Jpn. J. Cancer Res. **89**, 1113–1117, November 1998

Modulation of Dihydroxy-di-*n*-propylnitrosamine-induced Liver Lesion Development in *Opisthorchis*-infected Syrian Hamsters by Praziquantel Treatment in Association with Butylated Hydroxyanisole or Dehydroepiandrosterone Administration

Malcolm A. Moore,[1, 5] Witaya Thamavit,[2] Danai Tiwawech,[3] Nobuyuki Ito[4] and Hiroyuki Tsuda[1]

[1]Experimental Pathology and Chemotherapy Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, [2]Department of Pathobiology, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand, [3]Research Division, National Cancer Institute, Rama VI Road, Bangkok 10400, Thailand and [4]President, Nagoya City University, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-0001

The effects of praziquantel coupled with dehydroepiandrosterone (DHEA) or butylated hydroxyanisole (BHA) administration 16 weeks subsequent to dihydroxy-di-*n*-propylnitrosamine (DHPN) treatment and infection with *Opisthorchis viverrini* (OV) on lesion development in the liver of Syrian hamsters were investigated. Animals were given 80 OV metacercariae and then two i.p. injections of DHPN (500 mg/kg body weight) 4 and 5 weeks thereafter. At week 16, groups received praziquantel (250 mg/kg, i.g.) and were placed on normal diet or diet supplemented with BHA (1%) or DHEA (0.6%) until they were killed at week 24. Histopathological assessment revealed that, whereas antihelminthic treatment alone resulted in a clear reduction in hepatocellular lesion development, effects on cholangiocellular lesions were equivocal. BHA and DHEA, in contrast, were both associated with a significant reduction in frequency of cholangiofibrosis and cholangiocellular carcinoma. The former chemical, however, increased the numbers of liver nodules while the hormone brought about a decrease as well as a shift in the phenotype of the lesions. The results thus indicate that although cholangiocellular lesion development may, unlike generation of hepatocellular nodules, be to a certain extent independent of the continued presence of parasite, it can be influenced by exogenous treatments.

Key words: *Opisthorchis viverrini* — Liver cancer — Prevention — Butylated hydroxyanisole — Dehydroepiandrosterone

The great variation in incidence of liver cancer in different parts of the world is thought to be directly related to the presence or absence of predisposing factors, including exposure to viruses, carcinogens, dietary and cultural influences, and in some cases parasites. An especially strong correlation between cholangiocellular type carcinoma development and liver fluke infection in man has been demonstrated in Thailand, Korea and Hong Kong, the parasites causing a variety of proliferative and inflammatory changes in the affected tissues.[1–3)]

In Thailand, the liver fluke parasite *Opisthorchis viverrini* (OV) is endemic in the Northeast area, man becoming infected by ingestion of intermediate host fresh water fish bearing metacercariae.[4)] The human environment in this region is also contaminated with aflatoxins and nitrosamines[5, 6)] which are known to be able to induce hepatocellular or cholangiocellular tumor development in a number of rodent species. The extensive use of a fish sauce containing nitrites and other potential carcinogen precursors further suggests that significant exposure to chemical carcinogens may occur via endogenous formation in the gut.[7)] In the Syrian hamster, infection with liver flukes causes proliferative and inflammatory changes analogous to those observed in man[8, 9)] and results in liver tumors when associated with nitrosamine exposure.[10, 11)]

One approach to elimination of this problem has been removal of the parasite stimulus by a combination of education and chemotherapy, principally involving use of the antihelminthic drug, praziquantel (2-cyclohexylcarbonyl-1,2,3,6,7,11b-hexahydro-4H-pyrazino [2,1-a]isoquinolin-4-one EMBAY 8440, Biltricide@).[12)] However, one experimental study has shown that despite very effective killing of parasites, the influence on cholangiocellular tumor development may be limited.[13)] Therefore the present study was undertaken to assess whether administration of the adrenal hormone dehydroepiandrosterone (DHEA) or the synthetic phenolic antioxidant butylated hydroxyanisole (BHA) might exert modulatory effects. The reason

[5] To whom correspondence should be addressed.

