Immunohistochemical localization of inducible nitric oxide synthase and 3-nitrotyrosine in rat liver tumors induced by \textit{N}-nitrosodiethylamine

Byeongwoo Ahn\textsuperscript{1,2}, Beom Seok Han\textsuperscript{1}, Dae Joong Kim\textsuperscript{1} and Hiroshi Ohshima\textsuperscript{2,3}

\textsuperscript{1}Department of Pathology, National Institute of Toxicology Research, Korea Food and Drug Administration, 5 Nokbun-Dong, Eunpyung-Ku, Seoul 122-704, Korea and \textsuperscript{2}Unit of Endogenous Cancer Risk Factors, International Agency for Research on Cancer, 150 cours Albert-Thomas, 69372, Lyon Cedex 08, France

\textsuperscript{3}To whom correspondence should be addressed
Email: ohshima@iarc.fr

Human liver cancers have been associated mainly with chronic inflammations such as viral hepatitis B or C. This suggests that prolonged cell damage by chronic inflammation is critical in cancer development. Overproduction of nitric oxide (NO\textsuperscript{·}) and its derivative (NO\textsubscript{x}, peroxynitrite) has been implicated as a cause of tissue damage by inflammation, thus contributing to tumor promotion. We have demonstrated the expression of the inducible isoform of nitric oxide synthase (iNOS) and 3-nitrotyrosine, a marker of peroxynitrite formation, by immunohistochemistry in preneoplastic and neoplastic rat liver tissues induced by continuous infusion of \textit{N}-nitrosodiethylamine with minipumps. The preneoplastic lesions were characterized by proliferation of phenotypically altered hepatic foci (PAHF), dysplastic hepatocytes and oval cells. Histologically, the tumors were hepatocellular carcinomas (HCCs) of trabecular, (pseudo)glandular and solid types with or without cholangiocellular involvement. iNOS was located mainly in oval cells, capillary endothelial and muscular cells, epithelia of cholangiomas and glandular HCCs. 3-Nitrotyrosine was observed in the cytoplasmas of PAHF and dysplastic hepatocytes in preneoplasias and in the cytoplasmas of some living or apoptotic HCC cells, connective tissues, proteinaceous fluids, sinusoidal endothelia of tumorous hepatocytes and cholangiomas in tumors. From these observations, we suggest that: (i) chronic tissue damage by chemical carcinogens may act to induce iNOS and peroxynitrite formation; (ii) oval cells play a key role in development and/or growth of tumor tissues by producing NO\textsuperscript{·} via iNOS, which may also cause tissue damage by peroxynitrite; (iii) iNOS can be considered as a phenotypic marker in cells of oval cell lineage and neovascularized capillaries in tumor tissues.

Introduction

The role in carcinogenesis of prolonged cellular damage such as viral- or bacterial infection-related chronic inflammations has become widely recognized (1). In humans, hepatocellular carcinomas (HCCs) are usually associated with viral hepatitis B and C and cirrhosis resulting from chronic hepatitis (2). One of the major mediators in chronic inflammatory processes is nitric oxide (NO\textsuperscript{·}), which is produced by liver parenchymal and non-parenchymal cells from L-arginine via nitric oxide synthase (NOS). NO\textsuperscript{·} is considered to exert a hepatoprotective action against tissue injury and cytotoxic effects due to invading microorganisms, parasites and tumor cells. However, any situation that causes uncontrolled, prolonged and/or massive production of NO\textsuperscript{·} by inducible NOS (iNOS) may result in liver damage, leading to inflammation and even tumor development (3). Harmful effects of NO\textsuperscript{·} may be due to reaction with superoxide anion to yield peroxynitrite, which is a potent oxidizing and nitrating agent. Peroxynitrite can oxidize nuclear DNA and membrane phospholipid and also nitrate, free or protein-associated tyrosines and other phenolics (4). Thus, the occurrence of nitrotyrosine in tissues has been measured as a marker of peroxynitrite formation \textit{in vivo} (5,6).

