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Analysis of serum cytokine/chemokine profiles affected by aging and exercise in mice

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ABSTRACT

Aging could be the cause of inflammation involved in the progression of many degenerative diseases while physical exercise might reduce the inflammation. This study examined the effects of aging *versus* exercise on serum profiles of cytokines and chemokines in mice models. Male C57BL/6N mice with different ages (2 and 20 months old) were subjected to treadmill exercise for 4 weeks. The exercise did not affect the body mass gain of the young mice but significantly reduced that of the old mice. Of 50 cytokines/chemokines analyzed using a multiplexed bead-based sandwich immunoassay, Eotaxin, Interleukin-9 and Thymus and activation-related chemokine showed significantly higher serum levels in old mice compared with young mice ($p < 0.05$). The treadmill exercise did not alter serum cytokines/chemokines levels significantly. This study suggests that the cytokines and chemokines, whose serum levels were changed age-dependently, would provide useful markers of the systemic low-level inflammation associated with aging and age-related diseases.

1. Introduction

Chronic systemic low-level inflammation is involved in the pathogenesis of several prevalent age-related diseases such as atherosclerosis, heart disease and diabetes [1–3]. Various factors including genetic traits, life style, obesity, smoking, infection, etc. may contribute to low-level inflammation in old individuals. Circulatory levels of inflammatory markers including C-reactive protein, Tumor necrosis factor-α (TNF-α), Interleukin (IL)-1α, and IL-6 are known to increase in association with aging, and thus they are frequently measured in research and diagnosis [4,5].

Exercise is considered to be a readily accessible and safe "medicine" to attenuate inflammation and cellular senescence [6]. However, there are controversies regarding the effect of exercise intervention on circulatory markers of inflammation. Some previous studies have showed that aerobic exercise reduced circulatory C-reactive protein levels [7,8] while some other studies reported that exercise intervention had no such effects [9–11]. Thus, there is a need for concrete evidence supporting or refuting the anti-inflammatory effects of exercise intervention.

Cytokines and chemokines act as major players in innate immunity and inflammation [12,13], and thus their serum profiles could be relevant markers of systemic inflammation statuses [14,15]. Analysis of serum cytokines and chemokines has become more and more efficient due to advances in related technologies. Multiplexed bead-based immunoassay is one such a method that allows analysis of many cytokines and chemokines simultaneously using a small volume of serum samples. This method has recently been used in the analysis of human serum samples [15].

The purpose of the present study was to analyze serum cytokine/chemokine profiles affected by aging and exercise in animal models, using the highly advanced multiplexed immunoassay method. Although there have been previous studies that examined the effects of exercise on young and old animals [16], profiling data on serum cytokine/chemokine are not easily available. The results of the current study indicated that aging could have significant effects on serum levels of cytokine and chemokines that can mediate chronic and systemic low-level inflammation.

2. Materials and methods

2.1. Animal and treadmill exercise

Animal experiments were performed in accordance with the guideline of Kyungpook National University, Intramural Animal Use and Care Committee. Male C57BL/6N mice were purchased

Abbreviations: IFN-γ, Interferon-gamma; IL, Interleukin; SEM, standard error of mean; TARC, Thymus and activation-related chemokine; TIMP-1, Tissue inhibitor of metalloproteinase 1; TNF-α, Tumor necrosis factor-α.

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from Japan SLC, Inc. (Hamamatsu, Shizuoka Prefecture, Japan) and maintained under controlled environmental conditions (23 ± 1 °C, 55 ± 5% humidity, 12 h light/dark cycle) with free access to water and an ad libitum standard laboratory diet (Superfeed Co, Wonju, Kangwon-do, Korea). Young mice (2 months old) were randomly divided into a sedentary group (4 animals) and an exercise group (5 animals). Old mice (20 months old) were also divided in the same way.

The exercise mice groups were subjected to exercise on a home-made motorized treadmill for 4 weeks, following the protocol used in a previous study [17]. In the first week, mice were run at 15 m per min for 10 min, tree times at 60 min intervals, every other day. Over the following 3 weeks, they were run at the same speed for 30 min continuously, 5 days per week. The exercise was started at 6 pm to maintain daily activity cycle of the animals. The sedentary control mice groups were provided with similar environmental conditions. The body masses of each animal were measured in the morning.

2.2. Blood collection and serum preparation

On the next day after the last exercise, mice were anesthetized with 60 mg/kg body mass pentobarbital sodium (Sigma–Aldrich, St. Louis, MO, USA) and euthanized to collect the blood. The abdominal cavity of anesthetized mouse was opened and blood was collected using a 26 gage needle and 1 mL syringe (Shin Chang Medical, Gumi, Gyeongsangbuk-do, Korea) from the widest part of the posterior vena cava located between the kidneys. The collected blood was transferred to a serum separation tube (#365967, BD Bioscience, Franklin Lakes, NJ, USA). The tube was carefully inverted 5 times to mix the contents and was incubated at room temperature for 30 min to induce blood clotting. The tube was then spun at 6000*g* for 5 min to obtain clear serum (supernatant) which was moved to a new tube and stored at −80 °C until analysis.

