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Tenascin-C deficiency in apo E−/− mouse increases eotaxin levels: Implications for atherosclerosis

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ABSTRACT

Aim: To investigate the potential role of inflammatory cytokines in apo E−/− mouse in response to deletion of Tenascin-C (TNC) gene.
Methods and results: We used antibody array and ELISA to compare the profile of circulating inflammatory cytokines in apo E−/− mice and apo E−/− TNC−/− double knockout mice. In addition, tissue culture studies were performed to investigate the activity of cells from each mouse genotype in vitro. Cytokine array analysis and subsequent ELISA showed that circulating eotaxin levels were selectively and markedly increased in response to TNC gene deletion in apo E−/− mice. In addition, considerable variation was noted in the circulating level of eotaxin among the control apo E−/− mouse group. Inbreeding of apo E−/− mice with high or low levels of plasma eotaxin showed that the level of eotaxin *per se* determines the extent of atherosclerosis in this mouse genotype. While endothelial cells from apo E−/− mice had low level of eotaxin expression, cells derived from apo E−/− TNC−/− mice expressed a high level of eotaxin. Transient transfection of eotaxin promoter-reporter constructs revealed that eotaxin expression is regulated at the transcriptional level by TNC. Histochemical analysis of aortic sections revealed the massive accumulation of mast cells in the adventitia of double KO mice lesions whereas no such accumulation was detected in the control group. Plasma from the apo E−/− TNC−/− mice markedly stimulated mast cell migration whereas plasma from the apo E−/− mice had no such effect.
Conclusion: These observations support the emerging hypothesis that TNC expression controls eotaxin level in apo E−/− mice and that this chemokine plays a key role in the development of atherosclerosis.

1. Introduction

Substantial evidence from clinical and experimental studies supports the notion that atherosclerosis is a chronic immune-inflammatory disease. The continuous immigration and infiltration of inflammatory cells in lesions are prominent features in both human and experimental atherosclerotic disease. This process is guided by endothelial leukocyte adhesion molecules and chemoattractants, within the latter is a group of molecules called chemokines. These are structurally related, secretable, largely basic, chemotactic cytokines that play a key role in the initiation and progression of inflammatory disease [1].

Eotaxin (CCL11) is a potent chemokine that promotes migration and activation of eosinophils which participate in the pathogenesis of a broad range of allergic disorders [2]. Microarray analysis of human plaques showed that eotaxin is selectively and markedly overexpressed in human lesions [3]; although eosinophils are rare in atherosclerotic lesions, the receptor for eotaxin (CCR3) is present on macrophages, Th2 cells, and mast cells in lesions. Functional studies reveal that eotaxin induces CCR3-expressing endothelial cell migration in vitro and angiogenesis in vivo, as well as endothelial cell sprouting from aortic rings in the absence of an eosinophil infiltration [4]. Thus, in addition to potentially activating atheroma mast cells and macrophages, eotaxin may directly influence vascular inflammation through the endothelium [5]. While the underlying mechanism that regulates eotaxin expression in vivo remains unclear, inflammation induces marked upregulation of eotaxin.

The local microenvironment, or niche, of vasculature plays important roles in vascular pathologies. A major component of the

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niche is a matricellular protein, a complex network of macromolecules with distinctive physical, biochemical, and biomechanical properties. Although tightly controlled during embryonic development and organ homeostasis, the matricellular proteins are commonly remodeled in diseases such as atherosclerosis. TNC is a matricellular protein that is expressed in a rigidly controlled temporo-spatial pattern in the developing fetus, yet is undetectable or found only in low levels in the corresponding regions of the intact adult organs (for general discussion, see Midwood & Orend [6]).

We have previously shown that TNC is expressed in human vein grafts [7] and balloon catheterization induces arterial expression of different TNC isoforms [8,9]. We have also found that factors which are important in neointimal formation, such as angiotensin Ref. [10] and PDGF-BB [11], regulate the expression of TNC in vascular smooth muscle cells (SMCs). Others have reported that TNC is an essential factor for neointimal hyperplasia after aortotomy [12]. We have also shown that TNC is expressed in the macrophage rich regions of human atherosclerotic plaques [13] and in arterialized human vein grafts [7]. Both anti- and pro-inflammatory activities have been reported for TNC. To understand the function of TNC in atherosclerosis, we generated TNC−/−/apo E−/− double KO mice and have shown that deletion of the TNC gene promotes atherosclerosis, suggesting that TNC has an athero−protective activity [14]. We now report that deletion of TNC gene in apo E−/− mice leads to significant and specific upregulation of plasma eotaxin. Further evidence suggests that deletion of TNC leads to an upregulation of eotaxin in endothelial cells and the addition of TNC inhibit eotaxin promoter activity at the transcriptional level. These findings highlight the potential role of eotaxin in atherosclerosis and suggest that a clearer understanding of this pathway in atherosclerosis may have potential therapeutic implications.

