N-Nitrosodiethylamine Initiation of Carcinogenesis in Japanese Medaka (Oryzias latipes): Hepatocellular Proliferation, Toxicity, and Neoplastic Lesions Resulting from Short Term, Low Level Exposure

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To investigate relationships among carcinogen exposure, cell proliferation, and carcinogenesis, 14-day post-hatch Japanese medaka (Oryzias latipes) were exposed to 0, 10, 25, 50, or 100 ppm N-nitrosodiethylamine (DEN) for 48 h under static renewal conditions. They were then held in clean water until sampling at 3 and 6 months. The frequencies of hepatic lesions and neoplasms were determined from hematoxylin/eosin-stained paraffin sections. A significant (p < 0.0001) concentration-related increase in hepatic vacuolated foci occurred in 3- and 6-month samples, with males having a significantly (p = 0.02) higher incidence than females. Concentration-related increases in degenerative lesions were documented for spongiosis hepatis at 3 months (p = 0.053) and hepatic vacuoles at 6 months (p = 0.005). There was a significant (p = 0.0001) concentration-related increase in macrophage aggregates at 6 months. Basophilic foci were significantly related (p < 0.0001) to DEN concentration at 3 months post-exposure and were unaffected by gender or age. At both 3 and 6 months, there were significant concentration-related increases in hepatocellular carcinoma (p ≤ 0.02). Hepatocyte proliferation in 3-month whole specimens was quantified using an immunohistochemical assay for proliferating-cell nuclear antigen. Trend tests and a probit dose-response model showed a significantly positive correlation (p = 0.015) between proliferating hepatocytes and DEN concentrations. These results confirm that short-term exposure to low and moderate levels of DEN initiates concentration-dependent carcinogenic effects in medaka that are apparent at 3 months post-exposure. DEN could be an effective initiator in an initiation/promotion assay for medaka using a 48-h exposure period, DEN concentrations ≤ 10 ppm, and a 6-month sampling period.

Key Words: N-nitrosodiethylamine (DEN); Japanese medaka; hepatic lesion; hepatic neoplasms; hepatocellular proliferation; initiation/promotion.

The use of fish as models for carcinogenesis research has received considerable attention over the past decade (Bailey et al., 1996; Bunton, 1996; Hawkins et al., 1988, 1995; Naruse et al., 1994; Powers, 1989). Several species of fish, such as rainbow trout (Oncorhynchus mykiss) and Japanese medaka (Oryzias latipes), have been widely used in laboratory studies to further understand the mechanisms of carcinogenesis. The use of fish models in cancer research has the obvious advantages of economy, rapid response, and the opportunity to work with large numbers of specimens when compared with the more traditional rodent models. The Japanese medaka has been a favored model, because it develops a variety of neoplastic lesions following exposure to various chemicals (Bunton, 1996; Fabacher et al., 1991; Hatanaka et al., 1982; Hawkins et al., 1995). The sensitivity of medaka to many carcinogens, the large numbers that can be studied easily, their short life-span, and the ability to control many extraneous factors make this species a good model for studying the biology of hepatic neoplasia (Hinton et al., 1988).

Fish models can also serve in examining the roles of initiation and promotion in multi-stage carcinogenesis. Although most initiation/promotion models have utilized rodents (Carthew et al., 1997; Gelderbloom et al., 1996; Hasegawa et al., 1998; Hennings et al., 1993; Imaida and Fukushima, 1996), studies with rainbow trout have shown it to be a potential model for initiation, promotion, and factors that modify carcinogenic responses (Bailey et al., 1987, 1996; Dashwood et al., 1991; Nunez et al., 1988, 1989, 1992; Orner et al., 1996, 1995). The objective of the present study was to identify an initiation protocol for medaka that produces hepatic neoplastic lesions in the experimental model at no greater frequency than the background, or spontaneous rate, which is about 0.1 to 0.3% in medaka from our laboratory (Hawkins et al., 1995). N-nitrosodiethylamine (DEN) was chosen as the initiating agent because it has been the most widely used compound to investigate mechanisms of carcinogenesis in medaka (Bunton, 1990, 1996; Ishikawa and Takayama, 1979; Kyono-Hamaguchi, 1984; Lauren et al., 1990; Ortego et al., 1996), and it has the further advantage of producing predominantly hepatic lesions (Boorman et al., 1997; Bunton, 1990). Most previous DEN studies with medaka have been prolonged exposures lasting several weeks, which employed relatively high concentrations of DEN and had sampling times up to 1 year post-exposure.
exposure. Therefore, a secondary objective of our study was to investigate a rapid exposure/grow-out system so results would be available within 6 months of exposure. In the studies described here, the efficacy of a brief exposure to low to moderate concentrations of DEN, followed by an abbreviated grow-out time for production of hepatic lesions and neoplasms, was examined in medaka.