In order to elucidate the early hepatocellular lesions which may represent progenitor populations of HCCs, numerous investigations using rodent liver tumor models have been carried out (7–12). In particular, numerous efforts to induce proliferation of different cell types in the liver of rodents have been made. A single injection of the hepatocarcinogen \textit{N}-nitrosodiethylamine (NDEA) (200 mg/kg) induces phenotypically altered hepatic foci (PAHF) during the early stages of carcinogenesis without any notable involvement of other cell types. The development of PAHF is believed to be caused by mutations in normal cellular growth control genes and subsequent clonal growth (13). The progressive histological and enzymatic changes in hepatocytes developing into foci and nodules are typical features in the early stages of the carcinogenic process. However, the frequency of development of cancers from putative preneoplastic nodules in rodents is not high. Only 5–10\% of them persist and, of these, only a very small fraction develop into malignant tumors (14). Most of the remaining cells may redifferentiate to a normal hepatocyte phenotype (9). Meanwhile, in addition to the phenotypic alterations of hepatocytes, another outstanding event in the early chemical carcinogenic process is the proliferation of small epithelial cells with void nuclei, described as oval cells by Farber (15). These oval cells have been shown to express embryonic proteins, such as \(\alpha\)-fetoprotein, the M2 form of pyruvate kinase (M2-PK) and \(\pi\)-class glutathione \(\gamma\)-transferase (GST-\(\pi\)) (16). Oval cells usually proliferate following high doses or prolonged treatment with azo dyes, 2-acetylamino-fluorene (2-AAF) or a choline-deficient diet. They are bipotent in cellular differentiation activity, differentiating into either hepatocytes (17,18) or intrahepatic bile duct epithelial cells (19,20). Oval cells are believed to be progenitor cells of HCC and cholangiocellular carcinoma (CCC) (12,21,22). When cultured oval cells were injected into rats after transformation, CCC and undifferentiated carcinomas were induced (23). More recently, the importance of oval cells in other conditions, such as viral hepatitis B (24,25), alcohol-induced liver toxicity (26) and chronic iron overload (16) in rats, has been described. A common factor in these conditions is prolonged liver damage.

Abbreviations: 2-AAF, 2-acetylamino-fluorene; CCC, cholangiocellular carcinoma; DMSO, dimethyl sulfoxide; HCCs, hepatocellular carcinomas; iNOS, inducible NOS; NDEA, \textit{N}-nitrosodiethylamine; NOS, nitric oxide synthase; PAHF, phenotypically altered hepatic foci.
Therefore, we suspected that chronic liver damage by chemicals can be more effective than acute in experimental carcinogenesis. The basic goal in developing experimental tumors should be to simplify and simulate the major factors affecting human cancers. With this in mind, we tried to test the tumor-inducing potential of chronic tissue damage. Based on the Solt–Farber hepatocarcinogenesis protocol, rats were continuously given a relatively toxic dose of NDEA using osmotic mini-pumps and fed a basal or choline-deficient diet. These procedures enabled us to induce well-developed PAHF as well as oval cell proliferation at 5 weeks. To our surprise, HCCs, sometimes involving metastases to the lungs, and cholangiomas were induced in all treated rats within 6 months. Moreover, similar occurrences were observed in rats treated with NDEA by infusion only, which was intended to test whether it can be the major factor affecting tumor development. In the present study, we have investigated the immunohistochemical localization of iNOS and 3-nitrotyrosine in the induced preneoplastic and neoplastic rat liver tissues. On the basis of the results, we propose new roles for iNOS and reactive nitrogen species in chronic tissue damage as well as those for iNOS-expressing cells in carcinogenesis.

![Fig. 1. Experimental design. NDEA, i.p. implantation of minipumps containing 47.5 mg of NDEA dissolved in DMSO. DMSO, i.p. implantation of minipumps containing only DMSO as controls. Group 5 received the same dose level of NDEA as a single i.p. injection. After 3 weeks, 2-AAF, dissolved in corn oil, was given to all animals except those of group 4 for 2 weeks five times a week as a single i.p. injection of NDEA (47.5 mg/rat). 2-AAF, 1 mg/rat of 2-AAF dissolved in corn oil, by gavage for 2 weeks, five times a week. PH, two-thirds partial hepatectomy. □, basal diet; ■, choline-deficient diet. S*, five rats were killed; S, all remaining rats were killed.](image)

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*Cholangiomas developed concurrently with HCC.

