Effect of Ethyl Icosapentaenoate (EPA) on the Concentration of Tumor Necrosis Factor (TNF) and Interleukin-1 (IL-1) in the Carotid Artery of Cuff-sheathed Rabbit Models

Takeshi Shimizu¹, Toshihiko Iwamoto¹, Syozo Itou¹, Nobuhisa Iwata², Takahiko Endo², and Masaru Takasaki¹

¹Department of Geriatric Medicine and ²Department of Forensic Medicine, Tokyo Medical University, Tokyo, Japan.

To evaluate the effect of ethyl icosapentaenoate (EPA) on the metabolism of tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β), the concentrations of these cytokines in the carotids of rabbits sheathed in a cuff were studied. Japanese white rabbits were divided into two groups; the EPA group, in which 600 mg/kg/day EPA was administered forcibly p.o. for 1 week before cuff treatment, and the control group. Carotid artery samples were obtained just before, 3 days and 7 days after cuff treatment, and TNF-α and IL-1β were determined separately with the Western blot analysis method. In the control group, there were 43.5 (±3.0) pg/μg protein of TNF-α and 53.5 (±4.8) pg/μg protein of IL-1β just before cuff treatment. Compared to the control group, these concentrations of the EPA group were both significantly low. Three days after cuff treatment, TNF-α of the EPA group was still significantly low, while IL-1β showed no difference. There was no significant difference between the two groups 7 days after cuff treatment. These findings suggested that EPA could influence TNF-α and IL-1β metabolism in the arterial wall even at baseline. Furthermore, EPA suppressed TNF-α and IL-1β production in the early phase of intimal thickening, indicating a mechanism inhibiting the activation of smooth muscle cells such as their proliferation and migration, induced by the cuff-sheath method. J Atheroscler Thromb, 2001; 8: 45-49.

Key words: Cytokine, Atherosclerosis, Western blot analysis

Introduction

Several experiments regarding animals fed a cholesterol-rich diet (1) or subjected to balloon intravascular injury (2) have demonstrated that ethyl icosapentaenoate (EPA) inhibits intimal thickening of the arterial wall. Moreover, carotid arterial intimal thickening in rabbit carotid arteries, induced by cuff-sheathing, was also significantly suppressed by EPA (3). These findings suggested that EPA can inhibit intimal thickening in the early phase of arteriosclerosis. However, the mechanism by which EPA inhibits the arteriosclerotic process has not yet been fully understood because this process is associated with many other phenomena including interaction of cytokines and growth factors. From this point of view, the rabbit model in which the carotid arteries are cuff-sheathed is very useful to produce diffuse intimal thickening of the artery resembling the initial lesion of arteriosclerosis (4). Thus, to evaluate the effect of EPA on the metabolisms of tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β), the concentrations of TNF-α and IL-1β were studied in the carotid arteries of rabbits sheathed in a cuff.
Materials and Methods

Animal model

Japanese white rabbits (approximately 2 kg body weight) were divided into two groups; the EPA group (n = 15), in which 600 mg/kg/day EPA (90% purified, Mochida Pharmaceutical Co. Ltd., Tokyo, Japan) was administered forcibly p.o. for 1 week before cuff treatment, and the control group (n = 15), which did not receive EPA. After anesthetizing each animal with pentobarbital sodium i.v. and exposing the carotid artery, a polyethylene-tube cuff (2 mm in inner diameter and 15 mm in length, NOVA Biomedical Inc., Waltham, MA, USA) was placed around the bilateral arteries.

The animals were sacrificed, and carotid arteries after exposure and ligation were obtained just before, 3 days and 7 days after cuff treatment to determine TNFα, IL-1β and protein.

Determination of tissue TNFα, IL-1β and protein

After adding proteinase inhibitor with buffer to the specimens, they were immediately cut into small pieces and homogenized 10 times using a microwave for 10 seconds each time. They were centrifuged below 0°C for 20 minutes at 9,000 G to obtain supernatant, and were stored at −80°C until determination.

