High-fat diet- and angiotensin II-induced aneurysm concurrently elicits splenic hypertrophy

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ABSTRACT

Background Angiotensin II (Ang II) and high-fat diet are implicated in causing pathological changes in the vascular endothelium, brain, kidney and liver. The association of aneurysm leading to histopathological changes in the splenic compartment remains elusive. Further, the salubrious credentials of antioxidants, especially α-tocopherol and β-carotene in the resolution of splenic pathology have not been investigated.

Methods Four-month-old Apoe−/− mice were used in the induction of aneurysm by infusing Ang II, and subsequently were orally administered with α-tocopherol and β-carotene-enriched diet for 60 days.

Results We observed splenomegaly in Ang II-infused aneurysm and high-fat diet-supplemented mice as compared to normal mice. These observations were further confirmed through histopathological investigations, demonstrating splenic follicular hypertrophy. We observed a remarkable decrease in the size of spleen in α-tocopherol and β-carotene-treated Apoe−/− mice as compared with Ang II-treated animals. Furthermore, no marked changes in the histopathological splenic sections were seen in the β-carotene-treated group. However, hyperplasia and proliferation of immature lymphocytes in the follicles were observed in the α-tocopherol-treated animals. We found that CD4+ T-cell levels were increased in the high-fat diet group relative to the control group and were decreased in the β-carotene-treated animals.

Conclusions Our study provides evidence that Ang II infusion and high-fat supplementation induces abdominal aortic aneurysm that has pathological implications to the spleen. The use of β-carotene but not α-tocopherol as an antioxidant markedly ameliorates the pathological changes in spleen.

Keywords Aneurysm, angiotensin II, antioxidants, spleen, β-carotene.

Eur J Clin Invest 2014

Introduction

Abdominal aortic aneurysm (AAA) is a common chronic degenerative disease causing localized structural deterioration of the aortic wall, leading to aortic rupture [1,2]. Certain hematological factors, especially Angiotensin II (Ang II), are believed to play a vital role in the development of AAA, which reportedly triggers the generation of reactive oxygen species (ROS). This in turn leads to the initiation of certain cell death signalling cascades, apoptosis and cell migration, and the expression of pro-inflammatory and extracellular matrix (ECM) proteins. This assumption has been supported in several lab models where the infusion of Ang-II resulted in the induction of AAA in Apoe−/− mice [3–5]. Ang II and high-fat diet have been implicated in causing pathological changes in the vascular endothelium, brain, kidney and liver [6–9]. The association between pathologies occurring in visceral organs with secondary pathology related to arterial aneurysm has rarely been documented. In our recent publications, we demonstrated that during the onset of aneurysm, in addition to the vascular endothelium, pathological changes can also occur in certain other visceral organs such as liver, kidney and brain [6,7]. Further, our gross pathological examinations revealed certain clues into the existence of splenic damage [8,9]. To the best of our knowledge, aneurysms associated with histopathological changes in the splenic compartment as a result of Ang II and high-fat diet administration have not been previously described.

Antioxidants, especially α-tocopherol (vitamin E), β-carotene and C-phycocyanin have strong free radical scavenging properties [10, 11]. β-carotene supplementation has been shown to dramatically control the diffusion of inflammatory
macrophages into the aortic tunica intima, and circulating macrophages in addition to resolving the formation of atherosomatic plaque in experimental aneurysm [3,9,12]. Due to this, antioxidants are currently being used in the treatment of oxidative stress-related ailments especially atherosclerosis [13], cardiovascular and kidney diseases, and type I diabetes. Our recent investigations demonstrated that these antioxidants dramatically improved pathological lesions caused by Ang II infusion in the kidney, brain and hepatic compartments of Apoε−/− mice [6–8]. Although splenic injury has been reported to occur in 0.1–1% of AAA repairs in clinical settings, the damage occurring to spleen as a consequence of AAA and the potential effects antioxidants have on alleviating splenic damage has seldom been investigated [14,15]. To determine this, we conducted a study to determine the state of spleens in Apoε−/− mice-induced aortic aneurysm using Ang II treated with β-carotene and α-tocopherol.

