STEM CELLS AND DEVELOPMENT 15:118–123 (2006)

Original Research Report

Nutraceuticals Synergistically Promote Proliferation of Human Stem Cells

PAULA C. BICKFORD,[1,2] JUN TAN,[1] R. DOUGLAS SHYTLE,[1] CYNDY D. SANBERG,[3] NAGWA EL-BADRI,[1] and PAUL R. SANBERG[1]

ABSTRACT

A viable alternative to stem cell transplantation is to design approaches that stimulate endogenous stem cells to promote healing and regenerative medicine. Many natural compounds have been shown to promote healing; however, the effects of these compounds on stem cells have not been investigated. We report here the effects of several natural compounds on the proliferation of human bone marrow and human $CD34^+$ and $CD133^+$ cells. A dose-related effect of blueberry, green tea, catechin, carnosine, and vitamin D_3 was observed on proliferation with human bone marrow as compared with human granulocyte-macrophage colony-stimulating factor (hGM-CSF). We further show that combinations of nutrients produce a synergistic effect to promote proliferation of human hematopoietic progenitors. This demonstrates that nutrients can act to promote healing via an interaction with stem cell populations.

INTRODUCTION

STEM CELLS ARE FOUND in many organs of the adult human, including bone marrow, peripheral blood, umbilical cord blood, spleen, tooth pulp, and brain. These progenitor cells are being investigated for their potential use as transplanted tissues in the treatment of diseases such as cancer, diabetes, stroke, amyotrophic lateral sclerosis (ALS), and Parkinson's disease. However, little effort is being directed toward enhancing the endogenous stem cells in the adult as an avenue to promote healing.

Hematopoietic stem cells (HSCs) have been investigated for many years for their utility in cancer treatments. Experimental investigations of hematopoiesis and clinical approaches to correcting its deficiencies have focused on cytokine activity. Cytokines modulate hematopoiesis by maintaining the self-renewal of stem cells and stimulating the proliferation and maturation of committed progenitor cells required for the continuous replacement of mature blood cells (1–3).

In vitro, various combinations of cytokines including interleukin-1 (IL-1), IL-3, IL-6, stem cell factor (SCF), and erythropoietin (EPO) have been found to support the growth of multipotent progenitor cells (4,5). Individually, granulocyte–colony-stimulating factor (G-CSF) and EPO are growth factors for committed myeloid and erythroid progenitors, respectively (6). Clinically, G-CSF and EPO provide effective treatments for neutropenia and anemia (7,8) and are used to enhance peripheral blood progenitors as an alternative to bone marrow transplantation for cancer patients. However, such treatments are costly and are not without certain risks.

Although potentially better treatments are currently in development, few research studies have investigated the effects of natural products, vitamins, and other nutrients that may modulate self-renewal of stem cells. However, in recent years there has been an upsurge of interest on the effects of various dietary insufficiencies on hematopoietic and immune responsiveness. Folate, vitamin B_{12}, and iron have crucial roles in erythropoiesis. Erythroblasts require

[1]Center of Excellence for Aging and Brain Repair, University of South Florida College of Medicine, Tampa, FL 33612.
[2]James A. Haley Veterans Administration Hospital, Tampa, FL 33612.
[3]Natura Therapeutics, Inc., Tampa, FL 33618.

folate and vitamin B_{12} for proliferation during their differentiation. Deficiency of folate or vitamin B_{12} inhibits purine and thymidylate syntheses, impairs DNA synthesis, and causes erythroblast apoptosis, resulting in anemia from ineffective erythropoiesis (9). Recently, other studies have found that dietary fatty acids, particularly oleic acid and linolenic acid, actively promote the proliferation of hematopoietic stem cells (10,11) as well as modulate the self-renewal of intestinal epithelial cells (12).

