J Exp Clin Med 2011;3(2):80–84

Contents lists available at ScienceDirect

Journal of Experimental and Clinical Medicine

journal homepage: http://www.jecm-online.com

ORIGINAL ARTICLE

Alterative Expression of Angiogenic Proteins by Arginine in SW620 Cell-Inoculated Nude Mice

Shung-Sheng Tsou [1], Man-Hui Pai [2], Chiu-Li Yeh [3], Cheng-Chung Li [4], Sung-Ling Yeh [4]*

[1] Department of Surgery, Tung's Taichung MetroHarbor Hospital, Taichung, Taiwan
[2] Department of Anatomy, Taipei Medical University, Taipei, Taiwan
[3] Department of Food and Nutrition, Chinese Culture University, Taipei, Taiwan
[4] School of Nutrition and Health Sciences, Taipei Medical University, Taipei, Taiwan

ARTICLE INFO

Article history:
Received: Oct 14, 2010
Revised: Nov 19, 2010
Accepted: Jan 24, 2011

KEY WORDS:
angiogenic protein;
arginine;
CD31;
nude mice;
SW620

Background: Arginine (Arg) has been shown to possess numerous useful physiological properties. However, the effect of Arg on various cancers remains controversial. This study investigated the effect of Arg supplementation on highly metastatic human colorectal adenocarcinoma xenograft in nude mice.
Methods: Nude mice ($n = 20$) were inoculated with 1×10^7 SW620 cells and randomly assigned to two groups. The control group ($n = 10$) was fed a semipurified diet, the experimental group ($n = 10$) was supplied an identical diet except that part of the casein was replaced by Arg, which provided 2% of the total energy intake. After consuming the respective diets for 5 weeks, tumors and blood were obtained for angiogenic protein measurement, and immunocytochemical findings of CD31 expressions in tumor tissues were analyzed.
Results: Plasma matrix metalloproteinase-9, epidermal growth factor, vascular endothelial growth factor receptor concentrations, and tumor homogenate matrix metalloproteinase-9 and nitric oxide levels were higher in the Arg group than those of the control group ($p < 0.05$). There were more CD31-positive cells and higher immunoreactive intensities in mice fed with Arg than those fed with the control diet.
Conclusion: The results of this study suggest that dietary Arg supplementation may enhance angiogenic protein production in metastatic human colon cancer-implanted nude mice.

1. Introduction

Arginine (Arg), a nonessential amino acid for healthy adults, is required for the synthesis of proteins, creatinine, and polyamines.[1] Although Arg is synthesized in the body, it is not made in sufficient amount to support growth or meet metabolic requirements during periods of stress. Arg was shown to possess numerous useful physiological properties. Many studies have demonstrated the benefits of Arg supplementation on immune functions.[1,2] Arg is the substrate of nitric oxide synthase (NOS) and the precursor of nitric oxide (NO). NO is a neurotransmitter and vasodilator. Also, NO plays a critical role in antipathogen and tumoricidal responses of the immune system.[3,4] However, NO has been implicated as a deleterious agent in various pathophysiological conditions, including cancer.[5,6] The Arg-NO mediated modulatory effect on various cancers remains controversial.

Cancer was the first leading cause of death in Taiwan in 2009, and colorectal cancer (CRC) was the third leading cause of mortality among cancer patients.[7] Changing the diet or certain dietary components has become an important developing strategy to decrease the incidence of CRC and improve its prognosis. Arg is often used in immunonutrition regimens. Currently, Arg is added to enteral formulas at pharmacologic levels in an attempt to boost immune function and improve outcomes of critically ill patients. A previous study showed that immune-enhancing diets containing Arg reduced the infection rate in the postoperative period of head, neck, and esophageal cancer patients.[8] A study by Ma et al[9] revealed that Arg given during the initiation phase significantly reduced colorectal tumor production and crypt cell proliferation in rats. Our laboratory demonstrated that Arg supplementation inhibits the progression of primary colon cancer (SW480) in *in vivo* and *in vitro* models.[10] However, a report by Park et al[11] described an increase in tumor proliferation markers in patients with breast cancer treated with dietary Arg supplements. Yerushalmi et al[12] also found that Arg promotes colonic tumorigenesis in intestinal neoplastic mice. The contradictory results may have resulted from different features of the cancer cells and models. SW480 and SW620 are human colorectal adenocarcinoma cell lines which both derived from the same patient. SW480 was isolated from the primary adenocarcinoma arising in the colon, whereas SW620 was