TNFα and IL-1β was determined separately with the Western blot analysis method, using anti-TNFα and anti-IL-1β antibodies as well as the Lowry method for protein determination. In Western blot analysis, electrophoresis in 16% acrylamide gel solution was performed in ice water blotting onto polyvinylidene difluoride (PVDF) membrane (MILLIPORE Inc., Waltham, MA, USA) using semi-dry type translocation instruments (BIO-RAD Inc., Hercules, CA, USA). TNFα was reacted with each primary antibody (anti-human TNFα neutralizing antibody produced in goats, R & D systems, Minneapolis, MN, USA) and the second antibody [alkaline phosphatase-conjugated Affini Pure rabbit anti-goat IgG (H+L), Jackson Immuno-Research Labo Inc., West Grove, PA, USA] under Protoblott II AP system (Promega Inc., Madison, WI, USA), and determined with a densitometer based on the previously-prepared standard curve.

Figure 1 shows representative findings of electrophoresis for TNFα detection with Western blot analysis. As shown in this figure, there were 4 lanes, comparing tissue samples with standard samples. The lane ‘mix’ was a sample of mixed tissue and standard samples because the band level of tissue sample was not completely identical with those of standard samples.

IL-1β was also determined with anti-human IL-1β neutralizing antibody produced in goats (R & D systems, Minneapolis, MN, USA). Figure 2 shows electrophoretic findings of IL-1β using Western blot analysis. Two bands in the sample lanes were completely the same as those in the standard lanes.

The standard curves for TNFα and IL-1β were obtained before investigation with each standard sample of recombinant human TNFα and human IL-1β, which had approximately 70% homology to rabbit TNFα and 74% homology to rabbit IL-1β at the amino acid level and cross-reactivity between two species, respectively (5, 6). In this study, reproducibility of the standard curve for both cytokine concentrations was also studied with correlation analysis. TNFα and IL-1β concentrations of the standard samples were determined twice after stepwise three-fold dilution of each standard sample with the buffer, which was used in determining tissue samples, in order to plot curves. Both correlation coefficients (mean ± SE) of TNFα and IL-1β (0.995 ± 0.203 and 0.986 ± 0.221, respectively) revealed excellent correlation, suggesting good reproducibility.

Protein was determined photometrically with a scanner according to the Lowry method (7). Concentrations of TNFα and IL-1β were expressed as pg/µg protein.
Effect of EPA Arterial TNF and IL-1 Levels

Statistical analysis
Statistical analysis was performed using Student’s t-test with the Scheffe and Duncan test. A p-value of less than 0.05 was considered to indicate statistical significance.

Results

1. TNFα
1) Concentration at baseline in the control group
   There was 43.5 ± 3.0 pg/μg protein of TNFα just before cuff treatment (Table 1).
2) Comparison of the concentration between two groups
   The mean TNFα concentrations of the EPA group were significantly lower than those of the control group just before and 3 days after cuff treatment (Table 1). At baseline, the TNFα concentration of the EPA group was 1/14 of that of the control group. No significant difference was seen between the two groups 7 days after cuff treatment.
3) Changes of the TNFα ratio between two groups
   The ratio of TNFα concentration in the EPA group to that in the control group was lowest before cuff treatment and increased abruptly 3 days after cuff treatment (Fig. 3). Seven days after cuff treatment, the ratio was approximately 1.0.

2. IL-1β
1) Concentration at baseline in the control group
   There was 53.5 ± 4.8 pg/μg protein of IL-1β just before cuff treatment (Table 2).
2) Comparison of the concentrations between two groups
   As shown in Table 2, the mean IL-1β concentration of the EPA group was significantly lower than that of the control group just before cuff treatment. At baseline, the IL-1β concentration of the EPA group was 1/22 of that of the control group. There were no differences between the groups 3 and 7 days after cuff treatment.
3) Changes of the IL-1β ratio between two groups
   The ratio of IL-1β concentration in the EPA group to that in the control group was lowest before cuff treatment and increased gradually to approximately 0.8 within 7 days after cuff treatment (Fig. 4).

