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Time-dependent Modulation of Liver Lesion Development in Opisthorchis-infected Syrian Hamster by an Antihelminthic Drug, Praziquantel

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In the North-east of Thailand, repeated antihelminthic therapy has been introduced for control of the opisthorchiasis known to be a major risk factor for cholangiocellular carcinomas. What influence this may have on tumorigenesis, however, remains unclear. The effects of administration of praziquantel, an antihelminthic drug, at different time points subsequent to infection with *Opisthorchis viverrini* (OV) on 2,2′-dihydroxy-di-*n*-propylnitrosamine (DHPN)-initiated lesion development in the liver of female Syrian hamsters were therefore investigated. Praziquantel (250 mg/kg body weight, i.p.) was given 4, 12 or 20 weeks after infection of DHPN-treated animals (two 1000 mg/kg i.p. injections at weeks 0 and 2) with 60 OV metacercariae (at week 4). Survivors at week 38 were killed and examined. It was found that whereas praziquantel administration at the earlier two time points was effective at reducing hepatocellular nodule development, the results for cholangiocellular lesions were less pronounced, significant reduction only being evident in hamsters treated 4 weeks after parasite infestation. The findings thus indicate that enhancement of DHPN-initiated bile duct carcinogenesis by opisthorchiasis is both rapid and to a large degree irreversible. Hepatocellular lesion development in this model, on the other hand, appears to correlate more closely with the duration of parasite-associated proliferative stimulus.

Key words: Opisthorchis — Praziquantel — DHPN — Carcinogenesis — Hamster

A wealth of both epidemiological[1–5] and experimental[6–9] evidence points to a clear association between *Opisthorchis viverrini* (OV) fluke infestation and the high level of cholangiocarcinoma in the Northeast region of Thailand. One approach to control the problem has therefore centered on removal of the parasite influence through a concerted program of screening and antihelminthic drug treatment. *In vivo* eradication of live flukes can be effectively achieved by oral administration of the compound praziquantel (2-cyclohexylcarbonyl-1,2,3,6,7,11b-hexahydro-4*H*-pyrazino[2,1-*a*]isoquinoline-4-one, EMBAY 8440, Biltricide®)[10,11] with relief of the chronic symptoms associated with heavy worm load.[12] However, despite the prevailing attitude among the indigenous populations at risk that reinfection carries no danger because of the very availability of antihelminthic agents, what effects praziquantel treatment will have on tumor development remain unclear.

The present experiment was therefore undertaken to assess, in an experimental model based on the susceptibility of Syrian hamsters to both flukes and nitrosamine liver carcinogenicity,[6–9] the effects of antihelminthic treatment at different stages in the process of liver tumor generation. Dosage of praziquantel in terms of relative body weight and route of administration was comparable in both human and experimental cases.

MATERIALS AND METHODS

Cyprinoid fish harboring metacercarial cysts were purchased from market places in towns of North-eastern Thailand. 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 Tesque Inc. (Kyoto). Praziquantel was the generous gift of Bayer, Germany.

A total of 205 female Syrian golden hamsters (Armed Forces Research Institute of Medical Science, Bangkok, Thailand) aged 6 to 8 weeks at the commencement were kept 5 to a 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. The animals were divided into 7 groups: groups 1 to 5 (25–40 animals each) received two fortnightly injections of DHPN dis-

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solved in saline (1000 mg/kg body weight, i.p.) and groups 6 and 7 the vehicle alone. Two weeks later groups 1–4 and 6 were given 60 OV metacercariae by intragastric tube, the animals of groups 5 and 7 serving, respectively, as carcinogen-alone and untreated controls. Groups 1–3 received in addition a single 250 mg/kg dose of praziquantel suspended in saline by intragastric tube at 4, 12 and 20 weeks, respectively, subsequent to infection with parasite metacercariae. The animals were maintained on basal diet, then killed by exsanguination under ether anesthesia at the end of week 38.

After complete autopsy, the livers and other organs were removed and fixed in 10% buffered formalin or ice-cold acetone and embedded in paraffin. Sections cut at 4 μm were stained with hematoxylin and eosin as well as Alcian blue/PAS for mucins. Reactive lesions, hepatic nodules, areas of cholangiofibrosis and cholangiocellular carcinomas were diagnosed histopathologically as described earlier.[6,7] Incidence data were compared statistically by using the chi-square test and frequency data by using Students' t test.

RESULTS

During the experiment a number of hamsters infected with parasites rapidly became moribund and died. Due to cannibalism, autopsies could not be performed and these animals were not included in the effective numbers. Data for numbers of animals killed at the termination point as well as body and liver weights, and reactive proliferative lesions are summarized in Table I. All groups infected with parasites, irrespective of praziquantel treatment, demonstrated significant reduction in body weight. Liver weights, in contrast, were significantly increased in all groups receiving both OV and DHPN, and in the DHPN-alone group.