* Corresponding author. School of Nutrition and Health Sciences, Taipei Medical University, 250 Wu-Hsing St, Taipei 110, Taiwan.
E-mail: sangling@tmu.edu.tw (S.-L. Yeh).

1878-3317/$ – see front matter
doi:10.1016/j.jecm.2011.02.003

isolated some years later from a lymph node metastasis.[13] We have examined the effect of Arg on the progression of SW480; however, the role of Arg in the development of SW620 has not been clarified. In this study, the SW620 cell line was used in a xenograft model in athymic nude mice. We analyzed NO levels and several angiogenic factors including epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), VEGF receptor (VEGFR), matrix metalloproteinase (MMP)-2,9, and platelet-endothelial cell adhesion molecule (PECAM)-1 in plasma and tumors to evaluate the effect of Arg supplementation on the angiogenesis induced by SW620.

2. Materials and Methods

2.1. Cell culture

The SW620 human colorectal adenocarcinoma cell line was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan) and cultured in L-15 medium supplemented with 10% fetal bovine serum and a penicillin/ streptomycin mixture. Adherent monolayer cultures were maintained at 37°C in a mixture of 5% CO_2 and 95% air. Cells were routinely trypsinied (0.05% trypsin/EDTA) and subcultured in flasks.

2.2. Animals

Male 4-week-old ICR nu/nu mice were purchased from the National Laboratory Animal Center (Taipei, Taiwan). The mice were housed in a specifically designed pathogen-free isolation facility and maintained in a temperature- and humidity-controlled room with a 12-hour light-dark cycle. Care of the laboratory animals was established by Taipei Medical University, and protocols were approved by the Animal Care Committee. All mice were allowed free access to a sterilized standard chow diet for 1 week before the study.

2.3. Tumor cell inoculation and study protocol

Nude mice ($n = 20$) were weighed and anesthetized followed by inoculation with 1×10^7 SW620 cells in a total volume of 100 μL of culture medium in the right of left flank. We waited until the tumors were visible and then the mice were randomly assigned to a control group or an Arg group of 10 animals each. The control group was fed a common semipurified diet, and the Arg group was supplied an identical diet except that part of the casein was replaced by Arg (Sigma Chemical Co, St Louis, MO, USA), which provided 2% of the total energy intake (Table 1). This amount of Arg was previously found to enhance the immune response[14,15] and have effects on inhibiting the progression of primary colon cancer in rodents.[10] Although casein was isonitrogenously substituted by Arg, the amount of protein in the Arg group was adequate for growth and maintenance according to the reported nutrient requirements for mice.[16] The diets were sterilized and stored at −20°C in bags. The body weight was recorded weekly, and the growth rates of tumor were determined by weekly measurements of two diameters of the tumor with a vernier caliper. After consuming the respective diets for 5 weeks, mice were anesthetized with ether and sacrificed by cardiac puncture. Blood samples were collected in tubes containing heparin and were centrifuged for further measurements. Tumors were immediately harvested and stored at −70°C for further analysis.

Table 1 Composition of the experimental diets

Component (g/kg)	Control	Arginine
Soybean oil	100	100
Casein	200	158
Arginine	0	20.9
Salt mixture*	35	35
Vitamin mixture†	10	10
Methyl cellulose	31	31
Choline chloride	1	1
Methionine	3	3
Corn starch	620	641.1

* Salt mixture contained the following (mg/g): calcium phosphate dibasic, 500; sodium chloride, 74; potassium sulfate, 52; potassium citrate monohydrate, 20; magnesium oxide, 24; manganese carbonate, 3.5; ferric citrate, 6; zinc carbonate, 1.6; cupric carbonate, 0.3; potassium iodate, 0.01; sodium selenite, 0.01; and chromium potassium sulfate, 0.55; † Vitamin mixture contained the following (mg/g): thiamin hydrochloride, 0.6; riboflavin, 0.6; pyridoxine hydrochloride, 0.7; nicotinic acid, 3; calcium pantothenate, 1.6; D-biotin, 0.05; cyanocobalamin, 0.001; retinyl palmitate, 1.6; DL-α-tocopherol acetate, 20; cholecalciferol, 0.25; and menaquinone, 0.005.