Vitamin D has also received increasing attention over the past few years, in part, because recent studies suggest that nearly half the U.S. population may be vitamin D deficient (13). Recent laboratory studies demonstrate that vitamin D_3 has a dramatic effect on stimulating the proliferation of various forms of multipotent progenitor cells, particularly those involved with the immune system (14). Research on cellular senescence (the end of the life cycle of dividing cells) suggests that the dietary nutrient carnosine, found in muscle and brain of mammals, has the remarkable ability to rejuvenate cells approaching senescence, restoring normal appearance and extending cellular life span (15,16).

Still other studies suggest dietary supplementation with foods high in antioxidants, such as blueberries, can prevent and even reverse cellular and behavioral parameters that decline as a function of aging (17,18). For example, dietary supplementation with 2% blueberry extract has produced both neuroprotective and neurorestorative effects in aged animals, perhaps as a result of modulation of cell signaling cascades (19). Furthermore, blueberry extract has been shown to increase neurogenesis in the aged rat brain (20).

Green tea is a drink made from the steamed and dried leaves of the *Camellia sinensis* plant, a shrub native to Asia. Green tea has been widely consumed in Japan, China, and other Asian nations to promote good health for at least 3,000 years. Recently, scientists have begun to study its health effects in animal, laboratory, and observational human studies. Although active compounds within green tea extract have been shown to inhibit the growth of a number of tumor cell lines, they do not affect the growth of normal cells at similar concentrations (21,22) and actually may provide cellular protection from aging (23).

In light of such findings reviewed above, it appears that certain nutrients, vitamins, and flavonoids could have important roles in maintaining the self-renewal of stem cells and stimulating the proliferation and differentiation of committed progenitors required for the continuous replacement of mature cells in the blood, brain, and other tissues. Furthermore, it may be possible to use certain natural products, either alone or synergistically, for the treatment of conditions where the stem cell replacement appears warranted.

Thus, the purpose of the present study was to investigate the properties of various natural compounds for their ability to stimulate the proliferation of human HSCs. We investigated the effects of natural compounds on bone marrow cells, $CD34^+$ HSCs, and $CD133^+$ progenitor cells from peripheral blood. We show for the first time that certain natural compounds can promote proliferation of hematopoietic stem cells, and, more specifically, that a combination of blueberry extract, green tea extract, carnosine, and vitamin D_3 demonstrate synergistic activity.

MATERIALS AND METHODS

Reagents

All compounds were added to cell cultures as described in the Results sections. Sources of compounds were as follows: blueberry (freeze-dried powder, Van Drunen Farms, Momence, IL), green tea extract (Rexall), carnosine (Sigma), catechin (Sigma), and the activated form of vitamin D_3 (25-hydroxy-cholecalciferol; Sigma).

Cell cultures and MTT assay

For cell proliferation analysis, human bone marrow cells, human $CD34^+$ cells, or CD133 cells (BioWhittaker, Inc.) were cultured in 96-well plates (5×10^4/well) containing 100 μl of complete medium consisting of RPMI-1640 medium supplemented with 5% fetal calf serum (FCS). These cells were treated for 72 h with various extracts at a wide range of doses (8 ng/ml to 500 ng/ml) or molecular compounds (0.3125 μM to 20 μM). Five hours before the end of the treatment, 20 μl of MTT solution (MTT kit, Sigma) was added to each well. These plates were then incubated in a CO_2 incubator at 37°C for 5 h and the cultured medium was removed with needle and syringe. A total of 200 μl of dimethylsulfoxide (DMSO) was added to each well, with pipetting up and down to dissolve crystals. These plates were put back into the 37°C incubator for 5 min, transferred to a plate reader, and measured absorbance at 550 nM. Data were represented as the relative percentage mean proliferation, defined as the O.D. reading number of treated cells normalized to control cells (in the absence of treatment).

