Activation of Inducible Nitric Oxide Synthase Increases MMP-2 and MMP-9 Levels in ApoE-knockout Mice

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Objectives- Atheroma with reduced collagen content becomes fragile, but the underlying mechanisms have not been established. We investigated the influence of inducible nitric oxide synthase (iNOS) induction upon matrix metalloproteinases (MMP)s and collagen content in atheroma.
Methods and Results- ApoE$^{-/-}$ x iNOS$^{-/-}$ double knockout and ApoE$^{-/-}$ x iNOS$^{+/+}$ mice were fed a high-cholesterol diet for 15 weeks. Large atheromatous lesions of comparable size appeared in the roof of the aorta in both strains. Induction of iNOS mRNA was observed only in the atheroma of the ApoE$^{-/-}$/iNOS$^{+/+}$ mice. Collagen content was sparse and fat droplets were increased. Gelatin zymography and *in situ* zymography showed that pro- and active forms of MMP-2 and MMP-9 were more strongly expressed in ApoE$^{-/-}$/iNOS$^{+/+}$ than in ApoE$^{-/-}$/iNOS$^{-/-}$ mice, nitrotyrosine and MMP-9 were co-expressed in the atheroma.
Conclusion: We conclude that induction of iNOS in atheroma of high-cholesterol-fed ApoE$^{-/-}$/iNOS$^{+/+}$ mice leads to increased production and activation of MMPs, with a subsequent decrease in collagen content, affording fragile plaque.

Key words: Atheroma, Nitric oxide, Metalloproteinase, ApoE, Knockout mice

INTRODUCTION

The predominant underlying event in acute coronary syndromes is considered to be physical rupture of an atherosclerotic plaque. This exposes prothrombotic material from the core of the atheroma, and an occluding thrombus is formed on the surface of the atheroma, leading to partial or complete occlusion of the vessel. Rupture occurs preferentially in so-called unstable atheroma, which has a thin fibrous cap of scanty extracellular matrix (ECM) and is rich in lipid[1-3]. Since matrix metalloproteinases (MMPs) degrade collagen, a major component of ECM, MMPs are considered to have a key role in the formation of collagen-depleted, lipid-rich atheroma. MMPs are a family of approximately 30 structurally related endopeptidases, and strong local over-expression of MMPs has been observed in unstable regions of human atherosclerotic plaque[4, 5], and in the collagen-depleted plaque of animal models[6].

The influence of individual MMPs on plaque stability and growth has been examined by means of genetic manipulations[3, 7-9]. MMP-2 and MMP-9 are the major degraders of collagen in plaque, although MMP-1, -3, -8, -12, -13 and -14 also appear to make varying contributions in different models and animal models[7, 9]. The importance of MMP-9 in the formation of unstable atheroma was supported by studies using MMP-9-overexpressing macrophages in a mouse model of advanced atherosclerotic lesions[10, 11].

Another important issue is the role of nitric oxide in atherosclerosis. Among three nitric oxide synthases, endothelial NOS (eNOS) has been extensively studied and is generally accepted to be an important anti-atherogenic player[12-14]. However, the effects of inducible NOS (iNOS) on atherosclerosis are less well studied and published data are inconsistent[15-17]. Kuhlencordit et al. found that iNOS participates in the acceleration of atherosclerosis by using Apo E and iNOS double knockout mice (Apo E$^{-/-}$/iNOS$^{-/-}$) fed with an atherogenic diet[16]. However, the intimal hyperplasia of coronary arteries was markedly inhibited in the transplanted heart of iNOS$^{+/+}$ animals compared with that of iNOS$^{-/-}$ animals.[17] We examined the effect of iNOS on the characteristics of the atheroma and reported that extracellular collagen content was reduced and the volume of the lipid core was increased in the atheroma of iNOS$^{+/+}$ mice compared with that of iNOS$^{-/-}$ mice[18]. This is consistent with the formation of so-called unstable atheroma in the iNOS$^{+/+}$ mice. We have postulated that NO generated from iNOS in plaque reacts with superoxide to form peroxynitrite[19], which is a potent activator of MMPs[20-22].

The purposes of this study were to examine the influence of iNOS on the production and activation of MMPs in atheroma by using ApoE$^{-/-}$ x iNOS$^{+/+}$ mice fed a high-cholesterol diet, and to elucidate the pathogenic process leading to the formation of fragile

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plaque.

