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Annexin A2 expression and phosphorylation are up-regulated in hepatocellular carcinoma

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Abstract. Annexins (ANXs) constitute a family of Ca^{2+}-dependent membrane-binding proteins; at least 20 of them have been described to date. Among these, Annexin A2 (ANXA2) has been revealed as a multi-functional protein *in vitro*. Its actual role *in vivo*, however, requires further investigation. We already reported that ANX-I (ANXA1) was up-regulated in hepatocellular carcinoma (HCC). The role of ANXA2 in various liver diseases including HCC remains obscure. In the present study, the protein and mRNA levels of ANXA2, as well as its localization, were determined for the normal human liver, chronic hepatitis liver, and non-tumorous and tumorous portions of HCC tissues. ANXA2 was rarely detected in either normal or chronic hepatitis liver tissues, whereas it was overexpressed at both the transcriptional and translational levels in tumorous and non-tumorous regions of HCC. In addition, in many cases, more ANXA2 was expressed in the tumorous portion than in the non-tumorous portion of HCC. The expression of ANXA2 was mainly localized in cancer cells, especially in poorly differentiated HCC. Furthermore, ANXA2 was tyrosine-phosphorylated in HCC. These data suggest that overexpression and tyrosine phosphorylation of ANXA2 play important roles in the malignant transformation process leading to HCC and are related to the histological grade of HCC.

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Introduction

Annexins (ANXs) constitute a family of Ca^{2+}-dependent phospholipid-binding proteins with various membrane-related functions (1,2). At least 20 members of the Annexin family have been described to date (3). The proteins included in this family are lipocortins (4,5), calpactins (6,7), calelectrins (8-10), calcimedins (11), anticoagulant proteins (12), endonexins (13), macrocortin (14,15), and chromobindins (16). ANXs were first described as glucocorticoid-inducible inhibitory proteins of phospholipase A2 (14,15). These proteins have a unique N-terminal tail that is variable in length and sequence (17). ANXA2, also called Annexin II, is one of the best characterized of the group of ANXs (18). ANXA2 among ANXs is cleaved by chymotrypsin into a 33-kDa C-terminal core domain and 3-kDa N-terminal domain of 30 amino acids (19). The N-terminal domain of ANXA2 is a major substrate for phosphorylation by retroviral tyrosine-specific kinase ($pp60^{v\text{-}src}$) (20). Residues of Tyr 23 and serines 11 and 25 are phosphorylated in cells by c-Src and PKC, respectively (21), after activation of insulin receptor (22), insulin growth factor regulator (23), or platelet-derived growth factor-R (24). Therefore, ANXA2 may play a role as a second messenger for transduction of the growth, differentiation and transformation (3).

The role of ANXA2 in cellular transformation was first suggested by the identification of ANXA2 as the oncogene product v-src phosphorylation substrate in transformed fibroblasts (25). Subsequently, ANXA2 expression has been found to be up-regulated in several types of spontaneous neoplasms such as pancreatic cancer (26), gastric cancer (27), colorectal cancer (28) and high-grade glioma (29). On the other hand, its expression was down-regulated in prostate (30-32) and esophageal (33) cancer and in head and neck dysplasia and squamous cell carcinoma (34,35). In our previous report, ANXA2 was increased in proliferative or regenerative hepatocytes, suggesting that this protein plays a certain

role in the proliferation events of normal hepatocytes (36). However, its expression in cases of liver disease, including hepatocellular carcinoma (HCC), is not well known. Therefore, we analyzed the amount of ANXA2 at the protein and messenger RNA (mRNA) levels, as well as its localization in various liver tissues including HCC. We also investigated the relationship between ANXA2 expression and histological grade of HCC. In addition, we compared the tyrosine phosphorylation of ANXA2 in HCC with that in non-tumorous liver cirrhosis.

