A forest bathing trip increases human natural killer activity and expression of anti-cancer proteins: a comparison with a trip to a place without forest

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Abstract

We previously have reported that a forest bathing trip (Shinlinyoku) enhanced human natural killer (NK) activity; number of NK cells, and perforin, granzymes and granulysin-expression in peripheral blood lymphocytes (PBL). In the present study, we investigated how long the increased NK activity will be lasted and also compared the effect of a forest bathing trip on NK activity with a trip to a place without forest (general trip). 12 healthy male subjects, age 35-56 years, were selected with informed consent from four large companies in Tokyo, Japan. The subjects experienced a three-day/two-night trip at forest fields and a place without forest. On the first day, subjects walked for two hours in the afternoon in a forest field; and on the second day, they walked for two hours in the morning and afternoon, respectively, at two different forest fields. Blood was sampled on the second and third days during the trip, and on days 7 and 30 after the trip, and NK activity; numbers of NK, T cells, granulysin, perforin, and granzymes A/B-expressing cells in PBL were measured. Similar measurements were made before the trip on a normal working day as the control. The forest bathing trip significantly increased NK activity, the numbers of NK, perforin, granulysin, and granzymes A/B-expressing cells. The increased NK activity was lasted for more than 7 days after the trip. On the other hand, a general trip did not increase NK activity, the numbers of NK, and intracellular anti-cancer proteins. Taken together, these findings indicate that a forest bathing trip can increase NK activity, the number of NK cells and intracellular anti-cancer proteins, and that this effect at least lasts more than 7 days after the trip.

Key words: anti-cancer proteins, forest bathing, NK activity, granulysin, granzyme, perforin

Introduction

A forest bathing trip, called “Shinrinyoku” in Japanese, involves a visit to a forest field for the purpose of relaxation and recreation by breathing in the volatile substances, called phytocide released from tree, (Li et al., 2007). It was first proposed in the 1980s and has become a recognized relaxation activity in Japan (Ohtsuka et al., 1998; Yamaguchi et al. 2006; Morita et al., 2007; Li et al., 2007). Since forests occupy 67% of the land in Japan (Forestry Agency of Japan, 2002), participation in forest bathing trip is easily accessible. According to a public opinion poll conducted in Japan in 2003, 25.6% of respondents had participated in forest bathing trip, indicating the popularity of forest bathing trip in Japan (Morita et al., 2007). Moreover, forest bathing trip is possible in forest environments in the world. We previously have reported that phytoncytes enhanced human natural killer (NK) activity and intracellular levels of perforin, granulysin and granzyme A in NK cells *in vitro* (Li et al. 2006a). Komori et al. (1995) also reported that citrus fragrance found in the forest affects the human endocrine and immune systems as analyzed by measurement of urinary cortisol and dopamine levels, NK activity and CD4/8 ratios. These findings strongly suggest that forest bathing trip may have beneficial effects on human immune function, thus, we previously have investigated the effect of forest bathing trip on human NK activity, and found that a forest bathing trip increased human NK activity, number of NK cell, and intracellular levels of perforin, granulysin and granzymes A/B in peripheral blood lymphocytes (PBL) (Li et al., 2007). However, there are two questions remained to be resolved: one is that whether a trip to a place without forest (a general trip) also can increase NK activity? Another is that

how long the increased NK activity will be lasted after a forest bathing trip? In the present study, we conducted two investigations to resolve the above-mentioned two questions.

Subjects and methods

Subjects

Twelve healthy male subjects, aged 35-56 years (45.1±6.7), were selected from four large companies in Tokyo, Japan in the present study. The information gathered from a self-administered questionnaire including age, and lifestyle habits that asked about cigarette smoking, alcohol drinking habits, eating breakfast, sleeping hours, working hours, physical exercise, nutritional balance and mental stress, which have been reported previously (Li et al., 2006b; 2007). Written informed consent was obtained from all subjects after a full explanation of the study procedures. None of the subjects had any signs or symptoms of infectious disease, used drugs that might affect immunological analysis, or were taking any medications at the time of the study. The ethics committee of the Nippon Medical School approved this study (approval No. 16-1).