RESULTS

Promotion of bone marrow cell proliferation in a dose-dependent manner

Several whole food extracts, herbal extracts, and specific compounds were screened individually for proliferative activity on human bone marrow cells in culture. Spinach, spirulina, EGCG, epicatechin, withania, somnifera, carao, rehmania glutinosa, and astragulus membranoceaus did not show high activity on proliferation of

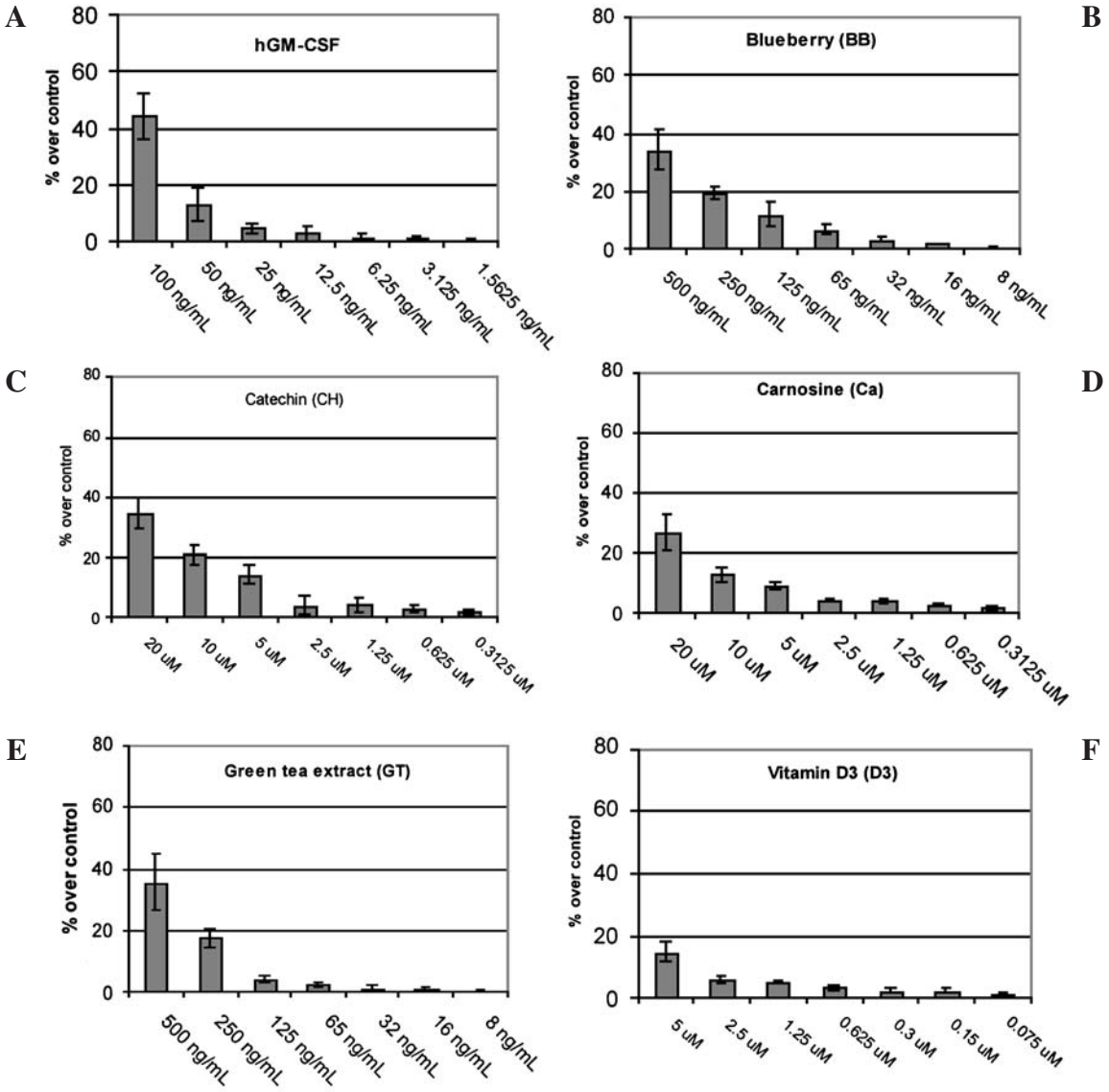


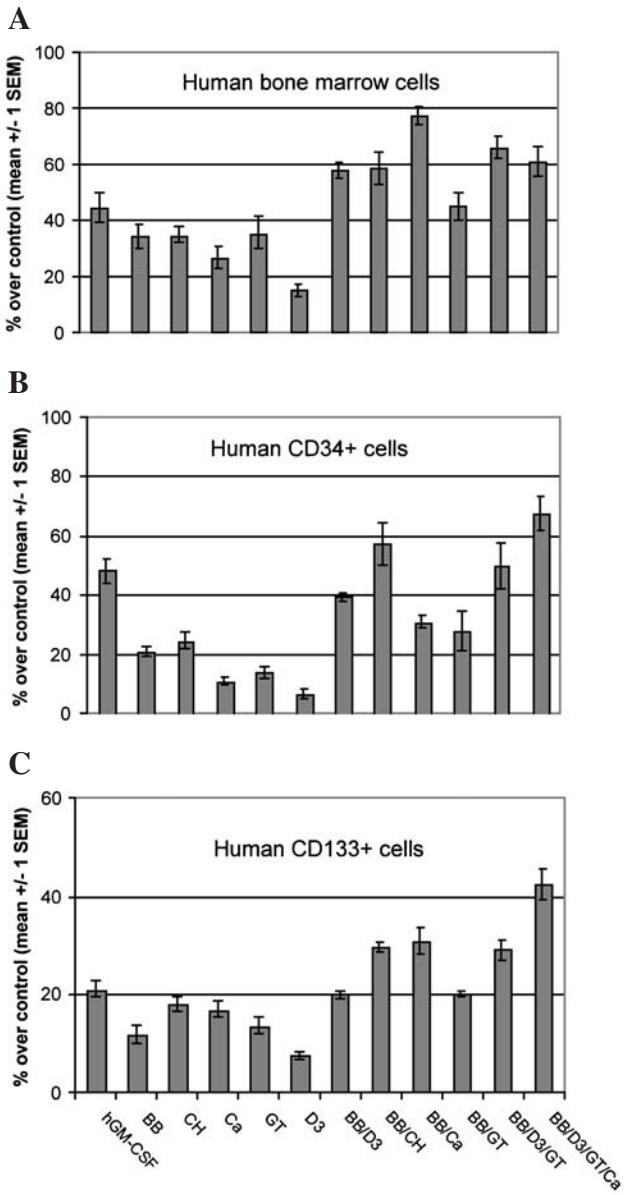
FIG. 1. Nutrition-related herb extracts or compounds promote cell proliferation for human bone marrow cells in the dose-dependent manner. Human bone marrow cells were cultured in 96-well tissue-culture plates (5×10^4/well) and treated with human hGM-CSF (**A**, as positive), blueberry extract (**B**), catechin (**C**), carnosine (**D**), green tea extract (**E**), and vitamin D_3 (**F**) at a wide range of doses as indicated for 72 h. After the treatment, these cells were prepared for MTT analysis of cell proliferation as described in Materials and Methods. Data were represented as the percentage over control (without any treatment under the same cultured condition). For **A–F**, analysis of variance (ANOVA) and post hoc testing show significant differences of mean percentage over control ($\pm$SD with $n = 3$ independent experiments) between high and low doses ($p < 0.005$).

human bone marrow cells in culture and were not tested further.