Materials and methods

Experimental animals

Experiments were conducted according to the guidelines formulated for the care and use of animals in scientific research (Indian National Science Academy, New Delhi, India) at a Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) registered animal facility. The experimental protocols were approved by the Institutional Animal Ethical Committee (IAEC) at CCMB (agreement no. IAEC72/07). We used 4-month-old male apolipoprotein E (Apoε−/−) knockout (n = 36) and C57BL/6j control mice in our experiments. All purchased from The Jackson Laboratory, ME, USA. Ang II was procured from Sigma-Aldrich (St. Louis, MO, USA), and ALZET osmotic pumps were from Charles River Laboratories

Induction of spleen damage

Ang II and high-fat diet were used to induce spleen damage, and histopathological changes were investigated in spleen of Apoε−/− mice [8,9]. Briefly, the mice were subcutaneously administered with Ang II via ALZET osmotic pump implantation [1,8,16,17] at a dose of 1 mg/kg/day for 45 days. Apoε−/− control mice (n = 6) received normal saline. After 45 days, six treated and six control Apoε−/− mice were sacrificed following standardized euthanastic protocols. The remaining 24 Ang II-treated Apoε−/− mice were grouped into four groups with six animals (n = 6) in each group. The first group received 800 mg α-tocopherol/kg of feed; the second received 800 mg β-carotene/kg of feed, the third group received 800 mg α-tocopherol and 800 mg β-carotene/kg of feed and the fourth group received normal chow diet. The corresponding antioxidant was mixed with normal chow diet ingredients that formed into pellets (National Institute of Nutrition, Hyderabad, India). After 60 days, antioxidant-treated and control Apoε−/− homzygous mice were sacrificed. The size of spleen was measured in control saline-treated, Ang II-treated and different antioxidant-treated Apoε−/− mice using a vernier caliper.

Flow cytometry

Spleen tissue was homogenized using a tissue homogenizer on 3% dextran in Hank’s balanced salt solution. Homogenized spleen samples were centrifuged at 1008 g. Then supernatant was collected and resuspended in fluorescence-activated cell sorting (FACS) staining medium (phenol red-free Hank’s balanced salt solution supplemented with 6% foetal bovine serum (FBS) and 0.01 M Na2-EDTA). Subsequently, samples were stained for 1 h at 4 °C in the dark with fluorescein isothiocyanate (FITC)-labelled anti-mouse CD45.2 and FITC-labelled anti-mouse MAC3 (1 : 100) separately. For each sample, washing and resuspending in FACS staining medium were conducted. Splenic CD4+ and CD8+ T cells were stained using FITC-labelled anti-mouse CD4 and CD8, respectively. Later, the samples were acquired on a BD FACSCalibur (Becton Dickinson, GmbH, Heidelberg, Germany). Results were analysed using FlowJo analysis software (Tree Star, Ashland, OR, USA).

Histopathology

Complete gross and histopathological evaluations were carried out in saline control, experimental control, Ang II- and antioxidant-treated Apoε−/− mice. After euthanasia, spleen was excised and dispersed in 10% buffered formalin (Sigma) from the control and treated Apoε−/− mice. Fixed and paraffin-embedded tissues were cut at 4 µm thickness, stained with haematoxylin and eosin (H & E) and examined under a light microscope (Zeiss, Oberkochen, Germany).

Statistical analysis

Values are expressed as percentage or mean ± SE where appropriate. Comparisons and correlations of treated and control samples were made with paired t-test. Statistical analysis was performed with GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). P values of <0.05 were considered significant.