Materials and methods

Animals

Male Fischer 344 (n = 35) and Sprague–Dawley (n = 9) rats, body weight 150–200 g, were supplied by the National Institute of Toxicology Research (Seoul, Korea). Rats were housed two or three to a polyethylene cage with hardwood chips for bedding. They were fed basal diet from Shirin Feed (Seoul, Korea) or choline-deficient diet from Purina Mills (St Louis, MO), depending on the experimental design, and were allowed free access to water throughout the experiment. The animals were kept in a temperature controlled room (21 ± 1°C) with a 12 h light/dark cycle.

Tumor incidence

As illustrated in Figure 1, the animals were divided into five groups. Groups 1–3 were Fischer rats and groups 4 and 5 were Sprague–Dawley rats. For induction of liver tumors, mini-pumps (Alzet model 2002; ALZA Scientific Products, Palo Alto, CA) providing a continuous infusion (0.5 µl/h for 2 weeks, nominal reservoir volume 200 µl) of NDEA (catalogue no. N 0756; Sigma, St Louis, MO) dissolved in dimethyl sulphoxide (DMSO) at a dose level of 47.5 mg/pump were implanted into the peritoneum of rats in groups 1, 2 and 4 under ether anaesthesia. Rats of group 3 received implants of mini-pumps containing only DMSO as controls. Group 5 received the same dose level of NDEA as a single i.p. injection. After 3 weeks, 2-AAF, dissolved in corn oil, was given to all animals except those of group 4 for 2 weeks five times a week by gavage at a dose level of 1 mg/rat/day. Half way through the 2-AAF treatment, two-thirds partial hepatectomy was performed on animals of groups 1–3 and 5. Group 4 rats were sham-operated. All of the mini-pumps implanted at the start of the experiment were removed during this operation. Throughout the experiment, groups 1, 4 and 5 were fed a basal diet and groups 2 and 3 the choline-deficient diet. Five rats were killed from groups 1 and 2 at 5 weeks and all the remaining animals at 26 weeks after the start of the experiment. The tumor incidences are summarized in Table I. Grossly identifiable tumors were observed in all the 25 rats of groups 1, 2 and 4, which were treated with NDEA by continuous infusion from mini-pumps. Animals of group 5, with almost the same Solt–Farber protocol, developed no tumors. The tumors were hepatocellular adenoma (HCA), HCC with or without cholangiomas and one case of primary lung cancer in group 4 and one mesothelioma on the surface of the testes and epididymis in group 1, respectively. Because two strains of rat (Sprague–Dawley and F344) were used in this study, it is not possible to directly compare the tumor incidence induced by different treatments in different strains (e.g. group 1 versus group 4). However, we recently repeated the group 4 protocol using F344 rats and obtained similar results (eight of nine rats developed HCC) to those shown here with Sprague–Dawley rats (manuscript in preparation). In the present study, however, we used liver specimens (preneoplastic and neoplastic) obtained from the two strains of rat for immunohistochemical localization of iNOS and 3-nitrotyrosine.

