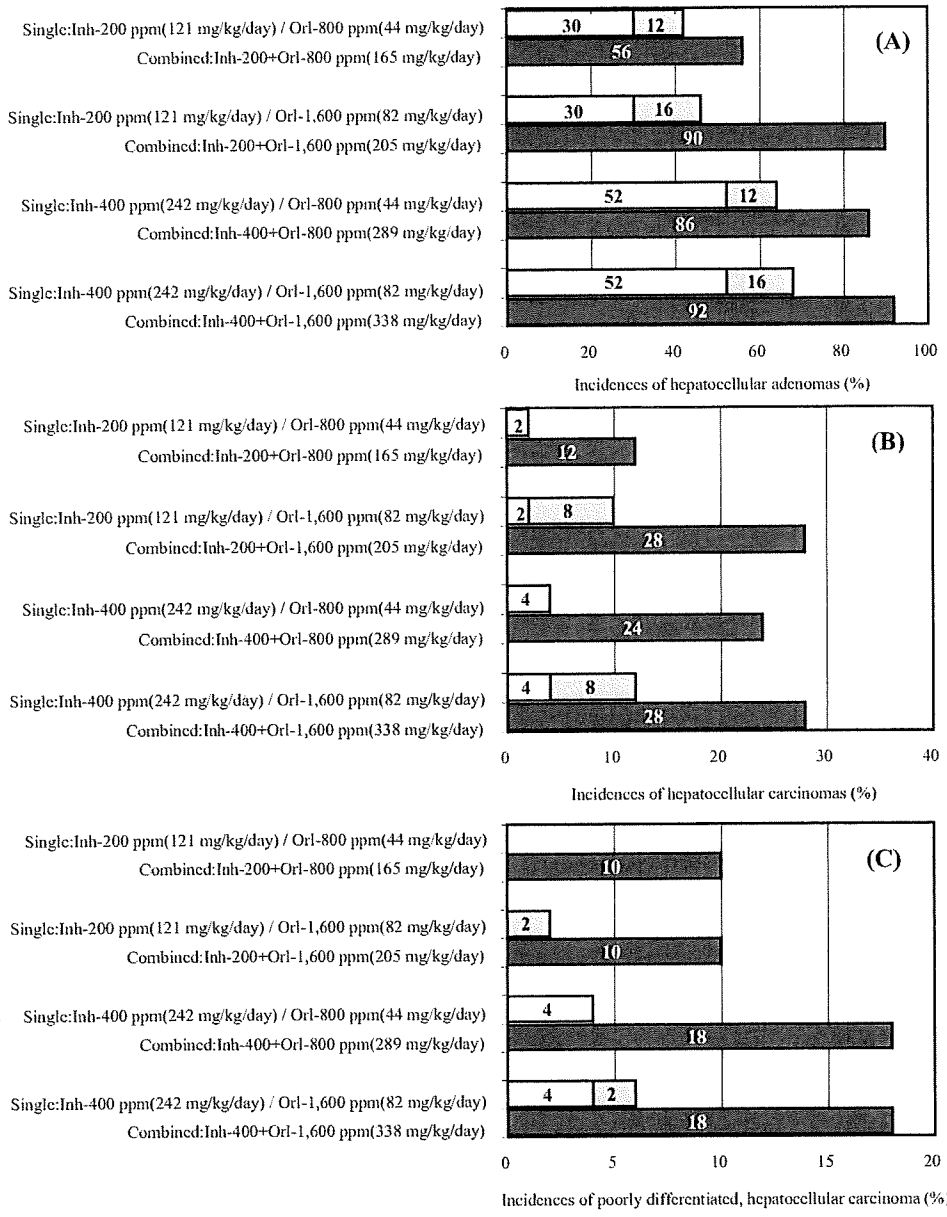

Fig. 1. Comparison of incidences of hepatocellular tumors ((A): hepatocellular adenomas, (B): hepatocellular carcinomas and (C): poorly differentiated hepatocellular carcinoma) in the combined-exposure groups (solid bars) with sum of the compornent incidences of the hepatocellular tumor in the single-route exposure groups through inhalation (open bars) and ingestion (shaded bars). The values in parenthesis indicate the total estimated amount of DMF uptake (mg/kg/day). The values in the bars represent the incidences of (A) hepatocellular adenomas, (B) hepatocellular carcinomas and (C) poorly differentiated, hepatocellular carcinoma.

were more remarkable than those of hepatocellular adenomas that are benign.