Results

High-fat diet supplementation and angiotensin II induces abdominal aortic aneurysm concurrently with splenic hypertrophy in Apoε−/− mice

Ang II-treated Apoε−/− mice showed balloon-like dilation and extensive plaque formation in abdominal aorta, whereas
control Apoe−/− mice did not demonstrate any notable signs of AAA [18]. High-fat-enriched diet-supplemented animals resulted in the degradation of elastin, infiltration of lymphocytes, chondrocytes and cellular migration towards the media layer of the aortic vessel wall in Apoe−/− mice [8]. High-fat diet induced aneurysms notably at the abdominal and/or thoracic aorta with extensive plaque formation. Our investigation on the gross and histopathological changes of the spleen surprisingly demonstrated an enlargement of spleen in both Ang II-infused and high-fat diet-supplemented Apoe−/− mice. However, control Apoe−/− mice did not demonstrate such changes.

Therefore, we conducted new experiments to examine the spleen pathology on the high-fat diet- and Ang II-infused animals. We observed enlarged spleen in the Ang II-treated group (Fig. 1a-E) as compared to the normal saline-treated Apoe−/− mice (Fig. 1a-A). In particular, the in situ images of Fig. 1-E showed an enlargement of the medullary portion of spleen in which lymphatic follicles are located. We also observed that the rim or edges of the spleen became sharp due to enlargement of the medullary region (Fig. 1a-D,E). In contrast, in the spleens of saline-treated control animals, none of these features were noticed (Fig. 1a-A).

Further, when we conducted gross examination on spleen of high-fat diet-treated group, we also observed enlarged spleen in the high-fat diet-treated group (Fig. 1b-B–D) as compared to the normal saline-treated Apoe−/− mice (Fig. 1b-A).

Angiotensin II and high-fat diet supplementation induces splenic hypertrophy by recruiting several splenic follicles and immature lymphocytes

Next, we conducted histopathological investigations on both high-fat diet supplementation- and Angiotensin II-administered spleens of Apoe−/− mice. Hypertrophies of splenic follicles were observed in the Ang II-treated animals (Fig. 2g,h) but not in the control animals (Fig. 2a,b). In addition, the number and size of the follicles in the medullary region were markedly increased in the Ang II-treated mice spleen as compared with controls. The proliferation of immature lymphocytes (arrow) within the lymphatic follicles of Ang II-treated mice appeared higher (Fig. 2h). The size of the periarteriolar lymphoid sheath (PALS) was increased in the Ang II-treated spleen in which a number of T lymphocytes reside. Further, when we investigated the histopathological changes in high-fat diet-treated animals, we found hypertrophy of lymphatic follicles and proliferation of lymphatic follicles in the hypertrophic spleen as compared to the control mice. Most of the follicles showed mature lymphocytes surrounding the central arteriolar sheet (Fig. 2c,d).

Administration of experimental Apoe−/− mice with high-fat diet leads to splenic enlargement and increased infiltration of T lymphocytes

When the weight and volume of spleen in the mice were measured, enlargement of spleen was seen in mice fed with high-fat diet. The details of spleen measurements are summarized in Table 1. The investigation of immunologic attributes of splenic enlargement revealed the presence of lymphocytes as evident from staining with anti-mouse CD4 (CD4+ T cells), anti-CD8 (CD8+ T cells) and anti-CD19 (B cells) antibodies (Fig. 3). We found that CD4+ T-cell
levels were significantly increased in the high-fat diet group and were decreased in the β-carotene-treated animals (Fig. 4).

Table 1  Relative weights of spleen in the different experimental Apoe−/− mice

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Weight of spleen (in gms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Types of treatment</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.123 ± 0.008</td>
</tr>
<tr>
<td>High-fat diet</td>
<td>0.282 ± 0.002**</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>0.128 ± 0.014</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>0.152 ± 0.01*</td>
</tr>
<tr>
<td>Combined antioxidant</td>
<td>0.138 ± 0.012</td>
</tr>
</tbody>
</table>

Statistical significance, **P < 0.001; *P < 0.01.

