Distant Skin Inflammation Induced by Chronic Bacterial Exposure to Abdominal Subcutaneous Tissue in Nonalcoholic Fatty Liver Disease-harboring Mice

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Background: The increasing prevalence of obesity has led to a higher incidence of metabolic syndrome. Nonalcoholic fatty liver disease (NAFLD) is thought to be a hepatic phenotype of metabolic syndrome. The prevalence of NAFLD increases with age among females. On the other hand, obesity is responsible for a variety of changes in skin physiology. For instance, psoriasis a multifocal skin disorder, and its severity has been found to be related to the level of obesity. To investigate whether a dermatologic disorder could be induced in NAFLD-harboring mice, we established a mouse model of obese mice with a skin disorder. Methods: Female C57BL/6 mice were ovarioctomized one month prior to the start of feeding with a high caloric diet (HCD). These HCD-fed mice were divided into two groups: the abdominal subcutaneous tissues in group 1 were inoculated with bacteria, while the same tissues in group 2 were inoculated with PBS. A total of 3 inoculations were performed over a period of three months. These mice were then fed with HCD for an additional 6 months and sacrificed to obtain the tissue samples. Results: In both groups 1 and 2, NAFLD-like hepatic alterations were observed in the liver. In group 1, but not in group 2, skin inflammation at the shoulder and neck, consisting of acanthosis, intracuticular edema, epidermal necrosis, and cellular inflammation in the dermis and subcutaneous tissues, was observed. In the inflamed skin of the group 1 mice, a marked thickness of the epidermis and lymphocyte infiltration were detected. Conclusions: Bacteria may trigger immune-mediated dermatologic disease in NAFLD-harboring mice.

Key Words: bacteria, metabolic syndrome, nonalcoholic fatty liver disease (NAFLD), dermatitis, acanthosis

Introduction

The increasing prevalence of obesity has led to a higher incidence of metabolic syndrome (visceral obesity, hyperglycemia, hyperlipidemia, and hypertension). Furthermore, obesity, type 2 (non-insulin dependent) diabetes mellitus, and hyperlipidemia are coexisting conditions that are frequently associated with nonalcoholic fatty liver disease (NAFLD). NAFLD covers a spectrum of disorders from simple steatosis to nonalcoholic steatohepatitis (NASH) and cirrhosis. NASH is defined as a subgroup of NAFLD where steatosis coexists with liver-cell injury and inflammation (steatohepatitis). In addition, NAFLD is thought to be a hepatic phenotype of metabolic syndrome. The prevalence of metabolic syndrome increases rapidly among women after menopause. Yatsu et al. reported that the severity of NASH increases with age, and among subjects older than 55 years, women were more common than men. In addition, if the patients were di-
vided according to their menopausal status, the difference was much more prominent[7]. Also, in a series of patients with NASH, more advanced cases were generally observed among women[8]. Furthermore, it was reported that among females, the ratio of the area of visceral fat to that of subcutaneous fat increases with age, especially after menopause[9]. Based on this clinical evidence, we prepared ovariectomized mice and used them in the present study.

Obesity is responsible for a variety of changes in skin physiology and has been implicated in some of dermatologic diseases, such as psoriasis, and acanthosis nigricans[10]. More than several decades ago, the relationship between skin manifestations and fatty infiltration in the liver had already been described (11 and therein). Zachariae et al. reported that significantly higher grades of steatosis, portal inflammation, and focal necrosis were found among patients with psoriasis than among controls[11]. In 1986, Lindegård described that psoriasis in women was associated with diabetes, obesity, and myocardial infarction[12], which are the major entities of metabolic disease. Lindegård also noticed that liver cirrhosis coexisting with psoriasis seemed to be related to diabetes, rather than alcoholism[13].

Recently, the involvement of bacteria in the pathogenesis of metabolic syndrome has been speculated. For example, Gordon et al. reported a relationship between obesity and microbiota[14,15]. Weak oral bacteria, such as periodontal bacteria, are thought to play an important role in the development of vascular disease, including atherosclerosis[16]. A relationship between Helicobacter pylori and diabetes mellitus, obesity, and dyslipidemia has also been reported[17]. On the other hand, we recently established two different autoimmune disease mouse models. Chronic exposure to a Gram-positive bacterium, Streptococcus intermedius, resulted in primary biliary cirrhosis-like nonsuppurative destructive cholangitis in the liver of BALB/c mice[18], while chronic exposure to a Gram-negative bacterium, Escherichia coli, resulted in autoimmune pancreatitis-like chronic inflammation in the pancreas of C57BL/6 mice[19]. These results indicate that bacterial infection abrogates normal immune responses. Based on these findings, we speculated that bacterial exposure might also trigger the onset of immune-mediated dermatitis in NAFLD-harboring mice.