Forest bathing trip and a trip to a place without forest (general trip)

In the forest bathing trip, the subjects experienced a three-day/two-night trip at three different forest fields in early September, 2006. On the first day, subjects walked for two hours in the afternoon in a forest field, and then stayed at a nearby hotel within the forest. On the second day, subjects walked for 2 hours in the morning and afternoon, respectively, at two different forest fields. Whereas, in the general trip, the subjects experienced a three-day/two-night trip

at a city in middle May, 2006. On the first day, subjects walked for two hours in the afternoon in a tourist rout in a city, and then stayed at a hotel in the city. On the second day, subjects walked for 2 hours in the morning and afternoon, respectively, at two different tourist routs in the same city. Each course in the both trips was 2.5 km, closely resembling normal physical activity for the subjects on normal working days. Daily physical activity of the subjects was monitored with a pedometer and the duration of sleep was measured with a piezo-electric accelerometer, Actiwatch(R) (Mini Mitter Co. Inc., Sunriver), worn on the wrist of the non-dominant arm. The validation study was previously reported (Kawada et al. 2001). Blood was sampled on the second and third days during the trips, and days 7 and 30 after the forest bathing trip and three days prior to the trips as a control. Since it has been reported that human NK cell activity shows circadian rhythms (Angeli 1992), all samples were obtained at 8:00 am. All blood samples were placed in an ice/water box at 4°C and assays performed within four hours of the blood draw. NK activity; proportions of NK, T cells, granulysin, perforin, and granzymes A/B-expressing cells in peripheral blood lymphocytes (PBL), counts of white blood cells were measured. Adrenaline concentration in urine also was determined.

Reagents

RPMI 1640 was purchased from Nissui Pharmaceutical (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from JRH Biosciences (Lenexa, KS), and heat-inactivated at 56°C for 30 min prior to use. Sodium ^{51}Cr-chromate was obtained from PerkinElmer (Boston, MA). Fluorescein isothiocynate (FITC)-mouse anti-human perforin, granzyme A (GrA), granzyme B (GrB) and FITC/phycoerythrin (PE)-CD16, PerCP-Cy5.5-CD3, FITC/PE-

negative isotypic control antibodies, and Cytofix/cytoperm solution were purchased from BD Pharmingen (San Diego, CA). Rabbit anti-human granulysin (GRN) polyclonal antibody was described previously (Hanson et al. 1999). PE-goat-anti rabbit IgG were purchased from Vector Laboratories Inc. (Burlingame, CA).

NK activity

Human peripheral blood lymphocytes (PBL) were separated from peripheral blood with BD Vacutainer CPT (Becton Dickinson, Franklin Lakes, NJ), and then adjusted to $4x10^6$ cells/ml for the assay of NK activity. The viability of the cells as determined by trypan blue dye exclusion was more than 95%. Human NK activity was assayed according to the traditional method (Li et al., 2007). Briefly, K-562 target cells were labeled with a sodium ^{51}Cr-chromate solution for 60 min at 37°C in 5% CO_2 and washed 4 times in RPMI 1640 containing 10% FBS. The target cells were plated into round-bottomed 96-well microplates, then the effector cells (PBL) at $4x10^6$, $2x10^6$ and $1x10^6$ cells/ml in 100 µl were added to the wells in triplicate at E:T ratios of 40:1, 20:1 and 10:1. Following a 4-hr incubation at 37°C in 5% CO_2, the microplates were centrifuged and 100 µl of supernatant from each well was collected and measured in a gamma counter. Then, the NK activity was calculated as described previously (Li et al., 2007).

Cell staining and flow cytometric analysis

The surface markers of PBL were stained with PE/FITC-CD16 for NK cells and PerCP-Cy5.5-CD3 for T cells for 30 min in the dark. Then, the cells were fixed/permeablized with Cytofix/cytoperm solution for 20 min at 4°C, and then the intracellular perforin and GrA/B

were stained with FITC- anti-human perforin and GrA/B, respectively, for 30 min at 4°C according to the manufacturer's instructions (BD PharMingen, San Diego, CA). Intracellular GRN was stained with rabbit anti-human GRN polyclonal Ab after fixation/permeablization with Cytofix/cytoperm solution, and then stained with PE-goat anti-rabbit IgG for 30 minutes at 4°C in the dark. After staining, the cells were washed twice with the fixative solution and once with PBS containing 1% FBS. Flow cytometric analysis was performed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA) as described previously (Li et al. 2005; 2006b; 2007). Lymphocytes were identified by their characteristic appearance on a dot plot of FSC versus SSC and electronically gated to exclude dead cells and granulocytes.