Patients and methods

Patients. Between 1997 and 2007, liver biopsy specimens were obtained from 39 patients with chronic hepatitis (CH) and 34 patients with HCC at Kagawa Medical University. Of those patients with CH, 31 patients were positive for HCV RNA, and others were positive for HBsAg. Seven patients were in F1, 9 patients in F2, 11 patients in F3, and 12 patients in F4 according to Desmet's classification (37). The mean age of the patients was 50.9±13.4 (mean ± SD; range 21-80). HCC specimens were obtained from 6 patients by surgical resection and the others were obtained by needle biopsy before therapy. Twenty-eight patients were males, and 6 were females. The mean age of the patients was 64.5±9.3 (mean ± SD; range 34-84 years). Thirty patients with HCC were positive for HCV-RNA, and 4 patients with HCC were positive for HBsAg. The histological grade of HCC was determined as well, moderately or poorly differentiated according to the criteria of the International Working Party (38). The numbers of patients with well, moderately and poorly differentiated HCCs were 4, 22 and 8, respectively. Five normal liver (NL) samples (4 males and 1 female; mean age 60.2±4.1 years; range 56-67 years) were obtained during surgery for colon cancer with liver metastasis. These patients were negative for both HCV-RNA and HBsAg. Informed consent was obtained from each patient prior to participation.

Chemicals. The monoclonal antibody against ANXA2 was purchased from Nippon Shinyaku Co., Ltd. (Tokyo, Japan). An anti-phosphotyrosine monoclonal antibody was purchased from Upstate Biotechnology Co., Ltd. (Tokyo, Japan). The monoclonal antibody (clone IgG2bκ) was produced *in vitro* by the mouse-mouse hybridoma, 4G10. This antibody reacts to phosphotyrosinated proteins, but not with those containing phosphoserine or phosphothreonine. Other chemicals were purchased from Sigma Chemical Co. (Tokyo, Japan) or Wako Pure Chemical Co. (Tokyo, Japan).

Gel electrophoresis and Western blot analysis. SDS (sodium dodecyl sulfate)-PAGE (polyacrylamide gel electrophoresis) was performed according to the method of Laemmli (39). Western blot analysis was performed according to the method of Towbin *et al* (40) using a mouse monoclonal antibody to ANXA2 in a 1:400 dilution, and a horseradish peroxidase-linked secondary antibody. Immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (Amersham Chemical Co, Tokyo, Japan) on X-ray film, as described in our previous reports (41-51).

Partial purification of ANXA2. ANXA2 was partially purified from liver tissues according to the procedure of the previous reports (36,44). Tissue samples were frozen on dry-ice within 20 min of collection. The samples were homogenized in TNE buffer [10 mM Tris HCl (pH 7.5), 1 mM EGTA, 150 mM NaCl, 1 mM Na_3VO_4, 50 mM Na_2MoO_4, 1% Nonidet P-40 and 100 U/ml aprotinin] and centrifuged at 29,000 g for 15 min at 4˚C. The livers were washed and homogenized in 10 mmol/l dithiothreitol, 100 μg/ml soybean trypsin inhibitor, 1 μg/ml leupeptin, 1 mmol/l diisofluorophosphate, and 10 mmol/l $CaCl_2$. After standing for 20 min at 4˚C, the homogenate was centrifuged for 15 min at 2,900 g, then the supernatant was centrifuged for 40 min at 27,000 g. The pellet was resuspended in buffer A (100 nmol/l NaCl, 1 mmol/l dithiothreitol, 1 mmol/l $CaCl_2$, 1% Triton X-100 and 10 mmol/l imidazole-hydrochloride, pH 7.4), stirred for 20 min, and centrifuged at 25,000 g for 30 min. The pellet was washed twice with buffer A without Triton X-100 and resuspended in buffer B (100 mmol/l NaCl, 1 mmol/l dithiothreitol, 1 mmol/l ethylene glycol tetraacetic acid, and 10 mmol/l imidazole-hydrochloride, pH 7.4), then centrifuged at 100,000 g for 70 min. The supernatant was extensively dialyzed against buffer C (1 mmol/l dithiothreitol, 10 mmol/l imidazole-hydrochloride, pH 7.4). The fractions were subjected to SDS-PAGE and Western blotting using ANXA2 monoclonal antibody. The protein concentration in partial purification of ANXA2 was measured by the bicinchoninic acid protein assay (52).

