Increased early atherogenesis in young versus old hypercholesterolemic rabbits by a mechanism independent of arterial cell proliferation

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Abstract We sought to determine the relative importance of aging and hypercholesterolemia on atherosclerosis. Although plasma cholesterol levels increased similarly in young and old rabbits fed an atherogenic diet for 2 months, aortic atherosclerotic lesions were more prominent in young animals. This finding was associated with an age-dependent reduction in the DNA-binding activity of the proinflammatory nuclear factor κB (NF-κB) in aortic tissue. Atherosclerotic lesions consisted mostly of macrophages, which displayed a similar proliferative response in both age groups. Independently of the age, medial cell proliferation was low and increased as a function of intimal lesion size. Thus, higher atherogenicity in young rabbits exposed to extreme hypercholesterolemia compared to old counterparts is associated with higher activity of NF-κB in the juvenile vessel wall without apparent age-dependent changes in arterial cell proliferation.

Key words: Atherosclerosis; Aging; Hypercholesterolemia; Proliferation

1. Introduction

Atherosclerosis is the leading cause of mortality and morbidity in developed countries. Animal and human studies indicate that atherosclerosis is initiated by alterations in the normal function of the endothelium [1]. Endothelial dysfunction triggers an inflammatory response that leads to the formation of fatty streaks, the earliest recognizable lesion of atherosclerosis, and ultimately to fibrous and fibrocalcified plaques [1].

Aging and hypercholesterolemia are major cardiovascular risk factors [1–3]. Numerous animal studies have shown that increasing dietary cholesterol content and the duration of exposure to cholesterol-rich diets results in augmented atherosclerosis. While aging is associated with structural and functional alterations in the cardiovascular system [2,3], it is unclear whether age-dependent intrinsic alterations in the arterial wall or increased exposure to risk factors contribute to the increased prevalence of atherosclerosis in the elderly. In this study we evaluated the relative contribution of aging and hypercholesterolemia to atherogenesis in New Zealand white rabbits. As both inflammation and excessive cell proliferation within the arterial wall have been implicated in atherogenesis [1], we also investigated whether age-dependent differences in atherosclerosis could be related to differences in the activity of the proinflammatory nuclear factor κB (NF-κB) and/or in arterial cell proliferation.

2. Materials and methods

2.1. Experimental design

Young (4–5 months old) and old (4–5 years old) male New Zealand rabbits fed a normal diet or a cholesterol-rich diet for 2 months were included in the following groups: YN (young–normal chow, $n=5$), YC (young–cholesterol-rich diet, $n=10$), ON (old–normal chow, $n=5$), and OC (old–cholesterol-rich diet, $n=10$). The atherogenic diet contained 10 g cholesterol (Sigma) and 60 ml peanut oil per kg of rabbit chow (1% cholesterol). Animal handling was in accordance with institutional guidelines. Blood was withdrawn before the onset of the atherogenic diet and 2 days before death to monitor plasma cholesterol and triglyceride levels. All animals received four intraperitoneal injections of 5-bromodeoxyuridine (BrdU) (20 mg/kg each) at 12-h intervals starting 48 h before death. Rabbits were killed with an overdose of pentobarbital (i.v. injection). A cut was made in the vena cava and the systemic circulation was thoroughly perfused with saline through the heart. The aortic arch, common carotid and femoral artery were fixed in 100% methanol for morphometric and immunohistological studies.

2.2. Immunohistochemistry, immunofluorescence and quantification of atheroma

Methanol-fixed arteries were paraffin-embedded and cut in 5-μm cross-sections. Intimal and medial areas were measured on specimens stained with elastic–trichrome or immunostained with anti-RAM11 antibody (see below). Quantification was done with the SigmaScan Pro 5.0 software (SPSS Science) using a hemocytometer for calibration as described previously [4]. For each rabbit, two to four sections were analyzed and the results were averaged. Immunohistochemistry using mouse monoclonal anti-BrdU (1:50, Dako) or anti-RAM11 (1:500, Dako) antibodies was done with a biotin/streptavidin-peroxidase detection system (Signet Laboratories) and 0.05% (w/v) 3,3′-diaminobenzidine tetrahydrochloride dihydrate substrate (DAB, Vector Laboratories). Double immunostaining for BrdU and smooth muscle