Measurements for adrenaline in urine

The levels of adrenalin in urine were measured by HPLC method using an analyzer HLC-725CAII. The instrument features a column-switching system composed of two pretreatment columns and one separation column and a high-sensitive detection unit based on a post-column reaction using a fluorogenic reagent, 1,2-diphenylethyleneamine. The detection limit of adrenaline in urine was 8 fmol/ml (Hirowatari et al., 1999).

White blood cell (WBC) count

WBC, RBC and platelet counts, the percentages of granulocyte, lymphocyte and macrophage, concentration of Hb, Hct, MCV, MCH and MHCH were determined by an automatic cell counter (LC-550, Horiba Co., LTD. Kyoto, Japan) as described previously (Li et al. 2007).

Measurements of phytoncide, and environmental temperature/ humidity in the forest fields

during the investigation

The volatile organic compounds (phytoncide) in forest air, and temperature and humidity in the forest fields were measured as previously reported (Li et al., 2007).

Statistical analysis

Multiple comparisons were made with the paired t-test if the analysis of variance was significant. The analysis was performed with the SPSS 11.5J software package for Windows. The significance level for p values was set at < 0.05.

Results

Effect of a forest bathing trip and the general trip on NK activity

Because the difference among individuals in NK activity is very big, we do not compare the difference in NK activity between the forest bathing trip and general trip, but compared the difference in NK activity between before and after the trips in the present study.

As shown in Fig. 1A, forest bathing trip significantly increased human NK activity, and this increase lasted more than 7 days after the trip. On the other hand, the general trip did not increase human NK activity (Fig. 1B).

Effect of a forest bathing trip and the general trip on CD16+ NK cells

As shown in Fig. 2A, forest bathing trip significantly increased the number of CD16+ NK cells, and this increase lasted more than 7days after the trip. On the other hand, although the general trip also slightly increased CD16+ NK cells, this increase was not significant (Fig. 2B).

The forest bathing trip did not affect lymphocytes and WBC counts.

Effect of forest bathing trip on the percentage of cells expressing cytolytic molecules

The forest bathing trip significantly increased the percentages of GRN, perforin, and GrA/B-expressing cells in PBL (Fig 3A). Whereas, although the general trip also slightly increased the percentages of GRN, perforin, and GrA/B-expressing cells in PBL, these increases were not significant (Fig. 3B).

Effect of forest bathing trip on T (CD3+) cells

Both forest bathing trip (Fig. 4A) and general trip (Fig. 4B) did not affect the number of T cells.

Effect of forest bathing trip on adrenalin concentration in urine

The forest bathing trip significantly decreased the concentration of adrenaline in urine, whereas, a general trip did not affect the level of adrenaline in urine, indicating that forest bathing trip has a relaxation effect (Fig. 5A, 5B).

There were no significant differences in daily physical activity before and during the trips (Fig. 6A, 6B). The hours of sleep were, increased during the trips compared with the control days (Table 2), however, the difference was not significant.

Lastly, phytoncides, such as alpha-pinene, beta-pinene and limonene were detected in the forest fields during the investigation (Table 1), and not detected in the urban area of Tokyo, and in the city for general trip. Weather during the forest bathing trip was excellent with

average temperatures and humidity in the forest fields during the walking of 16.31± 0.23°C, 99.77±0.44% on day 1 in the afternoon; 19.55± 0.61°C, 78.62±3.24% on day 2 in the morning; and 20.78± 0.42°C, 74.16±61.94% on day 2 in the afternoon, The average temperature and humidity in urban area of Tokyo on the control day was 28.5°C, 62%, respectively.

Discussion

We previously have found that a forest bathing trip increased human NK activity, number of NK cells, and intracellular levels of perforin, granulysin and granzymes A/B in PBL (Li et al., 2007). However, there are two questions remained to be resolved: one is that whether a trip to a place without forest (general trip) also can increase NK activity? Another is that how long the increased NK activity will be lasted after a forest bathing trip? The present study reconfirmed that a forest bathing trip can enhance the immune response as measured by human NK activity, and the numbers of NK cells, which is similar to our previous report (Li et al., 2007), whereas, a trip to a place without forest (general trip) has no effect on NK activity, indicating that forest bathing trip indeed can enhance human NK activity. Moreover, we also found that the increased NK activity and NK cells induced by forest bathing trip lasted more than 7 days, even 30 days after the trip. This suggests that if people go to a forest bathing trip once a month, they can always keep NK activity in a higher level. This has a very important significance in the health promotion from the viewpoint of preventive medicine.