for the choice of these compounds is that both can act as chemopreventive agents, but by different mechanisms,[14, 15] while also exerting carcinogenicity under certain conditions.[15, 16]

MATERIALS AND METHODS

A total of 140 male Syrian golden hamsters (Armed Forces Research Institute of Medical Science, Bangkok, Thailand) aged 6–8 weeks at the commencement, were kept 5 to a plastic cage in a temperature-controlled room at 24°C. They were fed on stock diet [Zuellig (Gold Coin Mills PTE, Ltd., Singapore)] and tap water *ad libitum* throughout the experimental period. Cyprinoid fish harbouring metacercarial cysts were purchased from market places in towns of Northeast Thailand and the muscles from the base of the pectoral fins and tail, as well as the fins and tails themselves, were removed and digested in pepsin solution overnight at 37°C. The whole digested content was then filtered, washed and resedimented several times. Metacercarial cysts of OV were identified under a dissecting microscope and collected.

The carcinogen, dihydroxy-di-*n*-propylnitrosamine (DHPN), was purchased from Nacalai Chemicals Ltd. (Kyoto), the hormone DHEA from Sigma Chemical Co. (St. Louis, MO) and the antioxidant BHA from Wako Pure Chemicals Industries Ltd. (Osaka).

The animals were divided into 2 main groups, each subdivided into four. All animals initially received 80 OV metacercariae by intragastric intubation, those in groups 1–4 (25 animals each) also being administered 800 mg/kg body weight injections of DHPN, twice, at weeks 4 and 5 (see Fig. 1). Hamsters of groups 5–8 received the saline vehicle alone. At week 16, animals of groups 2–4 and 6–8 were administered a suspension of praziquantel in salad oil, 250 mg/kg body weight via an intragastric tube. Starting at week 16, groups 3 and 7 received dietary supplementation with DHEA (0.6%) and groups 4 and 8 were administered BHA (1%). The animals in the other groups were maintained on basal diet. All surviving animals were killed by exsanguination under ether anesthesia at the end of week 24.

The livers were immediately removed and fixed in 10% buffered formalin for routine embedding in paraffin. Sections cut at 4 μm were stained with hematoxylin and eosin, and proliferative lesions, including areas of cholangiofibrosis and hepatic nodules, were diagnosed as described earlier,[10, 11] and counted under a microscope. Areas of liver sections analysed were also measured to allow calculation of numbers per unit area. Incidence data were compared statistically using the χ^2 test and frequency data with the Student's *t* test.

RESULTS

Survival in all groups was good, animals killed at week 24 providing the effective numbers. No significant intergroup differences in body weights were observed. Relative liver weights were heavier in the animals treated with OV and the carcinogen than in those receiving OV alone,