METHODS

Mice and Experimental Protocol

This study conforms with the Guide for the Care and Use of Laboratory Animals Published by the National Institute of Health (NIH Publication No. 85−23, revised 1996). The iNOS$^{-/-}$ mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and ApoE$^{-/-}$ mice were a gift from Dr N. Maeda[23]. The mice were backcrossed 10 times with C57BL/6 mice to ensure similar genetic backgrounds. ApoE$^{-/-}$/iNOS$^{-/-}$ double-knockout mice were generated by crossing ApoE$^{-/-}$ with iNOS$^{-/-}$ mice. ApoE$^{-/-}$/iNOS$^{+/+}$ mice were used for comparison. Mice were maintained on a 12-h dark/light cycle and were allowed access to food and water ad libitum. Experiments were started when the animals reached 8 weeks of age, and both strains were fed a high-fat diet containing 15% cocoa butter, 0.25% cholesterol and 0.5% cholic acid (Clea, Inc., Tokyo, Japan) for 15 weeks. Mice of both strains fed a normal chow (Lab Diet 5L65, PMI Feeds, Inc., St.Louis, MO) for the same period of time served as controls. Animals were sacrificed at 15 weeks with a pentobarbital overdose, and the heart and aorta were harvested for standard histological analysis, zymography and immunohistochemistry.

Measurement of Plasma Lipid Levels

Mice were fasted overnight and blood was collected via the abdominal aorta into heparin-coated tubes under pentobarbital-induced anesthesia. Plasma was separated and concentrations of total cholesterol and triglycerides were measured.

Tissue Preparation and Histological Analysis

The heart and proximal aorta were excised and embedded in OCT compound (Tissue-Tek) or snap-frozen, and stored at -80˚C until use. The OCT-embedded samples were serially sectioned at 4 m from the middle of the ventricle to the root of the aorta for histological analysis. Sections were counterstained with hematoxylin & eosin and Oil red O. For the quantitative evaluation of atherosclerotic lesion size, 9 sections were taken from each animal and the lesion size was quantified through microscopic examination with a computer-assisted image analysis system (KS 300; Carl Zeiss). The presence of collagen in the lesions was examined in sections stained with the standard Van Gieson method. For quantification of collagen fraction in the atheroma, 50 fields were microscopically examined at 40X magnification using a grid of 0.0625 mm^2 with 100 points. Values were expressed as the number of stained points/100 points. For evaluation of iNOS mRNA induction, the proximal aorta was snap-frozen in liquid nitrogen.

Reverse-transcription polymerase chain reaction (RT-PCR)

To determine iNOS mRNA levels, the frozen aorta was homogenized in ISOGEN (Nippon Gene, Tokyo, Japan). Total RNA was isolated and RT-PCR was performed; the primers for amplification of iNOS were described previously[18]. GAPDH mRNA was used as an internal control. The bands on the positive film were quantitated with National Institutes of Health (NIH) Image software.

Gelatin Zymography

Gelatin zymography was performed to determine expression of MMP-2 and MMP-9 proteins in the aorta in terms of gelatinase activity. Frozen aorta was homogenized in protein extraction buffer (50 mM Tris/HCl, pH 7.6, 150 mM NaCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.01% (v/v) Brij-35). The supernatant of a centrifuged sample (20 µg of protein extract per line) was mixed 1:3 with sample buffer and separated by SDS-polyacrylamide gel electrophoresis in 7.5% polyacrylamide gel which had been copolymerized with 1 mg/ml gelatin as described elsewhere[24]. Samples from the four groups were loaded separately and purified human MMP-2 and MMP-9 proteins in pro- and active forms served as positive controls.

Immunohistochemistry for Nitrotyrosine and MMP-9

Immunohistochemistry was performed using goat anti-nitrotyrosine antiserum (NITT13-S, Alpha Diagnostics Intl. Inc. USA, diluted 1:200) as previously described, with slight modifications[18], and using rabbit anti-human MMP-9 antibody developed in rabbit affinity-isolated antibody (M9555, Sigma-Aldrich Corp. USD, diluted 1:500) as previously described[25]. As a negative control, normal goat or rabbit IgG was used instead of the primary antibody. Sections were fixed with 4% PFAfor 5 min, blocked with 5% host serum (diluted 1:100) for 30 min, and incubated with the primary antibody for 2 hours at room temperature. The sections were then incubated with goat anti-mouse IgG Texas Red (1:100 dilution, alpha-goat Alexa 594, Invitrogen USA) for MMP-9 and with rabbit anti-mouse IgG FITC (1:100 dilution, alpha-goat Alexa 488, Invitrogen USA) for nitrotyrosine in the dark at room temperature for 30 min. Visualization was performed with a fluorescence microscope (Axiomager, Carl Zeiss, Germany).

