Fragrant environment with α-pinene decreases tumor growth in mice

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ABSTRACT

Stress is believed to be harmful to not only mental but also physical health. However, proving a link between stress and disease is difficult. A recent study reported that an environmental enrichment reduced cancer growth via the hypothalamic-pituitary-adrenal axis and leptin. Here, we report that mice kept in a fragrant environment enriched with α-pinene show reduced melanoma growth. Tumor volume of mice under the α-pinene environment was about 40% smaller than that in the control mice. α-Pinene had no inhibitory effect on melanoma cell proliferation in vitro, suggesting that this effect was not a direct effect of α-pinene. These results suggest that the provision of a fragrant environment may be an important factor in the therapeutic approach to cancer.

Many people who live under highly stressful conditions in modern societies consider that the stressful environment is harmful to their health. Social isolation (living alone instead of group breeding), a known stressful condition, was associated with a significant increase in the size of mammary tumors in rats (6). Similarly, social support is linked to improved health outcomes among cancer patients (4). Stressful conditions increase neuroendocrine signaling, including that via adrenal glucocorticoid and noradrenaline (3, 20). These findings suggest that glucocorticoid and noradrenaline might influence multiple aspects of tumor growth, including apoptosis, angiogenesis, invasion, metastasis, and immunological escape, and that they should accordingly be considered potential candidates which link stress to cancer (1). However, proving why or how the environment has an effect on health and outcome for cancer patients is difficult.

Recently, Cao et al. showed that environmental enrichment delayed tumor growth in several mouse models of cancer, including melanoma and colon cancer (2). The authors showed that housing mice in a more complex living environment (enriched environment, EE) increased the expression of brain-derived neurotrophic factor (BDNF) in the hypothalamus of the mouse brain. BDNF stimulated the sympathetic nervous system and hypothalamic-pituitary-adrenal axis to boost production of stress hormones such as glucocorticoid and noradrenaline. Noradrenaline activated white adipose tissue via β-adrenergic receptors to produce less leptin. Although the relationship of leptin with cancer proliferation is not fully elucidated, leptin significantly enhanced B16 melanoma and MC38 colon cancer cell growth in culture. When exogenous leptin was delivered via liposomes to offset the EE-induced decrease in endogenous leptin levels, the EE-induced tumor reduction was inhibited. The authors concluded that the enriched environment boosted hypothalamic BDNF, which increased activation of white adipose tissue by the sympathetic nervous system and decreased leptin secretion, and thereby decreased tumor growth and progression (2). Expressed simply, a small cramped environment exacerbated stress mediators and advanced tumor progression, whereas an enriched environment with

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physical and social complexity suppressed tumor growth.

The natural environment plays a comforting role in daily life. Given that human physiological functions have had to adapt to changes in the natural environment throughout the course of evolution, it appears reasonable that humans should feel a sense of comfort in this environment. In particular, it is a matter of common experience that walking in the forest confers a sense of refreshment and relaxation. In Japan, the ability of “Shinrin-yoku”, which can be defined as “taking in the forest atmosphere” or “forest bathing”, to induce a state of relaxation and to reduce stress has now reached popular acceptance (22). The forest environment or “Shinrin-yoku” has several effects on psychological, neuroendocrine, and immunological response. Using the profile of Mood States (POMS) test, one study demonstrated that a forest bathing trip significantly increased the score for vigor and decreased those for anxiety, depression, and anger, suggesting that the subjects were physiologically relaxed during their trip (23). Exposure to a forest environment has been associated with decreases in salivary cortisol concentration (18, 22) and urinary adrenaline and noradrenaline (10), while a 3-day/2-night stay in a forest increased NK activity, which lasted for more than 7 days following the trip (12). These reports suggested that subjects in a forest environment experience lower stress levels and a strengthened immune response.

Sensory engagement with the forest environment likely spans four of the five senses: vision (scenery), olfaction (smell of wood and other ambient organic gasses), audition (sound of running streams, rustle of leaves), and tactile sensation (feeling of vegetal and rocky surfaces). Of these four, olfaction has drawn most attention because of the many kinds of wood- and soil-derived volatile organic compounds present in forests, such as terpene. Although concentrations are very low, at no more than 1 ppm, they are thought to exert a range of effects on mood and health (7, 8, 15). In particular, a weak smell of α-pinene, a major component of wood scent which is generally detected in the air of coniferous forests, induces a relaxed physiological state (21, 24) and increases NK activity (11), suggesting that α-pinene and forest environment have similar effect on mental and immunological states.