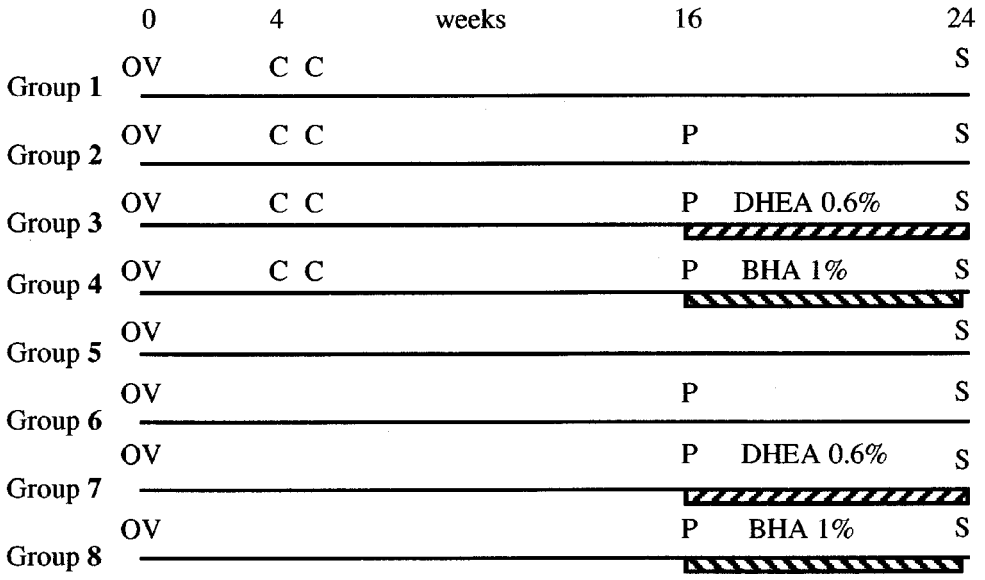

Fig. 1. Experimental protocol: DHEA and BHA effects on reversibility. C, dihydroxy-di-*n*-propylnitrosamine; OV, *Opisthorchis viverrini* metacercariae; P, praziquantel; S, animals killed.

Table I. Quantitative Data for Preneoplastic and Neoplastic Lesions

Group	No. animals	Cholangiocellular			Hepatocellular				
		Cholangiofibrosis		Cholangiocarcinoma	Foci		Nodules		
		Incidence (%)	No./cm^2	Incidence (%)	Incidence (%)	No./cm^2	Incidence (%)	No./cm^2	MC/B
1. OV control	24	21 (88)	5.1±1.8	4 (17)	23 (96)	5.8±1.3	9 (38)	11.3±3.2	2.0/9.3
2. OV+P	24	22 (92)	3.3±1.2^{+}	4 (17)	21 (88)	4.6±1.5	3 (13)	5.8±2.8^{++}	0.7/4.4
3. OV+P+D	23	14 (61)	1.3±0.8*	1 (4)	14 (61)	2.3±0.9*	5 (22)	3.5±1.9*	0.0/3.5
4. OV+P+B	23	10 (42)	0.5±0.5***	1 (4)	22 (92)	8.1±2.7**	9 (38)	10.2±2.5***	2.4/7.8

+,++ Significantly different from the respective OV control value at $P<0.05$, $P<0.01$, respectively.
*,**,*** Significantly different from the respective OV+P value at $P<0.05$, $P<0.01$, $P<0.001$, respectively.
MC/B: mixed cell/basophilic ratio.

but treatment with praziquantel, DHEA or BHA was without significant influence. In all cases, antihelminthic treatment was effective (less than 2 flukes per liver as opposed to 15 and above in the non-treated cases).

Preneoplastic and neoplastic lesion development was limited to groups 1–4 receiving the DHPN treatment (see Table I for incidence and frequency data). Hepatocellular foci and nodules demonstrating altered morphology were distinguished on the basis of presence or absence of compression. Mixed cell (MC) (mixtures of cells with clear, acidophilic and basophilic morphology) and homogeneously basophilic (B) types were encountered and quantified separately. Cholangiocellular lesions were divided into benign cholangiofibrosis and malignant carcinoma categories. Significant decrease in hepatocellular nodules and cholangiofibrosis, but not cholangiocellular carcinomas, was associated with praziquantel administration. Preneoplastic lesions of both cell types were further reduced in the hamsters receiving hormonal supplement, the proportional reduction in nodule development being greater for mixed cell lesions. BHA caused a decrease in cholangiocellular, but not hepatocellular lesion development. When compared with the praziquantel alone group, significant increase in both MC and B foci/nodules was apparent.

DISCUSSION

The present study demonstrated that removal of parasites by treatment with the antihelminthic drug praziquantel is not necessarily associated with a reduction in DHPN-initiated tumor development in the intrahepatic bile duct system, in line with earlier findings.[13] However, additional administration of either BHA or DHEA did bring about marked reduction in cholangiocellular lesions. An explanation for the lack of reversal with the anti-parasite treatment alone must remain speculative, but presumably this result reflects a certain autonomy in growth potential of early lesions in the bile ducts. They remain, however, susceptible to exogenous influence. The findings for hepatocellular foci/nodules suggest that continued proliferation is necessary for their appearance, while demonstrating clear modulatory differences between DHEA and BHA.