2. Materials and methods

2.1. Preparation of animals

The TNC−/−/apo E−/− mice were generated by crossing TNC−/− mice with apo E−/− mice, as previously reported [14]. Eotaxin KO mice were generously provided to us by Dr. Marc E. Rothenberg, Cincinnati Children's Hospital Medical Center, Cincinnati, OH. We also generated apo E−/− mice with high or low levels of circulating eotaxin. For the generation of eotaxin 'low' and eotaxin 'high' mice, 6 weeks old apo E−/− mice were purchased from Jackson labs and their plasma eotaxin levels were quantified by ELISA following an overnight fast. The median eotaxin level was 450 pg/ml and mice with an eotaxin level of $\leq$450 pg/ml were considered as the eotaxin 'low' group and mice with eotaxin level of $>$450 pg/ml were considered the eotaxin 'high' group. Mice form the 'low' group and mice from the 'high' group were interbred within their respective groups for 5 generations before they were used for additional experiments. In some experiments, the animals were fed a high fat diet (Harlan) as indicated starting when the mice were 6 weeks of age, with water taken ad libitum. The blood samples were collected at the indicated times, the plasma was separated from the red blood cells by low speed centrifugation (2000 rpm in a microcentrifuge for 20 min) and the plasma eotaxin level was quantified by ELISA kit from RD Systems essentially as described by manufacturer.

After the indicated time of being fed an atherogenic diet, terminal blood samples were collected by puncture of the right ventricle following euthanasia by an isoflurane overdose. Mice were perfused with PBS (20 ml) via the left ventricle, while the perfusate drained from the punctured right atria. The heart and ascending aorta to the iliac bifurcation were removed. The aortic tissue was placed in freshly prepared paraformaldehyde (4% [wt/vol] in PBS) overnight at room temperature and then embedded in paraffin. The sections were stained using standard protocols.

2.2. Atherosclerotic lesion analysis

The extent of the atherosclerotic lesions in the oil red O-stained aorta was quantified, as described previously [14,15]. Briefly, regions of each aorta quantified were defined as follows: (1) thorax, from the arch to the intercostal artery branch; and (2) abdominal region, from the thorax to the branch of the iliac bifurcation. The lesion size for each aorta was measured by Image-Pro Plus (version 4, Media Cybernetics). Lesions were reported as percentage of the total aortic area consisting of thoracic aorta (ending at the final intercostal artery), and abdominal aorta (ending at the iliac bifurcation).

2.3. Cytokine antibody array

The cytokine antibody array assessment was performed using antibody membrane purchased from RayBiotech Inc. (Norcross, GA). The membranes were processed essentially as described by the manufacturer. Briefly, membranes were blocked by addition of a blocking buffer and incubated at room temperature for 1 h. Membranes were then incubated with 1 ml diluted plasma (diluted 1: 1 with PBS) from 6 apo E groups or 6 TN/E groups (6 mice/group, $\sim$100 μl/mice) at 4 °C overnight. After washing, 1 ml of biotin-conjugated antibodies was added to each membrane and then incubated at room temperature for 1 h. After washing, 2 ml of diluted HRP-conjugated streptavidin were added to each membrane and then incubated at room temperature for 1 h. After washing, the membranes were developed by addition of buffers C and D, at room temperature for 1 h. The membranes were exposed to autoradiography film and the identity of the spots was determined using the template provided by the manufactures. According to manufacturer, the detection limitation for the cytokines/chemokines antibody array is around 15 pg/ml; therefore, any difference below this level is undetectable in our system.

2.4. Cell culture studies

Smooth muscle cells were isolated from mouse aortas by enzymatic digestion and cultured essentially as described [11,16]. The cells were used at passages 3–5. Endothelial cells were cultured on matrigel-coated culture dishes after enzymatic digestion essentially as described [17]. The cells were used at passages 3–5.

2.5. Transient transfection assay

A 1363-bp fragment of the promoter region of the eotaxin gene promoter luciferase construct was kindly provided by Robert P. Schleimer (Johns Hopkins Asthma and Allergy Center). Transient transfection experiments were performed with smooth muscle cells essentially as described [18]. Briefly, luciferase constructs expressing firefly luciferase, and pRL-β-actin, a plasmid expressing *Renilla* luciferase were mixed with Nucleofector solution V, and co-transfected into 1×10^6 smooth muscle cells. After transfection, cells were transferred to complete culture medium and treated with the indicated reagents. Cells were then harvested and lysed with lysis buffer. Luciferase activity was assayed using Dual Luciferase Reporter Assay System (Promega Corporation). All the transfection experiments were repeated at least three times, in triplicate.

2.6. Mast migration assay

Mast cell migration assay was performed using plasma from each mouse genotype. Plasma (pooled from 6 mice per genotype) was