DMF-induced hepatocellular carcinomas can be classified into two different types according to the histopathological characteristics described by Senoh *et al.* (2004). The first type of hepatocellular carcinomas is prima-

rily composed of thick trabeculae of hepatocytes with abundant cytoplasm and round nuclei, which are similar to normal hepatocytes in histological appearance (Fig. 2A). The second type of hepatocellular carcinomas is composed of extremely thick trabeculae of hepatocytes with little cytoplasm and spindle-shaped hyperchromatic nuclei (Fig. 2B). The second type is considered to be more atypical and poorly differentiated, hepatocellular carcinomas, since the appearance of the second type deviates to a greater extent from that of normal hepatocytes than does the first type. Morphological characteristics of the second type became more malignant along the tumor-developmental sequence than those of the first type. The first type of hepatocellular carcinomas was found in all DMF-treated groups including the oral-alone, inhalation-alone and combined-exposure groups. The second type of hepatocellular carcinomas found in single-route exposure groups occurred at low incidences: 2/50 cases (4%) for the Inh-400+Orl-0 ppm group and 1/50 case (2%) for the Inh-0+Orl-1,600 ppm group. However, the combined exposures were found to markedly increase the incidence of the second type of hepatocellular carcinomas: 5/50 cases (10%) for the Inh-200+Orl-800 ppm, 5/50 cases (10%) for the Inh-200+Orl-1,600 ppm, 9/50 cases (18%) for the Inh-400+Orl-800 ppm, and 9/50 cases (18%) for the Inh-400+Orl-1,600 ppm. Notably, the inci-

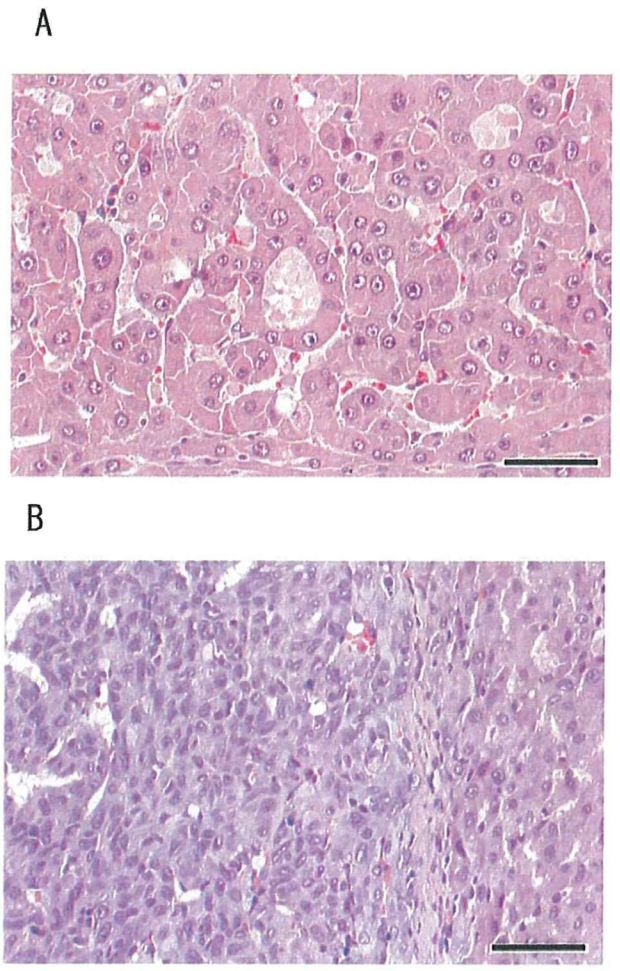

Fig. 2. (A): Hepatocellular carcinomas composed of thick trabeculae of hepatocytes with abundant cytoplasm and round nuclei in a male rat in the Inh-0+Orl-1,600 ppm group. (B): Poorly differentiated, hepatocellular carcinomas composed of extremely thick trabeculae of hepatocytes with little cytoplasm and spindle-shaped hyperchromatic nuclei in a male rat in the Inh-400+Orl-1,600 ppm group. H & E stain. Bar indicates 100 μm.