Immunohistology for human liver tissues. We prepared 2-μm-thick sections from formalin-fixed, paraffin-embedded tissue blocks. Sections of HCC tissue specimens were immunohistologically stained using the ABC method, as described in our previous reports (52,53). To detect ANXA2, sections were placed in 10 mM citrate buffer (pH 6.0) and processed at 500 W at 95˚C for 10 min in a microwave oven. Sections were deparaffinized in xylene, dehydrated in a graded series of alcohol solutions, and then mixed with a solution containing 0.5% hydrogen peroxide to block endogenous peroxidase activity. After washing with phosphate-buffered saline (PBS), the sections were processed for immunostaining. Primary incubation was performed overnight at 24˚C with the monoclonal antibody against ANXA2. Immunoreactivity products were visualized using diaminobenzidine, and the sections were counterstained with Mayer's hematoxylin. The specificity of immunostaining was examined using non-immune mouse IgG as a negative control for primary antibody.

Extraction of RNA and Northern blot analysis. The total RNA fraction was extracted from the liver using acid-guanidinium thiocyanate-phenol-chloroform. The probe used for Northern blot analysis was the cDNA clone 123, which has an open reading frame of 1,008 nucleotides and encodes a protein of 339 amino acids. This cDNA was kindly provided by Dr T. Hunter (Molecular Biology and Virology Laboratory, San Diego, CA). Total RNA isolated from liver tissues was denatured and separated by electrophoresis in formaldehyde agarose gel. RNA was transferred to a Hybond N membrane (Amersham, Chemical Co., Tokyo, Japan). After prehybridization at 42˚C for 4 h, the membranes were incubated at 42˚C

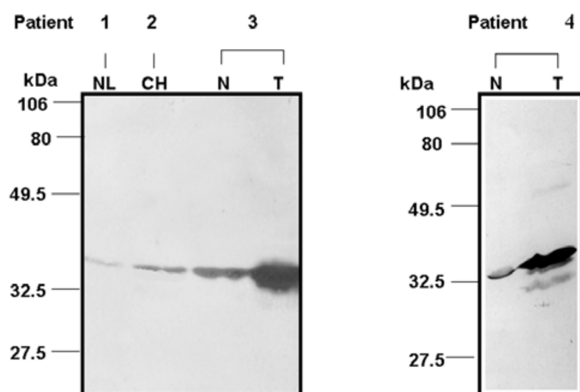

Figure 1. Western blot analysis of partially purified ANXA2 using a monoclonal antibody as a probe. Twenty micrograms of the partially purified ANXA2 fraction of tumorous (T) and non-tumorous (N) regions of HCC, chronic hepatitis (CH) and normal liver (NL) tissues were subjected to SDS-PAGE and analyzed as described in Materials and methods. A highly immunoreactive ANXA2 band is seen in the T portion of hepatocellular carcinoma. The histology of the N portion in cases 3 and 4 is cirrhosis and chronic hepatitis, respectively.

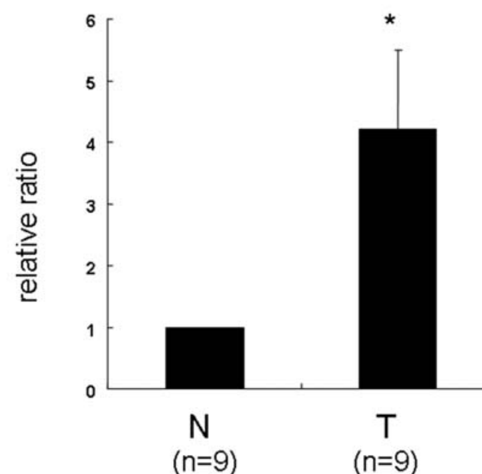

Figure 2. Densitometry of Western blot analysis of ANXA2 in tumorous (T) and non-tumorous (N) portions of HCC. The ratio in ANXA2 band density of T tissues to N tissues was 4.2±1.3. The T portions contained significantly more ANXA2 than the N regions ($P<0.01$).

for 15 h in a hybridization mixture containing 50% formamide, 5X standard saline citrate, 10 nmol/l sodium phosphate (pH 6.8), 0.5% SDS, 5X Denhart's medium, 20 μg/ml salmon sperm DNA, and the [^{32}P]-labeled cDNA probe. The membranes were washed three times with 0.2X standard saline citrate containing 0.1% SDS for 30 min at 42˚C, air-dried, and exposed to Kodak X-Omat film (Eastman Kodak, Rochester, NY) at -70˚C.

