BIOMEDICAL AND ENVIRONMENTAL SCIENCES 15, 41-47 (2002)

Antitumorigenic Potential of Diallyl Sulfide in Ehrlich Ascites Tumor Bearing Mice

R73 A

YOGESHWER SHUKLA[1], ANNU ARORA, AND ALKA SINGH

Environmental Carcinogenesis Division, Industrial Toxicology Research Centre, M. G. Marg, P. O. Box 80, Lucknow-226001, India

Objective To study the effects of diallyl sulfide (DAS), an organosulfur compound present in garlic (*Allium sativum*), on the life span of ehrlich ascites (EA) tumor bearing Swiss albino mice, cytotoxicity and angiogenesis. **Methods** EA tumor cells were maintained by serial transplantation in peritoneal cavity of male Swiss albino mice. EA tumor cells were inoculated at concentrations of 1×10^6 EA cells, 2.5×10^6 EA cells and 5×10^6 EA cells. DAS was given in 0.2 ml normal saline i. p., daily for seven days followed one hour later by inoculation with EA cells in respective groups. **Results** The results revealed that administration of DAS increased the life span of EA tumor bearing animals by more than 25 percent. A significant dose dependant cytotoxic response of DAS was also observed on EA tumor cells. DAS was also found to inhibit the angiogenesis in EA tumor bearing mice in a dose dependent manner. **Conclusion** It is suggested that DAS may exert its anticarcinogenic effects by more than one mechanism and is a useful chemopreventive and chemotherapeutic agent.

Key words: Garlic; Life span; Ehrlich ascites; EA tumor cells; Cytotoxicity; Angiogenesis

INTRODUCTION

Garlic and its constituents have been shown to have diverse biological activities including anticarcinogenic, antiatherosclerotic, antithrombotic, antidiabetic, anti-inflammatory, and fibrinolytic and other biological actions[1,2]. The mechanism responsible for these actions has been ascribed to the potent enzyme inhibiting activities[3-5] or antioxidant activities[6,7].

The prime active component in garlic is not only allicin but also its degradation products, a range of sulfides. Diallyl sulfide (DAS) is a lipophilic thioether derived from oxidized allicin, which is produced when garlic clove is crushed. Administration of allium compounds including diallyl sulfide inhibits tumor formation in a variety of organs in laboratory animal models[8-11]. DAS has also been shown to inhibit 1, 2-di-methylhydrazine induced colonic tumors in mice[12] and N-nitrosomethyl-benzylamine induced esophageal

[1]Correspondence should be addressed. E-mail: Yogeshwer_Shukla@hotmail.com; Fax: 0091-522-228227.

Biography of the first author: Dr. Yogeshwer Shukla, Ph. D, working as scientist in the Environmental Carcinogenesis Division at Industrial Toxicology Research Centre, working in the field of cancer chemprevention by the dietary constituents and their mechanism of action.

0895-3988/2002
CN11-2816

cancer in rats[11]. Earlier, we have reported the anticarcinogenic activity of DAS in mouse skin model of carcinogenesis[13,14]. Further, epidemiological studies suggested that a lower risk for gastric carcinoma is associated with higher consumption of garlic[15-18]. A similar protective effect against the incidence of colorectal cancer has been noted in the Japanese population in Hawaii[19].

Angiogenesis is a fundamental process of new vessel formation from pre-existing vasculature and is often activated during the early, preneoplastic stages of tumor development[20,21]. Increased vasculature in the peritoneal lining of the mice bearing ehrlich ascitis (EA) tumor is attributed to an angiogenic factor secreted by EA tumor cells and increased vascularization may be responsible for malignant progression or malignant drift. Thus, the inhibition of angiogenesis should be highly effective in inhibiting the growth of tumors. Tumor angiogenesis is controlled by a number of positive and negative regulators elaborated by tumor cells and tumor associated host cells, particularly by tumor associated macrophages[20,22].

Review of literature shows that some information is available on the antitumorigenic effects of DAS in solid tumors, but there is no information on the effect of DAS in transplantable tumors. Therefore, the present study includes the effects of DAS on EA transplantable tumors in Swiss albino mice. At the same time attempts are also made in the present study to investigate the effect of DAS on angiogenesis in order to understand the mechanism of DAS induced tumor inhibition in experimental models.

