論文の内容の要旨

論文題目 Effects of the ω-3 Polyunsaturated Fatty Acid, EPA, in Suppressing Abdominal Aortic Aneurysm Formation

（腹部大動脈瘤の形成における ω3 多価不飽和脂肪酸[EPA]の抑制効果）

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Abdominal aortic aneurysm (AAA) is a disease that can be defined as the gradual and irreversible dilatation of the abdominal aorta. It is common in men older than 65 years of age and has a reported prevalence of 4-9% in men and 1% in women. Surgical or endovascular repairs continue to be the only definitive treatment options for AAA, whereas pharmacological therapy for the prevention or slowing of AAA formation remains limited. Without treatment, natural disease progression of the AAA results in rupture. Given that the mortality rate for patients with AAA rupture remains extremely high (65-85%), new forms of pharmacological treatment are needed to improve patient outcomes for this common but silent and deadly disease.

Recent studies have revealed that inflammatory processes play a key role in the development of AAAs, which involves the infiltration of various immune cells (particularly macrophages and T cells) as well as activation of inflammatory pathways. Importantly, matrix metalloproteinase (MMP)-9 derived from macrophages and MMP-2 derived from vascular smooth muscle cells (SMCs) have been shown to be critical factors required for the elastin destruction and proteolytic degradation that are hallmark features of AAAs, thereby leading to gradual aortic dilatation. Interestingly, such vascular wall degradation in human AAAs is often also accompanied by calcification of the aneurysmal wall, suggesting a possible link between aneurysm formation and calcification.

The ω-3 polyunsaturated fatty acids (PUFAs) are a class of essential fatty acids required for normal biological activity and function in living organisms. These fatty acids can typically be either plant-derived (α-linolenic acid) or marine fish-derived [eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)]. From numerous clinical, epidemiological, and animal studies, ω-3 PUFAs have been demonstrated to possess anti-inflammatory, anti-fibrotic, and cardioprotective properties, and they are already being used widely as pharmacological agents and nutritional supplements in humans. They have been suggested to have various mechanisms of action, including the ability to reduce the production of inflammatory eicosanoids by competing with arachidonic acid, exertion of anti-inflammatory effects via ligand-receptor interactions with the G protein-coupled receptor 120, and activation of the resolution of inflammation by ω-3 PUFA metabolites such as resolvin E1 and protectin D1. However, the precise molecular mechanisms as to how ω-3 PUFAs exhibit beneficial effects in each pathological process still remain to be elucidated.

The role of ω-3 PUFAs in the management of AAAs has not been established. Given the pleotropic effects of ω-3 PUFAs, I hypothesized that ω-3 PUFA might suppress the formation of AAAs by attenuating tissue remodeling processes. In this study, AAA was induced by periaortic application of $CaCl_2$ in BALB/cA

mice that were fed either a control diet (Control diet group) or control diet supplemented with EPA (EPA diet group). Marked dilatation and calcification of the aorta in the Control diet group was clearly visible 6 weeks after $CaCl_2$ was applied to the infra-renal abdominal aorta; in contrast, the aortas of mice on the EPA-supplemented diet were dilated significantly less than those of mice on the control diet (Figure 1). The aortic diameters in the Control diet group were shown to have increased to approximately 1.6-fold that of sham-treated mice, which meets the definition for aneurysm formation (≥50% increase in aortic diameter), whereas the aortas in the EPA diet group only tended to increase by approximately 30%, a difference that was not statistically significant, indicating that EPA treatment attenuated the formation of $CaCl_2$-induced AAA (Figure 1). Histological examination of the AAAs demonstrated that the extensive matrix and elastic lamina destruction seen in AAAs of Control diet group mice was greatly suppressed in aortas from the EPA diet group, where elastic lamina strand breaks clearly seen in AAAs of the Control diet group were relatively absent in the EPA diet group. Taken together, the results support the notion that EPA attenuated aortic dilatation via suppression of vascular wall remodeling.