Immunoprecipitation. Aliquots containing 50 μg of the partially purified ANX fractions from HCC were incubated with 2 μl (2 μg) of monoclonal phosphotyrosine antibody for 4 h at 4˚C. Samples were then incubated with 50 ml of 50% (vol/vol) protein A sepharose CL-4B in buffer [20 mmol/l Tris-HCl (pH 7.5), 150 mmol/l NaCl, 1 mol/lethylene-diaminetetraacetic acid, 0.02% sodium azide (wt/vol), 0.5% NP40, and 10 μg/ml bovine serum albumin]. Immuno-precipitates were washed four times in 100 mmol/l Tris-HCl (pH 7.5) containing 0.5% Triton X-100, then resuspended in SDS-PAGE buffer [50 mmol/l Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 0.02% bromophenol blue]. Proteins were resolved in 12.5% SDS-PAGE, and phosphorylated ANXA2 was detected by Western blotting using monoclonal ANXA2 antibody.

Densitometry. The density of the immunoreactive band of 36 kDa for ANXA2 obtained on Western blotting using was analyzed by densitometric scanning (Tlc scanner, Shimizu, Co., Ltd., Kyoto, Japan).

Statistical analysis. The significance of the difference on Western blotting of ANXA2 was determined with the Student's t-test. Data are expressed as means ± SD. The quantitative variables were analyzed with the χ^2 test to compare the relationship between ANXA2 protein expression and HCC differentiation from immunohistochemical data. The significance of the differences between observations was determined at a $p<0.05$.

Results

Western blot analysis of AXNA2 in liver tissues obtained by surgery or biopsy. The level of ANXA2 expression was markedly increased in the primary tumorous (T) tissues compared with adjacent non-tumorous (N) tissues (Fig. 1, patients 3 and 4). The histological grades of patients 3 and 4 were moderately and poorly differentiated HCC, respectively. A very weak band was detected at 36 kDa in normal liver (NL) (no. 1) and chronic hepatitis (CH) (no. 2) patients.

Densitometry of Western blotting for ANXA2. The density of the immunoreactive 36-kDa band obtained by Western blotting was analyzed by means of densitometric scanning (Fig. 2). The densitometric ratio of the ANXA2 band of T tissues to N tissues was 4.2±1.3 ($p<0.01$), indicating that significantly more ANXA2 was present in the T parts than in the N regions of HCC.

Localization of ANXA2 in NL, CH, N and T portions. Expression of ANXA2 was not detected in NL (Fig. 3A, patient 1), but in CH, staining for ANXA2 appeared only in some hepatocytes adjacent to the area with the inflammation (Fig. 3B, patient 2, arrows). In cirrhotic liver tissues with HCC, ANXA2 was expressed only in some hepatocytes along the limiting plate and at the periphery of the regenerating nodules (Fig. 3C, patient 3, arrows). ANXA2 was also immunostained in biliary epithelial cells (Fig. 3C, patient 3, arrowhead). Typical ANXA2 staining in HCC is shown in Fig. 3D (patient 3). The expression of ANXA2 in HCC was detected not only in the cytoplasm (Fig. 3D), but also in the membranes (Fig. 3D, arrows) of cancer cells. In patient 4, ANXA2 expression was not seen in N tissue corresponding to NL, but was stained in cancer cells (Fig. 3E, arrows). All patient numbers correspond to those in Fig. 1. In addition, all HCC tissues expressed ANXA2, and poorly differentiated tissues were more strongly positive than well differentiated tissues ($P<0.01$) (Table I).