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Abbreviations: BrdU, 5-bromodeoxyuridine; EMSA, electrophoretic mobility shift assay; NF-κB, nuclear factor κB; OC, old–cholesterol-rich diet; ON, old–normal chow; YC, young–cholesterol-rich diet; YN, young–normal chow; SMC, smooth muscle cell

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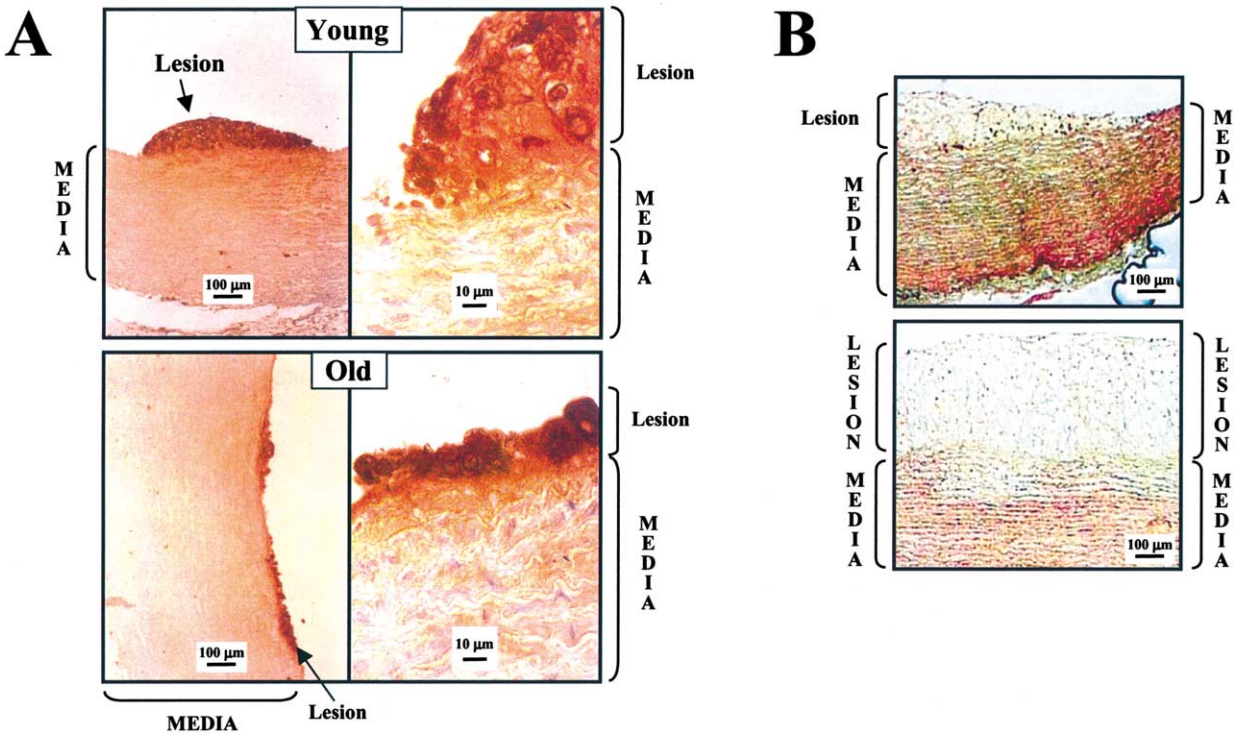

Fig. 1. Immunohistochemical analysis of cross-sections from the aortic arch of young and old fat-fed rabbits. A: Macrophages were detected using anti-RAM11 antibody. Specimens were counterstained with hematoxylin. Note that atherosclerotic lesions contained mostly RAM11-immunoreactive macrophages. B: Double immunostaining using alkaline phosphatase-conjugated anti-SMα-actin and anti-BrdU antibodies. Immunocomplexes were revealed with Fast red (SMα-actin) and DAB (BrdU). SMα-actin immunoreactivity was generally undetectable within intimal lesions and regions of the media near intimal lesions.

