Environ Health Prev Med (2009) 14:361–365
DOI 10.1007/s12199-009-0102-0

REGULAR ARTICLE

The modulation of immunological activities in human NK cells by extracts of ginkgo

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Received: 15 May 2009 / Accepted: 12 July 2009 / Published online: 12 September 2009

Abstract
Objective To investigate the association between extracts of ginkgo and the immune system by determining changes in natural killer (NK) cell activity and surface markers in human NK cells and by analyzing for surface markers.
Methods Natural killer cell activity was determined in peripheral blood samples of three subjects who received ginkgo daily (250 ml/day; (ginkgo concentration 40 mg/ml) for 14 days by the non-radioisotopic Europium (non-RI Eu) release assay. Peripheral blood samples were taken three times during the study period: before ginkgo sample ingestion, on day 7 after ginkgo ingestion, and on day 14 after ginkgo ingestion). The peripheral blood samples were also analyzed for surface markers (CD56, CD3, CD19, CD20, CD4, CD8) using a fluorescence-activated cell sorter (FACScalibur).
Results The non-RI Eu release assay revealed that the ingestion of ginkgo extracts elevated NK cell activity in the subjects, with the highest activity recorded following treatment with an extract at a concentration of 400–800 μg/ml. The analysis for surface markers using the FACScalibur showed that the expression of CD56 (NK cell surface marker) was elevated and the expression of CD19 had dropped in our subjects by day 14 of ginkgo ingestion. There was no significant difference in surface markers after 7 days of ginkgo ingestion.
Conclusion Ginkgo extracts were found to affect immunological activities and surface markers (CD56) in human NK cells. Our results also reveal an optimal range of ginkgo concentration—from 400 to 800 μg/ml—within which its immunopotentiating activity is highest. It took at least 2 weeks to affect surface markers in human NK cells after ginkgo ingestion, and surface markers were not affected after 7 days of ginkgo ingestion.

Keywords CD56 · Europium · Ginkgo · Immune system · NK cell

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Introduction

Ginkgo is known as the seed plant with the longest history of human use and has been called a "living fossil". The emergence of ginkgo is thought to date back about 250 million years. Ginkgo seeds are used as a foodstuff in Japanese and Chinese dishes, confectionaries, and other foodstuffs, while ginkgo leaves are used as an herbal medicine for treating headache, dizziness, shoulder stiffness, and other conditions [1, 2]. Ginkgo leaves have also been shown to be highly effective against the factors responsible for vascular and neuronal degeneration associated with dementia and Alzheimer's disease [3–5]. In 1994, ginkgo leaves were approved as an agent for the treatment of dementia and Alzheimer's disease in Germany and France.

Ginkgo has several effective ingredients, such as ginkgolic acid, terpenoids, and flavonoids. Ginkgolic acid has been reported to cause allergic reaction, while terpenoids have been reported to improve inflammatory reaction and allergic reaction. Flavonoids have been reported to remove activating enzymes and dilate blood vessels.

The ingestion of ginkgo has been reported to cause some side effects, such as dermatic symptoms, including itching and rash, and gastrointestinal symptoms, including nausea, abdominal pain, diarrhea, and poor appetite [6].

Natural killer (NK) cells are non-T, non-B lymphocytes that play critical roles in defense against virally infected cells and in tumor surveillance [7, 8]. These cells are considered to be part of the innate immune system because they do not require previous exposure to foreign, pathogenic, or dangerous antigens to act. The main function of these cells is to kill damaged or infected cells. Natural killer cells target any cell that is missing the "self" marker that identifies it as one of our own. Foreign cells and mutated cells, such as cancer cells, are without these "self" markers. The NK cells attach and destroy these cells and spare normal cells that have high levels of "self" markers. The killing process begins when the NK cell binds to the target cell and releases its lethal burst of chemicals that produce holes in the target cell membrane [9].

We have evaluated the immunopotentiating effects of ginkgo extracts by determining changes in NK cell activity and surface markers in human NK cells [10]. We also evaluated the side effects of ginkgo using a self-administered questionnaire after ginkgo ingestion.

Subjects, preparation of ginkgo extracts, and methods

Subjects

Peripheral blood samples from three individuals were used for the non-radioisotopic Europium (non-RI Eu) release assay and surface marker assay. None of the subjects had any signs or symptoms of infectious disease, used drugs that may have affected the immunological analysis, or were taking any medication at the time of the study. We obtained signed informed consent from these three individuals.