MATERIALS AND METHODS

Chemicals

Diallyl sulfide was purchased from Sigma Chemical Co., St. Louis, USA. The other chemicals obtained locally were of analytical grade of purity.

Animals and Treatment

Random bred Swiss albino mice (male, 20-22gm body weight) were obtained from Industrial Toxicology Research Center (ITRC) animal colony. They were kept in-group of 10 animals per polypropylene cage belonging to the same group under controlled temperature (22-25°C) and humidity (65-75%) with 12/12 h light/dark period. Animals were fed on a standard, solid pellet diet, containing all essential nutrients freshly obtained and stored under standard conditions and water *ad lib*.

In the first set of experiment, the protective effect of DAS was evaluated in EA tumor bearing mice. EA tumor cells were maintained by serial transplantation in peritoneal cavity of male Swiss albino mice. EA cells, extracted from peritoneal cavity of mice, were washed three times with 0.85% normal saline and suspended in 0.9 ml phosphate buffer saline pH 7.0, stained with 1% trypanblue and layered in haemocytometer and counted. Cells at 1×10^6 were injected i. p. per mouse for propagation.

Male Swiss albino mice were randomly divided into 6 groups. The treatment schedule is given below:

Gr. I: 0.2 ml normal saline i. p., daily for seven consecutive days followed 1 h later by inoculation with 1×10^6 EA cells.

Gr. II: 250 μg DAS in 0.2 ml normal saline i. p., daily for seven days followed 1 h later by inoculation with 1×10^6 EA cells as in Gr. I

Gr. III: 0.2 ml normal saline i. p. , daily for seven days followed 1 h later by inoculation with 2.5×10^6 EA cells.

Gr. IV: 250 µg DAS in 0.2 ml normal saline i. p., daily for seven days followed 1 h later by inoculation with 2.5×10^6 EA cells.

Gr. V: 0.2 ml normal saline i. p., daily for seven days followed 1 h later by inoculation with 5×10^6 EA cells.

Gr. VI: 250 µg DAS in 0.2 ml normal saline i. p., daily for seven days followed 1 h later by inoculation with 5×10^6 EA cells.

Body weight and mortality of animals were daily recorded throughout the entire period. The percentage of increased life span was calculated as described by Reibscheid *et al.* [23]

$$\% \text{ ILS} = \frac{T - C}{C} \times 100,$$

ILS = Increased life span; T = average life span of treated group; C = average life span of control group.

The Value More Than 20% is Taken as Significant Increase in ILS

In the second set of experiment, a comparison between animal's vasculature in the peritoneal lining was recorded in the animals exposed to DAS. The treatment schedule was the same as in the above-mentioned set of experiment. The animals from the treated (Gr. II, IV and VI) and control (Gr. I, III and V) groups were sacrificed after 28 days of inoculation with EA tumor cells and thereafter every week.

In the third set of experiment, the cytotoxic effect of DAS on EA cells *in vitro* was observed. EA cells were extracted from the peritoneal cavity of mice and washed with saline. About 200 cells were plated in 15 different plates. Different concentrations of DAS were added (0-30 µg) followed by incubation at 37°C for 3 h, stained with 1% trypan blue, layered in haemocytometer and counted as the following equation.

$$\text{Cytotoxicity} = \frac{\text{Dead Cells}}{\text{Viable+Dead Cells}} \times 100.$$

RESULTS

In the first set of experiment, the antitumorigenic effect of DAS was observed in Swiss albino mice. A significant increase in the survival rate of animals was recorded for the three groups given DAS prior to inoculation of EA tumor cells over respective controls (Fig. 1). By the end of the fourth week, an increased rate of mortality was evident in the animals of groups I, III and V over the animals inoculated with the same number of EA tumor cells and given DAS (Fig. 1). At the sixth week stage about 55% animals died in group V, while in the DAS exposed animals (group VI), only 30% mortality was recorded. The increased rate of mortality was also evident in other groups over DAS supplemented animals at the same period. At the end of the tenth week all the animals died in groups I, III and V, while few animals remained alive in the DAS supplemented groups. This observation indicates the anti-tumorigenic potential of DAS on EA transplantable tumors.