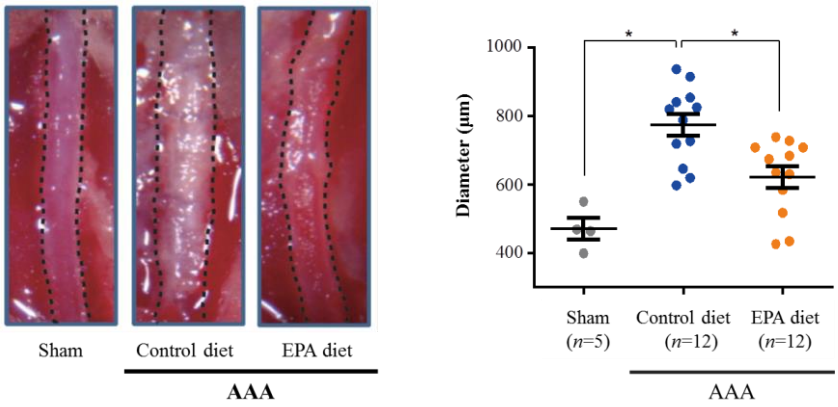

Figure 1. EPA reduced the diameter and macroscopic calcification of $CaCl_2$-induced AAAs. Representative photos of 6-week *in situ* AAAs. *P<0.05.

I subsequently began elucidating the molecular mechanism as to how EPA suppressed AAA formation, by first focusing on the expression of a set of genes related to tissue remodeling. Among the genes analyzed by real-time PCR, the expression levels of the matrix metalloproteinase (MMP) genes *Mmp2* and *Mmp9* were significantly increased in the aortas of control-diet-fed mice at 1 and 3 weeks after $CaCl_2$ application. In contrast, EPA-diet-fed mice had significantly lower levels of *Mmp2* and *Mmp9* expression. While tissue inhibitor of metalloproteinases (TIMP) *Timp1* and *Timp2* were also upregulated by the $CaCl_2$ treatment, EPA did not affect their expression. Previous reports have demonstrated that *Mmp9*-deficient mice are resistant to experimental AAA formation. Given that EPA seemed to impart a greater effect on *Mmp9* expression than on *Mmp2*, I decided to further analyze the effect of EPA on MMP9 in terms of AAA formation. Gelatin zymography (a method for assessing the functional enzymatic activity of MMPs in tissues) using 1-week AAA samples from Control diet and EPA diet groups was performed to examine the functional activity of MMP9. This showed that AAAs from

the EPA diet group exhibited only about 30% of the MMP9 activity observed in the AAAs from the Control diet group.

Since macrophages have been reported to be the major producer of MMP9 in AAA tissues, I therefore hypothesized that the decrease in MMP9 expression and activity might be due to a reduced number of macrophages recruited to the AAAs of EPA-treated mice. To this end, I analyzed the number of macrophages in 1-week AAAs by flow cytometry, but contrary to my hypothesis, there was no significant difference in the number of macrophages between the Control diet and EPA diet groups. However, when I subsequently sorted these macrophages and examined their *Mmp9* expression, there was significantly less *Mmp9* expressed by macrophages sorted from the AAAs of the EPA diet group, suggesting that an EPA-supplemented diet affected macrophage function, such as MMP production, within the AAA tissue. These *in vivo* results were further supported by my *in vitro* experiments, where I treated RAW264.7 macrophages (a well-established macrophage cell line) with EPA and then further stimulated these cells with TNF-α to induce *Mmp9* expression. The results demonstrated that EPA directly attenuated the TNF-α-induced upregulation of *Mmp9* compared to vehicle control in a dose-dependent manner. Taken together with the previous results, EPA appears to directly affect macrophages and reduce *Mmp9* expression.