Preparation of ginkgo leaf sample

A dried sample of ginkgo leaves (10 g) was mixed in 500 ml of sterilized distilled water and boiled until the volume was halved (250 ml), to a concentration of 40 mg/ml. The sample was then centrifuged at 1500 *g*, and the supernatant filtered through a 0.22-μm filter and sterilized, yielding the samples.

Immunological methods

For the non-RI Eu release assay, peripheral blood samples from the three subjects described above were used as effector cells. K562 cells served as target cells. The K562 erythroleukemia cell line derived from a patient with human chronic myeloid leukemia in blastic crisis was maintained in culture in RPMI 1640 medium supplemented with 10% v/v fetal bovine serum.

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by Ficoll Hypaque discontinuous gradient centrifugation and resuspended in complete medium consisting of RPMI 1640 medium, 10% (v/v) heat-inactivated fetal bovine serum, and 100 U/ml penicillin and 100 μg/ml streptomycin.

The PBMC were suspended in culture medium for each group to a density of 2×10^6 cells/ml. The final concentration of each ginkgo extract sample was set at 50–1600 μg/ml and added to each culture medium. Spontaneous release was determined by incubating labeled target cells in the medium alone. Samples containing interleukin (IL)-2 at a concentration of 500 U/ml served as positive controls. The 24-well plates containing each sample were incubated for 24 h in an incubator (5% CO_2, 37°C). Natural killer-sensitive K562 cells labeled with Europium (Eu3+) DTPA chelate served as target cells (T). The target cells were combined with PMBC (effector cells; E) obtained from each group at an E/T ratio of 40:1–20:1. Using the formula for calculating NK cell activity, we then measured cytotoxic activity [11].

$$\text{NK cell activity} = \frac{(\text{experimental release})\,\text{cpm} - (\text{spontaneous release})\,\text{cpm}}{(\text{maximal release})\,\text{cpm} - (\text{spontaneous release})\,\text{cpm}}$$

The *t* test was used to analyze the effect of ginkgo on NK cell activity. The significance level was set at $p < 0.05$.

A fluorescence-activated cell sorter, FACScalibur (BD Biosciences, San Jose, CA), was used to analyze the surface markers [12]. Three individuals ingested the prepared ginkgo leaf solution (250 ml/day; ginkgo concentration 40 mg/ml) each day for 14 days. Peripheral blood mononuclear cells were isolated three times: before ginkgo ingestion, on day 7 after ginkgo ingestion, and on day 14 after ginkgo ingestion) from heparinized venous blood by Ficoll Hypaque discontinuous gradient centrifugation and resuspended in complete medium consisting of RPMI 1640 medium, 10% v/v heat-inactivated fetal bovine serum, and 100 U/ml penicillin and 100 μg/ml streptomycin.

The PBMC were centrifuged at 6000 *g* for 45 s. Superior serum was absorbed and 50 μl serum saline added. After a 5-μl aliquot of surface marker (CD56, CD3, CD19, CD20, CD4, CD8) had been allowed to respond for 20 min at 4°C, the cells were washed twice with serum saline and for surface markers using FACScsalibur. The *t* test was used to analyze the expression of each surface marker, with significance set at $p < 0.05$.

The side effects of ginkgo ingested were assessed after 2 weeks using a self-administered questionnaire. We asked about dermatic symptoms, including itching and rash, and gastrointestinal symptoms, including nausea, abdominal pain, diarrhea, and poor appetite.