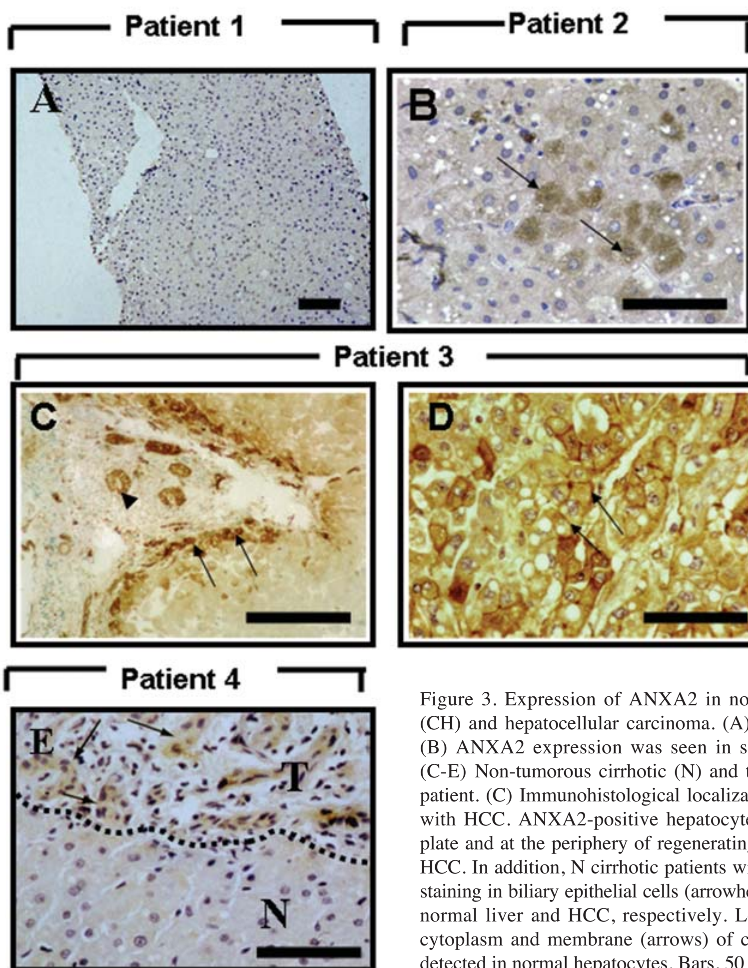

Figure 3. Expression of ANXA2 in normal liver (NL), chronic hepatitis (CH) and hepatocellular carcinoma. (A) ANXA2 was not detected in NL. (B) ANXA2 expression was seen in some hepatocytes (arrows) in CH. (C-E) Non-tumorous cirrhotic (N) and tumorous tissue (T) from the same patient. (C) Immunohistological localization of ANXA2 in cirrhotic tissues with HCC. ANXA2-positive hepatocytes were located along the limiting plate and at the periphery of regenerating nodules in N cirrhotic livers with HCC. In addition, N cirrhotic patients with HCC showed ANXA2 immunostaining in biliary epithelial cells (arrowhead). (D and E) 'N' and 'T' indicate normal liver and HCC, respectively. Localization of ANXA2 was in the cytoplasm and membrane (arrows) of cancer cells, while no staining was detected in normal hepatocytes. Bars, 50 μm.

Table I. Relationship between ANXA2 protein expressed and degree of HCC differentiation.

	ANXA2 protein status			
	-	+1	+2	+3
Well differentiated	0	4	0	0
Moderately differentiated	0	16	6	6
Poorly differentiated	0	0	0	8

The staining of cancer cells in HCC specimens was studied in 34 patients with HCC. ANXA2 was correlated to the degree of differentiation of cancer cells (P<0.01).

Northern blotting of ANXA2 in NL, CH and N and T portions of HCC. Equal amounts of total RNA were used for NL, CH, N cirrhotic tissues, and T tissues (Fig. 4A and C). Autoradiography analysis revealed a single band of the 1.6-kb mRNA of ANXA2 in T tissues and surrounding N cirrhotic tissues. More ANXA2-mRNA was expressed in T portions than in N portions (patients 3 and 4 in Fig. 4B and D), whereas it was not only undetectable in NL (patient 1 in Fig. 4B), but also in NL with HCC (patient 4 in Fig. 4). ANXA2 mRNA was also expressed at very low levels in CH (patient 2 in Fig. 4B). All patient numbers correspond to the numbers in Figs. 1 and 3.