dences of the second type of hepatocellular carcinoma in the four combined-exposure groups were markedly greater than the sum of the two incidences of the second type of hepatocellular carcinomas in the single-route exposure groups through inhalation and ingestion (Fig. 1C). Therefore, the combined inhalation and oral exposures to DMF were found to markedly enhance not only the incidences of hepatocellular adenomas and carcinomas but also their tumor malignancy that might progress from hepatocellular adenomas through carcinomas to poorly differentiated, hepatocellular carcinomas.

It was noteworthy that the DMF-induced hepatocellular carcinoma did not metastasize to any other organs.

DISCUSSION

IARC's overall evaluation that DMF is not classifiable as to its carcinogenicity to humans (Group 3; IARC, 1999) was partly based on experimental evidence indicating lack of carcinogenicity after 2-year inhalation exposure of Crl:CD rats and CD-1 mice to DMF at an inhalation concentration of 25, 100 or 400 ppm (Malley *et al.*, 1994). More recently, however, our carcinogenicity study (Senoh *et al.*, 2004) has demonstrated that 2-year inhalation exposure to DMF vapor at 200, 400 and 800 ppm produces hepatocellular adenomas and carcinomas in F344 rats and BDF_1 mice of both sexes, although the exposure to 200 and 400 ppm primarily elicited benign hepatocellular adenomas. Senoh *et al.* (2004) suggested that the difference in carcinogenicity between Senoh *et al.*'s study and Malley *et al.*'s study was attributed to the strain of rats used. The present study confirms our previous findings, and extends those to the induction of hepatocellular tumors by oral administration of DMF in drinking water. Notably, the present study demonstrated that the combined inhalation and oral exposures of male F344 rats to DMF for 104 weeks markedly increase the incidences of hepatocellular tumors and their malignancy, as compared with the single-route exposures through inhalation and ingestion.

The extent to which the combined exposures markedly enhance the incidences of hepatocellular tumors is characterized by the hepatocarcinogenic effect being greater than the sum of the two hepatocarcinogenic effects of the single-route exposures through inhalation and ingestion. The "greater than additive" effect of the combined exposures on hepatocellular tumors tended to be accompanied by an increase in the malignancy of hepatocellular tumors. That is, the incidences of benign hepatocellular adenomas in the four combined-exposure groups were increased only by 1.3- to 2.0-fold over the sum of the two incidences of hepatocellular adenomas in the single-route exposure groups through inhalation and ingestion (Fig. 1A). The incidences of hepatocellular carcinomas in the four combined-exposure groups were increased by 2.3- to 6.0-fold over the sum of the two incidences of hepatocellular carcinomas in the single-route exposure groups through inhalation and ingestion (Fig. 1B). Furthermore, the combined exposures enhanced by much greater folds the incidences of poorly differentiated, hepatocellular carcinomas, which are more malignant than the commonly observed hepatocellular carcinoma, over the sum of the two incidences of poorly differentiated, hepatocellular carcinomas in the single-route exposure groups through inhalation and ingestion (Fig. 1C). Therefore, the present findings suggest that the combined inhalation and oral exposures to DMF enhances the incidence of malignant hepatocellular tumors in a greater than additive manner, when we defined the additivity of carcinogenic responses as the same concept of "response additivity" applied to the effects of chemical mixture by the U.S. EPA (2000).