The aim of the present study was to investigate the above possibility by examining whether chronic persistent exposure to bacteria in mouse abdominal subcutaneous tissue could cause immune-mediated skin inflammatory change in elderly NAFLD-harboring mice with a postmenopausal status.

Methods

Experimental mouse model for dermatitis

C57BL/6 mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and bred in the animal facility at the Department of Microbiology and Immunology, Tokyo Women’s Medical University. For feeding, a maintenance feed diet (MF) (359.7 kcal/100 g: protein, 26.2%; carbohydrate, 60.5%; and fat, 5.3%) was used. Approximately 10-week-old female mice were used for the experiments; all the experiments were performed in accordance with the guidelines of the ethics review committee for animal experiments, Tokyo Women’s Medical University. Mice were ovariectomized under anesthesia using diethyl ether. One month after the ovariectomy, 12 mice were fed a high caloric diet (HCD) (450.8 kcal/100 g: protein, 15.8%; carbohydrate, 44.3%; and fat, 39.9%); one month later, 5 mice were inoculated with 1 µg of lipopolysaccharide (LPS) in 0.1 ml of PBS emulsified with the same amount of Freund’s complete adjuvant (CFA) into the abdominal subcutaneous tissue once a month for a total of three months (group 1). The remaining seven mice were continuously fed an HCD and inoculated with 0.2 ml of PBS without bacteria and with CFA (group 2). Group 3 (n = 4) was fed with MF without bacteria inoculation after ovariectomy. Ten months later, the mice were sacrificed under deep anesthesia using diethyl ether. The tissues were then removed, fixed in 10% formalin, and embedded in paraffin. Simultaneously, serum samples were taken and kept frozen at −80°C until use. Multiple 4-µm-thick sections were used for the pathological examinations.
Immunohistochemistry

Serial sections were subjected to immunohistochemical staining with monoclonal anti-CD3 antibody (clone SP7; Abcam, Tokyo, Japan), a B cell antibody specific for CD45R/B220 (clone RA3-6B2; BD Pharmingen, Franklin Lakes, NJ, USA), and anti-pan-monocyte/macrophages F4/80 antibody (clone BM8; BMA Biochemicals AG, Augst, Switzerland). The sections were then stained using a peroxidase technique with a Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer’s instructions, followed by staining with hematoxylin.

Results

Pathology of the liver

We sequentially examined histopathological changes in the liver in each of the groups. The livers obtained from both groups 1 and 2 mice showed NAFLD-like hepatic alterations. In contrast, almost no fatty change was observed in the livers obtained from the group 3 mice (Haruta et al., manuscript in preparation).

Induction of inflammation in the skin

We next investigated visible findings for distant skin lesion in each mouse. Marked erythemas with crusts were observed on the shoulder and neck and behind the ears in group 1 mice (Fig. 1), but not in group 2 or 3 mice (data not shown). The results are summarized in Table 1A.

Skin pathology

We next compared the pathological alterations in the skin of the animals in each group. In the group 1 mice, acanthosis (Fig. 2a), intercellular edema (Fig. 2b), and a crust (Fig. 2c) were observed in the epidermis. In addition, marked mixed cellular infiltration in the dermis (Fig. 2d) and cellular infiltration in the subcutaneous fatty tissues (Fig. 2e) were observed. In group 2 mice, on the other hand, the histopathological findings were much milder than those in group 1, and no changes were observed in the group 3 mice (Fig. 2; group 1, f; group 2, g; group 3, h). The results are summarized in Table 1B.

Thickness of the epidermis

Then, we performed an objective analysis to evaluate the thickness of the epidermis. We used
Table 1  Scoring of the severity of the shoulder skin lesions

<table>
<thead>
<tr>
<th></th>
<th>group 1 n=5</th>
<th>group 2 n=8</th>
<th>group 3 n=4</th>
</tr>
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<tbody>
<tr>
<td>A Visible skin inflammation</td>
<td>3/5 (60%)</td>
<td>0/8 (0%)</td>
<td>0/4 (0%)</td>
</tr>
<tr>
<td>B Epidermis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acanthosis</td>
<td>+ +</td>
<td>~ + ~ +</td>
<td>~ ~ + ~</td>
</tr>
<tr>
<td>Intercellular edema</td>
<td>+ +</td>
<td>~ ~ +/ ~</td>
<td>~ ~</td>
</tr>
<tr>
<td>Dermis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflammation</td>
<td>+ +</td>
<td>~ ~ + ~</td>
<td>~ ~</td>
</tr>
<tr>
<td>Subcutaneous tissue</td>
<td>+ +</td>
<td>~ ~ + ~</td>
<td>~</td>
</tr>
<tr>
<td>Inflammation</td>
<td>+ +</td>
<td>~ ~ + ~</td>
<td>~ ~</td>
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(+ + : severe, + : mild to moderate, ~ : none)

Fig. 2  Representative pathological findings of the mouse shoulder skin. In group 1 mice, acanthosis (a), intercellular edema (b), and a crust (c) were observed in the epidermis. Marked mixed cellular infiltration in the dermis (d) and mixed cellular infiltration in subcutaneous fat tissues (e) were observed. Findings of the shoulder skin of group1 (f), group 2 (g), and group 3 (h) mice (H&E-stained sections are shown).