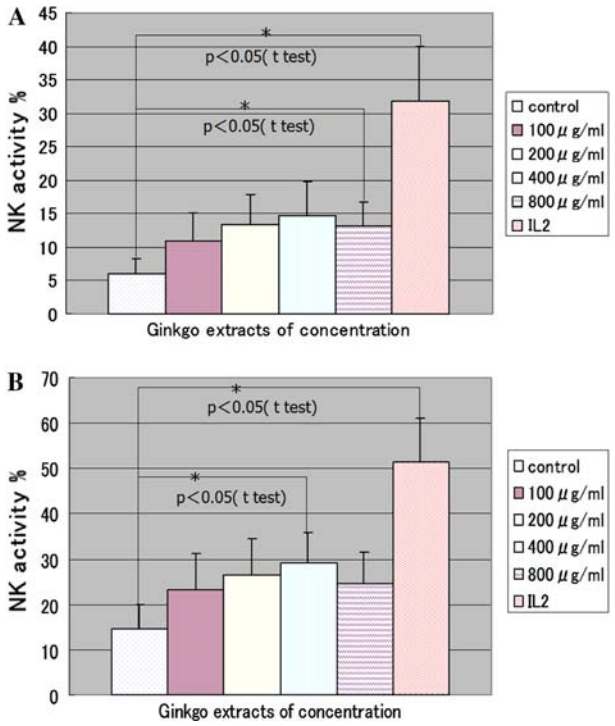

Fig. 1 Effect of ginkgo on natural killer (NK) cells after a 24-h incubation with the Europium release assay E/T20 (**a**) and E/T40 (**b**). Date are presented as the mean ± standard deviation (SD) ($n = 3$). NK cell activity in the three subjects was found to be elevated by the ingestion of ginkgo at a concentration 400 μg/ml (E/T40) and 800 μg/ml (E/T20). The *t* test was used to analyze the effect of ginkgo on NK cell activity, with the significance set at $p < 0.05$

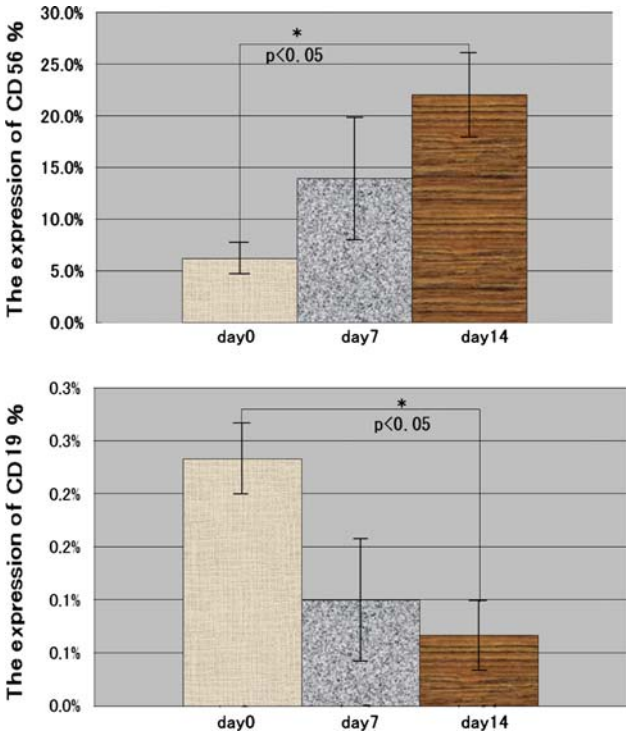

Fig. 2 Effect of ginkgo on NK cell surface markers using a fluorescence-activated cell sorter, FACScalibur. Data are presented as the mean ± SD ($n = 3$). The expression of CD56 (NK cell surface marker) was elevated and the expression of CD19 had dropped after day 14 of ginkgo ingestion. The *t* test was used to analyze the expression of each surface marker, with the significance set at $p < 0.05$

Results

Natural killer cell activity was measured by the non-RI Eu release assay, which revealed that NK cell activity had elevated in response to ginkgo ingestion at a concentration of 400–800 μg/ml (Fig. 1).

In the samples tested, peak NK cell activity tended to be recorded when the final concentration of ginkgo was about from 400 to 800 μg/ml. When the ginkgo level deviated from this level (either up or down), NK cell activity tended to gradually decrease. Ours results demonstrate that the ginkgo extracts were able to elevate immunological activities in human NK cells in our subjects. We also found an optimal range of ginkgo concentration (400–800 μg/ml) within which its immunopotentiating activity is highest (Fig. 1).

The FACScalibur analysis for surface markers revealed that the expression of CD56 (NK cell surface marker) was elevated and that of CD19 had dropped after day 14 of ginkgo ingestion. There was no significant difference in terms of surface markers after day 7 of ginkgo ingestion. It took at least 2 weeks of ginkgo ingestion to affect the surface markers in human NK cells ingestion (Fig. 2)

We also evaluated the side effects of ginkgo ingestion by our three subjects using a self-administered questionnaire at the end of the study period. The subjects did not report any side effects after 2 weeks of ginkgo ingestion.