Increase in the survival rate of animals of DAS supplemented groups was also evident when calculated in terms of ILS. At the eight-week stage 67% increase in the life span of animals of group II was observed over controls (Fig. 2). About 66% increase in the life span of animals of group IV was observed over group III. In group V all animals died at the eight-week stage and 36% increase in the longevity was recorded in the animals of group VI (Fig. 2).

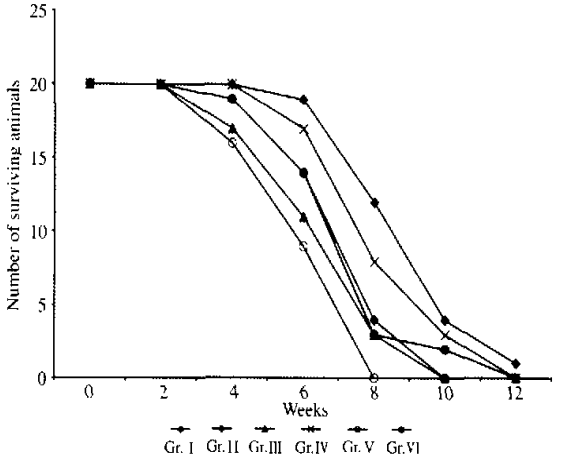

FIG. 1. Survival pattern of Swiss albino mice following DAS administration.

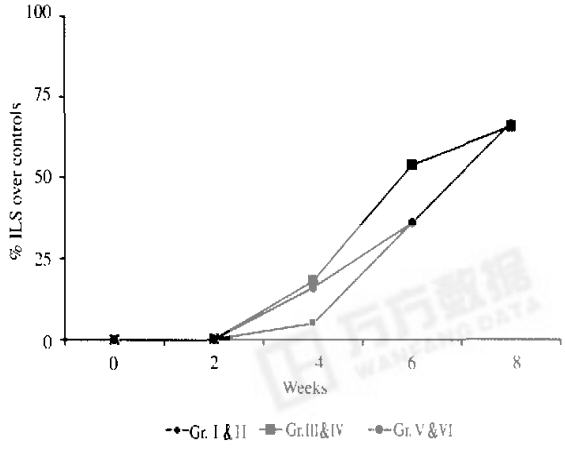

FIG. 2. Increase in the life span of Swiss albino mice following DAS administration.

In the second set of experiment, a marked inhibition in the peritoneal vasculature was observed in the animals of groups II, IV and VI over the respective controls (Table 1).

This may be attributed to the inhibition of neovascularization in the EA tumor bearing mice by DAS. The maximum inhibition of angiogenesis was observed in the animals of group II when compared with the respective controls (group I). The results indicates that DAS can effectively inhibit the formation of new blood vessels in the peritoneal lining of EA bearing Swiss albino mice.

In the third set of experiment the cytotoxic activity of DAS towards the EA tumour cells was observed in a dose dependent manner (Fig. 3). The maximum cytotoxic effect on the EA cells was observed at the dose of 25 µg DAS/ plate. This observation further confirmed the antitumorigenic potential of DAS in EA tumor bearing mice.

DISCUSSION

DAS, an organosulfur compound of garlic, is a well-known antitumor agent in rodent tumor models[11-14,24]. The results of the present study demonstrate its role in inhibiting the development of tumors in EA tumor bearing mice. Many *in vivo* and *in vitro* studies have shown that the percent increase in life span of EA tumor bearing mice and cytotoxicity towards EA tumor cells are well-established parameters for the evaluation of the antitumor efficacy of both synthetic and natural compounds[25,26]. In the present study, pretreatment with DAS significantly inhibited the development of ascites tumors, which in turn increased the survival rate of EA tumor bearing mice.