MATERIALS AND METHODS

Exposure and grow-out methodology. Medaka were obtained originally from brood stock purchased from Carolina Biological Supply, and which have been kept in continuous culture for 15 years at the Gulf Coast Research Laboratory, Ocean Springs, MS. Seven hundred fifty 14-day, post-hatch medaka were randomly divided into 10 groups of 75 fish each and replicate groups were exposed to 0, 10, 25, 50, and 100 ppm N-nitrosodiethylamine (DEN; CAS 55–18–5, lot number M0040191, Midwest Research Institute, Kansas City, MO). Exposures were static and were conducted in 1-L beakers for 48 h with renewal of DEN concentrations at 24 h. Beakers were placed in a water bath that was maintained at 26 ± 1°C. Fish were not fed during the exposure period.

At the end of the exposure period, fish were rinsed in clean water 3 times, removed from the treatment beakers, and transferred to a water bath in 39-L glass aquaria maintained at 26 ± 1°C. The photo period was maintained at 16-h light:8-h dark, with simulated dusk and dawn. Fish were fed brine shrimp (Artemia spp.) nauplii (Aquarium Products, Glen Burnie, MD) once daily and flake food (Prime Tropical Flakes–Yellow, Ziegler Brothers, Inc., Gardners, PA) 3 times daily during grow-out. Dissolved oxygen, temperature, and pH were measured weekly and nitrite and ammonia monthly in each grow-out aquarium.

Sampling methodology. At 3 months post-exposure, 50 fish from each replicate group (100 fish per DEN treatment) were removed from the grow-out aquaria and sacrificed by immersion in a lethal concentration of tricaine methane sulfonate (MS-222; Crescent Research Chemicals, Phoenix, AZ). At the end of the 6 month grow-out period, all the remaining fish in each tank were removed and similarly sacrificed. Fish were starved for 72 h prior to sacrifice, to facilitate sectioning whole specimens. Fish were examined for skeletal and external abnormalities, and length (SL to the nearest mm), wet weight (to the nearest 0.1 g), and sex of each fish was recorded. The tail was removed and similarly sacrificed. Fish were examined for early stages of granulomatous reactions.

Immunohistochemical procedures. Immunohistochemical staining for proliferating-cell nuclear antigen (PCNA) was performed on 20 specimens from each DEN treatment, from the 3-month samples. Two serial sections from the left mid-lateral plane were mounted on poly-L-lysine-coated slides (Fisher Scientific, Pittsburgh PA) and allowed to air dry overnight. The staining protocol used was a modification of Ortego et al., (1994). Sections were deparaffinized, rehydrated, and then incubated for 20 min in a 3% H₂O₂ solution to block endogenous peroxidase. Slides were briefly rinsed with horseradish peroxidase (HRP)-enhancing buffer (Innovex Scientific, Richmond, CA) to facilitate antigen retrieval and protein blocking before addition of the primary antibody. Slides were incubated with a 1:220 dilution of the primary antibody (PC10, Dako Corporation, Carpenteria, CA) for 30 min in the dark. Following a brief rinse with HRP buffer, slides were incubated with the secondary antibody (multivalent mouse and rabbit secondary linking antibody, Innovex Scientific) for 10 min in the dark, followed by a 10-min incubation with the linking reagent (HRP-labeled strepavidin, Innovex Scientific). Visualization was achieved by a 5-min incubation with a diaminobenzidine chromogen (Innovex Scientific). After a brief tap-water rinse, slides were counterstained in hematoxylin, dehydrated, cleared, and cover-slipped. Positive staining by PCNA in hepatocytes was indicated by a dark brown nucleus and occasionally brown cytoplasm. PCNA labeling indices (LI) were determined according to guidelines of Foley et al., (1991). The LI was expressed as labeled nuclei per 1000 hepatocyte nuclei (both labeled and non-labeled) per liver counted at 400× magnification. Nuclei were counted in indiscriminately chosen fields using a micrometer grid. Care was taken to count only hepatocytes and not hepatic supporting cells or blood cells. Nevertheless, in this study and other cell proliferation studies conducted in our laboratory, there may be confounding proliferation effects caused by tissue inflammatory reactions in early stages of granulomatous reactions.