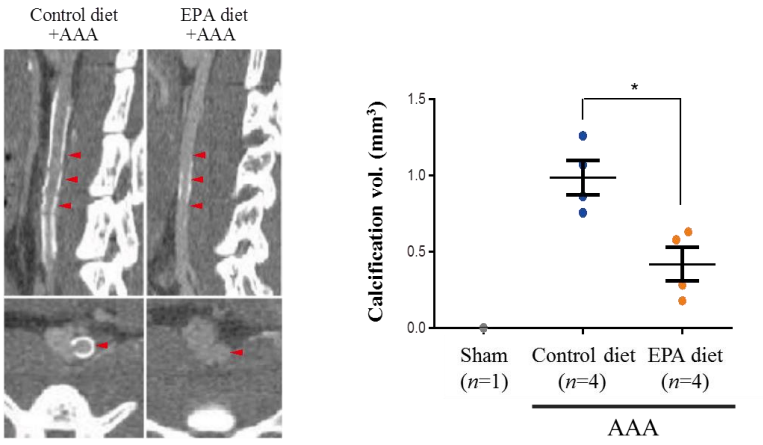

Figure 2. EPA reduced the calcification of $CaCl_2$-induced AAAs. Representative computed tomographic (CT) images of 6-week *in situ* AAAs with quantification of calcification volume. *$P<0.05$.

An interesting observation was that the aortic walls of $CaCl_2$-induced AAAs in mice from the Control diet group had clear, macroscopically visible calcification, whereas an EPA-supplemented diet attenuated this macroscopic calcification. Consistent with this observation, micro-computed tomography (CT) revealed that calcification along the area of the aorta to which $CaCl_2$ had been applied was significantly reduced in the EPA diet group compared to the Control diet group (Figure 2). When the expression levels of factors known to be implicated in this process were examined, Receptor Activator of Nuclear Factor κB Ligand (RANKL; encoded by *Tnfsf11*), a member of the tumor necrosis factor superfamily that is known to be a major factor that increases

vascular calcification and maintains bone homeostasis, was observed to be significantly up-regulated in AAAs of the Control diet group after $CaCl_2$-induction. In contrast, an EPA-supplemented diet significantly attenuated *Rankl* upregulation. Given that both tissue remodeling and vascular calcification were reduced by EPA, the next question was whether these two processes were somehow linked to each other. Indeed, the gene expression of *Rankl* in the first week of AAA formation revealed that it increased 4-fold one day after $CaCl_2$-induction and thereafter continued to increase in a time-dependent fashion. Most importantly, *Mmp9* up-regulation was temporally closely related to the increase in *Rankl*, supporting the possibility that RANKL could be actively linked to tissue remodeling *in vivo*.

In use clinically for over twenty years now, EPA alone or in combination with other omega-3 fatty acids has been shown to have pleiotropic benefits across a variety of diseases, such as the primary and secondary prevention of major coronary events, reduction of heart failure incidence, lowering blood pressure, improving outcomes of surgical and intensive care patients, and preserving renal function in patients with IgA nephropathy. Further adding to these reports, my findings here suggest that EPA may also be useful in slowing or preventing AAA formation as well as vascular calcification. My results suggest that inhibition of *Mmp2* and *Mmp9* expression is one potential mechanism by which EPA modulates tissue remodeling processes during AAA formation. In contrast to its effects on MMPs, EPA did not affect the expression of *Timp1* and *Timp2*, both of which are tissue inhibitors of a wide range of MMPs including MMP9 and MMP2. Given that the levels of both *Timp1* and *Timp2* in AAAs have also been shown to be associated with aneurysm formation, it is likely that administration of EPA shifted the AAA microenvironment from a pro-proteolytic to an anti-proteolytic milieu by altering the balance between MMP9, MMP2, and TIMP levels. Furthermore, the finding that vascular calcification and AAA formation could in fact be linked through RANKL is interesting from the point of view of both understanding AAA pathogenesis as well as the therapeutic implications.

It is clear that future studies are needed to evaluate EPA's use in AAA prevention in humans, and the fact that EPA is already in clinical use widely, both as a nutritional supplement in the form of unpurified fish oil preparations and as a pharmacological agent in the form of ultra-purified EPA, should facilitate this.