Certain whole-food extracts, such as blueberry, green tea, and specific compounds, including catechin, carnosine, and vitamin D_3, were found to increase cell proliferation of human bone marrow cells in a dose-dependent manner (Fig. 1). Cell proliferation as determined by MTT assay is displayed as the percent of cell proliferation over the control, which represents cells cultured in the same condition without any extract or compound added. The positive control, human granulocyte colony-stimulating factor (hGM-CSF; Fig. 1A), produced a 44.5 $\pm$ 8.1% proliferation at the highest dose of 100 ng/ml. Blueberry and catechin demonstrated a 34.5 $\pm$ 6.7 and 34.8 $\pm$ 5.2% increase in proliferation at 500 ng/ml and 20 μM, respectively (Fig. 1B,C). Carnosine displayed a 26.6 $\pm$ 6.0% increase at 20 μM (Fig. 1D), and vitamin D_3 displayed a lower percentage of proliferation, 14.8 $\pm$ 3.3% at 5 μM (Fig. 1F). Green tea produced a proliferation similar to blueberry and catechin with 35.6 $\pm$ 9.2% proliferation at 500 ng/ml (Fig. 1E)

Synergistic stimulatory effect of extracts and compounds on proliferation

To determine if the extracts and compounds displayed a synergistic effect on cell proliferation, we cultured human bone marrow cells with different combinations of the extracts and compounds. We also cultured the bone marrow cells with the individual extracts and compounds at the highest doses determined to promote the greatest amount of proliferation, which was represented by Fig. 1A–F. The positive control, hGM-CSF displayed 48.3 ± 7.4% proliferation, whereas blueberry, catechin, carnosine, green tea, and vitamin D_3 alone did not cause proliferation in a significantly different manner, as demonstrated in Fig. 1 (Fig. 2A). However, the combination of extracts and compounds resulted in a greater percentage of proliferation than observed with the individual extracts and compounds. For example, blueberry/vitamin D_3 exhibited a 62% increase in proliferation, blueberry/catechin a 70% increase, and blueberry/carnosine with the greatest synergistic affect of 83% (Fig. 2A). Blueberry/green tea, blueberry/vitamin D_3/green tea, and blueberry/vitamin D_3/green tea/carnosine also displayed significant increases in proliferation of 56%, 72%, and 70% respectively (Fig. 2A).



Promotion of CD34$^+$ cell proliferation and synergistic properties of extracts and compunds

To determine whether these extracts and compounds promoted cell proliferation of other progenitor cells, we cultured CD34$^+$ human hematopoietic stem cells under the same conditions as the bone marrow cells using different combinations of the extracts and compounds, and with the individual extracts and compounds at the highest doses determined to promote the greatest amount of proliferation in the bone marrow cell studies, as shown in Fig. 1A–F. The results revealed a 48.3 ± 7.4 increase for hGM-CSF, which was approximately a 5% increase in proliferation as compared to the hGM-CSF effect on the bone marrow cells (Fig. 2B). However, individually, blueberry, catechin, carnosine, green tea, and vitamin D_3 displayed a 20.9 ± 3.0, 24.8 ± 5.0, 11.05 ± 2.1, 14.0 ± 3.7, and 6.9 ± 2.6 increase in proliferation, respectively, which are much lower than observed in the bone marrow cells (Fig. 2B). However when combined, blueberry/vitamin D_3, blueberry/catechin, blueberry/carnosine, blueberry/green tea, and blueberry/vitamin D_3/green tea demonstrated a 39.3 ± 2.0%, 57.3 ± 10.4%, 30.9 ± 3.4%,