α-actin (SMα-actin) was performed using anti-BrdU and alkaline phosphatase-conjugated anti-SMα-actin (1:30, Sigma). The following antibodies were used for immunofluorescence: biotinylated anti-BrdU (1:60, Zymed Laboratories), FITC-conjugated anti-BrdU (1:20, Roche), anti-RAM11 (1:1000, Dako) and biotinylated anti-mouse (Signet Laboratories). Biotinylated antibodies were detected with streptavidin–Texas red (1:500, Molecular Probes). Hoechst 33258 (Roche) was used to counterstain the nuclei. Proliferation was quantified as the number of BrdU-immunoreactive cells per mm^2 of media or intimal lesion. For each rabbit, two or three sections were analyzed and the results were averaged.

2.3. Statistical analysis

Results are reported as mean ± S.E.M. Differences in body weight and triglyceride levels among the four groups were evaluated using ANOVA and Fisher's post-hoc test. Differences in intimal lesion size and in the density of BrdU-positive cells between YC and OC were assessed using a two-tailed, unpaired Student's *t*-test. Simple regression analysis was used for discerning relationships between lesion size (as measured by the intima/media ratio) and the density of BrdU-positive cells in the media and lesions. The *F*-test, which uses the sum of the squares and mean squares to calculate an *F*-statistic and an associated *P* value, was used to measure the quality (significance) of the regression analysis. In all cases, statistically significant differences were considered at $P < 0.05$.

2.4. Electrophoretic mobility shift assays (EMSA)

Aortic tissue from YC and OC rabbits ($n = 10$ each group) was pooled for the preparation of whole cell extracts [4]. EMSA using a radiolabeled consensus NF-κB probe was carried out as previously described [5]. Antibodies for supershift experiments were anti-p50 (sc-114) and anti-p65 (sc-372) (both from Santa Cruz Biotechnology).

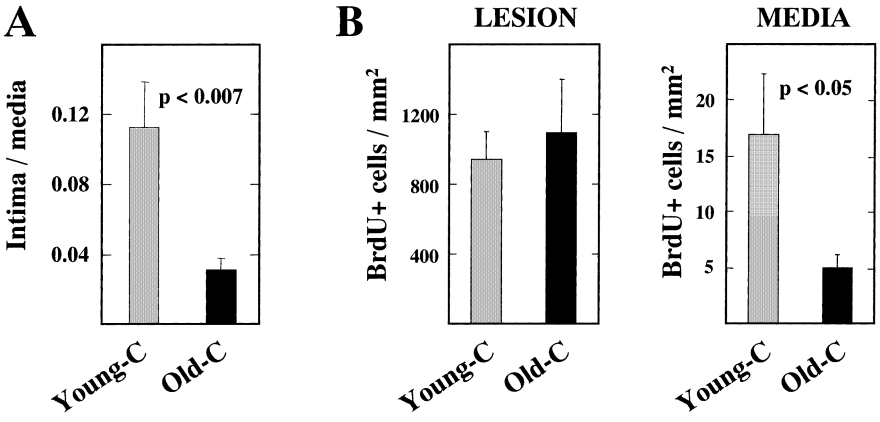

Fig. 2. Quantification of atherosclerosis and cell proliferation in the aortic arch of YC and OC animals ($n = 10$ each group). A: Intima-to-media ratio. B: Number of BrdU-immunoreactive cells per mm^2 of media and intimal lesion.