Discussion
The experimental model used in this study showed that, after cuff-sheathing, diffuse intimal thickening resulting from the migration and proliferation of smooth muscle cells became apparent within 1 week and reached a maximum level 3 weeks later (8). Histopathological findings showed that cellulosiferous intimal thickening with spindle-shaped mononuclear cells, mainly smooth muscle cells, resembling an initial lesion of arteriosclerosis (4, 8).

Table 1. TNFα concentration in each group.
<table>
<thead>
<tr>
<th></th>
<th>EPA group</th>
<th>control group</th>
<th>p value</th>
</tr>
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<tbody>
<tr>
<td>TNFα (pg/μg protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>before CT</td>
<td>2.0±0.7</td>
<td>43.5±3.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>after CT</td>
<td></td>
<td></td>
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<tr>
<td>3 days</td>
<td>2.9±0.9</td>
<td>15.4±3.8</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>7 days</td>
<td>13.6±3.0</td>
<td>12.7±1.3</td>
<td>ns</td>
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Scheffe and Duncan test (mean±SE) (Abbreviation) CT: cuff treatment, ns: not significant

Table 2. IL-1β concentration in each group.
<table>
<thead>
<tr>
<th></th>
<th>EPA group</th>
<th>control group</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β (pg/μg protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>before CT</td>
<td>3.6±1.1</td>
<td>53.5±4.8</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>after CT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 days</td>
<td>6.8±1.9</td>
<td>11.5±3.9</td>
<td>ns</td>
</tr>
<tr>
<td>7 days</td>
<td>19.1±4.8</td>
<td>22.7±3.5</td>
<td>ns</td>
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</table>

Scheffe and Duncan test (mean±SE) (Abbreviation) CT: cuff treatment, ns: not significant

Fig. 3. Changes of the TNFα ratio between two groups. TNFα ratio: ratio of TNFα concentration in the EPA group to that in the control group.

Fig. 4. Changes of the IL-1β ratio between two groups. IL-1β ratio: ratio of IL-1β concentration in the EPA group to that in the control group.
Leukocyte proliferation and an increase of leukotrien (LT) B4 in the arterial wall were also observed early after cuff-sheathing associated with partial desquamation of the endothelial cells. In the “response to injury” hypothesis on genesis of atherosclerosis proposed by Ross and Glomset (9, 10), it was considered that leukocytes play an important role in the initiation of arteriosclerotic lesions. These findings indicate that intimal thickening is not only the result of an inflammatory response, but also that this model could provide a sample of the early phase of atherosclerosis (11).

In this model, the histopathological and biochemical studies demonstrated that EPA significantly suppressed this intimal thickening of rabbit carotid arteries without lowering the levels of cholesterol (8). Furthermore, combined treatment with EPA and pravastatin reduced the serum levels of both cholesterol and triglyceride additively and complementarily and inhibited intimal thickening more strongly than did the respective monotherapy in this model (3). The mechanism of EPA to suppress atherothrombosis has been studied and it is well-known that EPA has a variety of actions on vascular biological phenomena. EPA, ω-3 fatty acid, enters the lipid bilayer of the cell membrane instead of ω-6 fatty acid through plasma lipid, and modifies the function of membrane receptors and the eicosanoids metabolism in the cells. For example, EPA changes arachidonic acid metabolism both in platelets (12, 13) and endothelial cells (14-16), resulting in an anti-thrombotic action on the interface between the blood and vascular wall. These mechanisms explained the epidemiological evidence that the incidence of cardiovascular disease was extremely low in Eskimos, who used to have fish oil instead of meat (17).

In addition to anti-platelet actions, EPA has anti-inflammatory actions, which can suppress the inflammatory response in the arterial wall due to the effect of EPA on the eicosanoids (LTB4) metabolism in neutrophils (18-20). Therefore, it was considered that one of the effects of EPA on intimal thickening after cuff-sheathing was due to suppression of LTB4 production. On the other hand, it has been reported that fish oil decreased not only production of TNF and IL-1 by peripheral monocytes (21), but also production of PDGF by macrophages (22). However, there is little information on cytokines in this model, although cytokines play an important role in inflammation.