FIG. 2. Blueberry extract synergistically affects cell proliferation in the presence of co-treatment with D3, CH, D3/GT, or D3/GT/Ca. (**A**) Human bone marrow cells were cultured in 96-well tissue-culture plates (5×10^4/well) and treated with blueberry extract (500 ng/ml) in the presence of D3 (5 μM), CH (20 μM), Ca (20 μM), GT (500 ng/ml), D3 (5 μM)/GT (500 ng/ml), or D3 (5 μM)/GT (500 ng/ml)/Ca (20 μM) for 72 h. (**B**) Human bone marrow-derived CD34$^+$ cells (5×10^4/well) and treated as same above (**A**). For the MTT assay, these cells were prepared for cell proliferation analysis. Data were also represented as the percentage over control. ANOVA and post hoc testing shows significant differences of mean percentage over control (±SD with $n = 3$ independent experiments) between individual and certain combined treatments, for **A**, BB/D3 combined treatment compared to BB or D3 individual treatment ($p < 0.005$), BB/CH compared to BB or CH ($p < 0.005$), BB/Ca compared to BB or Ca ($p < 0.001$), BB/D3/GT compared to BB, D3 or GT, BB/D3/GT/Ca compared to BB, D3, GT or Ca; for **B** BB/CH combined treatment compared to BB or CH individual treatment ($p < 0.005$), BB/D3/GT/Ca compared to BB, D3, GT or Ca ($p < 0.001$).

27.9 ± 10.0%, and 49.9 ± 13.1% increase in proliferation, respectively, which is at least additive and in some cases more than additive (Fig. 2B). Interestingly, the combination of blueberry/vitamin D_3/green tea/carnosine resulted in an increase of 67.6 ± 11.9%, a simple additive effect would have been 52% demonstrating a synergistic effect of this combination (Fig. 2B).

Promotion of CD133$^+$ cell proliferation and synergistic properties of extracts and compounds

Some of the compounds and combinations with the greatest activity on proliferation of the bone marrow derived CD34$^+$ cells were then used to treat CD133$^+$ (progenitor cells) collected from peripheral blood and cultured under the same conditions as above. Cell proliferation was determined by MTT assay (see Materials and Methods) and is displayed as the percent of cell proliferation over the control. The results revealed an 21.11 ± 2.9% increase after treatment with hGM-CSF (Fig. 2C). Individually, blueberry, carnosine, green tea, and vitamin D_3 displayed a 11.9 ± 3.1, 16.9 ± 3.3, 13.57 ± 3.0, and 7.6 ± 1.39% increase in proliferation, respectively (Fig. 2C). When combined, blueberry/vitamin D_3/green tea and blueberry/vitamin D_3/green tea/carnosine demonstrated a 29.2 ± 3.6 and 42.5 ± 5.9% increases in proliferation (Fig. 2C), reflecting an additive effect of combining these compounds together when examining proliferation of human CD133$^+$ cells.

DISCUSSION

In this study, we have demonstrated for the first time that various natural compounds and their combinations promote the proliferation of human bone marrow, human bone marrow-derived CD34$^+$, and human peripheral blood-derived CD133$^+$ cells. When tested individually, these compounds were most effective in promoting proliferation of the bone marrow cells and less effective when used to treat the progenitor populations. This finding may reflect an effect of the individual compounds on the mature cell populations that are also present in the bone marrow cell cultures. When the activity of the compounds was examined in combinations, the additive and synergistic effects were more profound in the progenitor CD34$^+$ and CD133$^+$ cells. Surprisingly, some of the combinations tested resulted in proliferation that exceeded that produced by the positive control, hGM-CSF. For example, the combination of blueberry extract, green tea extract, carnosine, and vitamin D_3 produced greater proliferation than that induced by hGM-CSF in all three cell types, with CD133$^+$ cells being most sensitive with a proliferation response twice that produced by hGM-CSF. Of all the compounds tested, blueberry extract most consistently produced significant proliferation when combined with the other compounds.

There are several caveats to the interpretation and scope of our findings. First, our assay for proliferation was solely the MTT assay, which reflects the activity of the mitochondrial respiratory chain. Although the MTT assay is a very accurate end point method used to measure the cell viability in vitro, its results can have alternative interpretations.