β-Carotene ameliorates splenic pathology but not α-tocopherol in Apoe−/− mice

β-carotene (Fig. 1B) and combined of β carotene + α tocopherol (Fig. 1a-C) treatment group showed remarkable decrease in the size of spleen. However, treatment using α-tocopherol (Fig. 1a-D) continued to demonstrate splenic hypertrophy. Investigations revealed no marked splenic changes in the histopathological sections of β-carotene-treated group (Fig. 2e,f) and combined antioxidant-treated spleen (Fig. 2c,d). The number of lymphatic follicles and size of follicles in the β-carotene-treated spleen were similar to control spleen, but the combined antioxidant-treated spleen showed slight increase in the size of lymphatic follicles. We could not find any proliferation of immature lymphocytes in the β-carotene-treated group (Fig. 2e,f) or combined antioxidant-treated groups (Fig. 2c,d). However, hyperplasia of lymphatic follicles and proliferation of immature lymphocytes in the follicles were observed in α-tocopherol-treated animals. Further, β-carotene-treated animals showed decreased spleen volumes and weights, but not the α-tocopherol-treated groups. These findings suggest that β-carotene may have a role in the reversal of splenic pathology (Fig. 5).

Discussion

Prior investigations have underpinned the association of the RAS pathway with Ang II-mediated tissue injury [9,19]. However, high-resolution studies are seldom available on Ang II-induced splenic damage heretofore, and moreover, studies on potential extra-primary targets besides the primary target organ and/or tissue involved in the corresponding disease pathology still remains a viable area of investigation. Particularly, high-fat diet or Ang II-induced aneurysm or spontaneously occurring aneurysmal conditions are directly associated with splenic hypertrophy and whether or not it concurrently elicits splenic hypertrophy has never been studied. The results of the present study indicate that increased levels of Ang II resulted in splenic damage, and our investigations on the gross
changes occurring in spleen have convincingly demonstrated an enlargement of spleen in both Ang II-treated Apoe\(^{-/-}\) mice and animals that received high-fat diet supplement relative to control Apoe\(^{-/-}\) mice that did not show any observable change. In Ang II-treated group, we also observed an enlargement in the medullary portion of spleen where lymphatic follicles were located, and the rim or edges of the spleen appeared sharp presumed to be owing to the enlargement of splenic medulla. Of note, histopathological investigations support the observations, as the investigation showed hypertrophy of splenic follicles. Furthermore, the number and size of the follicles in the splenic medullary region were markedly increased. We also observed the recruitment of numerous immature lymphocytes within the lymphatic follicles. In addition, the size of the periarteriolar lymphoid sheath (PALS) was found to be increased, PALS being the site where the T lymphocytes reside in the spleen [20]. In addition, in high-fat diet-treated group, enlargement of spleen and immunologic attributes of splenic enlargement demonstrated the presence of lymphocytes and that CD4\(^{+}\) T cells and CD8\(^{+}\) T-cell levels were seemingly increased [21]. These observations are strongly supports the view that high-fat diet and Ang II elicits splenic hypertrophy.

The effects of Ang II on splenic pathology are clearly apparent in our current investigation, with the assumption that this could lead to a number of immunological and haematological changes as suggested by a previous study [22]. It is known that in the event of liver dysfunction, the spleen takes charge of certain functions especially involving the haematological system [23,24]. Nevertheless, this can only be managed to a certain extent which otherwise could culminate in splenic pathology [24]. The present study demonstrates this very clearly, with marked splenomegaly observed in all Ang II-treated mice. It has been suggested that this may be the eventual outcome as the result of ongoing hepatic inflammation in the AAA scenario [9]. However, we cannot also exclude the fact that inflammatory mediators could be one of the likely causes of the splenic pathology observed. This can be inferred because similar lesions have been suggested to exist in brain, kidney and the vascular endothelium following the onset of AAA.

![Figure 3](image1.png)  
**Figure 3** FACS analysis of CD4\(^{+}\) and CD8\(^{+}\) T cells in spleen of high-fat-supplemented group compared with control Apoe\(^{-/-}\) mice.