NK cells kill tumor or virus infected cells by release of perforin, granzymes (Gr) (Shinkai

et al. 1988; Smyth et al. 2001; Beresford et al. 1997; Li et al. 2002), and granulysin (GRN) (Okada et al. 2003; Hanson et al. 1999; Stenger et al. 1998) via the granule exocytosis pathway. Cytotoxicity mediated by NK cells is greatly impaired in perforin-deficient mice (Kagi et al. 1994b; Li et al. 2004). GrA plays a critical role in triggering apoptosis in target cells either directly or *via* the activation of cellular caspases, and also cleaves IL-1β, the nucleosome assembly protein called putative HLA-associated protein II, TAF-Iβ, histones and lamins (Smyth et al. 2001; Zhang et al. 2001ab). GrB directly cleaves the downstream caspase substrates, nuclear matrix antigen, catalytic subunit of DNA-associated DNase inhibitor and lamins (Smyth et al. 2001; Zhang et al. 2001a). GRN, a lytic molecule expressed by human CTL and NK cells, is active against tumor cells and a variety of microbes. GRN can enter target cells in the absence of perforin and induce apoptosis, although GRN and perforin together are required to kill intracellular microbes like *Mycobacteria tuberculosis* (Okada et al. 2003; Hanson et al. 1999; Stenger et al. 1998). GRN is associated with diverse activities of NK cells and CTL in physiological and pathological settings and may be a useful marker to evaluate the status of host cellular immunity (Ogawa et al. 2003).

In order to explore the mechanism of enhancement of NK activity by forest bathing, we investigated the effect of forest bathing on the intracellular levels of perforin, GRN, and GrA/B in PBL. We found that the forest bathing trip significantly increased the proportion of perforin, GRN, GrA/B-expressing cells in PBL, which are similar to our previous report (Li et al., 2007), whereas, general trip had no effect on perforin, GRN, GrA/B-expressing cells, indicating that forest bathing trip indeed can increase perforin, GRN, GrA/B-expressing cells.

Moreover, we also found that increased perforin, GRN, GrA/B-expressing cells induced by forest bathing trip lasted more than 7 days, even 30 days after the trip. These cytolytic molecules contribute to NK and anti-tumor activity.

Activity of sympathetic nervous system causes adrenaline to be released into the circulation from the adrenal cortex. Therefore, concentration of adrenaline in urine has also been used as measures of autonomic nervous activity in response to mental demands (Hjemdahl et al. 1984). We found that forest bathing trip significantly decreased the adrenaline concentration in urine, however, the general trip had no effect on the adrenaline concentration in urine, suggesting that the parasympathetic nervous system of subjects was dominant, associated with relaxation and decreased stress (Mori et al., 2002). It has been reported that adrenaline inhibits human NK activity (Garland et al. 2003). The increased NK activity in forest bathing trip may be related to an attenuated stress hormone response (adrenaline) associated with forest bathing trip. Previous studies have reported that forest bathing reduces the concentration of cortisol in saliva, reduces prefrontal cerebral activity, reduces blood pressure and stabilize autonomic nervous activity in humans (Miyazaki and Motohashi 1996; Park et al., 2005, Yamaguchi et al. 2006).

Many factors, including circadian variation (Angeli 1992), physical exercise (Nieman 2000; Miles et al. 2002, Li et al., 2006b) and alcohol consumption (Ochshorn-Adelson et al. 1994, Li et al., 2006b) can affect human NK activity. In order to control the effect of circadian rhythms on NK activity, we sampled blood at 8 am on all days. To control for the effect of physical exercise on NK activity, we limited the walking steps during the trip to the normal

workday distances as monitored by a pedometer. To control the effect of alcohol on NK activity, the subjects did not consume alcohol during the study. The sleeping hours during the trips were a little longer than the average working days (Table 2), however, the difference was not significant in both forest bathing trip and general trip. There are several reports addressing the effect of sleeping hours on NK cell activity. Many reports suggest that sleep deprivation increases human NK activity (Dinges et al. 1994; Matsumoto et al. 2001), while others suggest that sleep deprivation decreased human NK activity (Moldofsky et al. 1989; Irwin et al. 1994); still other studies by Kusaka et al. (1992) and Inoue et al. (1996) reported that sleeping hours did not affect NK or LAK activity, or NK cell numbers under physiologic conditions. In fact, we also found that there was no difference in the numbers of NK cells, nor perforin, GRN, GrA/B-expressing cells in PBL among the subjects who slept 5, 6 or 7 hours, respectively (Li et al. 2006b). In addition, although the sleeping hours during the general trip were a little longer than the average working day (Table 2), however, the NK activities during trip were almost the same with the working day, indicating that the longer sleeping hours did not contribute to NK activity in the general trip. Taken together, although the sleeping hours during the trips were a little longer than that on the average working day, this difference did not affect either NK activity or numbers in the present study.

