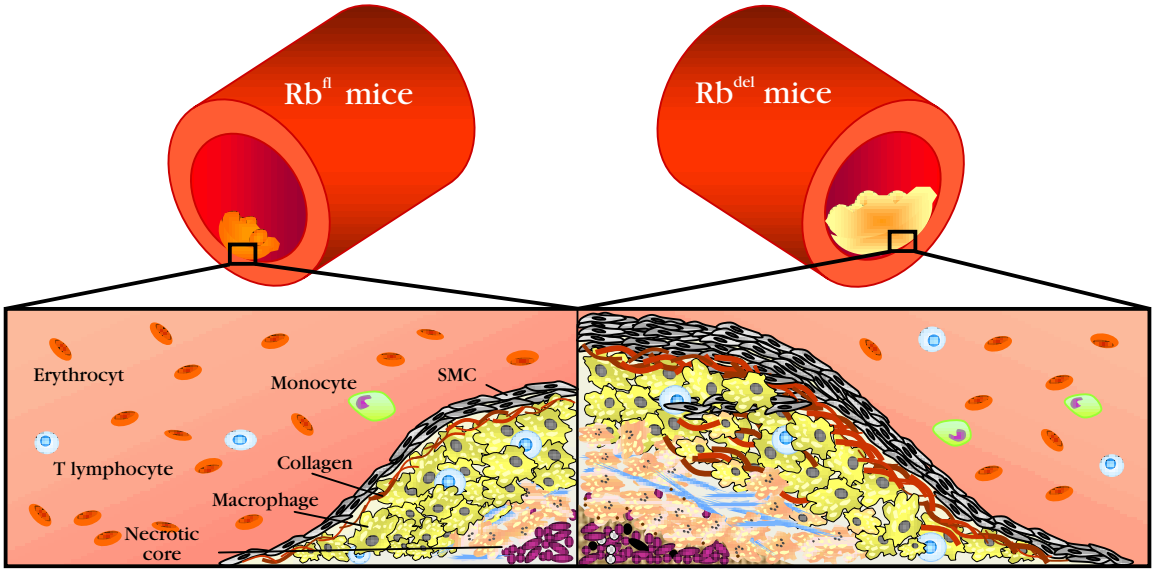

Figure 5. Schematic drawing illustrating lesion development upon macrophage-restricted Rb deletion. Macrophage-restricted Rb deletion leads to enhanced atherosclerosis development characterized by increased lesion area and the presence of more advanced lesions rich in smooth muscle cells and collagen and poor in macrophages.

the systemic inflammation markers SAA and fibrinogen, monocyte differentiation or macrophage apoptosis, lesional macrophage proliferation was strongly increased. These studies demonstrate that macrophage Rb is a suppressing factor in the progression of atherosclerosis.

Bennett et al. demonstrated that human plaque-derived VSMCs show reduced proliferation and earlier senescence due to an increased ratio of the active form of Rb.[29] In addition, localized infection of the arterial wall with an adenovirus encoding a constitutively active non-phosphorylatable form of Rb significantly reduced medial vascular smooth muscle cell proliferation and restenosis in two animal models of balloon angioplasty.[30] Moreover, a phosphorylation-competent full-length and a truncated form of Rb inhibited vascular smooth muscle cell proliferation and neointima formation.[31] These data, together with our data, indicate that Rb can be a strong modulator of vascular disease both at the level of SMCs and macrophages.

To define the molecular pathways underlying Rb function in vascular disease our findings may support an initial mechanistic explanation for a role of macrophage Rb in atherosclerosis. We showed that increased macrophage proliferation may underlie the formation of more advanced lesions in Rb^{del} mice. Surprisingly, increased macrophage proliferation did not coincide with increasing effects on macrophage area or number. Remarkably, macrophage area was even decreased upon Rb deletion. Detailed analysis of individual lesions showed that the decrease in macrophage area reflected the more advanced state of the lesions in Rb^{del} mice, rather than an additional effect of Rb deletion on lesion (macrophage) composition.[4] In general, macrophages produce growth factors, cytokines, chemokines and metallopoteinases which play an important role in the development and progression of atherosclerotic lesions. This diverse array of bio-active molecules activates the surrounding endothelium and SMCs. Following, the lesion becomes increasingly complex with the presence of SMCs, lipid-laden macrophages, T-lymphocytes, a necrotic core and

cholesterol crystals covered by a fibrous cap.[2,3,32] During atherosclerosis development, the macrophage population in Rb^{del} mice exhibited an increased proliferative state. We hypothesize that this increased proliferative state resulted in enhanced lesion pathology via increased production of growth factors, cytokines, chemokines and metalloproteinases. This array of proteins stimulated the formation of an advanced atherosclerotic lesion, characterized by a relative decrease in macrophage content and a relative increase in SMC content. Hence our data suggest that Rb protects against excessive macrophage proliferation and thereby against enhanced atherosclerosis progression.