2.4. Measurement of tumor volume

Tumors were measured with a microcaliper, and the ellipsoid tumor volume was calculated using the formula: volume = length × width × height × π/6.

2.5. Plasma EGF, VEGF, VEGFR, and MMP-9 concentrations

Plasma EGF, VEGF, VEGFR, and MMP-9 concentrations were measured by using commercially available enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN, USA). Antibodies specific for mouse EGF, VEGF, VEGFR, and MMP-9 were coated onto the wells of the microtiter strips provided. Procedures were followed according to the manufacturer's instructions.

2.6. VEGF; VEGF-R; MMP-2, 9; and NO_2^-/NO_3^- levels in tumors

Tumor homogenates were prepared by adding 1 mL of 0.1M Tris-HCl (pH 7.5) containing 10 mM of a $CaCl_2$ solution to 0.1 g tissue using a homogenizer. The homogenates were centrifuged to remove cell debris. The supernatant was used for the analysis of MMP-9, VEGF, and VEGFR concentrations in tumors using commercially available enzyme-linked immunosorbent assay kits. Antibodies specific for human MMP-9, VEGF, and mouse VEGFR were coated onto the wells of the microtiter strips provided (R&D Systems, Minneapolis, MN, USA).

NO is highly unstable in solution and cannot readily be assayed. However, NO is converted to stable nitrite and nitrate ions in an aqueous solution. After conversion of nitrate to nitrite using nitrate reductase, nitrite concentrations were measured using the Griess reagent. Concentrations of NO_2^-/NO_3^- in tumor homogenates were determined with a commercial kit (Assay Designs, Ann Arbor, MI, USA). Protein concentrations of homogenates were measured by Lowry's method. The MMP-2, 9, VEGF, VEGFR and NO levels in tumors were based on milligram protein of tumor homogenates. Procedures were followed according to the manufacturer's instructions.

2.7. CD31 immunocytochemistry

To demonstrate PECAM-1 (CD31) immunoreactivity, consecutive frozen sections of tumors (at a thickness of 10 μm) were obtained using a Bright Cryostat (Bright, Huntingdon, UK) at −20°C and preincubated in a blocking solution containing 10% normal goat serum and 0.3% H_2O_2 in 0.1M phosphate buffer for 1 hour to block endogenous peroxidase activity and the nonspecific binding of antibodies. Sections were then incubated with a mouse monoclonal primary antibody against CD31 (AbD Serotec, Martinsried, Planegg, Germany), diluted 1:100 in 0.1M phosphate buffer, for 24 hours at 4°C. After washing in buffer, sections were next incubated in biotinylated goat anti-mouse immunoglobin G (diluted 1:300,

Chemicon, Temecula, CA, USA) for 1 hour at room temperature. After reaction with the peroxidase-linked Avidin-Biotin complex (Vector, Burlingame, CA, USA) for 1 hour at room temperature, a diaminobenzidine solution kit (Vector) was used to detect CD31 immunoreactivity. Hematoxylin (Sigma, St Louis, MO, USA) nuclear staining was also applied to contrast the cell nucleus with cytoplasm. All tissue sections were mounted on silane-coated slides and coverslipped by Permount (Fisher Scientific, Pittsburgh, PA, USA), examined with a Zeiss Axiphot light microscope equipped with a digital camera (Carl Zeiss, Oberkochen, Germany), and photographed. Image of immunoreactive areas were assessed with the digital image analysis system Image Pro Plus 5.1 (Media Cybernetics, Silver Spring, MD, USA). We used the "count/size" and "area" commands to perform the intensity of CD31 immunoreactivity. The automatic object counting and measuring process was used to quantify the immunoreactive areas in the sections. The values were expressed as square micrometer. At least eight microscopic fields per section and three independent samples for each group were analyzed and the averaged areas were obtained for each group.

