RESEARCH ARTICLE

Increased Expression of Epithelial Cell Adhesion Molecule (EpCAM) in Rat Hepatic Tumors Induced by Diethylnitrosamine

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Abstract

The epithelial cell adhesion molecule (EpCAM) is a pan-epithelial differentiation antigen that is expressed on almost all carcinomas. However, a role in rat liver carcinogenesis has never been reported previously. Thus, its expression was investigated herein in rat liver tumors induced by diethylnitrosamine (DEN). Twenty male 5-week-old F344 rats were used in this experiment. Mini-osmotic pumps containing doses of 47.5 mg of DEN were inserted into the abdominal cavity of each animal to initiate liver carcinogenesis. All animals were sacrificed at 26 weeks after DEN treatment. At necropsy, hepatic masses were processed for histopathological examination, which revealed forty-four hepatocellular adenomas (HCAs) and twenty hepatocellular carcinomas (HCC). Tumors were immunohistochemically analyzed for EpCAM, proliferating cell nuclear antigen (PCNA) and co-localization of the two. EpCAM expression was mainly detected in hepatic tumor cells, showing a cytoplasmic staining pattern. However, expression was also slightly observed in normally-appearing surrounding hepatic cells. PCNA expression was highly detected in tumor cells, showing nuclear staining. Double staining of EpCAM and PCNA in tumors showed many cells with co-localization. Taken together, EpCAM and PCNA expression were increased in DEN-induced tumors and many tumor cells showed co-expression. It is suggested that EpCAM may increase during DEN-induced tumors, possibly associated with cell proliferation.

Key words: Hepatocarcinogenesis - DEN - EpCAM - PCNA - rat

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Introduction

Epithelial cell adhesion molecule (EpCAM) is a membrane glycoprotein that is expressed on most normal epithelial cells. It was first proposed to function as a cell adhesion molecule since it mediates homophilic adhesion interaction, which might prevent metastasis (Litvinov et al., 1994). In contrast, EpCAM has been reported to abrogate E-cadherin-mediated cell-cell adhesion, indicating that is may have a different role in some other tumor type by promoting metastasis (van der Gun et al., 2010). In humans, EpCAM-expression in liver cells was rare in early stages of liver diseases and was increasingly prominent in later stages, consistently arrayed around the periphery of cords of hepatobiliary cells (Yoon et al., 2011). On the other hand, it was shown recently that EpCAM-positive cells were defined as novel prognostic subtypes of hepatocellular carcinomas (HCCs) in human (Yamashita et al., 2008).

Moreover, EpCAM-positive HCCs displayed a distinct molecular signature with features of hepatic progenitor cells including the presence of known stem cell markers, whereas EpCAM-negative HCCs displayed genes with features of mature hepatocytes (Yamashita et al., 2008).

Hepatic stem cells are located in the ductal plate in the embryonic and in the Hering canal of young and adult animals, and in the hepatic portal area (Turner et al., 2011). Because stem cells have the potential to survive, DNA damage induced by carcinogens may be fixed when cell proliferation occurs. Stem cells with a loss of DNA repair may be susceptible to malignant transformation either directly or through the emergence of cancer-prone stem cells (Kenyon and Gerson, 2007). In rodents, hepatic stem and/or progenitor cell origin of HCCs has been also postulated (Sell and Dunsford, 1989). Actually, many stem cell markers have been detected in solid tumors (Visvader and Lindeman, 2008).

Although EpCAM expression was shown to be elevated during human liver tumor progression, there are no reports about EpCAM expression in rat tumors yet. Therefore, the present study has investigated its

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expression in hepatic tumors of rats induced by DEN and its relationship with cell proliferation.

Materials and Methods

Animals and treatment

Twenty male 5-week-old F344 rats (Koatec Inc, Korea) were housed in a room maintained on a 12 h light/dark cycle, at constant temperature and humidity. Rats were allowed free access to a pellet chow diet (Koatec Inc, Korea) during the experiment. For the induction of liver tumors, mini-osmotic pumps were used (Alzet 2002; Durect, Cupertino, CA) providing a continuous infusion (0.5 μl/hour for 2 weeks) of DEN (N0756, Sigma, St Louis, MO) dissolved in dimethyl sulphoxide (DMSO). The mini-osmotic pumps were inserted into the abdominal cavity of each animal under ether anesthesia at 6 weeks of age to provide a total dose of 47.5 mg for each rat. All animals were sacrificed at 26 weeks after DEN treatment. At necropsy, hepatic masses were processed for histopathological examination.

Histopathological examination

At necropsy, hepatic masses were routinely processed for histopathological examination. Briefly, tumors were fixed in 10% neutral phosphate buffered formalin, embedded in paraffin, sectioned to a thickness of 4 μm, and stained with hematoxylin and eosin. Tumor characteristics were classified based on both histopathological and cytological criteria.