Discussion

Ginkgo is a genus of highly unusual non-flowering plants with one extant species, *G. biloba*, which is regarded as a "living fossil". Fossil records date the emergence of ginkgo to about 250 million years ago. Ginkgo seeds are used as a foodstuff in Japanese and Chinese dishes, confectionaries, and other foodstuffs, and ginkgo leaves are used as an herbal medicine for treating many ailments [13]. Ginkgo leaves are highly effective against the factors responsible for vascular and neuronal degeneration associated with dementia and Alzheimer's disease. Consequently, ginkgo, as a foodstuff, herbal medicine and agent for the treatment of disease, is closely associated with various aspects of human nutrition and health.

Ginkgolic acid, terpenoids, and flavonoids are the major effective agents in ginkgo leaves. Ginkgolic acid has been

reported to cause allergic reaction, and terpenoids have been reported to improve the inflammatory reaction and allergic reaction. Flavonoids have been reported to remove activating enzymes and dilate blood vessels. These agents as well as other unknown ones from ginkgo leaves may induce both good and bad responses in the human body. Our study was not aimed at determining which agent is effective in elevating NK cell activity and CD56 expression (NK cell surface marker). Rather, our results show the total effect of ingesting an aqueous solution of boiled dried ginkgo leaves, which is a traditional ginkgo treatment.

Ginkgo has been reported to induce a number of side effects, such as dermatic symptoms, including itching and rash, gastrointestinal symptoms, including nausea, abdominal pain, diarrhea, and a poor appetite. We also evaluated the side effects by asking the three subjects to complete a self-administered questionnaire after 2 weeks of ginkgo ingestion. It has been reported that ginkgo can cause dermatic symptoms in >30% of those ingesting it and gastrointestinal symptoms in >20%, but we did not find any side effects, which may partially be due to the small size of our study population ($n = 3$). It is very possible that side effects would have been reported if more subjects had participated.

Natural killer (NK) cells are non-T, non-B lymphocytes that play critical roles in defense against virally infected cells and in tumor surveillance [14]. These cells are considered to be part of the innate immune system because they do not require previous exposure to foreign, pathogenic, or dangerous antigens to act [15, 16]. The main function of these cells is to kill damaged or infected cells. Natural Killer cells target any cell that is missing the "self" marker that identifies it as one of our own. Foreign cells and mutated cells, such as cancer cells, are without these "self" markers. The NK cells attach and destroy these cells and spare normal cells that have high levels of "self" markers. The killing process begins when the NK cell binds to the target cell and releases it's lethal burst of chemicals that produce holes in the target cell membrane [17]. The NK cell function is very important to the maintenance of good health and prevention of many diseases [18, 19]. It is therefore very important to improve our life style so as to elevate NK cell function as much as possible [20, 21]. Cigarette smoking, alcohol consumption, and mental stress have been reported [22, 23] to significantly affect human NK cell activity [24–26]. To date, there have been no reports on the effect of ginkgo on human NK cell function. We evaluated the immunopotentiating effects of ginkgo extracts by determining changes in NK cell activity and surface markers in human NK cells. Natural killer cell activity was first determined by the non-RI Eu release assay, which revealed that NK cell activity was elevated following the ingestion of ginkgo at a concentration from 400 to 800 μg/ml. Peak NK cell activity tended to be recorded when the final concentration of ginkgo was about from 400 to 800 μg/ml; deviations from this level tended to result in a gradual decrease in NK cell activity. These results demonstrate that ginkgo extracts are able to elevate immunological activities in human NK cells. They also reveal an optimal range of ginkgo concentration (from 400 to 800 μg/ml) within which its immunopotentiating activity is the highest.

Peripheral blood samples were analyzed for surface markers (CD56, CD3, CD19, CD20, CD4, CD8) using a fluorescence-activated cell sorter, FACScalibur. This analysis revealed the expression of CD56 (NK cell surface marker) was elevated and the expression of CD19 had dropped after day 14 of ginkgo ingestion. There was no significant difference in terms of surface markers at day 7 of ginkgo ingestion—i.e., it took at least 2 weeks of ginkgo ingestion to affect the surface markers in human NK cells in our subjects.

In summary, we have evaluated the immunopotentiating effects of ginkgo extracts by determining changes in NK cell activity and surface markers in human NK cells. Based on our results, we conclude that ginkgo does elevate the immune system. However, we have to pay attention to the side effects of ingesting ginkgo. It takes at least 2 weeks of ginkgo ingestion, on a daily basis, to elevate immune system.

Acknowledgments This work was supported by the facility of Education, Science and medical. We are grateful to all staffs at the Department of Hygiene and Preventive Medicine Osaka University School.

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