2.8. Statistical analysis

All data are expressed as the mean ± standard deviation. Student's *t* test was used to analyze the significance of differences between mean values. Linear regression analysis was used to determine the correlation between plasma concentrations of EGF and MMP-9 and the MMP-9 levels between plasma and tumors. A *p* value less than 0.05 was considered statistically significant.

3. Results

3.1. Body weights and tumor size

There were no differences in the initial body weights and weights after feeding the diets for 5 week (control, 20.2 ± 0.8 g *vs.* Arg, 21.1 ± 0.5 g, $p > 0.05$). The tumor size of the Arg group tended to be higher than that of the control group at the end of the experiment; however, no statistically significant difference was observed between the two groups (control, 4076 ± 891 mm^3 *vs.* Arg, 4538 ± 931 mm^3, $p = 0.06$).

3.2. Plasma EGF, VEGF, VEGFR, and MMP-9 concentrations

Plasma EGF, VEGFR and MMP-9 levels in the Arg group were significantly higher than those in the control group. There was a positive correlation between EGF and MMP-9 levels ($p = 0.018$). No differences in VEGF concentrations were observed between the Arg and control groups (Table 2).

3.3. VEGF; VEGF-R; MMP-2, 9; and NO levels in tumors

The MMP-9, VEGFR, and NO levels were significantly higher in the Arg group than those of the control group. Tumor MMP-9 was positively related to plasma MMP-9 levels ($p = 0.001$). No differences in MMP-2 and VEGF concentrations were observed between the Arg and control groups (Table 3).

Table 2 Plasma EGF, VEGF, VEGFR, and MMP-9 concentrations of the two groups

Variable	Control group (pg/mL)	Arginine group (pg/mL)
EGF	2.26 ± 0.01	5.01 ± 2.68*
VEGF	27.2 ± 1.9	31.5 ± 4.9
VEGFR	494.3 ± 69.5	549.3 ± 62.3*
MMP-9	0.126 ± 0.014	0.295 ± 0.113*

* Significantly differs from the control group at $p < 0.05$. Data are presented as the mean ± SD.
EGF = epidermal growth factor; MMP-9 = matrix metalloproteinase; SD = standard deviation; VEGF = vascular endothelial growth factor; VEGFR = VEGF receptor.

3.4. CD31 expression in tumor

The distribution of CD31-immunoreactive cells with hematoxylin stained-nuclei in tumor tissues is shown in Figure 1. The arrows point to CD31-positive cells with brown cytoplasm and hematoxylin-stained nuclei. The immunocytochemical findings showed that the brown CD31-positive cells were few and less intense in the control group, and most of them were found in the vicinity of blood vessels (Figure 1A). In the Arg-fed group, CD31-immunoreactive cells with stained nuclei were randomly distributed throughout the whole tissue. As shown in Figure 1, CD31-positive cells exhibited higher immunoreactive intensities in mice fed with Arg than those fed with the control diet (Figure 1B). The quantification of CD31 immunoreactive areas between groups were shown in Figure 1C, and the immunoreactive areas were significantly higher in the Arg group than in the control group ($p = 0.0036$).

4. Discussion

In the present study, we found that although the tumor size tended to be greater in the Arg group, no significant difference was observed between the two groups. Because tumor growth was noted for only 5 week, whether longer period may differentiate the growth rates between groups requires further investigation. However, we did observe some angiogenic protein expressions were higher in the Arg-supplemented group in xenograft nude mice with CRC. Angiogenesis is a prerequisite for tumor growth and metastasis. Vascular endothelial cell proliferation, migration, and capillary formation are stimulated by angiogenic growth factors.[17] EGF and VEGF are potent proangiogenic proteins. Levels of EGF and VEGF are positively associated with angiogenesis.[18,19] VEGF activates VEGFR expressed by vascular endothelial cells. VEGFR activation also plays an important role in the progression of tumor growth.[20] MMPs are a family of zinc-dependent neutral endopeptidases collectively capable of degrading essentially all matrix components. MMP-2 and MMP-9 degrades components of the basement membranes and is believed to be crucial for invasion by malignant tumors.[21]