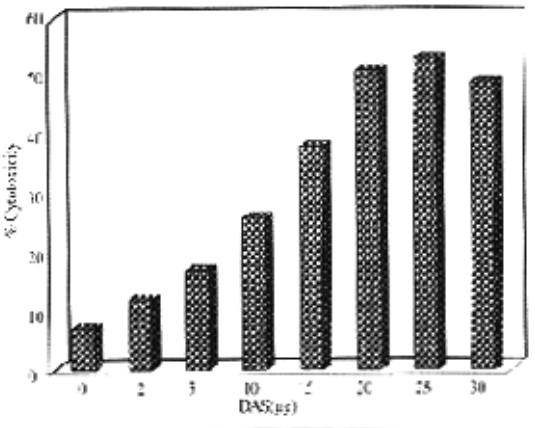

FIG. 3. Dose dependent cytotoxic response to DAS of EA cells.

It is a well-known fact that chemopreventive agents act through a variety of mechanisms and can prevent *in vivo* carcinogenesis. Investigations for tumor angiogenesis have focused on inhibition of tumor neovasculature as yet another possible mechanism for impairing tumor progression. Numerous studies have demonstrated that tumor growth and formation of metastases are angiogenesis-dependent and can be inhibited by angiogenesis inhibitors[27-30]. Many natural and synthetic compounds have been categorized as angiogenesis inhibitors[31-33]. Dietary glycine prevents the growth of B-16 tumor mela-

TABLE 1

Effect of DAS on Angiogenesis in Ehrlich Ascites Tumor Bearing Mice

Weeks	Control	1×10^6 EA Cells	2.5×10^6 EA Cells	5×10^6 EA Cells
2	-	-	-	-
4	+	-	-	-
6	+++	+	+	++
8	++++	++	+++	+++
10	*	+++	+++	*

Note. (-)= negative; (+)= weak; (++) =moderate; (+++) = high; (++++) very high.

(*) = all the animals of this group died.

noma in mice by inhibiting angiogenesis through mechanisms involving inhibition of endothelial cell proliferation[34]. Similarly, the antiangiogenic effect of retinoids, 1, 2, 5-dihydroxyvitamin D3 and their combination has also been demonstrated in experimental system *in vivo*[35]. Sola *et al.*[36] have shown that sulphonic acid derivatives of distamycin A are capable of inhibiting tumor development both *in vitro* and *in vivo* by inhibiting formation of new capillary blood vessels. Similar results were obtained in the present study in case of DAS treated EA tumor bearing mice. Pretreatment of DAS effectively decreased the number of blood vessels and a consequent reduction in blood flow. Furthermore, DAS (0-30 μg) also inhibited the growth of EA cells *in vitro* in a dose dependent manner. Based upon these observations on the protective effects of DAS on EA tumor cells, it is suggested that DAS possess cytotoxic and antitumor potential in EA transplantable tumors by modulating angiogenesis.

ACKNOWLEDGEMENTS

The authors wish to extend their thanks to Dr. P. K. Seth, Director, Industrial Toxicology Research Centre, Lucknow, for his keen interest in the study. The authors are also thankful to Mr. Nikhil Garg, scientist for statistical analysis of the data.