In situ zymography

The procedures for *in situ* zymography were essentially based on the methods described previously,[26] and followed the manufacturer's instructions (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Fresh tissue was embedded in OCT compound and sectioned at 4 µm. These thin sections were placed on a polyethylene terephthalate base film coated with cross-linked gelatin (7 µm thickness; *MMP in situ Zymo-Film*, Wako Pure Chemical Industries, Ltd., Osaka, Japan) as described previously[26]. The film with the sections was incubated in a moist chamber at 37°C for 6 h, then stained for 4 min with Biebrich Scarlet (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The film was then rinsed for 10 min with distilled water. Gelatinolytic activity was identified as faded areas on the red background. In order to distinguish MMPs activity from nonspecific proteinase activities in the tissue, a counter-tissue section was placed on *MMP-PT in situ Zymo-Film*, which contains an MMP inhibitor, 1, 10-phenanthroline and the areas which are faded

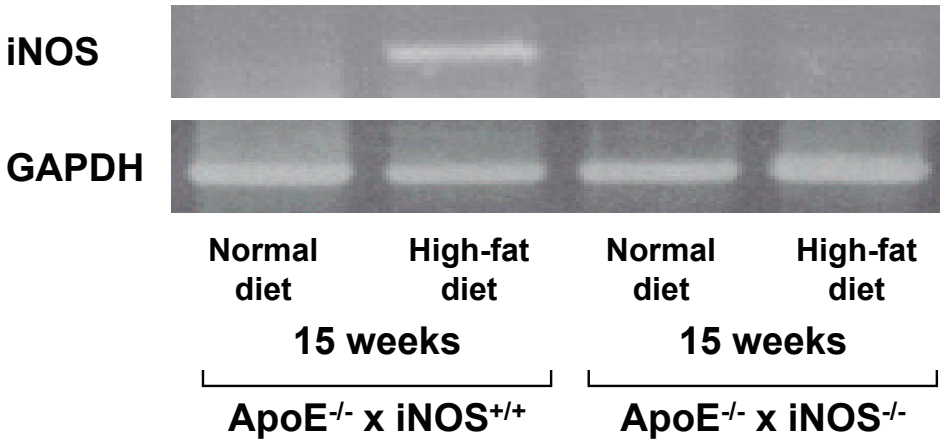

Fig. 1. iNOS mRNA expression in the aorta. mRNAs of iNOS and the housekeeping gene GADPH were evaluated with RT-PCR in ApoE$^{-/-}$/iNOS$^{+/+}$ and ApoE$^{-/-}$/iNOS$^{-/-}$ mice. Marked induction of iNOS was seen in ApoE$^{-/-}$/iNOS$^{+/+}$ mice fed a high-fat diet.