In this study, we found that plasma EGF and MMP-9 concentrations were correlated. EGF, VEGFR, and MMP-9 were all higher in the Arg group than in the control group. These results were comparable with higher MMP-9 levels and PECAM-1 expressions observed in tumors of the Arg group. A study performed by Ohnishi et al[19] also reported that MMP-9 expression was positively correlated with EGF. EGF increased the promoter activities of MMP-9 genes and may consequently promote the invasion activity of cancer cells.[19] PECAM-1 (CD31) is a member of the immunoglobin gene superfamily of cell adhesion molecules. It is highly expressed on the surface of endothelial cells.[22] Previous study showed that PECAM-1 deficient mice exhibited defects in their angiogenesis and

Table 3 VEGF, VEGFR, MMP-9, and NO concentrations in the tumor homogenates

Variable	Control group	Arginine group
MMP-2 (ng/g protein)	382.4 ± 141.9	450.5 ± 23.1
MMP-9 (ng/g protein)	8.14 ± 13.9	47.0 ± 27.1*
VEGF (pg/g protein)	20.9 ± 9.5	22.3 ± 8.6
VEGFR (pg/g protein)	314.1 ± 140.2	521.3 ± 123.1*
NO (μM/g protein)	7.9 ± 6.7	15.7 ± 8.8*

* Significantly differs from the control group at $p < 0.05$. Data are presented as the mean ± SD.
EGF = epidermal growth factor; MMP = matrix metalloproteinase; NO = nitric oxide; SD = standard deviation; VEGF = vascular endothelial growth factor; VEGFR = VEGF receptor.

inflammatory responses to foreign body challenges.[23] PECAM-1 plays an important role in survival, migration, and functional organization of endothelial cells during vascular development and angiogenesis.[24] The higher EGF, VEGFR, and MMP-9 expressions in plasma and more CD31-positive cells and higher immunoreactive intensities in tumors of mice fed with Arg suggest that dietary Arg supplementation may enhance angiogenesis in metastatic colon cancer-implanted nude mice.