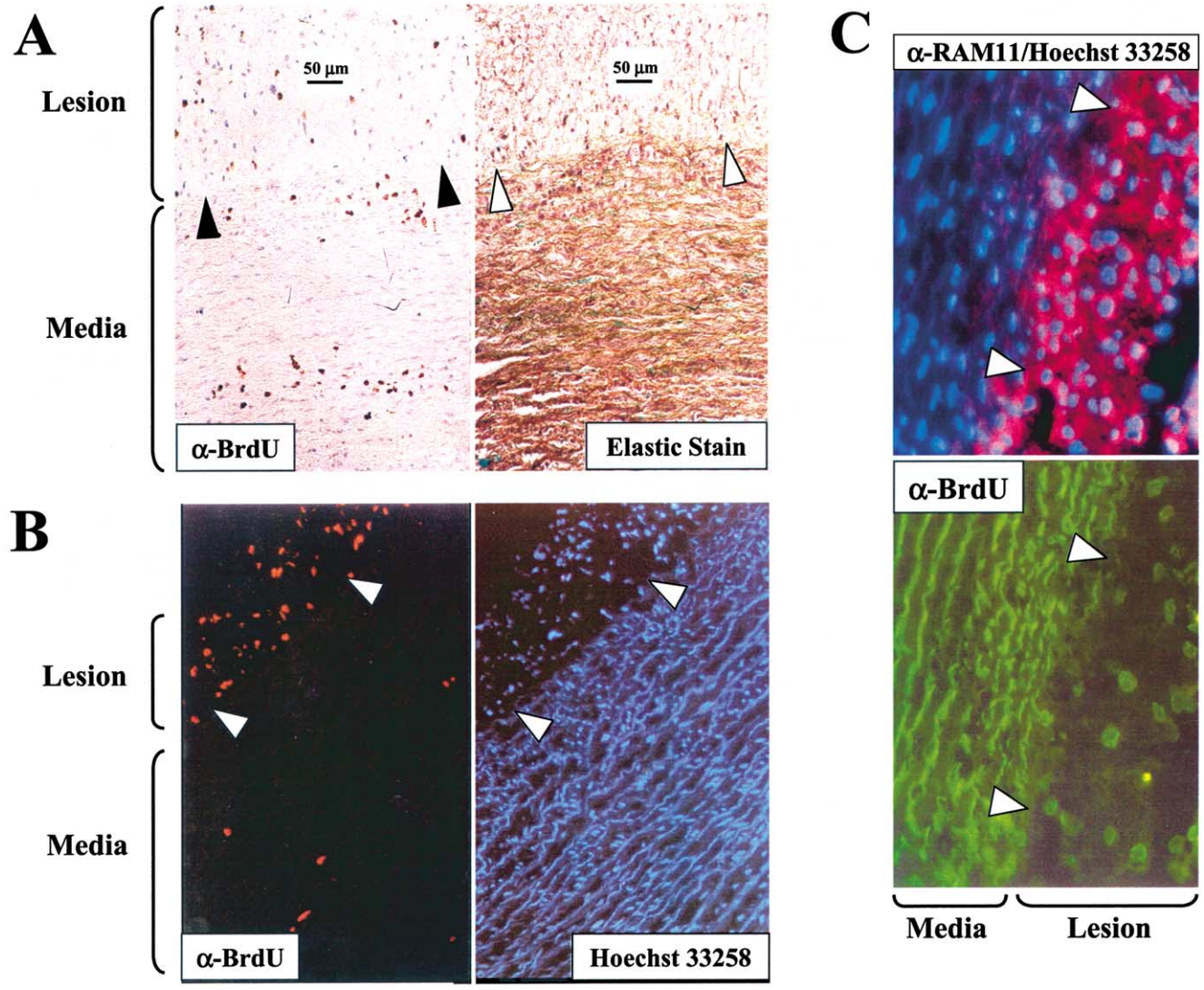

Fig. 3. Aortic arch cell proliferation as indicated by BrdU incorporation. Arrowheads point to the internal elastic lamina. A: Left: BrdU immunostaining detected with streptavidin-peroxidase (brown nuclear signal) and hematoxylin counterstain. Right: Adjacent section stained with elastic–trichrome. This photomicrograph of a YC rabbit shows a region of the media underneath a prominent lesion, where medial cell proliferation prevailed. Note that, in both age groups, average cell proliferation within the intimal lesion greatly exceeded that seen in the media (see Fig. 2B). B: Left: Same as in A, but BrdU-containing immunocomplexes were detected using streptavidin–Texas red. Right: Hoechst 33258 nuclear staining of the same microscopic field. Elastic fibers disclosed blue autofluorescence. C: Double immunofluorescence using anti-RAM11 antibody (revealed with biotinylated anti-mouse antibody and streptavidin–Texas red) and FITC-conjugated anti-BrdU antibody (green signal). Nuclei are counterstained with Hoechst 33258 (blue signal). Top: Double exposure showing RAM11 and Hoechst 33258 staining. Bottom: BrdU signal in the same microscopic field. Elastic fibers disclosed green autofluorescence. Note the abundance of proliferating macrophages within the intimal lesion (co-localization of RAM11 and BrdU).

3. Results