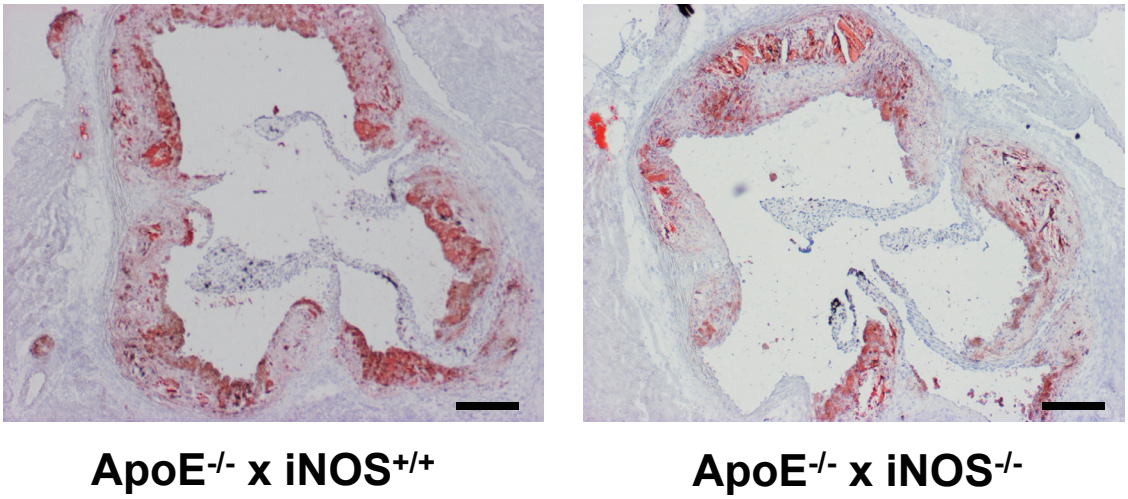

Fig. 2. Photomicrographs of aorta sections stained with Oil-red O. Fat droplets are evident in the atheroma, being more marked in ApoE$^{-/-}$/ iNOS$^{+/+}$ than in ApoE$^{-/-}$/ iNOS$^{-/-}$ mice.

without the MMP inhibitor but remain red (unfaded) with the MMP inhibitor (on MMP-PT Zymo-Film) were defined to represent MMPs-specific activity.

Statistical evaluation

Values are expressed as mean ± SEM. The significance of differences was evaluated by using Student's *t* test for parametric data and the Mann-Whitney *U* test for nonparametric data. A value of $P<0.05$ is considered to be statistically significant.

RESULTS

Plasma Lipids Levels

Table 1 shows that the plasma total cholesterol levels were markedly elevated in both ApoE$^{-/-}$/iNOS$^{+/+}$ and ApoE$^{-/-}$/iNOS$^{-/-}$ mice fed the high-fat diet. Total cholesterol levels in mice fed normal chow were 361± 41.6 mg/dl in ApoE$^{-/-}$/iNOS$^{+/+}$ and 339±19.1 mg/dl in ApoE$^{-/-}$/iNOS$^{-/-}$ mice. HDL cholesterol and triglyceride levels remained low. iNOS knockout status had no effect on the levels of total cholesterol, HDL cholesterol and triglyceride. Body weight remained in the same range in both groups.

iNOS Expression in Aorta

As shown in Figure 1, marked induction of iNOS occurred in ApoE$^{-/-}$ /iNOS$^{+/+}$ mice fed a high-fat diet. iNOS was not induced in ApoE$^{-/-}$/iNOS$^{-/-}$ mice fed a high-fat diet, as expected or in the aorta of mice fed normal chow.

Histological evaluation

Mice of both strains fed the high-fat diet developed significant atherosclerotic lesions along the proximal aortic wall at the aortic valve cusps. There was no consistent difference in lesion size between the two strains, as evaluated based on hematoxylin and eosin staining. The average cumulative lesion sizes were $212 \pm 26 \times 10^3$ μ m^2 in ApoE$^{-/-}$/iNOS$^{+/+}$ and $201 \pm 43 \times 10^3$ μ m^2 in ApoE$^{-/-}$/iNOS$^{-/-}$ mice. Oil-red O staining showed that accumulation of fat droplets in red-stained areas was more marked in ApoE$^{-/-}$/iNOS$^{+/+}$ mice (Fig. 2). Collagen, detected with the Van Gieson method as pink-stained areas, was sparse in the lesions of