Histology and immunohistology

The livers and other tumor-bearing tissues were removed at sacrifice, fixed in formalin for 24 h and processed routinely for paraffin embedding. Tissue sections (4–5 µm thickness) were mounted on poly-L-lysine-coated slides and stained with hematoxylin and eosin for routine histology. For immunohistochemical study, the sections were incubated with proteinase K (0.02 µg/ml of 10 mM Tris, pH 8) for 20 min for antigen unmasking and then with 1.5% normal goat serum to the slides for iNOS or 5% skimmed milk for 3-nitrotyrosine for 30 min at room temperature to block non-specific binding. IgG fractions
purified from rabbit polyclonal antibody against rat liver iNOS (27,28) or monoclonal antibody against 3-nitrotyrosine (Upstate Biotechnology, Lake Placid, NY) was applied in phosphate-buffered saline containing 0.1% bovine serum albumin overnight at room temperature. The slides were subsequently incubated with biotinylated secondary antibodies and avidin–biotin–peroxidase complex in Elite ABC kit (Vector, Burlingame, CA) according to the manufacturer’s instructions. Binding was visualized by incubation with 0.06% diaminobenzidine (Sigma D5637) dissolved in tap water containing 0.01% H₂O₂ for 3–5 min. The nuclei were counterstained with hematoxylin. The specificity of the iNOS antibody was verified previously (28). It does not cross-react with either the neuronal or the endothelial isoform of NOS. Western blot analysis of liver from a rat stimulated with Propionibacterium...
Fig. 3. Immunohistochemistry of iNOS. Oval cells surrounding PAHF and dysplastic hepatocytes express iNOS in preneoplastic tissues from group 2 at 5 weeks (A). In tumor tissues from group 1 at 26 weeks, iNOS is mainly stained in glandular HCC (B and C) and cholangioma (D). Note the glandular HCC cells with hepatocellular (B and C, arrows), transitional (B, empty arrows) and cholangiocellular (C, empty arrow) phenotype. Bile ductule-like oval cells (E) and capillary endothelial cells (F) in HCC also show specific reactions. ABC stain. Original magnification: A, C and D ×100; B, E and F ×200.

acnes and lipopolysaccharide showed the presence of a single band of ~135 kDa, corresponding to iNOS. Pre-absorption of the antibody with purified iNOS from rat liver diminished immunostaining. Purified IgG fractions from pre-immune serum gave no immunoreactivity.

Results

Histopathology of preneoplastic and neoplastic lesions

Most liver tissues from groups 1 and 2 were losing their normal architecture at 5 weeks due to development of numerous well-developed PAHF characterized by white miliary spots on the surface in gross findings. The PAHF, mostly of clear and/or eosinophilic cell type, were well demarcated by actively proliferated oval cells and fibrous tissues. In between these foci, the remaining hepatocytes showed a dysplastic appearance, forming cell clusters or adenoid structures. These dysplastic hepatocytes were also surrounded by oval cells. Normal hepatocytes were rarely seen in the lesions (Figure 2A and B). However, the lesions from rats which were not given the choline-deficient diet were less severe in terms of dysplastic hepatocytes and oval cell proliferation (data not shown). In the rats killed at week 26, the lesions comprised putative preneoplastic foci, HCAs, HCCs [showing mixed or single types of trabecular, (pseudo)glandular and solid patterns] and cholangiomas (Figure 2). Apoptotic tumor cells were frequently observed in solid areas, but rarely in other subtypes. Vascular invasion by HCC was frequently observed. HCCs of typical glandular or adenoid pattern contained blood cells or cell debris in the lumens. The epithelial cells were columnar to
Fig. 4. Immunohistochemistry of 3-nitrotyrosine. In preneoplastic lesions from group 2, nitrotyrosine-containing proteins are observed in both PAHF and dysplastic hepatocytes (A). In tumor tissues from group 1 at 26 weeks, nitrotyrosine is detected in some iNOS-expressing or neighboring tumor cells (B) and apoptotic cells (C). ABC stain. Original magnification: A, B, ×200; C, ×100.

cuboidal or even flat with a moderately differentiated appearance, whereas pseudoglandular HCCs showed higher differentiation to hepatocytes and were present within areas of trabecular HCC. Usually glandular HCCs showed the cellular phenotypes of hepatocytes, transitional and/or cholangiolar cells and were surrounded by cancer cells with hepatocytic phenotype (Figure 2D–F). Cholangiomas were of well-differentiated cystic type (Figure 2G) and sometimes intermingled with HCC cells. HCC metastases in the lungs also showed all the cell subtypes seen in livers and compressed surrounding pulmonary tissues.