We thus conducted the present study to clarify the effect of EPA on the metabolism of inflammatory cytokines (IL-1 and TNF).

Applying this model, the effect of EPA on these cytokines was investigated within 1 week after cuff-sheathing, because it was clear that intimal thickening became apparent within a week (8). Moreover, animals were given a high dose of EPA for 1 week before cuff-sheathing so that their serum levels of EPA were the same as those of individuals treated with EPA.

In our study, there were 43.5 (±3.0) pg/μg protein of TNFα and 53.5 (±4.8) pg/μg protein of IL-1β in the control group without cuff treatment, and significantly low concentrations of both TNFα and IL-1β in the EPA group. This suggested that these cytokines were present even in the normal vessel wall under preparation, and EPA could influence the metabolism of those cytokines in the arterial wall at baseline just before cuff-sheathing. Furthermore, EPA suppressed the production of TNFα in the early phase of intimal thickening, indicating a mechanism inhibiting the activation of smooth muscle cells such as their migration and proliferation induced by the cuff-sheath method. However, this gives rise to two questions; one concerning what kind of vascular cells are influenced by EPA, and the other regarding what the origin of these cytokines in the control group.

In general, TNFα is produced by macrophages, smooth muscle cells, and T-cells when they are activated. Although the origin and distribution of TNFα was not identified in this study, it is considered that some physical stimuli due to the exploration and ligation of the carotid arteries might activate these cells, especially the macrophages existing and circulating in the sample. The origin of IL-1β, produced by macrophages, smooth muscle cells, and endothelial cells, is also unknown, but physical stimuli might activate these cells as well as TNFα because mRNA of IL-1β could be immediately produced by the stimuli. Low concentrations of these cytokines in the EPA group suggested that paracrine and autocrine interactions between vascular cells secondary to cuff-sheathing stimuli could be weak. Further study will be needed to compare the mRNA levels of TNFα and IL-1β before cuff-sheathing stimuli to those after these stimuli, and to clarify the origin of these cytokines by immunohistochemical techniques.

In this study, the concentrations of these cytokines could not be compared directly between the samples obtained on different days. One of reason was that the concentrations were expressed as the ratio of cytokine weight to protein weight, each of which could influence the other. After cuff-sheathing, the vessel wall becomes swollen with the increase of the extracellular matrix as well as cell proliferation and formation of mural thrombi. Therefore, the ratios of TNFα and IL-1β concentrations in the EPA group to those in the control group were used as a marker, instead of these concentrations.

These findings suggested that EPA can inhibit intimal thickening in the pre-phase and the early phase of arteriosclerosis by suppression of the TNFα and IL-1β metabolisms. EPA has also been reported to directly suppress smooth muscle cell proliferation (23) by inhibiting the binding of cytokines to their receptors on the surfaces of these cells. From this point of view, further study on the interactions among EPA, cytokines, and smooth muscle cells in the vessel wall should be performed coupling with each receptor, and using cytokines and antibodies specific for species.
Effect of EPA Arterial TNF and IL-1 Levels

Conclusion
To evaluate the effects of EPA on TNF$_\alpha$ and IL-1$\beta$ metabolisms in the thickening intima, the concentrations of these cytokines in the carotid arteries of rabbits sheathed in a cuff were studied using the Western blot method. The concentrations of both TNF$_\alpha$ and IL-1$\beta$ in the group pretreated with EPA for 1 week were significantly lower than in the control group even at baseline. Furthermore, EPA suppressed TNF$_\alpha$ production 3 days after cuff-sheathing. These findings suggested that EPA could influence the TNF$_\alpha$ and IL-1$\beta$ metabolisms in the arterial wall in the pre-phase and early phase of intimal thickening, indicating a mechanism inhibiting the activation of smooth muscle cells induced by the cuff-sheath method.

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Reference