All rabbits appeared healthy throughout the experimental protocol and were included in all analysis. By the end of the study, body weight in ON and OC rabbits was similar (4.30 ± 0.16 and 4.29 ± 0.14 kg, respectively) and greater than respective young animals (YN = 3.34 ± 0.08 kg, $P < 0.003$ vs. ON; YC = 3.45 ± 0.18 kg, $P < 0.0004$ vs. OC). Neither age nor the type of diet affected the level of plasma triglycerides (YN = 88.4 ± 9.7, YC = 107.7 ± 15.5, ON = 87.2 ± 24.9, OC = 120.4 ± 29.9 mg/dl). Total plasma cholesterol in control animals was < 45 mg/dl, and was exceedingly increased in fat-fed rabbits without significant differences between YC and OC (2488 ± 315 and 1747 ± 212 mg/dl, respectively). With the exception of one YC rabbit that developed intimal thickening in all arteries examined (aortic arch, common carotid and femoral arteries), atherogenesis was mostly restricted to the aortic arch of fat-fed rabbits. Therefore, we focused all subsequent studies on the aortic arch.

Intimal lesions in both YC and OC animals consisted predominantly of macrophages, as indicated by abundant RAM11 immunoreactivity throughout the lesions (Figs. 1A and 3C, top). In marked contrast, expression of the smooth muscle cell (SMC) marker SMα-actin was generally undetectable within intimal lesions, and reduced or absent in medial cells underlying atherosclerotic lesions (Fig. 1B). Whereas medial cross-sectional area was similar in YC and OC rabbits (4.58 ± 0.43 and 5.69 ± 0.35 mm^2, respectively, $P > 0.05$), inti-