As detailed in Table 1, we detected several phytoncides such as alpha-pinene, beta-pinene and limonene in the forest fields during the trip. We previously found that phytoncides, such as alpha-pinene, d-limonere significantly enhanced human NK activity and increased expression of intracellular cytolytic molecules, perforin, GrA and GRN *in vitro* (Li et al.

2006a), suggesting that phytoncide may partially contribute to the enhanced NK activity during the forest bathing trip.

Taken together, these findings indicate that forest bathing trip can increase human NK activity, the number of NK cells and intracellular perforin, GrA/B and GRN, and that increased NK activity, number of NK cells and intracellular perforin, GrA/B and GRN at least can last more than 7 days after a trip.

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Figure legends

Figure 1. Effect of the forest bathing trip (A) and a general trip (B) on NK activity. Data are presented as the mean+SE ((n=12 in A and n=11 in B). ANOVA indicated that the forest bathing trip significantly affected the NK activity ($p<0.01$). *: $p<0.05$, **: $p<0.01$, significantly different from before the trip by the paired t-test. The activity values for an E/T ratio of 20/1 are shown, and the similar results were also obtained with E/T ratios of 40/1 and 10/1.

Figure 2. Effect of the forest bathing trip (A) and a general trip (B) on the number of NK cells. Data are presented as the mean+SE (n=12 in A and n=11 in B). ANOVA indicated that the forest bathing trip significantly affected the percentage and number of NK cells (all $p<0.01$). *: $p<0.05$, **: $p<0.01$, #: $p=0.054$ significantly different from before the trip by the paired t-test.

Figure 3. Effect of the forest bathing trip (A) and a general trip (B) on GRN, perforin, GrA/B-expressing cells in PBL. Data are presented as the mean+SE (n=12 in A and n=11 in B). ANOVA indicated that the forest bathing trip significantly affected the GRN, perforin, GrA/B-expressing cells in PBL (all $p<0.01$). *: $p<0.05$, **: $p<0.01$, significantly different from before the trip by the paired t-test.

Figure 4. Effect of the forest bathing trip (A) and a general trip (B) on the total number of T cells. A: Data are presented as the mean+SE (n=12 in A and n=11 in B). ANOVA indicated that

both the forest bathing trip and general trip did not affect the number of T cells.

Figure 5. Effect of the forest bathing trip (A) and a general trip (B) on adrenaline concentration in urine. Data are presented as the mean+SE (n=12 in A and n=11 in B). ANOVA indicated that the forest bathing trip significantly affected the adrenaline concentration in urine ($p<0.05$). *: $p<0.05$, significantly different from before the trip by the paired t-test.

Figure 6. Consumption of energy of the subjects before and during the forest bathing trip (A) and general trip (B). Data are presented as the mean+SE (n=12 in A and n=11 in B). ANOVA indicated that there was no significant difference before and during the trips in the consumption of energy of the subjects.

Table 1. Concentration of volatile substances in the air of forest fields calculated as alpha-pinene (ng/m^3)

Measuring points	Field 1 Day 1 pm	Field 2 Day 2
Kind of Trees	Chamaecyparis	Chamaecyparis White cedar
Tricyclene	299.7	805.5
α-Pinene	**2,886.7**	**1,281.7**
Camphene	375.6	486.8
β-Pinene	**137.5**	**66.9**
Myrcene	109.4	71.8
δ-3-Carene	66.6	25.3
α-Terpinene	43.8	26.9
p-Cymene	109.4	67.3
Limonene	**111.1**	**48.4**
γ-Terpinene	n.d	33.2
Terpinolene	87.5	13.4
Camphor	32.8	14.8
Bornyl acetate	54.7	43.4

n.d: not detected.

Table 2. Sleeping hours of the subjects before and during the forest bathing trip and general trip (Mean±SD)

	Control day (Before trip)	Day 1 (During trip)	Day 2 (During trip)	1 Week (After trip)	1 Month (After trip)
Forest bathing trip	6.18±1.63	7.29±1.69	7.03±1.44	5.38±0.88	5.63±0.96
General trip	5.98±1.79	7.22±1.52	6.59±1.41		