Our earlier study revealed the 4-week time point after OV infection to be a threshold for significant inhibitory effects of praziquantel on cholangiocellular lesion development in the Syrian hamster model.[13] The antihelminthic agent was administered 12 weeks after DHPN in the present case and again the findings imply that a relatively short exposure to the inflammatory and proliferative influence of liver fluke parasites is sufficient to effectively enhance carcinogenesis in the bile duct tree. While carcinogenicity testing has shown that praziquantel administration is safe[17] and as a preventive measure, its use is clearly advisable to control parasite exposure in affected human beings, additional intervention would appear warranted if a suitable chemopreventive agent is available.

The present results for BHA suggest a positive influence on cholangiocellular lesions but are surprising in terms of the apparent promotion of their hepatocellular counterparts, given the fact that clear inhibitory effects in the rat and hamster have earlier been shown for this compound.[15, 18] One possible explanation for the anomalous findings is that the effects of the antioxidant are altered under the conditions of cirrhosis prevalent with OV infection. Although this question remains to be elucidated, the possibility of an analogy with the effects of β-carotene on lung cancer development in smokers[19] deserves consideration in this respect.

While the underlying mechanisms remain unclear, the adrenal hormone DHEA demonstrated significant inhibition of both hepatocellular and cholangiocellular lesions, in line with that earlier reported for the rat liver.[20] Similar second-stage inhibition by DHEA has previously been described for the skin, colon, lung and thyroid.[14, 21] Since DHEA is known to be an uncompetitive inhibitor of glucose-6-phosphate dehydrogenase,[22] it is conceivable that a

reduction in ribose unit production via the pentose phosphate pathway may be directly involved in inhibition of neoplastic development. This was earlier indicated by the reduced generation of nodules in the Solt-Farber short-term induction brought about by DHEA treatment[23] and its reversal by provision of ribo- and deoxyribonucleosides. However, the hormone could also cause a decrease in the level of NADPH generated by the same pentose phosphate system, and this could be involved. It is, moreover, far from clear whether the short-term inhibition of the key pentose phosphate enzyme actually persists with long-term administration, and in the rat liver many other enzyme species are also affected.[24] The fact that DHEA shares some characteristics with the peroxisome proliferator group of compounds is also of interest,[25] particularly in the light of the shift in phenotype of hepatic nodules observed in the present experiment. Thus, the increase in the proportion of B type lesions is directly comparable to the shift towards amphophilia reported earlier in the rat.[20] Whether this morphological change was associated with alteration in enzyme phenotype, as described for both dimethylaminobenzene-[26] and nitrosomorpholine-induced lesions,[27] requires further investigation. Similarly, clarification of any influence of DHEA on proliferation kinetics in the *Opisthorchis*-treated livers is necessary. The hormone is known to suppress cell turnover in the mouse epidermis and breast epithelium.[29]

In conclusion, while praziquantel is very effective for removal of OV parasites in the Syrian hamster liver, the concomitant influence of this antihelminthic agent on tumor development may be only limited. This has an obvious bearing on the human situation, where continued stress must be placed on surveillance of high-risk populations for early cholangiocellular tumors as the most effective approach to control. However, further investigation of the interaction between other exogenous factors and praziquantel in bringing about a reversal of fibrotic changes similar to that earlier reported for mice infected with schistosomes[29, 30] appears warranted.

ACKNOWLEDGMENTS

This study was supported by NIH grant 5 RO1 CA50216-02 and in part by the Society for Promotion of Pathology in Nagoya, by a Grant-in-Aid for the 2nd Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health and Welfare, a Grant-in-Aid from the Minstry of Health and Welfare and a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture, Japan. During the drafting of this paper Malcolm Moore was the recipient of a Foreign Research Fellowship from the Foundation for Promotion of Cancer Research Program for Invitation of Foreign Researchers.