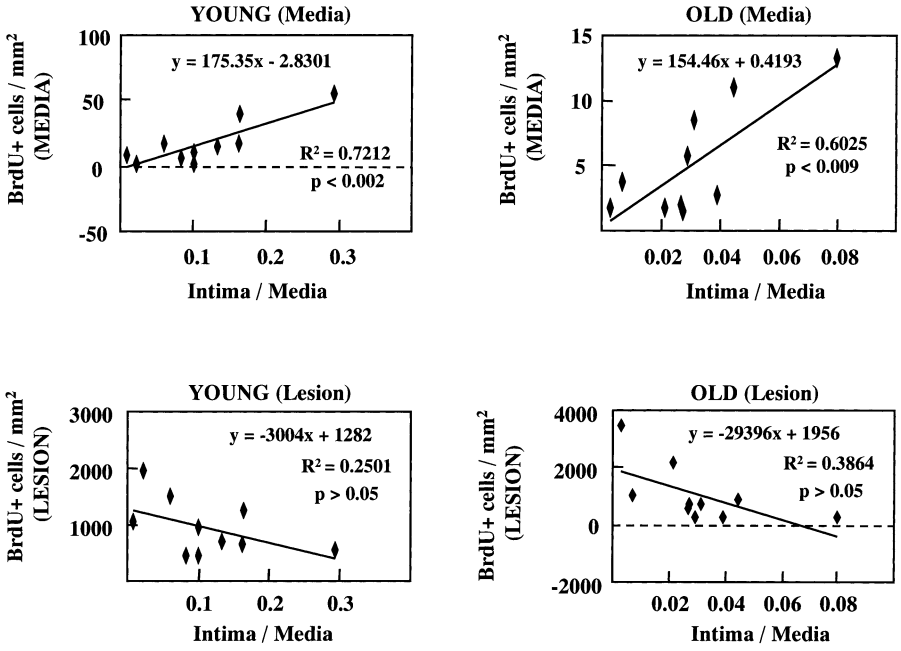

Fig. 4. Simple regression analysis for discerning relationships between lesion size (as measured by the intima/media ratio) and the density of BrdU-positive cells in the media and lesions of young and old fat-fed rabbits. Each point represents one animal. The *F*-test was used to measure the significance of the regression analysis to calculate an *F*-statistic and an associated *P* value. Each plot shows the equation for linear regression, the coefficient of determination (R^2) and the *P* value.

mal thickening was significantly higher in YC compared to OC rabbits (Fig. 2A, $P < 0.007$). Generally, lesions in YC rabbits were thicker and more localized than in OC rabbits.

Using genetically modified mice, we have recently shown that excessive arterial cell proliferation plays a critical role during atherogenesis [4]. Because arterial cell proliferation is abundant at the onset of atherosclerosis in hypercholesterolemic rabbits [6–9], we investigated whether age-dependent differences in cell replication might contribute to augmented atherosclerosis in juvenile rabbits. As expected, arterial BrdU incorporation in the vessel wall of normolipemic rabbits was scattered (not shown). In contrast, BrdU immunoreactivity in the aortic arch of YC and OC rabbits was abundant in both medial and intimal cells (Figs. 2B and 3A,B). Double immunofluorescence experiments demonstrated that proliferating intimal cells were predominantly RAM11-immunoreactive macrophages (Fig. 3C). The major proliferating component within the media appeared to be SMCs, as suggested by abundant medial SMα-actin immunoreactivity in this compartment (Fig. 1B).

When expressed relative to the area of lesion, cell replication within the atheroma was similar in young and old rabbits (Fig. 2B). Moreover, simple regression analysis and *F*-test disclosed a lack of significant relationship between intimal cell proliferation (as revealed by the density of BrdU-positive cells) and lesion size (Fig. 4, bottom panels, $P > 0.05$). In both age groups, intimal cell proliferation greatly exceeded that seen in the media (60–200-fold increase, Fig. 2B). Medial cell proliferation occurred predominantly near intimal lesions (Fig. 3A) and was approximately three times higher in YC

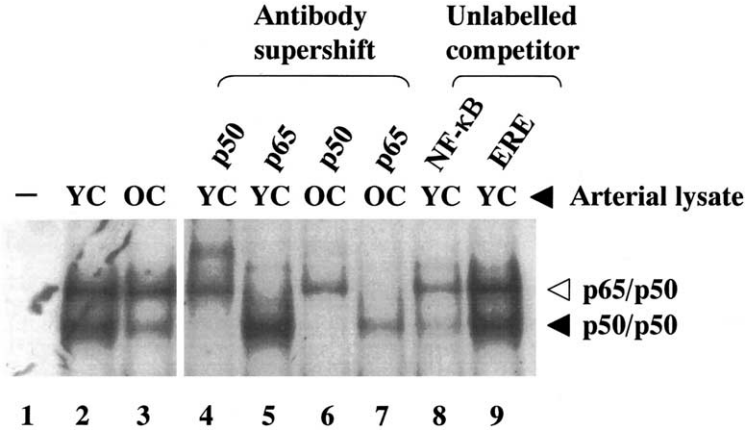

Fig. 5. DNA-binding activity of NF-κB in aortic tissue of cholesterol-fed young (YC) and old (OC) rabbits. EMSA was performed using a radiolabeled NF-κB consensus binding site. Only retarded nucleoprotein complexes are shown. Lane 1 corresponds to binding reaction without arterial lysate. Lanes 4–7: Binding reactions included the indicated antibodies. Lanes 8, 9: Competition with a 100-fold excess of unlabeled NF-κB or unrelated oligonucleotide (ERE: estrogen-responsive element).

than in OC rabbits (Fig. 2B). Simple regression analysis and *F*-test revealed a statistically significant direct relationship between the density of BrdU-positive medial cells and intimal lesion size in both age groups (Fig. 4, top panels, $P < 0.002$ and $P < 0.009$ in young and old rabbits, respectively).