REFERENCES

1. Agarwal, K. C. (1996). Therapeutic actions of garlic constituents. Med. *Res. Rev.* **16**, 111-124.
2. Augusti, K. T. (1996). Therapeutic values of onion (*Allium cepa L.*) and garlic (*Allium sativum L.*). *Indian J. Exp. Biol.* **34**, 634-640.
3. Sendle, A., Elbl, G., Stenke, B., Breu, W., and Wanger, H. (1992). Comparative pharmacological investigations of *Allium ursinum and Allium sativum*. *Planta Med.* **58**, 1-7.
4. Yangs, C. S., Smith, T. J., and Hong, J. Y. (1994). Cytochrome P-450 enzymes as targets for chemoprevention against chemical carcinogenesis and toxicity: opportunities and limitations. *Cancer Res.* **54**, 1982s-1986s.
5. Yeh, Y. Y. and Yeh, S. M. (1994). Garlic reduces plasma lipids by inhibiting hepatic cholesterol and triacylglycerol synthesis. *Lipids* **29**, 189-193.
6. Perchellet, J. P., Perchellet, E. M., Abney, N. L., Zimstein, J. A., and Belman, S. (1986). Effects of garlic oils on glutathione peroxidase activity, the ratio of reduced/oxidized glutathione and ornithine decarboxylase induction in isolated mouse epidermal cells treated with tumour promoters. *Cancer Biochem. Biophys.* **8**, 299-312.
7. Phelps, S. and Harris, W. S. (1993). Garlic supplementation and lipoprotein oxidation susceptibility. *Lipids* **28**, 475-477.
8. Meng, C. L. and Shyu, K. W. (1990). Inhibition of experimental carcinogenesis by painting with garlic extract. *Nutrition and Cancer* **14**, 207-217.
9. Nagabhushan, M., Line, D., Polverini, P. J., and Solt, D. B. (1992). Anticarcinogenic action of diallyl sulfide in hamster buccal pouch and fore stomach. *Cancer Lett* **66**, 207-216.
10. Unnikrishnan, M. C., Soudamini, K. K., and Kuttan, R. (1990). Chemoprotection of garlic extract toward cyclophosphamide toxicity in mice. *Nutrition and Cancer* **13**, 201-207.