Statistical analysis. Standard lengths of fish sampled at 3 and 6 months were analyzed using ANOVA. Significant differences among treatment means were determined with a Student Neuman Kuell multiple range test using SPSS-PC version 5.0 (1992, SPSS, Inc, Chicago, IL). Careful examination of the histopathological data confirmed the absence of clustering among fish within a concentration group. Probit dose-response models were fit to the incidences of various hepatic lesions for each sampling time, using a binomial distribution. Dose-response relationships between hepatic lesions and DEN concentration, adjusting for age at sampling and sex, were analyzed using a probit model with a binomial distribution. For these analyses, the 3- and 6-month data were combined.

RESULTS

Survival and Gross Morphological Observations

The survival rate of medaka was high, with only 15 of the initial 750 fish dying prematurely during the 6 months of the test. Mortalities were not concentration-related. Similarly, few fish sampled at 3 and 6 months exhibited skeletal abnormalities or external lesions. Lordosis occurred in one fish from the 10-ppm DEN exposure and one from the 100-ppm exposure at the 3-month sample and in a single control specimen from the 6-month sample. Exopthalmia was noted in a single specimen from the 10-ppm DEN exposure, 6-month sample. Medaka in all concentration groups showed normal reproductive development with spawning occurring in all tanks by 3 months of age.

Small differences in the standard lengths were noted in fish sampled at 3 and 6 months (Fig. 1). Control fish sampled at 3 months were significantly (p < 0.05) longer by 1 mm than fish exposed to 100 ppm DEN (Fig. 1A). There were no significant
differences in standard length among any treatments for fish sampled at 6 months.

**Histopathological Observations and Analysis**

Only a few histological changes were seen in organs and tissues other than the liver. In the 3-month sample, one male fish exposed to 10 ppm DEN had a primary oocyte in the testis. Enlarged swim-bladder gas glands were observed in 5 fish sampled at 3 months, 1 exposed to 25 ppm DEN and 2 each exposed to 50 and 100 ppm DEN. Additionally, 1 specimen exposed to 100 ppm DEN with an enlarged gas gland also exhibited granulomatous inflammation in the swim bladder. Neoplastic lesions originated from hepatocytes except for 1 specimen sampled at 6 months and exposed to 25 ppm DEN that had a cholangiocarcinoma in the liver. A high percentage of the fish sampled at 6 months had macrophage aggregates in the kidney, ranging from 22% of the control fish to 50% of the fish exposed to 100 ppm DEN. However, macrophage aggregate occurrence in the kidney did not follow a prominent dose-response relationship.

The principal hepatic histopathological conditions that were evaluated are illustrated in Figures 2–6. Inflammatory, hyperplastic and degenerative lesions included macrophage aggregates (MAs; Fig. 2), vacuolated foci (Fig. 3), eosinophilic foci (Fig. 3), spongiosis hepatis (Fig. 4) and hepatic vacuoles (Fig. 5). Other lesions included basophilic foci (Fig. 6), considered to be preneoplastic, and hepatocellular adenomas and carcinomas (Fig. 5), the latter 2 of which were analyzed as a single group. Each of these lesions occurred in fish sampled at both 3-
and 6-month periods. Additionally, 10% of fish exposed to 100 ppm DEN and sampled at 6 months had scattered hyaline cells in the liver. Only 4 fish sampled at 3 months and one fish sampled at 6 months had granuloma in the liver. One of these cases was an advanced condition probably caused by a mycobacterial infection (Teska et al., 1997; unpublished observations).