**Immunohistology of iNOS and 3-nitrotyrosine**

In untreated liver tissue, iNOS was very weakly localized in bile duct epithelium and endothelium and the muscle layer of vessels. 3-Nitrotyrosine was not detected. In preneoplastic lesions, iNOS was not expressed in hepatocytes of PAHF but proliferated oval cells were weakly positive (Figure 3A). 3-Nitrotyrosine-containing protein was localized predominantly in dysplastic hepatocytes and some altered hepatocytes in the foci. The cytoplasm had a homogeneous appearance (Figure 4A). In tumor tissue, expression of iNOS was strongly localized in bile duct epithelia, proliferated ductule-like oval cells and epithelia of cholangiomas. In contrast to the lack of immune response in trabecular HCC and/or pseudoglandular HCC, which were not lined by basement membrane, the epithelia of glandular HCCs stained extensively for iNOS (Figure 3). iNOS was strongly expressed in cells with hepatocellular, transitional or cholangiocellular phenotype (Figure 3B and C). Ductule-like oval cells and neovascularized capillary endothelial cells in HCCs also showed high staining intensity (Figure 3). iNOS was also expressed in the smooth muscle layer of relatively large vessels. 3-Nitrotyrosine immunoreactivity was relatively weak in cells of trabecular HCC, glandular HCC and cholangioma, connective tissues of glandular HCC, proteinaceous serous fluids and sinusoidal endothelia of tumorous hepatocytes, but was strongly localized in apoptotic cells (Figure 4). The expression of iNOS from groups 3 and 5 at 26 weeks was similar to the pattern from untreated liver tissues. Likewise, 3-nitrotyrosine was not detected in these tissues.

**Discussion**

The hepatocarcinogenic protocols used in this study were designed to induce tumors by causing prolonged cell damage by chemicals. Although two different rat strains were used, they were very effective, at least in terms of tumor incidence (100%), relatively short induction periods (<6 months) and malignancy (diverse tumor patterns and metastasis). Since the protocol using NDEA infusion only induced cancers similar to those using combined treatment with NDEA and 2-AAF, in conjunction with partial hepatectomy with or without choline deficiency, the fundamental cancer-causing factor clearly appears to be chronic toxic damage by continuous infusion of NDEA. One of the features of the tumor incidences is the concurrent development of both HCCs and intrahepatic biliary tumors, although the latter did not show malignancy at the time of death. As in general CCCs are rarely induced in rodents by chemicals, the continuous infusion protocol will be useful to study tumor cell lineages between oval cells, ductular epithelial cells and hepatocytes.

Mechanisms that have been proposed to play roles in NDEA carcinogenicity in hepatocytes include DNA adduct formation followed by gene mutation (29–31), cytolethality following regenerative proliferation and oxidative stress (32) or damage by impairment of mitochondrial respiration by free radicals (33,34). Oxidative stress increases iNOS gene transcription and promoter activity in hepatocytes (35). Many studies have recently focused on the role of NO· in carcinogenesis. The toxicity of NO· has been attributed to the potent nitrating and oxidizing agent peroxynitrite, which is formed by a near-diffusion-limited reaction between NO· and superoxide. Per-
oxytnitrite has been shown to induce DNA strand breakage, nitration and oxidation of proteins and nucleic acids and cell death in vitro (4,36,37). There is no appropriate method to detect peroxynitrite formation in vivo directly, but its reaction product, 3-nitrotyrosine, has been measured as a marker of peroxynitrite production in vivo (5,6). This product has been detected immunohistochemically in acute lung injury in humans and rats (38) and human atherosclerotic coronary arteries (39). In liver, hepatocytes have been reported to generate superoxide intracellularly, which reacts with NO· to produce peroxynitrite, resulting in cytotoxicity (40). Inhibition of NO· attenuated peroxynitrite generation by lowering NO· production (41). Conversely, overproduction of NO· by induction of iNOS in the liver may be related to cytotoxicity in hepatocytes.