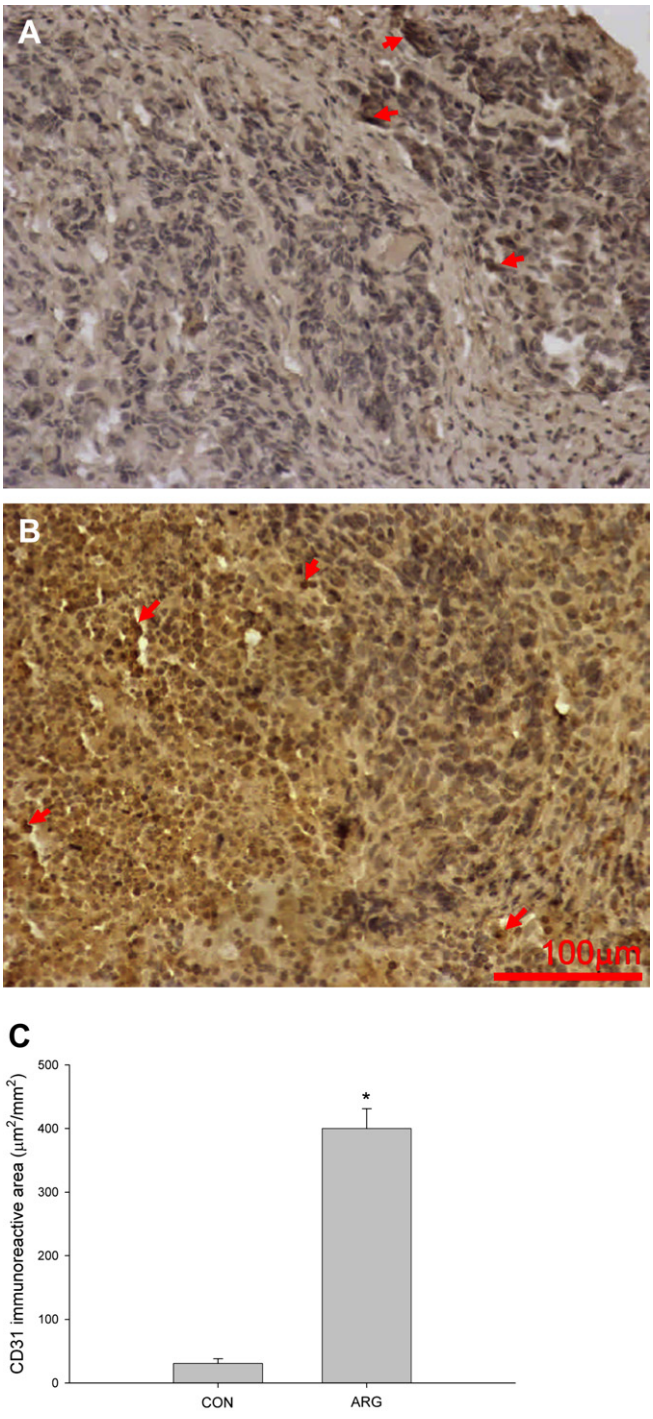

Figure 1 Expression of CD31 immunoreactivity in tumor tissues in the (A) control group and (B) arginine group. The arrow points to CD31-positive cells with brown cytoplasm and hematoxylin-stained nuclei. Only a few brown CD31-labeled cells were detected in the control group, and most of them were found in the vicinity of blood vessels. The expression of CD31 exhibited higher immunoreactive intensities in mice fed with arginine, and the CD31-positive cells were distributed randomly throughout the tissue. Scale bars = 100 μm. (C) Indicates the quantification of immunoreactive areas between groups in the target region ($n = 3$). The areas were assessed with Image Pro Plus 5.1 (Media Cybernetics, Silver Spring, MD, USA) and were calculated as described in the Materials and methods section. They are significantly different between the control group and arginine group ($p = 0.0036$).

In this study, we observed that NO levels were higher in the Arg group than in the control group, which was consistent with higher expression of MMP-9 in tumors. NO has multifaceted roles in cancer and the impact of NO on the progression of various cancers remains controversial. Some studies suggested that expression of NOS was correlated with reduced metastasis,[25,26] others showed that there is greater expression of iNOS in higher tumor grades, which tend to be more invasive.[27,28] The results of this study support the description that NO promotes angiogenesis of colorectal adenocarcinoma cells in xenograft nude mice. Our results were consistent with reports by Yerushalmi et al[12] and Yeatman et al.[29] The former study found that iNO derived from Arg promoted colonic tumorigenesis in congenital multiple intestinal neoplastic mice. Yeatman et al[29] examined the effects of dietary Arg on the growth of murine colon cancer metastatic to the liver and found that Arg supplementation may stimulate the growth of tumor *in vivo*. However, the results of this study contradict a previous report performed by our laboratory, which showed that Arg supplementation increased NO secretion and may consequently inhibit the progression of colon cancer.[10] In that experiment, colon primary adenocarcinoma cells (SW480) were used, whereas metastatic colon carcinoma SW620 cells were selected in this study. Zhou et al[28] revealed that SW620 cells had higher tissue factor expression than did SW480 cells. Tissue factors are believed to play important roles in tissue repair, angiogenesis, and tumor metastasis. Cianchi et al[30] found that iNOS activity was higher in metastatic colorectal tumors than in nonmetastatic ones, and there was a significant correlation between iNOS and VEGF expressions. A previous study performed by Cendan et al[31] compared the L-Arg transport system between SW480 and SW620 cells. They found that L-Arg transport occurs primarily through the sodium-independent system y^+, and the y^+ system activity in SW620 cells was almost twofold higher than that of SW480 cells. They concluded that the increased Arg transport y^+ activity may be a mechanism to provide continuous substrate for tumor growth.[31] We speculated that an Arg-supplemented diet increases L-Arg transport and endogenous NO synthesis in SW620 tumor cells and may consequently upregulate the expressions of angiogenic proteins. Because the angiogenic process is very complicated, factors other than endogenous NO may also have played roles in tumor angiogenesis in this study.