Treatment with praziquantel proved very effective for removal of parasites, as shown by the data for incidences and numbers of OV (see Table I). In the case of anthelminthic application 4 weeks, and to a lesser extent 12 weeks, after dosing with parasites this was associated with a reduction in the degree of development of biliary

Table I. Quantitative Data for Body and Liver Weights and Incidence Data for Histopathological Findings for Reactive Lesions

Group	Effective No. of animals	Mean body weight	Mean liver weight	Billary cirrhosis	Bile duct hyperplasia	Cystic lesions	Cholangitis	Main duct hyperplasia	Parasites No./hamster (%incidence)
1	22	112±20	15.7±6.6[a]	+	++	++	+	+	0[b] (0)
2	22	105±21	10.3±4.0	++	++	++	+++	+++	0.1[b] (5)
3	16	110±18	10.0±3.3	+++	+++	++	+++	+++	0.1[b] (6)
4	16	107±29	11.9±3.5	+++	+++	++	+++	+++	13.8 (100)
5	15	136±21	17.6±6.0	−	−	+++	−	−	0 (0)
6	18	119±31	7.4±1.5	+++	+++	−	+	+++	14.9 (100)
7	15	148±28	8.2±1.5	−	−	−	−	−	0 (0)

a), b) Significantly different from group 4 value at $P<0.05$, $P<0.001$.
+, Slight; ++, moderate; +++, marked; −, absent.

Table II. Incidence Data (%) for Histopathological Findings for Preneoplastic and Neoplastic Lesions

Group	Treatment[a]	Effective No. of animals	Cholangiocellular		Hepatocellular		
			Cholangiofibrosis incidence (%)	Carcinoma incidence (%)	Nodules incidence (%)	Nodules No./cm²	Carcinoma incidence (%)
1	DHPN+OV+PZ(4)	22	8 (36)[c]	4 (18)[b]	20 (91)	3.6±2.1[c]	1 (5)
2	DHPN+OV+PZ(12)	22	22 (100)	6 (28)	22 (100)	7.4±3.2[b]	1 (5)
3	DHPN+OV+PZ(20)	16	16 (100)	10 (63)	16 (100)	9.8±4.5	0 (0)
4	DHPN+OV	16	16 (100)	8 (50)	16 (100)	13.6±5.0	0 (0)
5	DHPN	15	5 (33)	0 (0)	14 (93)	3.8±2.3	0 (0)
6	OV	18	10 (56)	2 (11)	4 (22)	0.5±0.8	0 (0)
7	None-treated	15	0 (0)	0 (0)	0 (0)	0	0 (0)

a) PZ: praziquantel (weeks).
b), c) Significantly different from group 4 value at $P<0.05$, $P<0.001$.

cirrhosis, characterized by bile duct proliferation accompanied by fibrotic changes and pseudolobule formation. Acute and chronic inflammatory changes involving the ducts (cholangitis), as well as epithelial hyperplasia of the main ducts in which the parasites were observed to reside, were less prevalent in group 1.

Quantitative data for preneoplastic and neoplastic lesions developing from both cholangiocellular and hepatocellular compartments of the liver are summarized in Table II. In the former, development of benign cysts lined by extremely flattened epithelium, associated with DHPN initiation, did not appear to be affected by parasite infection. In contrast, the incidence and degree of cholangiofibrosis development was clearly increased in the OV-treated animals. Praziquantel application was only linked to a reduction in this lesion type when given 4 weeks after metacercariae. Similar but not statistically significant tendencies were observed for mucinous cystadenomas and cholangiocellular carcinomas (see Table II).

With regard to hepatocellular lesions, large numbers of foci of clear, mixed or basophilic morphology were observed in all groups receiving DHPN, together with almost 100% incidences of hepatic nodules demonstrating compression of the surrounding parenchyma. The numbers of these latter were significantly reduced in group 1 as compared to group 4.

DISCUSSION

The present experiment clearly showed that whereas parasite stimulus can be effectively removed by treatment with the antihelminthic drug, praziquantel, inhibitory effects on OV enhancement of neoplastic development initiated by administration of DHPN are limited. Only when the drug was given at the early 8-week time point was a significantly lower incidence of cholangiofibrosis associated with praziquantel administration. A tendency for concomitant reduction of cystadenoma and cholangiocellular carcinoma development was evident, although this was not statistically significant. Indeed, the data suggest that just 8 weeks of exposure to parasite influence is sufficient to promote irreversibly the process of intrahepatic bile duct neoplasia formation.

The present experiment was designed so that the influence of parasite removal at different stages within the sequence of cholangiocellular lesion development in the Syrian hamster could be assessed. Bile duct hyperplasia and biliary cirrhosis as well as cholangitis and main duct hyperplasia are relatively early changes appearing within the first two months of *Opisthorchis* infestation.[13] The present results demonstrating differences in the degree of these reactive lesions only when antihelminthic treatment was performed at the earliest time point are therefore in line with expectation, as well as pointing to a basically irreversible nature for such proliferative changes in the hamster liver. However, this question deserves further investigation in view of the potential reversibility of hepatic fibrotic changes in the mouse associated with praziquantel removal of schistosomal parasites.[14, 15] It should also be remembered that early treatment of OV-infected hamsters was previously reported to bring about a reduction in the procollagen prolyl hydroxylase activity.[16] Since cholangiofibrosis generally develops approximately two months after exposure to both nitrosamine carcinogen and parasite, and cholangiocarcinomas one month later, the present findings would further suggest that the praziquantel administration did not reduce the ability of the lesions present to develop further along the line to malignancy. The histogenesis of cholangiocarcinomas is now generally accepted to involve a cholangiofibrotic stage in both the rat and the hamster.[17-19]

In conclusion, while a battery of toxicity/carcinogenicity tests, both *in vivo* and *in vitro*,[20-23] have indicated praziquantel use to be safe, the present results would suggest that removal of parasite load in itself may not be sufficient to block cholangiocellular carcinoma development. On the other hand, development of the hepatic nodules associated with opisthorchiasis, like those arising in the livers of mice and men heavily infected with *Schistosomiasis japonica*,[24, 25] appears to be more dependent on continued proliferative stimulus.

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