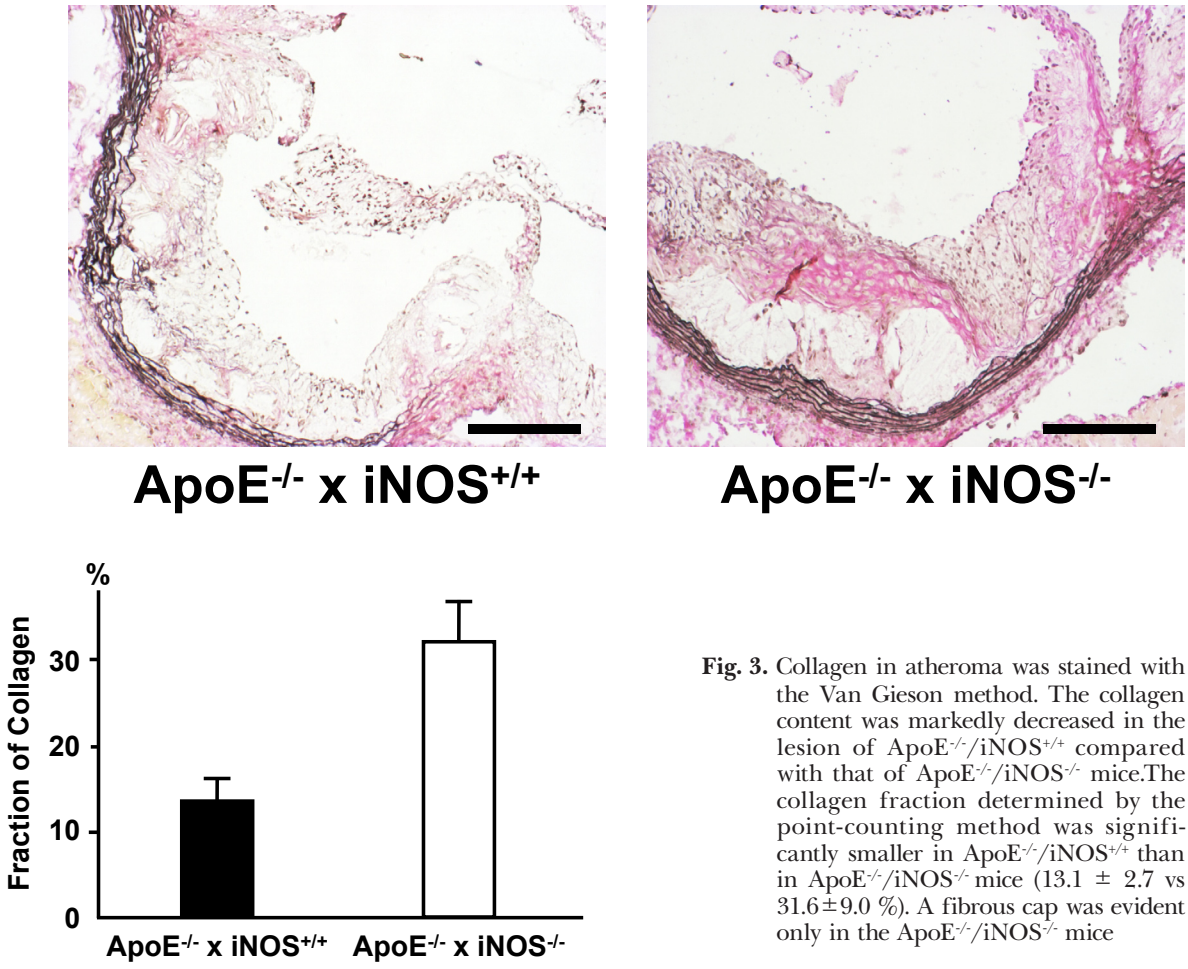

Fig. 3. Collagen in atheroma was stained with the Van Gieson method. The collagen content was markedly decreased in the lesion of ApoE$^{-/-}$/iNOS$^{+/+}$ compared with that of ApoE$^{-/-}$/iNOS$^{-/-}$ mice.The collagen fraction determined by the point-counting method was significantly smaller in ApoE$^{-/-}$/iNOS$^{+/+}$ than in ApoE$^{-/-}$/iNOS$^{-/-}$ mice (13.1 ± 2.7 vs 31.6±9.0 %). A fibrous cap was evident only in the ApoE$^{-/-}$/iNOS$^{-/-}$ mice