Second, these findings need to be replicated in vivo before we can make statements regarding the application of our findings. For example, our studies were conducted in vitro and therefore do not have the added complexities associated with the pharmacokinetics of these various compounds when administered orally. Third, future studies should determine whether the proliferation caused by the substances and their combinations increase the risk of tumorgenicity. Because most of these compounds have been found to either (1) inhibit the growth of tumor cell lines, (2) induce tumor cell differentiation, or (3) not affect or increase the growth of normal cell types (22,24–26), our working hypothesis is that this combination will promote the growth of normal stem cells needed for healing while also reducing tumorgenicity potential. Finally, although the present study demonstrates enhanced proliferation, we do not know what the final phenotypes of these cells were following treatment. One goal of future studies would be to determine whether we can identify natural compounds that can not only increase proliferation of normal stem cells, but also push them into desired phenotypes specific to certain disorders.

In conclusion, we demonstrated for the first time that certain natural compounds can promote proliferation of hematopoietic stem cells in vitro, and more specifically that a combination of blueberry extract, green tea extract, carnosine, and vitamin D_3 demonstrate synergistic activity in these assays. Future studies will examine the effects of these compounds on nonhematopoietic derived cells.

DISCLOSURES

P.B. and P.R.S. are founders, and R.D.S., J.T., and N.D. are consultants for Natura Therapeutics, Inc. (Tampa, FL), a USF spin-out company.

REFERENCES

1. Ogawa M. (1993). Differentiation and proliferation of hematopoietic stem cells. Blood 81:2844–2853.
2. Socolovsky M, SN Constantinescu, S Bergelson, A Sirotkin and HF Lodish. (1998). Cytokines in hematopoiesis: specificity and redundancy in receptor function. Adv Protein Chem 52:141–198.