Table 1 summarizes the significant histopathological findings for medaka sampled at 3 months. Control fish, as well as those exposed to 10 and 25 ppm DEN, showed low incidences of hepatic vacuolation, vacuolated foci, spongiosis hepatitis, MAs and eosinophilic foci at 3 months post-DEN exposure. Additionally, no fish in these first 3 treatments exhibited evidence of a pre-neoplastic or neoplastic response. In the 50 ppm DEN exposure group there was a notable dose-dependent increase in the percentage of livers having vacuolated foci. A modest increase was noted for spongiosis hepatitis and eosinophilic foci at 50 and 100 ppm DEN exposure as well. A small increase in the percentage of fish with hepatic vacuolation was noted at 100 ppm DEN. Finally, a small percentage of fish exhibited evidence of neoplastic development at 50 and 100-ppm DEN. Neoplastic lesions at 50 ppm DEN were all diagnosed as adenomas whereas lesions at 100-ppm DEN were all diagnosed as carcinomas. No fish from the 3-month sample had multiple pre-neoplastic or neoplastic hepatic lesions.

Probit dose-response models fit to the incidence of the hepatic lesions at 3 months sampling showed that the concentration-response relationship for vacuolated foci ($p < 0.0001$) was highly significant and the relationship for spongiosis hepatitis was marginally significant ($p = 0.053$). There was no significant concentration-dependent relationship for hepatic vacuoles ($p = 0.206$), MAs ($p = 0.458$) or eosinophilic foci ($p = 0.091$). The dose-dependent relationships of the pre-neoplastic and neoplastic lesions were both significant ($p < 0.001$ for basophilic focus, $p = 0.022$ for adenoma/carcinoma).

Table 2 summarizes the principal hepatic histopathological findings for medaka sampled at 6 months post-DEN exposure. Percentages of vacuolated foci and eosinophilic foci in control fish were similar to control values at 3 months. There was a low percentage of MAs and spongiosis hepatitis in control fish and a relatively high percentage of vacuoles. Additionally, one control fish (2%) developed an hepatic carcinoma. Vacuoles, vacuolated foci, and MAs exhibited a dose-response pattern in fish from the 6-month sample. Evidence of neoplastic lesions, in the form of altered foci and adenoma/carcinoma, appeared at 25 ppm DEN exposure and was noticeably increased compared to the 3-month sample. All the large neoplastic lesions examined (adenoma/carcinoma) were determined to be carcinomas with the exception of 1 fish exposed to 25 ppm DEN, in which the lesion was diagnosed as an adenoma. As was the case with fish sampled at 3-months post-exposure, no medaka sampled at
6 months displayed multiple pre-neoplastic or neoplastic hepatic lesions.

Probit dose-response models fit for various hepatic lesions at 6 months post-DEN exposure showed that the concentration-response relationships for hepatic vacuolation ($p < 0.005$), vacuolated foci ($p < 0.0001$) and MA ($p < 0.0001$) were highly significant, whereas the relationships for eosinophilic focus ($p = 0.122$) and spongiosis hepatis ($p = 0.656$) were not. The results for neoplastic lesions indicated the increasing occurrence of adenomas/carcinomas; there was a significant concentration-response relationship for adenoma/carcinoma ($p < 0.0003$) whereas the relationship for basophilic focus ($p = 0.072$) was only marginally significant.

Statistical models were developed for each of the histopathological lesions observed by considering the concentration-response relationship between a lesion and the DEN concentration while adjusting for the confounding effects of gender and age at sampling. There was no evidence of clustering among fish in the analysis. In all models ($n = 6$) there was a significant relationship between lesion type and DEN concentration ($p < 0.01$) with the exception of spongiosis hepatis.