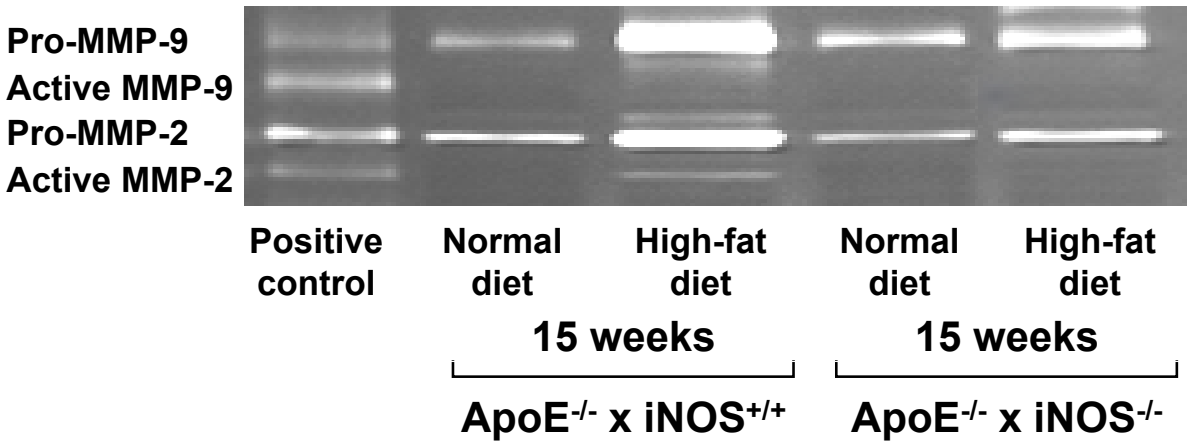

Fig. 4. Expression of MMP-2 and MMP-9 protein in the aorta was examined by means of gelatin zymography. Homogenized aorta from the four groups and purified human MMP-2 and MMP-9 proteins as controls were loaded in separate lanes. Pro-MMP2 and MMP-9 were expressed in all groups, but were more highly expressed in mice fed a high-fat diet than in mice fed a normal diet. The increase of both pro- and active-forms of MMP-2 and MMP-9 was more marked in ApoE$^{-/-}$/iNOS$^{+/+}$ mice than in ApoE$^{-/-}$/iNOS$^{-/-}$ mice.

ApoE$^{-/-}$/iNOS$^{+/+}$ mice compared with those of ApoE$^{-/-}$/iNOS$^{-/-}$ mice (Fig. 3). Atherosclerotic lesions were not observed in ApoE$^{-/-}$/iNOS$^{+/+}$ or ApoE$^{-/-}$/iNOS$^{-/-}$ mice fed normal chow.

Expression of MMP-2 and MMP-9 in the aorta

Figure 4 shows the result of gelatin zymography of pro- and active forms of MMP-2 and MMP-9 in the four groups. The pro-form of both MMPs was observed in all groups, but the levels were markedly increased in both groups fed a high-fat diet. Of particular importance is the profound increase of pro-MMP-2 and pro-MMP-9 with clear expression of the active forms in the ApoE$^{-/-}$/iNOS$^{+/+}$ mice fed a high-fat diet.

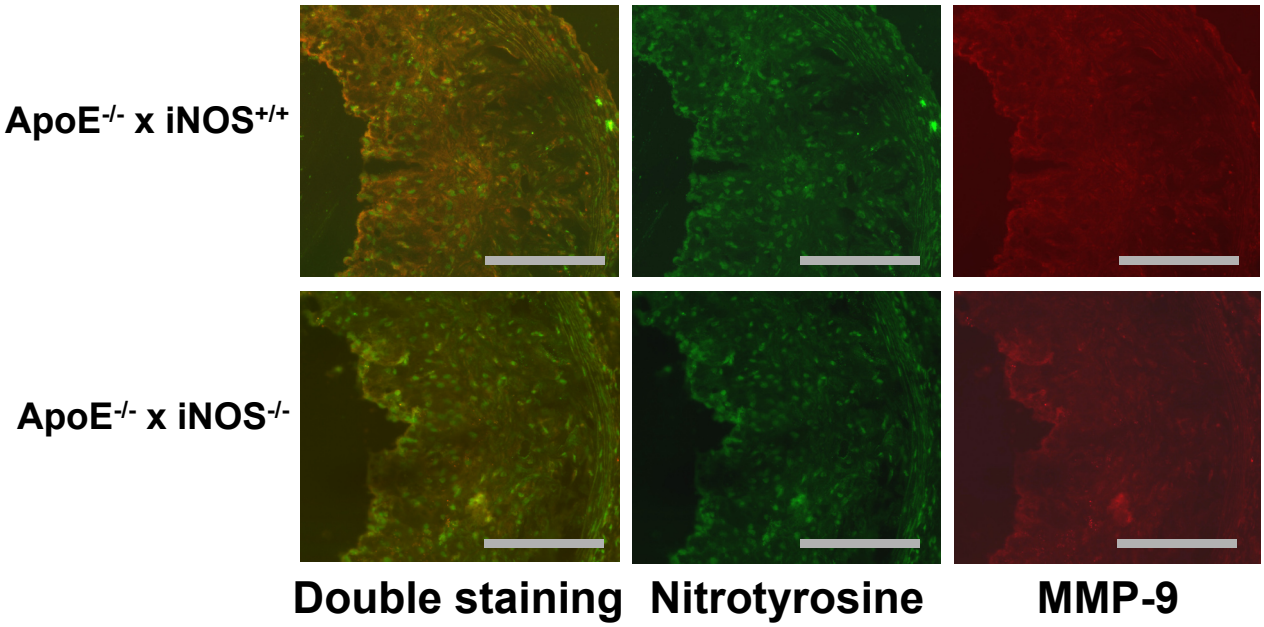

Fig. 5. Staining of nitrotyrosine and MMP-9 in atheroma. Nitrotyrosine (green) and MMP-9 (red) were increased in ApoE$^{-/-}$/iNOS$^{+/+}$ mice as compared with ApoE$^{-/-}$/iNOS$^{-/-}$ mice. Double staining showed that nitrotyrosine and MMP-9 are co-localized.