Most studies on the pathogenic effects of NO· production have examined inflamed tissues induced by bacterial, viral or parasitic infection. Only a few published data are available concerning the role of NO· in carcinogenesis in vivo. Elevated formation of nitrate and N-nitrosodimethylamine has been shown to occur in woodchucks infected with woodchuck hepatitis virus (42). It has also been shown that hepatitis B virus surface antigen induces iNOS in cultured hepatocytes which may play a role in woodchuck hepatocarcinogenesis (43). Another example is CCC development in humans and in hamster models, which is closely associated with chronic inflammation due to infections with the liver flukes Clonorchis sinensis and Opisthorchis viverrini (1,44–46). Bile duct epithelial cells infected with the parasites produce NO·, which may induce acute or chronic damage to the cells. From the current study, it is evident that iNOS is produced in liver tumors of rats treated with NDEA by chronic infusion. iNOS may contribute to tumor promotion via NO· production and the subsequent action of peroxynitrite. The role of NO· in normal liver has been considered as a means of host defense. iNOS is induced in rat liver in the Kupffer cells, sinusoidal endothelium, bile duct epithelium and hepatocytes in response to lipopolysaccharide or acetaminophen treatment (27,47–49). The NO· formed in these cells may then be primarily used to attack invading organisms and also to regulate physiological functions. However, the expression of iNOS and formation of nitrotyrosine-containing protein that we observed in tumor tissues may be related to the promoting action of overproduced NO· and peroxynitrite. We detected iNOS expression mainly in proliferating oval cells in preneoplastic and neoplastic lesions. This suggests that NO· produced in the proliferating oval cells may play an important role in tumor cell growth and differentiation. NO· has been reported to enhance tumor cell growth by stimulating angiogenesis (50,51). It has also been shown that NO· contributes to cell differentiation (52), which may provide a molecular basis for aberrant differentiation of hepatocytes and oval cells to HCCs and/or biliary epithelial tumors. Usually, ductular reactions caused by cholestatic conditions, including bile duct ligation, or those caused by D-galactosamine injury rarely lead to tumors (10,53). This suggests that the proliferation of oval cells itself is not the main factor in tumor formation and that additional promotional stimuli may be necessary. Since iNOS is present in oval cells and capillary endothelial cells in tumor tissues and nitrotyrosine-containing protein in preneoplastic hepatocytes (PAHF and dysplastic hepatocytes), as shown in this study, NO· may act in cancer promotion by stimulating aberrant differentiation of the cells as well as angiogenesis. In order to further study the role of NO· in oval cell differentiation and hepatocarcinogenesis, it would be interesting to examine the expression of iNOS and nitrotyrosine in the liver of rats from the above non-carcinogenic models (10,53).

The histogenetic origin of HCC has been a matter of controversy for decades. In particular, HCCs of glandular pattern (adenoid type, cholangiomatous type and adenocarcinoma) have an intermediate phenotype between those of hepatocytes and of duct-like oval cells. They are believed to be derived either from differentiation of pre-existing ductular cells, on the one hand, or from transdifferentiation of hepatocytes toward glandular formation, on the other (54). iNOS has not been considered as a specific cell marker, like cytokeratin-19, M2-PK, GST-π and monoclonal antibody OV-6 in oval cells. However, the expression of iNOS in both oval cells and glandular HCCs suggests a common cellular lineage between these two cell types. The fact that iNOS was strongly expressed in proliferated bile ductules in HCC tissue and that mainly glandular HCCs showed iNOS localization in all HCC subtypes suggests that iNOS-positive tumor cells may have a biliary epithelial cell lineage. Furthermore, hepatocytes connected with oval cells by junctional complexes in a ductular structure were observed in preneoplastic lesions by transmission electron microscopy (not shown). This may also be a significant finding supporting the suggestion in terms of cellular differentiation. However, we, of course, do not exclude 3-nitrotyrosine-expressing PAHF and dysplastic hepatocytes in preneoplastic tissues as potential precursor cells of HCCs.

In conclusion, in rat tumor tissues induced by continuous infusion of NDEA, iNOS was often found in oval cells, capillary endothelial cells and glandular HCC cells and nitrotyrosine-containing protein in cells and/or tissues neighboring the iNOS-expressing cells. It therefore appears that NO· produced in these cells may play a role in carcinogenesis by inducing tissue and/or DNA damage, stimulating angiogenesis and impairing normal cellular differentiation.

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