The model describing the concentration-response relationship for hepatic vacuolation showed that, at a younger age, male medaka were more likely to have hepatic vacuolation than were females but that female medaka had a higher age-related risk of developing the vacuoles than did males ($p < 0.0011$, Fig. 7A). The model for vacuolated foci (Fig. 7B) showed that male fish had a higher incidence rate overall ($p = 0.02$). Additionally, whereas older fish were more likely to develop vacuolated foci in the absence of exposure, younger fish were at a higher risk of developing the foci as the DEN concentration increased ($p = 0.04$). The model for MA showed that gender appeared to be unrelated to MA and that the risk of developing MA following exposure to DEN appeared to be the same regardless of age or gender ($p = 0.0001$). However, the risk of developing MA increased with age when analyzed in the absence of DEN exposure ($p = 0.001$, Fig. 7C). The occurrence of spongiosis hepatis was not significantly related to age or gender of the fish and did not show a significant concentration-dependent response when 3- and 6-month data were combined. However, the incidence of spongiosis hepatis increased dramatically at 6 months across all DEN exposure groups when compared to 3-month results. Neither gender nor age had an effect on the concentration-response relationships for eosinophilic foci or basophilic foci although both models showed a significant concentration-response relationship (eosinophilic focus, $p = 0.03$; basophilic focus, $p < 0.0001$, Fig. 8A). The model for adenoma/carcinoma showed that older fish had a significantly ($p < 0.0001$) higher incidence of adenoma/carcinoma and that the risk of developing a neoplastic lesion

### TABLE 1

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*Note.* Values are expressed as percentages.

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*Note.* Values are expressed as percentages.
increased with exposure level of DEN \( (p < 0.0001; \text{Fig. 8B}) \). However, there were no differences in the development of an adenoma/carcinoma based on gender of the fish.

**PCNA Immunohistochemistry**

Figure 9 shows the results of quantification of the PCNA immunohistochemical assay. Many individual specimens showed no hepatocyte proliferation, resulting in large variations in labelling index (LI). Overall, control fish exhibited less hepatocyte proliferation than did fish exposed to DEN. These results suggest that hepatocyte proliferation was sustained in medaka for at least 3 months after the brief exposure to DEN, at each concentration.

Three commonly used trend tests (beta-binomial, GLM, and transform) indicated a significant association between increasing LI in medaka and DEN concentration at 3 months post-exposure \( (p < 0.02 \text{ for all 3 tests}) \). Labeling indices leveled off at the highest DEN concentration (100 ppm). Trend tests excluding the highest DEN concentration yielded slightly more significant results. The probit dose-response model of LI and DEN concentration fit the data well, yielding a Wald statistic of \( z = 2.18 \) \( (p = 0.015) \) and a deviance reduction of 1.05 with 1 degree of freedom and \( p = 0.026 \). This model also showed a

**FIG. 8.** Statistical models depicting the rate of hepatic pre-neoplasm and neoplasm formation in medaka after 48-h exposure to varying concentrations of DEN by age and gender. (A) Concentration-response of basophilic focus. There were no significant age or gender effects. (B) Concentration-response of neoplasms (adenoma and carcinoma combined). There were no significant gender effects.

**FIG. 9.** PCNA labeling indices of medaka sampled at 3 months post-DEN exposure showing the rate of hepatocyte proliferation. Bars represent mean ± 1 SD. \( n \), 20 fish per DEN concentration.
significantly positive correlation between LI and DEN concentration and accounted for cell clustering within the same fish, lending certain evidence that hepatocyte proliferation was induced by DEN. However, LI had a very weak association with the incidence of the various histopathological lesions examined and could not be used to predict lesion incidence in fish sampled at 3 months \((p > 0.3\) in all cases). The low occurrence of neoplastic lesions in 3-month fish limited the statistical power to detect a significant association between LI and a particular lesion. However, the few fish with neoplastic lesions that were stained for PCNA showed increased staining in the lesion relative to the surrounding hepatocytes.