In this study, we established a forest environment using α-pinene as a representative comfortable environment and examined the effects of this fragrant environment on tumor growth in mice. Male 4-week-old C57/BL6 mice were purchased from CLEA Japan (Tokyo, Japan). All mice were housed and used for experiments in accordance with standard ethical guidelines for the care and use of laboratory animals (Science Council of Japan; Guidelines for Proper Conduct of Animal Experiments, 2006), and this study was approved by the Animal Experiment Ethics Committee of Shizuoka Cancer Center. A 90-L polyethylene bag (Shimojima, Tokyo, Japan) was used as a ventilation bag, with the open side of the bag shielded with tape following emplacement of a Tygon tube (Saint-Gobain K.K., Tokyo, Japan). We dropped 20 μL (= 17 μg) of 1R-(+)-alpha-pinene (Tokyo Chemical Industry Co., Ltd. Tokyo, Japan) on to fragrance testing strip blotter cards (Tree Of Life Co., Ltd, Tokyo, Japan) and fixed them in the tube. α-Pinene on the cards was evaporated by injecting 90 L of high purity air (O₂: 21% ± 0.5%) (Toatsu Yamazaki, Tokyo, Japan) into the bag via the tube, where it was kept for 1 h at room temperature for further stabilization. The estimated final concentration of α-pinene in the bag air was 180 ng/L, which is 10 times the human olfactory threshold for α-pinene (16). To expose mice to α-pinene, mouse cages were transferred into an anesthesia chamber (chamber size: 500W × 350D × 300H mm, 60 L) (Sanplatec Corporation, Osaka, Japan). Air in the anesthesia chamber was changed by attaching a bag to one outlet, and removing all the air from the bag by suction with a DCI-NA flexible pump (Techjam, Osaka, Japan) via the outlet on the other side. After suctioning, we exchanged the empty bag for new filled one and then ventilated it by suction with the pump for 10 min per hour (estimated volume of ventilated air = 18 L/10 min) (Fig. 1a, b). After 5 h, the mice cages were removed from the anesthesia chamber and returned under standard laboratory conditions.

The C57BL/6 mice were randomized into two groups, one exposed to the environment containing α-pinene for 5 h per day (n = 8), and the other to room air (n = 8), beginning from 4 weeks before melanoma implantation until 17 days after implantation. After 4-week treatment with inhalation of α-pinene or air, they underwent subcutaneous implantation of a syngeneic melanoma cell line B16F10 on the flank (1 × 10⁵ cells/mouse), and were then returned to their respective cages. Tumor dimensions were measured with calipers at 10, 14, and 17 days post-inoculation, and tumor volume was calculated by the formula for an ellipsoid (volume = length × width² × π/6). At 17 days, mean tumor volume in the mice under the α-pinene environment was about 40% smaller than that in the
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control mice (control 1814 ± 753.7 mm$^3$ vs. α-pinene 1084 ± 535.8 mm$^3$, mean ± SD, $P < 0.05$; Fig. 2). This result indicated that fragrant environment with α-pinene inhibited melanoma cell growth in mice. Interestingly, this efficacy shown by the forest environment was equivalent to that reported previously for an enriched environment (2).

The concentration of α-pinene we used in this experiment was 180 ng/L in air. When given in ppm (mL gas per m$^3$ gas-air mixture), in which 5.56 mg/m$^3$ (μg/L) at 25°C corresponds to 1 ppm (mL gas per m$^3$ gas-mixture) (17), 180 ng/L in air corresponds to 0.035 ppm. This level is considerably below that at which an irritant effect on the airway or whole body might be expected (17). Concentrations of volatile compounds from woods in forests are typically expected to be about 0.001–1 ppm, indicating that the concentration we used was reasonable. Although the mouse olfactory threshold for α-pinene is unknown, mice exposed to it showed no abnormal behavior or agitation.