Double staining for nitrotyrosine and MMP-9 in atheroma

NO-induced oxidant activates MMPs[20-22] and its presence is detectable by measurement of nitrotyrosine, an NO-induced nitration reaction product[19]. Thus, the distribution of nitrotyrosine staining was compared with that of MMP-9 in atheroma of ApoE$^{-/-}$/iNOS$^{+/+}$ and ApoE$^{-/-}$/iNOS$^{-/-}$ mice fed a high-fat diet (Fig. 5). Nitrotyrosine is stained green, and MMP-9 is stained red. There was a clear increase of both nitrotyrosine and MMP-9 in atheroma of ApoE$^{-/-}$/iNOS$^{+/+}$ mice, and the two were colocalized (Fig. 5 upper left).

***In situ* Zymography of atheroma**

Figure 6 shows gelatinolytic activity in atheroma of ApoE$^{-/-}$/iNOS$^{+/+}$ and ApoE$^{-/-}$/iNOS$^{-/-}$ mice fed a high-fat diet. Areas of degraded gelatin are seen as white faded areas on the red background stained with Biebrich Scarlet on *MMP in situ ZymoFilm*; these areas represent both nonspecific and MMPs-specific proteinase activities. Red areas newly emerged in the white faded areas represent areas having MMPs-specific gelatinolytic activities. MMPs-specific gelatinolytic activity was evident in the ApoE$^{-/-}$/iNOS$^{+/+}$ mice (Fig. 6 B). All histological, immunostaining and molecular results suggested that the augmentation of the expression and activities of MMPs occurs in the atheroma with increased iNOS expression and the atheroma is collagenless and lipid rich.

DISCUSSION

A comparison of ApoE$^{-/-}$/iNOS$^{+/+}$ and ApoE$^{-/-}$/iNOS$^{-/-}$ mice in this study showed that the induction of iNOS in atheroma of ApoE$^{-/-}$/iNOS$^{+/+}$ mice did not influence the size of atheroma, but was associated with remarkable and important changes in the nature of the atheroma. Namely, the fat composition was increased and the collagen content was decreased, which are typical characteristics of fragile atheroma[1-3]. To clarify the mechanisms underlying the increased fat and decreased collagen levels in atheroma of the ApoE$^{-/-}$/iNOS$^{+/+}$ animals, we examined changes of the collagen degrading enzymes MMP-2 and -9. These matrix metalloproteinases were increased in both ApoE$^{-/-}$/iNOS$^{+/+}$ and ApoE$^{-/-}$/iNOS$^{-/-}$ mice fed a high-fat diet, but the increase was greater in ApoE$^{-/-}$/iNOS$^{+/+}$ animals. Furthermore, expression of the active forms of MMP-2 and MMP-9 was clear in the ApoE$^{-/-}$/iNOS$^{+/+}$ mice. Nitrotyrosine was colocalized with MMP-9 in atheroma of ApoE$^{-/-}$/iNOS$^{+/+}$ mice, suggesting that the iNOS-catalyzed NO production enhanced MMP activation, since NO-induced oxidant is known to be a potent activator of MMPs[20-22]. The activated MMPs would then degrade collagen.

It is generally accepted that eNOS is an important anti-atherogenic enzyme[12-14], though only a few studies have examined the effect of iNOS on atheroma, and the results are inconsistent[15, 16, 17, 18, 27]. Moreover, no study has focused on the relationship between iNOS, MMPs, and the composition of atheroma. The present findings suggest that iNOS is responsible for generating collagen-depleted and lipid-rich atheroma through increasing MMP production and activity. High concentrations of NO generated by iNOS yield peroxynitrite, which is a potent activator of MMPs[20-22]. In addition, peroxynitrite nitrates tyrosine residues of proteins, and enhanced formation of nitrotyrosine has been demonstrated in various pathological conditions in which iNOS induction occurs[13, 19, 24]. We also showed that the MMP-9 staining in atheroma was colocalized with nitrotyrosine staining (Fig. 5). As both pro- and active forms of MMP-2 and MMP-9 were increased (Fig. 4), iNOS induction may enhance the production of these MMPs, as well activating them.