In summary, this study showed that dietary Arg supplementation resulted in higher EGF, VEGFR, and MMP-9 levels and higher NO secretion. Also, tumor CD31 expressions were higher in SW620 cancer cell-implanted nude mice. These results suggest that Arg administration may promote angiogenesis of metastatic colon cancer. This study implies that dietary Arg supplementation in metastatic colon cancer should be carefully evaluated.

Acknowledgments

This study was supported by a research grant TTM-TMU-97-02 from Tung's Taichung MetroHarbor Hospital, Taichung, Taiwan.

References

1. Nieves Jr C, Langkamp-Henken B. Arginine and immunity: a unique perspective. *Biomed Pharmacother* 2002;**56**:471–82.
2. Evoy D, Lieberman MD, Fahey III TJ, Daly JM. Immunonutrition: the role of arginine. *Nutrition* 1998;**14**:611–7.
3. Fukumura D, Yonei Y, Kurose I, Saito H, Ohishi T, Higuchi H, Miura S, et al. Role in nitric oxide in Kupffer cell-mediated hepatoma cell cytotoxicity in vitro and ex vivo. *Hepatology* 1996;**24**:141–9.

4. Cifone MG, Festuccia C, Cironi L, Cavallo G, Chessa MA, Pensa V, Tubaro E, et al. Induction of the nitric oxide-synthesizing pathway in fresh and interleukin 2-cultured rat natural killer cells. *Cell Immunol* 1994;**157**:181–94.
5. Mordan LJ, Burnett TS, Zhang LX, Tom J, Cooney RV. Inhibitors of endogenous nitrogen oxide formation block the promotion of neoplastic transformation in C3H 10T1/2 fibroblasts. *Carcinogenesis* 1993;**14**:1555–9.
6. Gottke M, Chadee K. Exogenous nitric oxide stimulates mucin secretion from LS174T colonic adenocarcinoma cells. *Inflamm Res* 1996;**45**:209–12.
7. Department of Health, Taiwan. Top 10 leading cause of death in Taiwan, www.doh.gov.tw [accessed 03.06.10].
8. Casas-Rodera P, Gomez-Candela C, Benitez S, Mateo R, Armero M, Castillo R, Culebras JM. Immunoenhanced enteral nutrition formulas in head and neck cancer surgery: a prospective, randomized clinical trial. *Nutr Hosp* 2008;**23**:105–10.
9. Ma Q, Williamson KE, O'Rourke D, Rowlands BJ. The effects of L-arginine on crypt cell hyperproliferation in colorectal cancer. *J Surg Res* 1999;**81**:181–8.
10. Yeh CL, Pai MH, Li CC, Tsai YL, Yeh SL. Effect of arginine on angiogenesis induced by human colon cancer: in vitro and in vivo studies. *J Nutr Biochem* 2010;**21**:538–43. Epub ahead of print.
11. Park KG, Heys SD, Blessing K, Kelly P, McNurlan MA, Eremin O, Garlick PJ. Stimulation of human breast cancers by dietary L-arginine. *Clin Sci (Lond)* 1992;**82**:413–7.
12. Yerushalmi HF, Besselsen DG, Ignatenko NA, Blohm-Mangone KA, Padilla-Torres JL, Stringer DE, Cui H, et al. The role of NO synthases in arginine-dependent small intestinal and colonic carcinogenesis. *Mol Carcinogen* 2006;**45**:93–105.
13. Leibovitz A, Stinson JC, McCombs WB, McCoy CE, Mazur KC, Mabry ND. Classification of human colorectal adenocarcinoma cell lines. *Cancer Res* 1976;**36**:4562–9.
14. Yeh CL, Lee CH, Chen SC, Hou YC, Yeh SL. Effects of arginine-containing total parenteral nutrition on N balance and phagocytic activity in rats undergoing a partial gastrectomy. *Br J Nutr* 2005;**93**:267–72.