![Figure 4](image2.png)  
**Figure 4** Percentage of CD4\(^{+}\) and CD8\(^{+}\) T cells in spleen of different treatment group compared with control Apoe\(^{-/-}\) mice by FACS.
which clearly points to the role of infiltration of inflammatory macrophages [6,7,9].

With strong evidences suggesting that a poor prognosis could be the expected outcome following Ang II administration, it is not unexpected that the development of angiotensin-converting enzyme (ACE) inhibition is regarded as a milestone in modern medicine[13]. ACE inhibitor prevents the release of monocytes from splenic reservoirs in mice with myocardial infarction [25]. Further, others have shown that ovariectomy had no effect on Ang II-induced reductions in plasma renin concentration or spleen [18,25]. Our investigations on the immunologic attributes of splenic pathology also appears to rely on the potential recruitment of CD4+ T cells and CD8+ T-cell levels in the spleen as evident from the increased levels of these cells in the high-fat diet group relative to the control mice.

Several studies have established that α-tocopherol and β-carotene confers protection from oxidative stress and Ang II-induced pathology in vitro [26–28]. Here, we have convincingly demonstrated that dietary supplementation of α-tocopherol and β-carotene resulted in substantial protection of Apoe−/− mice from splenic hypertrophy. Our current results showed remarkable recovery of the splenic hypertrophy in β-carotene-treated Apoe−/− mice. Furthermore, no marked splenic follicular hypertrophy was seen in the histopathological splenic sections of β-carotene-treated Apoe−/− mice. In addition, CD4+ T cells and CD8+ T cells that increased in the high-fat diet group were lowered in the β-carotene-treated Apoe−/− mice. Hence, it is clearly evident that β-carotene treatment has a beneficial effect in alleviating splenic pathology. Further, even though α-tocopherol treatment showed a decrease in splenic hypertrophy (enlarged spleen size), it however, failed to control splenic hyperplasia and proliferation of immature lymphocytes in the follicles suggesting that α-tocopherol treatment likely has minimal beneficial effects on splenic pathology. Furthermore, one pitfall of the current study is the lack of establishment of the antioxidant functions of β-carotene in alleviating splenic damage, although we assume that the role of β-carotene as an antioxidant as a neutralizer of ROS has been well established [6,7,9,13]. In addition, it is also not clear as to why the protective effect α-tocopherol was relatively lesser compared to β-carotene in ameliorating splenic damage.

In conclusion, our study demonstrates that Ang II and high-fat diet supplementation induces AAA in Apoe−/− mice that results in secondary pathology involving the spleen and that use of antioxidants in particular β-carotene, markedly ameliorates these pathological changes following Ang II administration. Our
study also warrants potential investigations on extra-primary targets besides the primary target organ and/or tissue involved in the corresponding disease pathology, as exemplified from our current investigations on Ang II- and high-fat diet supplementation-induced AAA and splenic hypertrophy. Further, the currently observed splenic pathology in Apoe−/− mice following Ang II and high-fat diet supplementation occurring in murine AAA, potentially mimicking the same in human remains to be elucidated. Hence, our current investigation marks the beginning of an exciting and novel therapeutic strategy using β-carotene against splenic damage.

**Acknowledgements**

We are indebted to N. Dwarkanath and J. Jose for expert technical assistance and animal care. The authors also acknowledge National Institute of Nutrition for Preparation and Supply (NCLAS) for helping with preparation of antioxidant supplements used in our experiments. This work was supported by the Indian Council of Medical Research (ICMR), Government of India (Project IRIS ID. 2005-04430). This research is supported by HIR-MoE Grant (Reference number - UM.C/625/1/HIR/ MOHE/MED/04, account number – E00003-20001).

**Conflict of interest**

The authors have declared that no conflict of interest exists.

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Received 28 April 2014; accepted 12 October 2014

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