Immunohistochemical analysis of EpCAM

The avidin-biotin complex method was used to stain EpCAM in 4 μm liver sections. Sections were dewaxed with xylene and hydrated through using a graded ethanol series, and were then boiled in a sodium citrate buffer (pH 6.0) in an autoclave for 20 min. After that, the sections were sequentially treated with 0.3% hydrogen peroxide, blocking skim milk, and anti-EpCAM antibody (ab32392; Abcam) (diluted 1:400) for overnight at 4°C. They were then washed with TBS-T and subjected to ABC-peroxidase procedures (ABC Kit; Vector Laboratories). Skim milk was used instead of the primary antibody as a negative control.

Immune complexes were visualized using 3,3'-diaminobenzidine tetrahydrochloride as the chromogen. The sections were counterstained with Mayer's hematoxylin to facilitate their examination under a light microscope.

Immunohistochemical examination of PCNA and apoptosis

The avidin-biotin complex method was used to demonstrate PCNA in 4 μm sections of liver tissues. Sections were dewaxed with xylene and hydrated through a graded ethanol series. Sections in sodium citrate buffer (pH 6.0) were boiled in an autoclave for 25 min, and then were treated with 0.3% hydrogen peroxide, normal horse serum, anti-PCNA antibody (M 0744, Dako) at 1:500 dilution for overnight at 4°C, followed by ABC-peroxidase procedures (ABC kit, Vector Laboratories, Burlingame, CA). Immune complexes were visualized with 3,3'-diaminobenzidine tetrahydrochloride as a chromogen. As a negative control, normal serum was used instead of primary antibodies. The sections were counterstained with Mayer's hematoxylin to facilitate examination under a light microscope.

The avidin-biotin complex method was used to demonstrate apoptosis in 4 μm sections of liver tissues. Sections were dewaxed with xylene and hydrated through a graded ethanol series. Sections were then treated sequentially with proteinase K and 3% hydrogen peroxide, and were treated with equilibration buffer, TdT enzyme and anti-digoxigenin peroxidase conjugate according to the manufacturer's instruction (ApopTag® Peroxidase In Situ Apoptosis Detection Kit, Chemicon, CA). Immune complexes were visualized with 3,3'-diaminobenzidine tetrahydrochloride as a chromogen. As a negative control, phosphate buffered saline was used instead of TdT enzyme treatment. The sections were counterstained with Mayer's hematoxylin to facilitate examination under a light microscope.

Double staining of EpCAM and PCNA

Sections were dewaxed with xylene and hydrated through a graded ethanol series, and were boiled in a sodium citrate buffer (pH 6.0) in an autoclave for 20 min, and then were sequentially treated with 0.3% hydrogen peroxide, blocking horse serum, and EPOS anti-PCNA antiboy (M879; Dako) (diluted 1:500) for 1 h. Afterwards, sections were washed with TBS and incubated with HRP-Polymer (MRT621; BIOCARE) and immune complexes were visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB) as the chromogen.

Sections were then washed with TBS and incubated with goat serum and anti-EpCAM antibody (ab32392, Abcam) for 1-h and incubated with AP-Polymer (RMR625; BIOCARE) for 30 min. They were then washed with TBS and subjected to Vulcan fast Red 200μl (FR805H; BIOCARE. The sections were washed with TBS and counterstained with hematoxylin to facilitate their examination under a light microscope.

Results

Histopathological findings of liver tissues and hepatic masses

Histopathological examination for hepatic masses showed that forty-four samples were HCAs and twenty ones were HCCs. There were no cholangioma or cholangiocarcinoma. The tumor tissues exhibited nuclear pleomorphism and alteration of cellular structure, with or without fatty liver and inflammatory cell infiltrations.

Immunohistochemical examination of EpCAM

EpCAM expression was highly detected in tumor

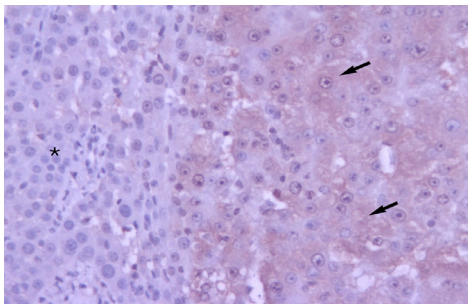

Figure 1. Epithelial Cell Adhesion Molecule (EpCAM) Expression in a Diethylnitrosamine (DEN)-induced Hepatocellular Tumor. Note the high levels of expression of EpCAM in tumor cells, showing cytoplasmic staining (arrow), but no expression observed in normally-appearing hepatocytes (asterisk), ×400.

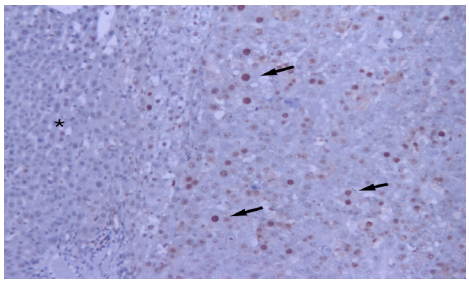

Figure 2. Proliferating Cell Nuclear Antigen (PCNA) Expression in a DEN-induced Hepatocellular Tumor. Note the high levels of expression of PCNA in tumor cells, showing nuclear staining (arrow), however its expression was rarely observed in normally-appearing hepatocytes (asterisk), ×200.