(Received July 10, 1998/Revised August 7, 1998/Accepted August 12, 1998)

REFERENCES

1) Hou, P. C. The relationship between primary liver cancer and infestation with *Clonorchis sinensis*. *J. Pathol. Bact.*, **72**, 239–246 (1956).
2) Kim, Y., II, Yang, D. H. and Chang, K. R. *Clonorchis sinensis* infestation and cholangiocarcinoma of the liver in Korea: epidemiological and pathological reappraisal of 495 consecutive primary carcinomas of the liver in Seoul and Pusan areas. *Seoul J. Med.*, **15**, 247–253 (1974).
3) Kurathong, S., Lerdvirasirikul, P., Wougpaitoon, V., Pramoolsinsap, C., Kanjanapitak, A., Varawithaya, W., Phupradit, P., Bunyaratvej, S., Upatham, S. and Brockelman, W. Y. *Opisthorchis viverrini* infection and cholangiocarcinoma: a prospective, case-control study. *Gastroenterology*, **89**, 151–156 (1985).
4) Harinasuta, C. and Harinasuta, T. *Opisthorchis viverrini*: life cycle, intermediate hosts, transmission to man and geographical distribution in Thailand. *Arzneim-Forsch/Drug Res.*, **34**, 1164–1167 (1984).
5) Migasena, P. and Changbumrung, S. The role of nitrosamines in the causation of primary carcinoma. *J. Med. Assoc. Thailand*, **57**, 175–178 (1974).
6) Shank, R. C., Gordon, J. E., Wogan, G. N., Nondasuta, A. and Subhmani, B. Dietary aflatoxins and human liver cancer. III. Field survey of rural Thai families for ingested aflatoxins. *Food Cosmet. Toxicol.*, **10**, 71–76 (1972).
7) Migasena, P., Reaunsuwan, W. and Changbumrung, S. Nitrates and nitrites in local Thai preserved protein foods. *J. Med. Assoc. Thailand*, **63**, 500–505 (1980).
8) Bhamarapravati, N., Thamavit, W. and Vajrasthira, S. Liver changes in hamsters infected with a liver fluke of man, *Opisthorchis viverrini*. *Am. J. Trop. Med. Hyg.*, **27**, 787–794 (1978).
9) Evans, H., Bourgeois, C. H., Comer, D. S. and Keshamras, N. Biliary tract changes in opisthorchiasis. *Am. J. Trop. Med. Hyg.*, **20**, 667–671 (1971).
10) Thamavit, W., Bhamarapravati, N., Sahaphong, S., Vajrasthira, S. and Angsubhakorn, S. Effects of dimethylnitrosamine on induction of cholangiocarcinoma in *Opisthorchis viverrini*-infected Syrian golden hamsters. *Cancer Res.*, **38**, 4634–4639 (1978).
11) Thamavit, W., Kongkanuntn, R., Tiwawech, D. and Moore, M. A. Level of *Opisthorchis* infestation and carcinogen dose dependence of cholangiocarcinoma induction in Syrian golden hamsters. *Virchow Arch. B Cell Pathol.*, **54**, 52–58 (1987).
12) Bunnag, D. and Harinasuta, T. Studies on the chemotherapy of human opisthorchiasis: I. Clinical trial of praziquantel. *Southeast Asian J. Trop. Med. Public Health*, **11**, 528–531 (1980).
13) Thamavit, W., Moore, M. A., Sirisinha, S., Shirai, T. and