**DISCUSSION**

In this study, a 48-h exposure to non-toxic levels of DEN resulted in significant increases in hepatic neoplasia in Japanese medaka \((Oryzias latipes)\). Effects of DEN exposure occurred in concentration-related patterns seen within 3 months post-exposure. This study, along with longer protocols frequently used for medaka carcinogenesis experiments that involve almost a 30-day exposure followed by 9-month to 1-year grow-out \((Boorman et al., 1997; Hinton et al., 1988; Ishikawa and Takayama, 1979; Lauren et al., 1990)\), shows that DEN is a potent and complete carcinogen in medaka. Additionally, DEN requires only a brief exposure at relatively low concentrations and a short latency to initiate carcinogenesis in medaka. Results from the 6-month post-exposure data statistically confirm the efficacy of this short-term, moderate level DEN exposure for initiation of hepatic neoplasia in medaka. The study supports findings by Bunton \((1989, 1990)\), which showed that a 48-h exposure of medaka to relatively high DEN concentrations \((100, 200, and 400 \text{ ppm})\) resulted in a variety of neoplastic lesions at 6 months post-exposure.

In addition to hepatic neoplasia, degenerative, inflammatory, and hyperplastic lesions \((hepatic vacuolation, spongiosis hepatis, macrophage aggregates, vacuolated foci and eosinophilic foci)\) were induced in medaka liver by exposure to DEN. Hepatic non-neoplastic lesions frequently co-occur with neoplastic ones and some of the lesions are related to hepatic carcinogenesis in the laboratory \((Bunton, 1996; Koza et al., 1993)\) and in wild fishes \((Moore et al., 1989; Myers et al., 1994)\). With the exception of the eosinophilic focus, the non-neoplastic lesions observed were all significantly concentration-dependent at 3 or 6 months post-exposure, further confirming long-term hepatic effects of a brief exposure to moderate concentrations of DEN. Bunton \((1989)\) found similar dose-response relationships in degenerative lesions in medaka briefly exposed to high DEN concentrations. Furthermore, Boorman et al. \((1997)\) reported an apparent dose-response relationship in hepatic vacuolation and spongiosis hepatis in medaka exposed to low levels of DEN for 28 days. The relatively high percentage of vacuolated foci present 6 months after DEN exposure in this study, as well as the highly significant concentration-response pattern of this lesion evident at both 3- and 6-month samples suggests that short-term exposure to moderate levels of DEN causes sustained hepatic toxicity. These results support those found in 6- and 9-month-old medaka following a longer \((28\text{-day})\) exposure to 5 to 20 ppm DEN \((Boorman et al., 1997)\).

Cell proliferation, as evidenced by DEN-induced PCNA staining at 3 months post-exposure, was significantly elevated. Increased hepatocyte proliferation has been demonstrated in medaka exposed to DEN concentrations of \(\leq 20\text{ ppm}\) for 28 days for fish sampled immediately and one month post-exposure \((Ortego et al., 1996)\). The sustained hepatocyte proliferation in medaka at 3 months post-exposure suggests an alteration in the replicative control of hepatocytes that could indicate poor DNA repair capabilities in medaka and an increased risk of developing neoplastic lesions later. Cell proliferation is considered a toxic response that is important in development of neoplasia when it is sustained over time rather than being brief and transient \((Swenberg, 1993)\). Statistical analyses showed no association between LI values and hepatic lesions at 3 months, possibly because of the low incidence of neoplastic lesions observed at this time period. Ortego et al. \((1996)\) found a correlation between sustained hepatocyte proliferation in medaka sampled at 1 month post-DEN exposure and hepatocellular carcinoma incidence in fish 9 months post-DEN exposure. In fishes, as in rodents, effects of interrelationships among age, gender, and exposure to a specific carcinogen on the patterns of neoplastic and non-neoplastic lesions are complex. Non-neoplastic lesions, such as macrophage aggregates and spongiosis hepatis, appear to occur more commonly in medaka as they age \((Boorman et al., 1997)\). The present study provides further evidence for this assumption, as the incidence of both MA and spongiosis hepatis was greater at 6 months than at 3 months post-exposure. However, there were no significant differences in the occurrence of MA, spongiosis hepatis, or eosinophilic focus by gender. In contrast, female medaka had lower incidences of both vacuolated foci and hepatic vacuolation than did males, which is interesting in light of the highly significant correlation of vacuolated foci with DEN concentration. Regarding neoplastic lesions, the incidence of spontaneous tumors in pond-reared medaka was low in both male and female fish under 3 years of age, but after that, hepatic tumor incidence was higher in females than males \((Masahito et al., 1989)\). There may be a similar gender bias related to age in spontaneous tumor formation in younger medaka as well \((Teh and Hinton, 1998)\). Our results show that neoplastic lesion development, in the form of both basophilic foci and adenoma/carcinoma, did not differ between male and female medaka exposed to low concentrations of DEN. This differs from some reported studies. Nakazawa et al., \((1985)\) found that DEN-induced hepatic basophilic lesions were more frequent in males and that the ratio of the enzyme-altered areas in these lesions was greater in male than female medaka. In contrast, Teh and
Hinton (1998) found that female medaka exposed to DEN had a significantly greater incidence and more rapid development of hepatic neoplasia (both basophilic foci and adenoma/carcinoma) than males. The differences in results could be related to differences in DEN-exposure protocols as well as to the age of the medaka at exposure. Teh and Hinton (1998) exposed 21-day-old medaka to 250 ppm DEN for 48 h, whereas Nakazawa et al. (1985) exposed 1-year-old medaka to 50 ppm DEN for 6 weeks. Perhaps there is a differential susceptibility to neoplasia by gender in medaka that is related to a combination of age at exposure as well as to the dosage and duration of exposure to DEN.