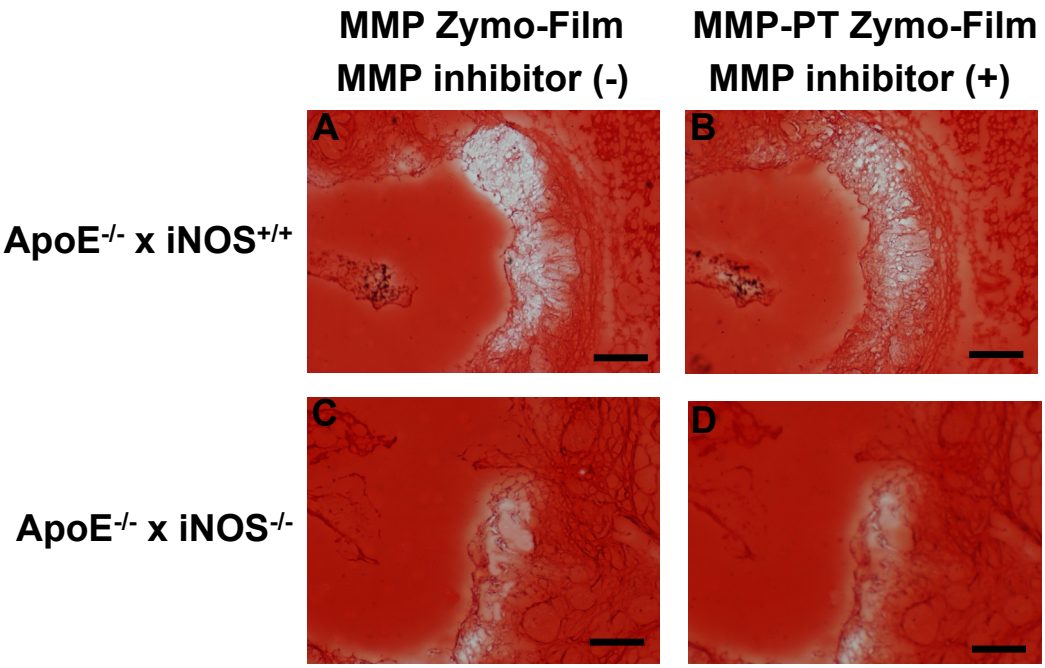

Fig. 6. *In situ* zymography of the aorta.
MMP Zymo-Film: gelatin-coated film,
MMP-PT Zymo-Film: gelatin-coated film containing MMP inhibitor (1, 10-phenanthroline).
White faded areas on the red background in A and C represent areas of nonspecific and MMPs-specific proteinase activities. Red areas newly emerged in the white faded areas of B and D show MMPs-specific gelatinolytic activity.

Inflammatory cells, including activated macrophages and T cells, secrete MMPs and other cysteine proteases, as well as producing cytokines, radicals and coagulation factors, which, in concert, reduce the levels of endogenous inhibitor proteins of MMPs and cysteine proteases[1, 2, 27-29]. The decrease in inhibitor proteins, such as tissue inhibitors (TIMP) and cystatins, may have accounted for the activation of MMPs in this study, since these inhibitors play key roles in the formation of fragile atheroma. Cystatin C is the most efficient endogenous inhibitor of elastases and collagenases, and Sukhova et al.[3] have shown that cystatin C and Apo E double-knockout mice develop a dilated aorta with disrupted elastin. Thus, cysteine proteases, TIMP and cystatins, besides MMP-2 and MMP-9, may all influence the formation of fragile atheroma. Indeed, the influence of other MMPs remains to be established, as existing data on the roles of MMP-1, -3, -8, -12, -13, -14 in atheroma are conflicting[3, 7-9],

Regarding the effect of iNOS on the size of atheroma, our previous study with iNOS$^{+/+}$ mice lacking any manipulation of the ApoE gene showed that the size was similar in iNOS$^{+/+}$ and iNOS$^{-/-}$ mice[18]. Chen J et al. also found that lesion size was not affected by the presence or absence of iNOS, using ApoE/iNOS double-knockout mice[27]. Our findings were consistent with this.

In conclusion, our results indicate that iNOS does not affect atheroma size, but plays an important role in inducing fragile atheroma by initiating the activation of MMPs. Therefore, inhibition of iNOS is a potential therapeutic target.

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