Ito, N. Time-dependent modulation of liver lesion development in Opisthorchis-infected Syrian hamsters by an antihelminthic drug, praziquantel. *Jpn. J. Cancer Res.*, **84**, 135–138 (1993).
14) Gordon, G. B., Shantz, L. M. and Talalay, P. Modulation of growth, differentiation, and carcinogenesis by dehydroepiandrosterone. *Adv. Enzyme Reg.*, **26**, 355–382 (1987).
15) Ito, N., Hirose, M., Fukushima, S., Tsuda, H., Shirai, T. and Tatematsu, M. Studies on antioxidants; their carcinogenic and modifying effects on chemical carcinogenesis. *Food Chem. Toxicol.*, **24**, 1071–1082 (1986).
16) Rao, M. S., Subbarao, V., Kumar, S., Yeldandi, A. V. and Reddy, J. K. Phenotypic properties of liver tumors induced by dehydroepiandrosterone in F-344 rats. *Jpn. J. Cancer Res.*, **83**, 1179–1183 (1992).
17) Thamavit, W., Moore, M. A., Ruchirawat, S. and Ito, N. Repeated exposure to *Opisthorchis viverrini* and treatment with the antihelminthic praziquantel lacks carcinogenic potential. *Carcinogenesis*, **13**, 309–311 (1992).
18) Moore, M. A., Tsuda, H., Thamavit, W., Masui, T. and Ito, N. Differential modification of development of preneoplastic lesions in the Syrian golden hamster initiated with a single dose of 2,2′-dioxo-N-nitrosodipropylamine: influence of subsequent butylated hydroxyanisole, alpha-tocopherol, or carbazole. *J. Natl. Cancer Inst.*, **78**, 289–293 (1987).
19) The Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study Group. The effects of vitamin E and beta-carotene on the incidence of lung cancer and other cancers in male smokers. *N. Engl. J. Med.*, **330**, 1029–1035 (1994).
20) Weber, E., Moore, M. A. and Bannasch, P. Phenotypic modulation of hepatocarcinogenesis and reduction in N-nitrosomorpholine-induced hemangiosarcoma and adrenal lesion development in Sprague-Dawley rats by dehydroepiandrosterone. *Carcinogenesis*, **9**, 1191–1196 (1988).
21) Moore, M. A., Thamavit, W., Tsuda, H., Sato, K., Ichihara, A. and Ito, N. Modifying influence of dehydroepiandrosterone on the development of dihydroxy-di-n-propylnitrosamine initiated lesions in the thyroid and liver of F344 rats. *Carcinogenesis*, **7**, 311–316 (1986).
22) Schwartz, A. G., Whitcomb, J. M., Nyce, J. W., Lewbart, M. L and Pashko, L. L. Dehydroepiandrosterone and structural analogs: a new class of chemopreventive agents. *Adv. Cancer Res.*, **51**, 391–424 (1988).
23) Garcea, R., Daino, L., Pascale, R., Frassetto, S., Cozzolino, O. P., Ruggiu, M. E., Vannini, M. G., Pascale, R., Lenzerini, L., Simile, M. M., Puddu, M. and Feo, F. Reversal by ribo- and deoxyribonucleosides of dehydroepiandrosterone-induced inhibition of enzyme altered foci in the liver of rats subjected to the initiation-selection process of experimental carcinogenesis. *Carcinogenesis*, **9**, 931–938 (1988).
24) Mayer, D., Weber, E., Moore, M. A., Letsch, I., Filsinger, E. and Bannasch, P. Dehydroepiandrosterone induced enzyme alterations in rat liver carbohydrate metabolism. *Carcinogenesis*, **9**, 2039–2043 (1988).
25) Sakuma, M., Yamada, J. and Suga, T. Comparison of the inducing effect of dehydroepiandrosterone on hepatic peroxisome proliferation-associated enzymes in several rodent species. *Biochem. Pharmacol.*, **43**, 1269–1273 (1992).
26) Moore, M. A., Weber, E. and Bannasch, P. Modulating influence of dehydroepiandrosterone administration on the morphology and enzyme phenotype of dimethylaminoazobenzene-induced hepatocellular foci and nodules. *Virchow Arch. B Cell Pathol.*, **55**, 337–343 (1988).
27) Weber, E., Moore, M. A. and Bannasch, P. Enzyme histochemical and morphological phenotype of amphophilic foci and amphophilic/tigroid cell neoplastic nodules in rat liver after combined treatment with dehydroepiandrosterone and N-nitrosomorpholine. *Carcinogenesis*, **9**, 1049–1054 (1988).
28) Pashko, L. L., Schwartz, A. G., Abou-Garbia, M. and Swern, D. Inhibition of DNA synthesis in mouse epidermis and breast epithelium by dehydroepiandrosterone and related steroids. *Carcinogenesis*, **2**, 717–721 (1981).
29) Andrade, Z. A. and Grimaud, J. A. Evolution of schistosomal hepatic lesions in mice after curative chemotherapy. *Am. J. Pathol.*, **124**, 59–65 (1986).
30) El-Badrawy, N. M., Botros, S. S., Nagy, F. M., Abdallah, N. M. and Herbage, D. Effect of praziquantel on hepatic fibrosis in experimental *Schistosomiasis mansoni*. *Exp. Mol. Pathol.*, **49**, 151–160 (1988).