H&E-stained samples obtained from the shoulder skin as shown in Figure 3a in each mouse. Using Image J software, the thickness of the epidermis was evaluated as schematized in Figure 3b. The thickness of the epidermis in group 1 was significantly greater than that in group 2 (Fig. 3c). These results indicated that although bacteria were injected into the subcutaneous tissues of the abdomen, distant shoulder and neck skin inflammation was observed in these mice. Thus, in mice suffering
from NAFLD-like liver pathological alterations, bacterial loading can easily lead to the establishment of systemic inflammation including distant skin lesions.

**Immunohistochemical findings for the shoulder skin**

To clarify the cell subsets infiltrating the shoulder skin, we next performed immunohistochemical staining. Although in the epidermal lesion, CD3-positive and B220-positive cells were occasionally observed in all the groups, F4/80-positive cells were more pronounced in the group 1 mice (Fig. 4A). In the group 1 mice, CD3-positive, B220-positive and F4/80-positive cells had markedly infiltrated the subcutaneous fat tissue (Fig. 4B). On the other hand, in group 2 and 3 mice, cellular inflammation in the subcutaneous fat tissue was much milder or not present (Table 1B). This observation suggests that bacterial stimulation in a local area can induce the up-regulation of systemic inflammatory responses in group 1 mice via an immune-mediated mechanism.

**Discussion**

The present study demonstrated a novel mouse model of chronic immune-mediated dermatitis in association with NAFLD-like hepatic alteration-harboring mice. The livers obtained from both groups 1 and 2 mice showed NAFLD-like hepatic alterations. In contrast, almost no fatty change was observed in the livers obtained from the group 3 mice (Haruta et al., manuscript in preparation). Although neither steatotic changes nor inflammation was observed in the livers of the MF-fed mice (group 3), steatotic changes were observed in the livers of both HCD-fed groups (group 1 and 2). However, severe skin eczematous change at the shoulder and neck lesions were only observed in group 1 mice, with some mild change observed in group 2 mice (Fig. 2). We found marked visible skin eczematous change in the shoulder neck lesions of 3 out of 5 (60%) of the mice in group 1 but not in the group 2 or 3 mice, and these areas of inflammation occurred far from the bacteria-inoculated site, i.e., the subcutaneous tissues of the abdomen. The visible skin inflammatory change consisted of acanthosis, intercellular edema as well as inflammation of the dermis and subcutaneous fat tissues, histologically (Fig. 2).
Fig. 4  Histological findings of the skin lesions. A. Immunostaining of the shoulder skin in group 1 (a, d, g), group 2 (b, e, h) and group 3 (c, f, i) mice. The immunoreactivity of the mouse epidermis to CD3 (a, b, c), B220 (d, e, f) or F4/80 (g, h, i) in the shoulder skin is shown. B. Inflammatory cellular infiltrates in the subcutaneous fat tissue in the shoulder skin of the group 1 mice. The immunoreactivity to CD3 (a, b), B220 (c) or F4/80 (d) is shown.
Table 1). Taken together, these findings suggest that HCD feeding alone in the ovariectomized mice was not sufficient to trigger skin changes, but that an additional bacteria-mediated innate immune response might be needed to trigger the progression of dermatitis associated with steatohepatitis.

As for obesity-associated skin disorders, some disease entities have been reported. An association between obesity and psoriasis, one of the most common chronic inflammatory skin disorders, has been identified in several studies (19 and therein). Established psoriasis has been associated with many of the components of metabolic syndrome including hypertension, dyslipidemia, obesity and impaired glucose tolerance. Matsumoto et al. reported a case of psoriasis vulgaris associated with NASH. Unfortunately, in our experiments, there were no elongated club-shaped rete ridges of the epidermis and parakeratosis in the shoulder and neck skin of the group 1 mice. Therefore, the pathological alterations observed in psoriasis were not observed in the present study. Acanthosis nigricans should be considered as another possible obesity-based complication. Among the types of acanthosis nigricans, pseudoacanthosis nigricans is known as a complication of obesity, possibly arising from insulin resistance.