According to the response to injury hypothesis, continued inflammation results in chemotaxis and accumulation of macrophages in fatty streaks [1]. Given that NF-κB appears to play a critical role in the induction of several proinflammatory chemokines during atherogenesis [10], we performed EMSAs using a consensus NF-κB probe to examine the DNA-binding activity of this family of transcription factors in aortic tissue (Fig. 5). These analyses revealed the presence of two nucleoprotein complexes in both YC and OC rabbits (lanes 2, 3), which were identified as p50/p50 homodimers and p50/p65 heterodimers using specific antibodies (lanes 4–7). An excess of unlabeled NF-κB, but not unrelated estrogen-responsive element oligonucleotide efficiently competed DNA-binding activity (lanes 8, 9). Averaged over three independent assays, densitometric analysis revealed a 35% decrease in both p50/p50 homodimers and p50/p65 heterodimers in OC versus YC aortic tissue.

4. Discussion

The major finding of the present study is that juvenile rabbits receiving a short-term high-cholesterol diet (1% cholesterol) developed more aortic atherosclerosis than did old counterparts. Our findings appear to disagree with previous studies reporting that old rabbits receiving short- (2 months) and long-term (18 months) exposure to a low-dose hypercholesterolemic diet (0.25% cholesterol) developed more atherosclerosis than young counterparts [11,12]. This discrepancy may be due to differences in the dose of dietary cholesterol used in each study, which resulted in striking differences in total plasma cholesterol. Indeed, young and old rabbits receiving the 0.25% cholesterol diet for 2 months averaged a plasma cholesterol level of 148–178 mg/dl [12], whereas animals challenged for the same period with a 1% cholesterol diet averaged 1747–2488 mg/dl (this study). Thus, in a mildly proatherogenic environment, age-dependent intrinsic alterations in the vessel wall appear to increase the susceptibility to atherosclerosis in aged rabbits. In contrast, the predisposition to atherosclerosis upon extreme dietary hypercholesterolemia was significantly higher in young rabbits.

Previous studies have shown that short-term hypercholesterolemia in the rabbit induces the formation of fatty streaks containing mostly macrophage-derived foam cells. [13]. We also found scarce SMα-actin immunoreactivity (Fig. 1B) and abundant expression of RAM11 (Figs. 1A and 3C) within intimal lesions, suggesting that macrophages are the primary atheromatous cellular component in the present study. Medial SMC proliferation in fat-fed rabbits is likely to result from the action of inflammatory cytokines produced by intimal macrophages, consistent with the response to injury hypothesis [1]. This notion is supported by the prevalence of proliferating medial cells in regions of the media underneath intimal lesions, together with the statistically significant direct relationship observed between medial cell proliferation and lesion size in both age groups.

Our results suggest that an age-dependent difference in arterial cell proliferation does not account for increased intimal lesion growth in young versus old fat-fed rabbits. First, intimal cell proliferation was similar in both age groups. Second, the prevalence of macrophages within the atheromatous lesions suggests that augmented medial SMC proliferation and migration of 'activated' medial SMCs into the intima contributes little to intimal lesion growth at the early stages of atherosclerosis examined here. Additional studies are thus required to elucidate the molecular mechanisms underlying the stronger atherogenic effect in juvenile rabbits exposed to extreme hypercholesterolemia. These might include augmented chemotaxis and accumulation of macrophages in young animals as a result of higher DNA-binding activity of NF-κB, a proinflammatory factor which is thought to contribute to chemokine production during atherogenesis [10]. Moreover, bearing in mind the important role of apoptosis during atherosclerosis [14], it appears appropriate to examine whether increased rates of apoptosis in the old vessel wall might contribute to the observed age-dependent reduction in intimal lesion growth during atherosclerosis.

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