The present study has implications for the development of initiation/promotion assays using medaka. Currently, most initiation/promotion studies using fish models are conducted with rainbow trout (Bailey et al., 1987, 1996). However, data from this study show that an initiation protocol for the medaka model might involve exposure to 10 ppm DEN or lower for 48 h. This protocol resulted in no hepatic neoplasia at 6 months post-exposure, a requirement for an initiating dose in an initiation/promotion study (Maronpot, 1991). DEN has been shown to effectively initiate hepatocarcinogenesis in rats (Carthew et al., 1997; Imaida and Fukushima, 1996), rainbow trout (Hendricks et al., 1994; Shelton et al., 1984), and zebrafish (Danio rerio) (Spitsbergen et al., 1997), as well as medaka (Boorman et al., 1997; Bunton, 1989, 1990; Lauren et al., 1996), thus demonstrating the efficacy of this compound as a complete carcinogen in several model organisms. However, a complete carcinogen, given in a single low dose, can be an effective initiating agent requiring subsequent promotion for the expression of neoplasms (Maronpot, 1991). The low-level, short-term exposure of DEN used in our studies suggests that DEN can be used as a true initiator in medaka, since neoplastic lesions did not develop after 6 months in fish briefly exposed to 10 ppm DEN. However, this short-term, low level exposure did result in sustained hepatocyte proliferation and cytotoxic damage to the liver, suggesting hepatocytes may be susceptible to promotion and the subsequent development of neoplasms.

A variety of promotional agents has been tested in the rainbow trout model (Bailey et al., 1996). Identifying the appropriate promoter for use in an initiation/promotion assay can be challenging, since promoter activity is often initiator- and tissue-specific (Maronpot, 1991). For example, in trout initiated with MNNG, dehydroepiandrosterone (DHEA) acted as a promoter for liver and kidney tumors, had no effect on stomach tumors, and inhibited the formation of swim bladder tumors (Orner et al., 1996). Furthermore, DHEA was a much more effective promoter of liver tumors in trout initiated with aflatoxin B1 than those initiated with MNNG (Orner et al., 1995, 1996). Thus, choosing an effective promoter for the medaka model will require studies involving a variety of test compounds. Studies by Cooke and Hinton (1999) suggest that estradiol and hexachlorocyclohexane are effective promoters of DEN-initiated hepatocarcinogenesis in medaka. The results obtained from the present study ensure that an appropriate initiation dose and exposure time for DEN have been identified for the continuation of initiation/promotion experiments using the medaka small fish model.

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