11. Wargovich, M. J., Woods, C., Eng, V. W. S., Stephens, L. C., and Gray, K. (1988). Chemoprevention of N-nitrosomethylbenzylamine induced esophageal cancer in rats by the naturally occurring thioether, diallyl sulfide. *Cancer Res.* **48**, 6872-6875.
12. Wargovich, M. J. (1987). Diallyl sulfide, a flavor component of garlic (*Allium sativum*), inhibits dimethylhydrazine induced colon cancer. *Carcinogenesis* **8**, 487-489.
13. Singh, A. and Shukla, Y. (1999). Evaluation of antitumour activity of diallyl sulfide in two- stage mouse skin model of carcinogenesis. *Biomedical and Environmental Sciences* **11** (3), 258-263.
14. Singh, A. and Shukla, Y. (1998). Antitumour activity of diallyl sulfide in polycyclic aromatic hydrocarbon induced mouse skin carcinogenesis. *Cancer Lett.* **131**, 209-214.
15. Han, J. (1993). Highlights of the cancer chemoprevention studies in China. *Preventive Med.* **22**, 712-722.
16. You, W. C., Blot, W. J., Chang, Y. S., Ershow, A., Yang, Z. T., An, Q., Henderson, B. E., Fraumeni, J. F., Jr., and Wang, T. G. (1989). Allium vegetables and reduced risk of stomach cancer. *J. Natl. Cancer Inst.* **81**, 162-164.
17. You, W. C., Blot, W. J., Chang, Y. S., Ershow, A. G., Yang, Z. T., An, Q., Henderson, B., Xu, G. W., Fraumeni, J. F., Jr., and Wang, T. G. (1988). Diet and high risk of stomach cancer in Shandong, *China. Cancer Res.* **48**, 3518-3523.
18. Buiatti, E., Palli, D., Decarli, A., Amadore, D., Avellini, C., Bianchi, S., Biserni, R., Cipriani, F., Coco, P., Glacosa, A., Marubini, E., Puntoni, R., Vindigni, C., Fraumeni, J., and Blot, W. (1989). A case-control study of gastric cancer and diet in Italy. *Int. J. Cancer* **44**, 611-616.
19. Haenszel, W. and Kurihara, M. (1972) Cancer among Japanese in Hawaii. *J. Natl. Cancer Inst.* **49**, 969-988.
20. Folkman, J. (1995). Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat. Med.* **1**, 27-31.
21. *Hanahan, D. and Folkman, J. (1996). Patterns and emerging mechanisms of angiogenic switch during tumorigensis. Cell* **86**, 353-364.
22. Polverini, P. J. and Leibovich, S. J. (1987). Effect of macrophage depletion on growth and neovascularization of hamster buccal pouch carcinoma. *J. Oral Pathol.* **16**, 436-441.
23. Reibscheid, E. M., Zyngier, S., Maria, D. A., Mistrone, R. J., Sinisterra, R. D., Couto, L. G., and Najjar, R. (1994). Antitumour effects of rhodium (I) complexes on mice bearing Ehrlich Tumours Braz. *J. Med. Biol. Res.* **27**, 91-94.
24. Sparnins, V. L., Barany, G., and Wattenberg, L. W. (1988). Effect of organosulfur compounds from garlic and onions on benzo (a) pyrene induced neoplasia and glutathione S-transferase activity in the mouse. *Carcinogenesis* **9**, 131-134.
25. Mitsui, T., Yamada, K., Yamashita, K., Matsus, N., Okuda, A., Kimura, G. and Sugano, M. (1995). EIA-3Y1 cell specific toxicity of tea polyphenols and their killing mechanism. *Int. J. Oncol.* **6**, 377-383.
26. Nishida, H., Omori, M., Fuhutomi, Y., Ninomiya, M., Nishiwaki, S., Suganuma, M., moriwaki, H., and Muto, Y. (1994). Inhibitory effects of (-)-epigallocatechin gallate on spontaneous hepatoma in C3H/HCNCri mice and human derived PLC/ PRF/5 cells. *Jap. J. Cancer Res.* **85**, 221-225.
27. Verheul, H. M., Bom, J. B., Hoekman, K., and Pinedo, H. M. (1999). Inhibition of tumour angiogenesis as a cancer treatment. *Ned. Tijdschr. Geneeskd.* **143**. 1549-1555.
28. Folkman, J. and Ingber, D. (1992). Inhibition of angiogenesis. *Semin. Cancer Biol.* **3**, 89-96.
29. Seon, B. K., Matsuno, F., Haruta, Y., Kondo, M. and Barcos, M. (1997). Long lasting complete inhibition of human solid tumours in SCID mice by targeting endothelial cells of tumour vasculature with antihuman endoglin immunotoxin. *Clin. Cancer Res.* **3**, 1031-1044.
30. Shalinsky, D. R., Brekken, J., Zou, H., Bloom, L. A., McDermott, C. D., Zook, S., Varki, N. M., and Appelt, K. (1999). Marked antiangiogenic and antitumour efficacy of AG3340 in chemoresistant human non-small cell lung cancer tumours: single agent and combination chemotherapy studies. *Clin. Cancer Res.* **5**, 1905-1917.
31. Joussen, A. M., Kruse, F. E., Volcker, H. E. , and Kirchhof, B. (1999). Topical application of methotrexate for inhibition of corneal angiogenesis. Graefes. Arch. *Clin. Exp. Opthal.* **237**, 920-927.
32. Cai, T., Fassina, G., Morini, M., Aluigi, M. G., Masiello, L., Fontanini, G., D'Agostini, F., De-Flora, S., Noonan, D. M., and Albini, A. (1999) N-acetyl cysteine inhibits endothelial cell invasion and angiogenesis. *Lab. Invest.* **79**, 1151-1159.
33. Keane, M. P., Belperio, J. A., Arenberg, D. A., Burdick, M. D., Xu, Z. J., Xue, Y. Y., and Strieter, R. M. (1999). IFN-gamma-inducible protein-10 attenuates bleomycin-induced pulmonary fibrosis *via* inhibition of angiogenesis. *J. Immunol.* **163**, 5686-5692.
34. Rose, M. L., Madren, J., Bunzendahl, H., and Thurman, R. G. (1999). Dietary glycine inhibits the growth of B16 melanoma tumours in mice. *Carcinogenesis* **20**, 793-798.
35. Majewsky, S., Szmurlo, A., Marczak, M., Jablonska, S., and Bollag, W. (1993). Inhibition of tumour cell-induced angiogenesis by retinoids, 1, 25-dihydroxyvitamin D3 and their combination. *Cancer Lett.* **75**, 35-39.
36. Sola, F., Biasoli, G., Pesenti, E., Farao, M., Della-Torre, P., Mongelli, N., and Grandi, M. (1992). *In vivo* activity of novel sulphonic derivatives of distamycin A. *Exs.* **61**, 459-462.

(Received October 24, 2001 Accepted December 25, 2001)