Acanthosis nigricans is characterized by papillomatous elevations and pigmentation on body-fold lesions clinically and by hyperkeratosis and papillomatosis with no inflammation histologically. Consequently, our model was not compatible with acanthosis nigricans. We suspected that not only HCD-feeding but also bacterial stimuli might induce eczematous change and even panniculitis, which is different from psoriasis and acanthosis nigricans. In particular, chronic bacterial exposure may have affected the pathogenesis of this mouse dermatitis model, since the skin inflammation in group 1 was much severer than that in group 2. Thus, the differences between a group inoculated with bacteria and a group inoculated without bacteria should be strictly examined not only in HCD-fed mice, but also in MF-fed mice. In addition, visible eczematous change was observed not exactly at the point of LPS and CFA injection, but on the neck and shoul-
der skin. These findings suggest that distant skin inflammation can be induced by focal bacterial infection. Some reports have discussed the relationship between chronic focal infection and dermatological disorders, pustulosis palmaris et plantaris (PPP) and prurigo chronica multiformis (PCM). The dermatological features of the group 1 mice were not compatible with these disorders. The mechanisms that severe inflammatory changes in group 1 mice were induced in distant area from the point injected with LPS and CFA are unknown at this time.

Adipose tissue is an active endocrine organ that secretes numerous adipokines, cytokines and chemokines including adiponectin, tumor necrosis factor α (TNF-α), interleukin (IL)-1β, and IL-6. Elevated levels of pro-inflammatory cytokines have been noted in the serum of NAFLD patients. In PCM, the levels of TNF-α and interferon-γ are increased in affected mucosal lesions, suggesting that T-helper cell (Th) 1-type effector immune responses are evoked. Human leukocyte antigen (HLA) class II antigens are expressed by infected gastric mucosal epithelia, which may stimulate the immune system. Such immune reactions elicited by H. pylori might even cause various diseases, including PCM. Similar immune reaction induced by persistent bacterial infection might occur severe eczematous change and even panniculitis in distant skin lesion, synergized on this NAFLD-harboring ovarectomized mouse model. To clarify the pathogenesis of the skin inflammatory change, a study of cytokine regulation in this NAFLD-harboring ovariec-tomized mouse model is needed.

In conclusion, bacteria stimulation in HCD-fed and NASH-like steatohepatitis-harboring mice surely induced immune-mediated distant skin eczematous change and even panniculitis, accompanied by a significant thickening of the epidermis. Especially, chronic bacterial exposure might affect the pathogenesis of distant skin inflammation in our NAFLD-like hepatic alteration-harboring mice. Further studies are needed to confirm our speculation.
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Declarations
The authors declare no conflict of financial interest.

References
非アルコール性脂肪性肝炎モデルマウスにおける、慢性細菌曝露が誘因となる遠隔部皮膚病変の検討

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[背景] 近年肥満の増加によりメタボリック症候群の増加が問題となっている。非アルコール性脂肪性肝疾患（NAFLD）は肝障害におけるメタボリック症候群の表現形であり、NAFLD は閉経後の女性で悪化することが知られている。一方、肥満に伴う皮膚疾患も近年注目されてきており、中でも尋常性乾癬は肥満の程度と皮膚所見の程度の関連も示唆されている。本研究では、閉経後に相当する状態を作製した NAFLD モデルマウスを作製し、皮膚病変の誘因に細菌の関与が認められるかを検討した。[方法] 10 遅未, 雌 C56BL/6 マウスに卵巣摘出術を施行した。術後, 高カロリー食 (HCD, 450.8 kcal/100 g) を 1 カ月間投与後, HCD 投与を継続した期間内毒素 (LPS) + Freund's complete adjuvant (CFA) を 1 回/月, 計 3 回腹部皮下注射した群（group1）と, HCD 継続投与のみの群（group2）に分け, 更に 6 カ月間飼育し皮膚所見を観察した。観察終了後マウスを深麻酔下にて sacrifice し, 病変部皮膚の組織学的検討を行った。[結果] group1, 2 もとに肝に NAFLD の所見が観察された。group1 マウス皮膚では, 耳介後部から皮膚にかけて発赤を認め, 本部位の皮膚を group1, 2 マウスで比较検討した結果, group1 マウスでは表皮の肥厚, 細胞間浮腫, 壊死, 著明な細胞浸潤を認め, 皮下組織に著明な脂肪織炎を認めた。一方, group2 マウスではこれらの所見は軽微であった。また, 皮膚の免疫染色を行った結果, group1 マウス表皮では細胞浸潤部に F4/80 陽性の macrophage/macocytes が優位に認められ, 皮下脂肪織炎部では CD3, B220, F4/80 陽性細胞が認められた。[結論] 卵巣摘出後の NAFLD 様脂肪性肝疾患を有するマウスで, 細菌曝露が誘因になって遠隔部皮膚病変が生じることが明らかとなった。