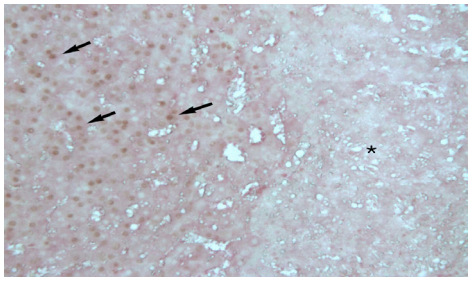

Figure 3. Co-expression of PCNA and EpCAM in DEN-induced Tumor. Note co-expression of PCNA (nuclear staining) and EpCAM (cytoplasmic staining) in tumor (arrow) but its expression not observed in normal appearing hepatocytes (asterisk), ×200.

masses, showing cytoplasmic staining pattern. However, its expression was slightly observed in normally-appearing surrounding hepatic cells, also cytoplasmic staining pattern (Figure 1). EpCAM expression was not found different between HCAs and HCCs.

Immunohistochemical examination of PCNA and apoptosis

PCNA expression was highly detected in tumor masses, showing nuclear pattern. However, its expression was rarely observed in normally-appearing surrounding hepatic cells (Figure 2). The apoptotic cells were slightly observed in tumor masses. However, its expression was not different between normally-appearing surrounding hepatic cells and tumor tissues (data not shown).

Double staining of EpCAM and PCNA

Double staining of both EpCAM and PCNA showed that many tumor cells had obvious co-localization, however, synergistic expression was not detected in normally appearing hepatocytes (Figure 3).

Discussion

In this study, EpCAM expression was strongly expressed in DEN-induced hepatic tumor cells of rats, but was slightly detected in the surrounding normally-appearing cells.

EpCAM is considered as one of the stem cell markers and its expression has been identified as an additional marker of cancer-initiating cells (van der Gun et al., 2010) and was considered as hepatic stem/progenitor cells in HCCs subtype (Yamashita et al., 2009). In this study, EpCAM expression showed diffuse cytoplasmic staining in tumor cells. Previous data has shown that EpCAM expression was found in the cytoplasm of hepatic stem cells (Turner et al., 2011). It has been hypothesized that stem cells may be transformed into cancer stem cells (Wicha et al., 2006), by a process involving the dysregulation of stem cell self-proliferation (Al-Hajj and Clarke, 2004). As EpCAM-positive HCCs exhibited stem cell and progenitor cell markers (Yamashita et al., 2008), further studies are warranted to investigate the co-expression of EpCAM and other stem cell markers in the context of rat hepatocarcinogenesis.

Diethylnitrosoamine (DEN) is used to induce rat liver cancer models (Peto et al., 1991; Pitot et al., 1996) and was also reportedly to be hydroxylated by P450 isozymes in the liver to a bioactive metabolite (Verna et al., 1996). This appears to be associated with early hepatocyte injury and liver carcinogenesis (Kang et al., 2007). Hepatic tumors were generated as a result of DEN treatment in animals and about 1% cells were shown to be developed into altered hepatic foci when treated with tumor promoter(s) (Pitot et al., 1996). Carcinogenesis is believed to be a multistage process involving initiation, promotion, and progression, and tumor cells are believed to arise as a result of several cellular events such as dysregulation of proliferation and/or apoptosis. In this study, tumor cell showed high expression level of PCNA, representing high proliferation stage of hepatic tumors, however, no expression in normal appearing hepatocytes. A recent report showed that regulated intramembrane proteolysis activates EpCAM as a mitogenic signal transducer, resulting elevated expression of c-myc and cyclin A and E (Maetzel et al., 2009).

Furthermore, liver injury in response to DEN exposure elicits an inflammatory response in nonparenchymal cells, which secrete several cytokines and growth factors that promote compensatory proliferation of quiescent hepatocytes carrying DEN-induced mutations (Arsura and Cavin, 2005). Double staining of EpCAM and PCNA

had a co-localization of both antibodies in DEN-induced tumors, but its expression was not or rarely observed in normally-appearing hepatocytes. Thus it is thought in the present study that EpCAM-expressed hepatocytes may be associated with high cellular proliferation.

Even though no direct evidence is available, it is assumed that liver stem cells could be more easily transformed by DNA damage than mature hepatocytes, yielding cancer stem cells. This process may allow the transmission of genetic alterations to daughter cells, thereby favoring neoplastic progression in the liver. Further studies are warranted to investigate the role and fate of stem cells after DNA damage and co-localization of stem cell markers and DNA damage markers and the possibility of transformation of stem cells into cancer stem cells.

In conclusion, EpCAM expression was increased in DEN-induced tumors and it is suggested that EpCAM may be gradually increased during rat hepatocarcinogenesis. The data point to an early and gradual effect of DEN in the tumor initiating stem cell which is believed to be accompanied with synergistic initiation of cellular proliferation leading to liver carcinogenesis.

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