Bergh et al. showed that Rb plays a critical role in monocytic and neutrophilic lineage commitment of normal human bone marrow progenitor cells.[27] In addition, macrophages play a central role in red blood cell development. Erythroblast islands, required for red blood cell development, are present in the liver of fetuses and in the bone marrow of adults. These erythroblast islands consist of a central macrophage that supplies nutrients to the surrounding erythroblasts and degrades the nuclei from the enucleated circulating red blood cells. Recently, it was shown that Rb-deficient murine fetuses have a severe defect in macrophage maturation and fail to form functional erythroblast islands resulting in lethal anemia characterized by the persistence of nucleated erythroid cells in the peripheral blood.[33] However, we did not observe differences in monocyte differentiation analyzed using different macrophage markers (e.g. FA-11, Mac1, ERTR9, and F4/80) or in macrophage maturation characterized by an aberrant blood composition (e.g. nucleated red blood cells) as analyzed by FACS, hematocrite levels and May-Grunwald Giemsa stained blood smears. Thus, differences in either monocyte differentiation or macrophage maturation during embryonic development or in adult life that might affect atherosclerosis development in Rb^{del} mice have not been found.

In addition to defining the molecular pathways underlying Rb function in atherosclerosis our findings may also have direct clinical significance. Unregulated cell proliferation has been implicated in the etiology of a variety of vascular proliferative diseases including atherosclerosis and (in-stent) restenosis after PTCA or placement of a stent.[34-37] Recently, the use of drug-eluting stents has emerged as a highly promising approach to reduce in-stent restenosis.[38] In addition to the vascular smooth muscle cells (VSMCs), macrophages also play a crucial role in the formation of neointima via the stimulation of VSMC migration and proliferation at the injury site.[39] The different drugs used in drug-eluting stents most often target proliferative state of the cells. Therefore, regulation of the Rb gene via drug-eluting stents might prove a promising approach since activation of Rb both at the level of VSMCs [30,31] and macrophages (present study) is shown to be beneficial for inhibition of vascular disease.

Our observation that Rb^{del} mice after 12 weeks feeding a high-fat diet have a tendency towards increased plasma SAA levels, may point towards a role for Rb in modulating the inflammatory status in apoE-deficient mice. This might not be a very plausible explanation, however, for the following reasons. Firstly, our *in vitro* experiments showed that Rb^{del} macrophages do not respond differently from Rb^{fl} macrophages regarding LPS-induced production of TNFα and Il-10. Secondly, SAA and fibrinogen levels on a standard chow diet (data not shown) and fibrinogen levels

after 12 weeks feeding a high-fat diet (Table 1) did not differ between Rb^{del} and Rb^{fl} mice. Finally, correlation analysis for SAA (and fibrinogen) and the total lesion areas in both Rb^{del} and Rb^{fl} mice negated that inflammation contributes to atherosclerosis development in these mice. The absence of a correlation between SAA and total area in this mouse model also excludes the possibility that the observed increased SAA level is the result of an increase in lesion area as recently described by O'Brien et al.[40] Thus, changes in inflammatory status due to macrophage specific Rb deletion as an explanation for the observed increase in atherosclerosis is highly unlikely.

In contrast to conditional $Rb^{loxP/loxP}$ mice, conventional homozygous Rb deficient mice die at mid-gestation, displaying impaired neurogenesis, fetal liver erythropoiesis and lens development, which was related to a defect in proliferation and end-stage differentiation in several lineages.[14-16] Pituitary-specific inactivation of the Rb gene resulted in pituitary tumors due to hyperproliferation and impairment of apoptosis.[21] In the present study, LysMCre-mediated deletion of the floxed-Rb allele did not result in any abnormality at the level of circulating $CD45^+/CD11b^+$ blood leukocytes or resident cells in liver, spleen or bone marrow (data not shown). Hence, the Rb gene product has a pivotal role in controlling cell division, cell death and cell differentiation of neuronal, erythropoetic, lens and pituitary cells, but not in controlling these cellular processes in the blood granulocyte and monocyte population. Moreover, the application of the site-specific recombinase technology (i.e. the LysMcre mouse model in combination with the $Rb^{loxP/loxP}$ mouse model) creates a first and unique opportunity to study the role of macrophage Rb in pathophysiology of disease in general.

In conclusion, we demonstrate that deletion of the tumor suppressor gene Rb specifically in macrophages enhances atherosclerosis development. Combined with our previous and comparable findings for p53 in macrophages,[8] we conclude that Rb and p53, in addition to their suppressing function in cancer, have a suppressing function for atherosclerosis development.

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