3. Whetton AD and E Spooncer. (1998). Role of cytokines and extracellular matrix in the regulation of haemopoietic stem cells. Curr Opin Cell Biol 10:721–726.
4. Henschler R, W Brugger, T Luft, T Frey, R Mertelsmann and L Kanz. (1994). Maintenance of transplantation potential in ex vivo expanded CD34(+)-selected human peripheral blood progenitor cells. Blood 84:2898–2903.
5. Miller CL and CJ Eaves. (1997). Expansion in vitro of adult murine hematopoietic stem cells with transplantable lympho-myeloid reconstituting ability. Proc Natl Acad Sci USA 94:13648–13653.
6. Demetri GD and JD Griffin. (1991). Granulocyte colony-stimulating factor and its receptor. Blood 78:2791–2808.
7. Adamson JW and JW Eschbach. (1990). Treatment of the anemia of chronic renal failure with recombinant human erythropoietin. Annu Rev Med 41:349–360.
8. Eschbach JW, NR Haley and JW Adamson. (1990). The anemia of chronic renal failure: pathophysiology and effects of recombinant erythropoietin. Contrib Nephrol 78:24–36.
9. Koury MJ and P Ponka. (2004). New insights into erythropoiesis: the roles of folate, vitamin B12, and iron. Annu Rev Nutr 24:105–131.
10. Hisha H, H Yamada, MH Sakurai, H Kiyohara, Y Li, C Yu, N Takemoto, H Kawamura, K Yamaura, S Shinohara, Y Komatsu, M Aburada and S Ikehara. (1997). Isolation and identification of hematopoietic stem cell-stimulating substances from Kampo (Japanese herbal) medicine, Juzentaiho-to. Blood 90:1022–1030.
11. Hisha H, U Kohdera, M Hirayama, H Yamada, T Iguchi-Uehira, TX Fan, YZ Cui, GX Yang, Y Li, K Sugiura, M Inaba, Y Kobayashi and S Ikehara. (2002). Treatment of Shwachman syndrome by Japanese herbal medicine (Juzen-taiho-to): stimulatory effects of its fatty acids on hemopoiesis in patients. Stem Cells 20:311–319.
12. Holehouse EL, ML Liu and GW Aponte. (1998). Oleic acid distribution in small intestinal epithelial cells expressing intestinal-fatty acid binding protein. Biochim Biophys Acta 1390:52–64.
13. Meyer C. (2004). Scientists probe role of vitamin D: deficiency a significant problem, experts say. J Am Med Assn 292:1416–1418.
14. Mathieu C, EE van, B Decallonne, A Guilietti, C Gysemans, R Bouillon and L Overbergh. (2004). Vitamin D and 1,25-dihydroxyvitamin D3 as modulators in the immune system. J Steroid Biochem Mol Biol 89–90:449–452.
15. Hipkiss AR, JE Preston, DT Himsworth, VC Worthington, M Keown, J Michaelis, J Lawrence, A Mateen, L Allende, PA Eagles and NJ Abbott. (1998). Pluripotent protective effects of carnosine, a naturally occurring dipeptide. Ann NY Acad Sci 854:37–53.
16. Holliday R and GA McFarland. (2000). A role for carnosine in cellular maintenance. Biochemistry (Mosc.) 65: 843–848.
17. Joseph JA, B Shukitt-Hale, NA Denisova, D Bielinski, A Martin, JJ McEwen and PC Bickford. (1999). Reversals of age-related declines in neuronal signal transduction, cognitive, and motor behavioral deficits with blueberry, spinach, or strawberry dietary supplementation. J Neurosci 19:8114–8121.
18. Gemma C, MH Mesches, B Sepesi, K Choo, DB Holmes and PC Bickford. (2002). Diets enriched in foods with high antioxidant activity reverse age-induced decreases in cerebellar beta-adrenergic function and increases in proinflammatory cytokines. J Neurosci 22:6114–6120.
19. Williams RJ, JP Spencer and C Rice-Evans. (2004). Flavonoids: antioxidants or signalling molecules? Free Radic Biol Med 36:838–849.
20. Casadesus G, B Shukitt-Hale, HM Stellwagen, X Zhu, HG Lee, MA Smith and JA Joseph. (2004). Modulation of hippocampal plasticity and cognitive behavior by short-term blueberry supplementation in aged rats. Nutr Neurosci 7:309–316.
21. Chen ZP, JB Schell, CT Ho and KY Chen. (1998). Green tea epigallocatechin gallate shows a pronounced growth inhibitory effect on cancerous cells but not on their normal counterparts. Cancer Lett 129:173–179.
22. Wang YC and U Bachrach. (2002). The specific anti-cancer activity of green tea (−)-epigallocatechin-3-gallate (EGCG). Amino Acids 22:131–143.
23. Song DU, YD Jung, KO Chay, MA Chung, KH Lee, SY Yang, BA Shin and BW Ahn. (2002). Effect of drinking green tea on age-associated accumulation of Maillard-type fluorescence and carbonyl groups in rat aortic and skin collagen. Arch Biochem Biophys 397:424–429.
24. Bomser J, DL Madhavi, K Singletary and MA Smith. (1996). In vitro anticancer activity of fruit extracts from Vaccinium species. Planta Med 62:212–216.
25. McFarland G and R Holliday. (1999). Differential response of embryonic stem cells and teratocarcinoma cells to carnosine. In Vitro Cell Dev Biol Anim 35:15–16.
26. Hussain EA, RR Mehta, R Ray, TK Das Gupta and RG Mehta. (2003). Efficacy and mechanism of action of 1alpha-hydroxy-24-ethyl-cholecalciferol (1alpha[OH]D5) in breast cancer prevention and therapy. Recent Results Cancer Res 164:393–411.

Address reprint requests to:
Dr. Paula C. Bickford
Center of Excellence for Aging and Brain Repair
MDC-78
University of South Florida College of Medicine
12901 Bruce B. Downs Boulevard
Tampa, FL 33612

E-mail: pbickfor@hsc.usf.edu

Received November 18, 2005; accepted December 20, 2005.