15. Shang HF, Wang YY, Lai YN, Chiu WC, Yeh SL. Effects of arginine supplementation on mucosal immunity in rats with septic peritonitis. *Clin Nutr* 2004;**23**:561–9.
16. National Research Council. *Nutrient Requirements of Laboratory Animals*. 3rd ed. Washington D.C.: National Academy of Sciences; 1978.
17. Rose DP, Connolly JM. Regulation of tumor angiogenesis by dietary fatty acids and eicosanoids. *Nutr Cancer* 2000;**37**:119–27.
18. Dobbs SP, Hewett PW, Johnson IR, Carmichael J, Murray JC. Angiogenesis is associated with vascular endothelial growth factor expression in cervical intraepithelial neoplasia. *Br J Cancer* 1997;**76**:1410–5.
19. Ohnishi Y, Lieger O, Attygalla M, Iizuka T, Kakudo K. Effects of epidermal growth factor on the invasion activity of the oral cancer cell lines HSC3 and SAS. *Oral Oncol* 2008;**44**:1155–9.
20. Ebos JM, Lee CR, Bogdanovic E, Alami J, Van Slyke P, Francia G, Xu P, et al. Vascular endothelial growth factor-mediated decrease in plasma soluble vascular endothelial growth factor receptor-2 levels as a surrogate biomarker for tumor growth. *Cancer Res* 2008;**68**:521–9.
21. Vihinen P, Kahari VM. Matrix metalloproteinases in cancer: prognostic markers and therapeutic targets. *Int J Cancer* 2002;**99**:157–66.
22. Albelda SM, Oliver PD, Romer LH, Buck CA. EndoCAM. A novel endothelial cell-cell adhesion molecule. *J Cell Biol* 1990;**110**:1227–37.
23. Solowiej A, Biswas P, Graesser D, Madri JA. Lack of platelet endothelial cell adhesion molecule-1 attenuates foreign body inflammation because of decreased angiogenesis. *Am J Pathol* 2003;**162**:953–62.
24. Dimaio TA, Wang S, Huang Q, Scheef EA, Sorenson CM, Sheibani N. Attenuation of retinal vascular development and neovascularization in PECAM-1-deficient mice. *Dev Biol* 2008;**315**:72–88.
25. Xie K, Dong Z, Fidler I. Activation of nitric oxide synthase gene for inhibition of cancer metastasis. *J Leukoc Biol* 1996;**59**:797–803.
26. Radomski MW, Jenkins DC, Holmes L, Moncada S. Human colorectal adenocarcinoma cells: differential nitric oxide synthesis determines their ability to aggregate platelets. *Cancer Res* 1991;**51**:6073–8.
27. Jenkins DC, Charles IG, Thomsen LL, Moss DW, Holmes LS, Bavlis SA, Rhodes P, et al. Roles of nitric oxide in tumor growth. *Proc Natl Acad Sci USA* 1995;**92**:4392–6.
28. Zhou H, Hu H, Shi W, Ling S, Wang T, Wang H. The expression and the functional roles of tissue factor and protease-activated receptor-2 on SW620 cells. *Oncol Rep* 2008;**20**:1069–76.
29. Yeatman TJ, Risley GL, Brunson ME. Depletion of dietary arginine inhibits growth of metastatic tumor. *Arch Surg* 1991;**126**:1376–82.
30. Cianchi F, Corteshni C, Fantappie O, Messerini L, Schiavone N, Vannacci A, Nistri S, et al. Inducible nitric oxide synthase expression in human colorectal cancer: correlation with tumor angiogenesis. *Am J Pathol* 2003;**162**:793–801.
31. Cendan JC, Souba WW, Copeland III EM, Lind DS. Increased L-arginine transport in a nitric oxide-producing metastatic colon cancer cell line. *Ann Surg Oncol* 1996;**3**:501–8.