We next examined the direct effect of α-pinene on B16F10 melanoma cell growth in vitro, following previous reports that α-pinene induced apoptosis and inhibited cell growth via a ras-dependent mechanism (13). B16F10 melanoma cells ($5 \times 10^5$ cells) were seeded in 100-mm dishes in RPMI1640 medium plus 10% fetal bovine serum. On the following day, the cells were exposed to several concentrations of α-pinene (0.01 ng/mL to 10 ng/mL) for 48 h, after which cell number was counted. The proliferation of B16F10 cells was not inhibited by α-pinene at any concentration (Fig. 3), suggesting that α-pinene shows no direct effect on tumor growth under the
and exposure to carcinogens. In the future, however, consideration should be given to patient environment both as a determinant of the initiation and growth of cancer and as a factor in its treatment and outcome.

Several limitations of the study warrant mention. First, it is unclear whether mice have a preference for or aversion to the scent of α-pinene. We used α-pinene as a comfortable odor in this experiment. The presence of aversive behavior in our experimental condition was examined by observation only. Yamaoka et al. reported that α-pinene had a sedative effect on sleep in rats, suggesting that it is at least an uncomfortable odor for rats (24). In addition, we set only one duration of exposure and concentration of α-pinene in this experiment. Although a high concentration of α-pinene is an irritant in both human and mice (17, 21), the concentration used in this experiment was below that at which an irritant effect was induced. However, minimum and maximally effective durations and concentrations have not been established. Second, we did not assess the possible effect of other fragrances on tumor growth. Many kinds of volatile wood-derived compounds are present in the forest, as are many kinds of scents derived from non-wood sources, such as flowers. Other kinds of compounds should also be tested. Third, we evaluated the effect of α-pinene in only one murine cancer model, and thus the results cannot be generalized. Further, we did not test whether forest environment-induced tumor suppres-

Matsuo et al. found that α-pinene induced apoptosis and inhibited metastasis in a melanoma model with a relatively high concentration of α-pinene (100 μg/mL = 100 mg/L) (14). In contrast, we used α-pinene at the very low concentration of 180 ng/L in air. Although it is difficult to estimate exact α-pinene concentrations in tissue, α-pinene had no effect on cell growth in vitro at concentrations up to about 50 times higher than that in air (10 ng/mL = 10,000 ng/L vs. 180 ng/L). This level was relatively low compared with that reported by Matsuo et al., suggesting that our results were not the result of any direct antitumor effect of α-pinene.

The mechanism of the inhibitory effect of a fragrant environment on tumor growth is unknown. Two possible explanations are activation of the immune or endocrine system (or both). Further study is required to elucidate the molecular pathway of the link between a forest environment and the decrease in tumor size seen here. The tumor microenvironment—the balance between factors that stimulate cell growth and induce cell survival and those that inhibit cell proliferation and lead to apoptosis—influences the progression of most cancers (5) and has been extensively examined. The effect of the macroenvironment on systemic cancer, in contrast, specifically the individual’s interaction with their physical living and social environment, is much less well defined. Studies of environmental effects in cancer research to date have largely focused on diet and exposure to carcinogens. In the future, however, consideration should be given to patient environment both as a determinant of the initiation and growth of cancer and as a factor in its treatment and outcome.

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sion in tumor implant models was effective in spontaneous tumorigenesis models more relevant to human disease. Finally, we did not investigate the possibility that unpleasant or uncomfortable odors might stimulate tumor growth. Many diseases are considered to be characterized by specific odors, particularly cancer, a characteristic known as byoshu in Japan (9). Recently it was reported that odors from patients with colorectal cancer could be identified by dogs (19). Byoshu from cancer patients can become so strong that it adversely effects not only the patient himself, but also his family and medical staff. If these stressful conditions might exacerbate the growth of their cancer, malodor elimination might be an important component of care for cancer patients.

In summary, our results demonstrated that a fragrant environment with α-pinene was associated with the significant inhibition of cancer growth. Since α-pinene shows no direct effect on cancer cell proliferation, further study will be necessary to clarify the mechanism of this effect. At a clinical level, efforts to improve the environment during hospitalization might have a favorable effect on the treatment of patients with cancer.

REFERENCES