The characteristic relationship of total doses versus carcinogenic responses expressed as the incidences of hepatocellular tumors can be found in the two combined-exposure groups of Inh-200+Orl-800 ppm and Inh-200+Orl-1,600 ppm, both of which had total estimated DMF uptakes of 165 and 205 mg/kg/day, respectively. It is noteworthy that the incidences of hepatocellular carcinomas were greater in these two combined-exposure groups than in the Inh-400+Orl-0 ppm group having the total estimated DMF uptake of 242 mg/kg/day, although the former two combined-exposure groups had lesser uptakes than the latter single-route exposure group did. This finding also indicates that the hepatocarcinogenic effect induced by the combined inhalation and oral exposures would be enhanced in a greater than additive manner as expected from sum of the two effects induced by the single-route exposure through inhalation and ingestion.

The present carcinogenic effect of the combined exposures is in sharp contrast to that found in an inhalation study by Senoh *et al.* (2004) who reported 13/50 cases of hepatocellular adenomas with null case of hepatocellular carcinomas following 2-year inhalation exposure of male F344 rats to 400 ppm DMF vapor, which corresponded to an estimated DMF uptake of 242 mg/kg/day. Comparison of the estimated DMF dose-carcinogenic response relationships between the present combined-exposure study and Senoh *et al.*'s inhalation study (2004) also indicates that the combined inhalation and oral exposures to DMF produce greater incidences of hepatocellular tumors with higher malignancy than the inhalation-alone exposure

does.

We have not yet obtained any experimental evidence to elucidate the markedly enhanced hepatocarcinogenic effect of the combined inhalation and oral exposures to DMF. However, a clue to understanding the intriguing carcinogenic responses by the combined exposures can be seen in the finding in our previous study (Ohbayashi *et al.*, 2008) that the combined inhalation and oral exposures of male rats to DMF for 4 weeks markedly enhanced the proliferation index expressed as the percentage of PCNA-positive hepatocytes in a greater than additive manner as compared with the sum of the two proliferation indices by the single-route exposures through inhalation and ingestion. Since a broad range of *in vitro* and *in vivo* genotoxicity assays showed negative genotoxicity responses to DMF (IARC, 1999), a nongenotoxic-cytotoxic-proliferative mode of action can be hypothesized to operate for the DMF-induced hepatocarcinogenesis. This hypothesis suggests that hepatocellular death by the toxic insult of DMF and/or its active metabolites and the subsequently increased regenerative proliferation of hepatocytes play a crucial role in DMF-induced hepatocarcinogenesis (Butterworth *et al.*, 1992). Since both *N*-methylformamide (NMF), which was metabolized from DMF through *N*-(hydroxymethyl)-*N*-methylformamide and methyl isocyanate, which was possibly biotransformed from NMF were reported to be the most potent hepatotoxicants (Kestell *et al.*, 1987; Gescher, 1993; Mráz *et al.*, 1989, 1993), these two metabolites might cause severe hepatocellular damage that would result in enhanced cell proliferation in DMF-induced hepatocarcinogenesis. Further quantitative investigations such as physiological based pharmacokinetic modeling in consideration of the hepatic levels of DMF and its toxic metabolites are needed to examine greater than additive effects of the combined exposures to DMF on the hepatocellular tumors.

In conclusion, the present study demonstrated that the combined exposures of male rats to DMF at approximately similar dose levels each through inhalation and ingestion enhance induction of hepatocellular tumors and their malignancy in a greater than additive manner (i.e., possibly synergistic). In addition to the reported nongenotoxicity of DMF suggesting the presence of a threshold level for the tumor induction, sufficient consideration should be paid to delineation of the relationship between the dose levels given through the multiple routes and carcinogenic responses, in order to estimate quantitatively carcinogenic risks of humans exposed to an environmentally ubiquitous carcinogen such as DMF. Indeed, combined exposures to DMF at environmentally relevant levels is anticipated to occur for community residents living near a neighboring factory where DMF is used (Amster *et al.*, 1983) and/or public water contaminated with DMF, although a general population may be exposed to DMF at far lower levels through inhalation and ingestion.

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