2.3. Multiplexed immunoassay of serum cytokine/chemokines

Mouse serum was subjected to a multiplexed capture sandwich immunoassay using MILLIPLEX™ MAP (Multi-Analyte Profiling) Mouse Cyotokine/Chemokine Panel I, II, III (kit No. MPXMCYTO-70 K, Millipore Corporation, Billerica, MA, USA), as per the manufacturer's instruction. Panel I includes Eotaxin, G-CSF (Granulocyte-colony stimulating factor), GM-CSF (Granulocyte–macrophage colony-stimulating factor), IFN-γ (Interferon-gamma), IL-1α, M-CSF (Macrophage colony-stimulating factor), IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12 (p40), IL-13, IL-15, IL-17, IP-10 (IFN-γ-induced protein 10), MIP-2 (Macrophage inflammatory protein 2), KC (Keratinocyte-derived cytokine), LIF (Leukemia inhibitory factor), LIX (Lipopolysaccharide-induced CXC chemokine), MCP-1 (Monocyte chemotactic protein 1), MIP-1α, MIP-1β, MIG (Monokine induced by IFN-γ), RANTES (Regulated upon Activation, Normal T-cell Expressed, and Secreted), TNF-α, IL-12 (p70), VEGF (Vascular endothelial growth factor), and IL-9; Panel II includes EPO (Erythropoietin), Exodus-2, MCP-5, MIP-3β, TARC (Thymus and activation-related chemokine), MIP-3 α, IL-16, Fractalkine, IL-21, IL-22, IL-25, and IL-28β; Panel III includes MDC (Macrophage-derived chemokine), IL-23, IL-27, TIMP-1 (Tissue inhibitor of metalloproteinase 1), IL-20, and IL-33.

Briefly describing the analysis procedure, either standards or diluted (1/2) serum samples in triplicate were incubated with the pre-mixed capture antibody-coupled bead sets in 96 well plates overnight at 4 °C, followed by a wash step and incubation with the biotinylated secondary antibodies for 1 h. Then, streptavidin–phycoerythrin was added and incubated for 30 min. After the beads were washed and suspended in sheath fluid, fluorometric analysis was performed using a Luminex 100™ (Luminex, Austin, TX), an analyzer based on the principles of flow cytometry. Data were reported as Median Fluorescent Intensity. Serum concentrations of cytokines and chemokines were estimated based on standard curves constructed using high purity recombinant cytokines and chemokines ranging from 0.64 to 10,000 pg mL^{-1}.

2.4. Statistical analysis

Data are presented as the Means ± SEM (standard error of mean) of experiments. Significant differences among the groups were determined using one-way ANOVA at a significance level ($p < 0.05$). The Pearson's correlation was used to find a correlation between two continuous variables, using SPSS 11.5 (SPSS Inc., Chicago, IL, USA).

3. Results

Young and old mice were subjected to exercise on a motorized treadmill for 4 weeks (Fig. 1A). During this period, the young mice grew and their body mass increased significantly (Fig. 1B). Relative body mass gains (% of the initial body mass) were + 11.0% in the sedentary group and +14.3% in the exercised group. These changes were not statistically different from each other (Fig. 1C). Compared with the young mice, the old mice showed only a little change in body mass (Fig. 1B). It is worthy to noting that the body mass of the exercised old mice decreased (body mass gain, −2.9%) while that of the sedentary old mice increased (body mass gain, +1.8%) (Fig. 1C). These changes were statistically different from each other. This suggests that exercise could have greater effects on old animals than young animals.

After a four week-exercise, serum samples were obtained from the sedentary and the exercised, young and old mice, and used in a multiplexed immunoassay. The assay method utilizes special beads that carry primary antibodies specific to a cytokine or chemokine. They are also labeled with mixed dyes at a specified ratio to identify which beads carry which antibodies. A mixture of various kinds of beads, each captures a specific cytokine or chemokine, is incubated with a serum sample and secondary detector antibodies. Flow cytometry-based analysis is performed to quantify the fluorescent intensity of each kind of beads. Using this method, total 50 cytokines and chemokines were analyzed simultaneously using a small volume of serum sample less than 150 μL.

As shown in Fig. 2A, absolute serum concentrations were provided for more than 30 of the 50 cytokines and chemokines analyzed, although data for some other analytes could not be obtained due to low detection signals. The highest serum concentration (in pg mL^{-1}) in sedentary young mice was observed with IL-16 (15,142) followed by LIX (12,951), Fractalkine (2,301), TIMP-1 (1,188), Exodus 2 (1,091), Eotaxin (713), IL-1α (463) and so on. Serum levels of most cytokines and chemokines were not significantly different between the sedentary and the exercised, young and old mice groups. Only TARC, IL-16 and TIMP-1 showed statistical differences between these groups (Fig. 2B–D).

Statistical analysis was performed to compare relative effects of aging and exercise on serum cytokine/chemokine levels. As shown in Fig. 3A, when all the tested mice were divided into two groups, young and old mice groups, regardless of exercise treatments, the serum levels of Eotaxin, IL-9, TARC were significantly higher and those of IL-16 and TIMP-1 were lower in old mice compared to young mice. IL-9 showed the biggest fold change. In contrast, no such significant differences were observed when the mice were divided into the sedentary and exercised groups regardless of age (Fig. 3B). These results indicate that the intensity and duration of exercise provided in this study might not be enough to induce a significant change in the serum cytokine/chemokine levels,