Detection of tyrosine-phosphorylated ANXA2 in HCC. In one of the nine cases examined in this study, the ANXA2 levels in T and N cirrhotic tissues were similar on Western blotting (Fig. 5A). Overexpressed ANXA2 in T portion was shown to have phosphotyrosine residues by means of Western blotting using anti-phosphotyrosine monoclonal antibody (PY) (Fig. 5B). However, the ANXA2 overexpressed in N cirrhotic tissue was not tyrosine-phosphorylated (Fig. 5B).

Discussion

ANXA2 is thought to be involved in the cellular signal transduction associated with inflammation, differentiation and proliferation (3). We have already reported that ANXA2 levels increase in proliferative (or regenerative) hepatocytes suggesting that it plays a role in the proliferative events (36). Although Frohlich *et al* reported that ANXA2 was up-regulated in HCC (54), the expression in CH and liver cirrhosis was not clear, and the phosphorylation of ANXA2 was not

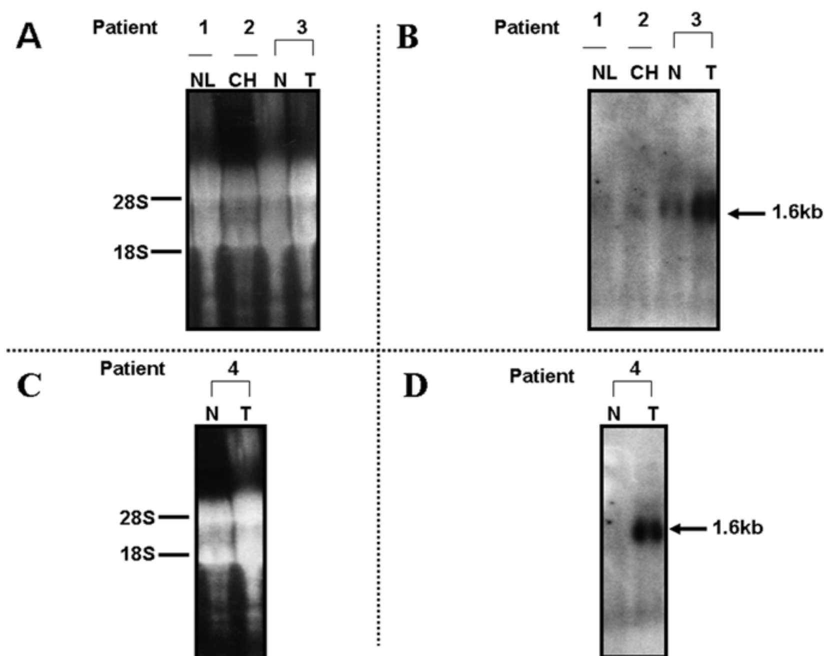

Figure 4. Northern blot analysis of ANXA2 in normal liver (NL), chronic hepatitis (CH), non-tumorous (N) and tumorous (T) portions of hepatocellular carcinoma. Patient numbers correspond to those in Fig. 1. (A and C) Twenty micrograms of total RNA was loaded in each lane, by the intensity of the bands of 28S and 18S ribosomal RNA in agarose gel stained with ethidium bromide and visualized under UV illumination. (B) Northern blot analysis of ANXA2 mRNA using ANXA2 cDNA as a probe. A single band of ANXA2 mRNA at 1.6 kb (arrows) was detected in CH (patient 2), non-tumorous cirrhotic tissue (N) and the tumorous portion (T) (patient 3). The expression of ANXA2 mRNA in the T portion was higher than that in the N portion. However, no bands were detected in NL (patient 1 and the N portion from patient 4). The histology of the non-tumorous portion in cases 3 and 4 is cirrhosis and normal tissue, respectively.

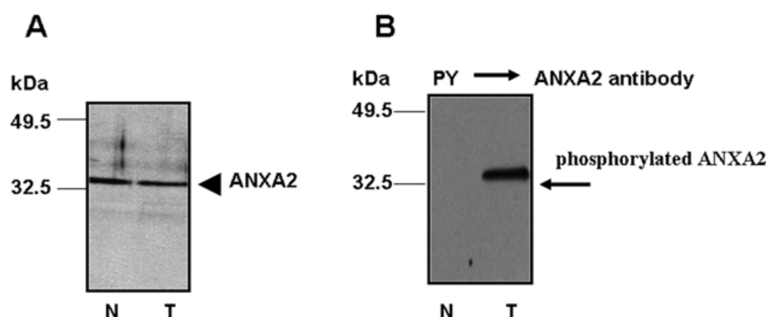

Figure 5. Tyrosine phosphorylation of ANXA2 in non-tumorous (N) and tumorous tissue (T) of HCC. (A) In one case, the ANXA2 levels in N and T tissues from the same patient were similar on Western blotting (arrowhead). (B) Western blotting of ANXA2 after immunoprecipitation using an anti-phosphotyrosine antibody (PY) in the N and T portions of HCC. The tyrosine phosphorylation of ANXA2 was detected in T portions of HCC (arrow), but not in N cirrhotic tissue.

studied. Therefore, the expression of ANXA2 in various liver diseases including HCC has not been adequately analyzed.

In this study, we showed that the expression ANXA2 was abundant in HCC and N cirrhotic tissues at both the transcript and protein levels, and that more ANXA2 was expressed in the T tissues than in the N cirrhotic tissues. On the other hand, ANXA2 was expressed at very low levels in NL and CH tissues. Moreover, ANXA2 was expressed more in poorly differentiated than in well and moderately differentiated HCC. We also showed that the enhanced ANXA2 in HCC was tyrosine-phosphorylated.

The levels of ANXA2 are highest in pancreas and skin tissues, intermediate in lung, colon, heart and spleen tissues, and low in muscle, kidney, stomach, esophagus and liver tissues (54). In the present study, we also showed that the protein and mRNA levels of ANXA2 were extremely low in NL and CH (Figs. 1, 3 and 4). However, the expression of ANXA2 was enhanced in and the T and N cirrhotic portion of HCC. The amount of ANXA2 in the T portion of HCC was 4.2±1.3 times higher than that in the N portion (Fig. 2). These observations suggest that induction of ANXA2 is associated with a malignant transformation process leading to HCC. In previous reports, overexpression of ANXA2 has been reported in pancreatic (26), gastric (27), brain (29), breast (55), lung (56), hematologic (57,58), and colonic cancer (59). Considering our data and these previous reports, the induction of ANXA2 may be related to malignant processes in various malignant tumors.

ANXA2 is involved in cell differentiation (3). We analyzed the relationship between ANXA2 expression and the histological grades of HCC. ANXA2 staining was more intense in moderately or poorly differentiated HCC, rather than in well differentiated specimens (Table I). These data suggest that the expression of ANXA2 may also be closely related to the histological grade of HCC.

We also showed that the overexpressed ANXA2 in HCC was tyrosine-phosphorylated. The ANXA2 level in HCC was similar to N cirrhotic tissues in only 1 case. Interestingly, the tyrosine phosphorylation of ANXA2 was detected in HCC but not in N cirrhotic tissue. Hunter *et al* have reported that chicken cells transformed by Rous sarcoma virus exhibit up to 8-fold more tyrosine phosphorylation than uninfected cells (60). These data suggest that tyrosine phosphorylation is an important event in hepatocarcinogenesis. It has been reported that ANXA2 is an excellent substrate for Src. We have reported that the level of Src kinase activity in HCC is higher than that in N cirrhotic tissues (61). These data suggest that ANXA2 in HCC may be tyrosine-phosphorylated via the elevated tyrosine kinase activity of Src or other kinases, and that increased ANXA2 amounts and its tyrosine phosphorylation may be related to human hepatocarcinogenesis.

In conclusion, ANXA2 is almost undetectable in normal liver and chronic hepatitis tissues, but its synthesis is induced in transformed hepatocytes. The overexpression and tyrosine phosphorylation of ANXA2 may be among several factors that contribute to malignant transformation, progression and differentiation in HCC. Therefore, HCC may offer a novel model for studying the functions of ANXA2 in malignant transformation.

Acknowledgements

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