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Decreases of Metallothionein and Aminopeptidase N in Renal Cancer Tissues[1]

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Good molecular markers for investigating the biochemical differences between renal cancer and surrounding tissues have not yet been developed. Sixteen kidney samples (clear cell RCC) were investigated to determine the differences in the protein components between renal cancer and surrounding tissues, using HPLC analysis. The metallothionein (MT) and zinc levels were consistently lower in renal cancer tissues compared with in surrounding tissues. The mean concentration of MT in normal tissues surrounding renal tumors was about 15 times higher than that in cancer tissues. An immunohistochemical study confirmed that the expression of MT in renal cancer tissues was lower than that in adjacent normal tissues. The activities of aminopeptidases (APs) were significantly decreased in renal cancer tissues compared with in adjacent normal tissues. An immunohistochemical study and Western blot analysis confirmed that the expression of AP-N in renal cancer tissues was also lower than in adjacent normal tissues. These results suggest that the immunohistochemical detection of MT and AP-N could provide useful information as a pathological diagnostic tool for classifying renal cancer and surrounding tissues.

Key words: aminopeptidase N, metallothionein, renal cancer, zinc.

Many molecular markers, which indicate the biochemical differences between cancer and surrounding normal tissues, have been studied. Zinc is an essential heavy metal in the human body, and is most abundant in prostate tissue (*1*) and is a constituent of more than 200 enzymes. Zinc plays an important role in nucleic acid metabolism, cell replication, tissue repair, and growth, and is required for tumor growth (*2*). Although several studies have focused on the evaluation of the zinc concentration in renal cell carcinomas, the concentration has been reported to vary, as it is high and low. This seems to result from the great fluctuation of the zinc concentration in kidney tissues.

Metallothionein (MT) is a small, cysteine-rich protein that acts as a reservoir for essential metals, and contributes to the homeostatic regulation of essential metals such as zinc and copper. This protein is also involved in the detoxification of nonphysiological metals and plays a protective role in metal-stressed individuals (*3*). Both serum and urinary MT have been observed to increase significantly in metal-stressed individuals. Exposure to heavy metal cations, both *in vivo* and *in vitro*, has been associated with alteration of immune responses and immunotoxicity. The long term result of this can be autoimmunity, immunosuppression, or even cellular transformation (*4*). Most MT binding of heavy metals is intracellular, but extracellular forms of the protein are released from injured or dead cells. Tumor cells can cause injury to and death of the surrounding normal tissues, thereby increasing the MT level in the extracellular space. The biosynthesis of MT has been reported to be regulated by steroid hormones (*5*). MT also seems to participate in the scavenging of oxygen free radicals (*6*), and has also been implicated in drug resistance in cancer chemotherapy (*7*).

Several studies have been performed to evaluate new molecular markers in renal cell carcinomas, however, few of the various parameters appear to be better predictive prognostic factors than clinical criteria such as staging. Thrasher (*8*) recently reported that the cellular adhesion molecule CD44, as an independent prognostic parameter, is useful for predicting the progression tendency of renal cell carcinomas. CD44 represents a large family of adhesion molecules involved in cell–extracellular matrix interactions.

Aminopeptidase N (AP-N; CD13, EC 3.4.11.2) is a cell surface glycoprotein similar to CD44. This molecule is a zinc ion–dependent exopeptidase which cleaves neutral amino acids from the N-terminals of oligopeptides. It is well known to be highly expressed on the brush border membranes of the small intestine and renal proximal tubules (*9*). In fact, AP-N constitutes 8% of the total protein in the intestinal brush border membrane, where it participates in the final hydrolysis of ingested nutrients. This enzyme is also present in the breast (*10*), colon (*11*), and synaptic

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Abbreviation: RCC, renal cell carcinoma.

membranes of the central nervous system (*12*). It has been reported that the neuropeptides, enkephalin and endorphin, are principally degraded by AP-N. AP-N might also act as a corona virus receptor (*13*), and has been implicated in antigen processing (*14*). Further reported functions of AP-N include degradation of collagen type IV with a proposed role in tumor invasion (*15*). Although AP-N expression in tumor progression is involved in growth (*16*) and metastasis (*17, 18*), the precise relation of AP-N to renal cancer remains unclear.

Since we first found the difference between the chromatograms when the protein components in human renal cancer and surrounding tissues were analyzed by HPLC, this difference is biochemically investigated in this paper. We demonstrate that the expression levels of MT and AP-N decrease markedly in renal cancer tissues compared with in surrounding normal tissues, and discuss that these proteins may be good markers in pathological diagnosis.

EXPERIMENTAL PROCEDURES

Materials—Kidney tissues (clear cell RCC) were obtained from the archives of the Department of Urology of the Aichi Cancer Center Research Institute, through a protocol approved by the Institutional Review Board, and stored at −80°C until use. Fluorogenic peptide substrates such as Met-MCA and Gly-Pro-MCA were purchased from the Peptide Institute (Osaka). 2-(5-Bromo-2-pyridylazo)-5-(*N*-propyl-*N*-sulfopropyl-amino) phenol disodium salt dihydrate (5-Br-PAPS) was obtained from Dojindo Laboratory (Kumamoto). Purified AP-N from human prostate was used. All other chemicals were of analytical grade.

Preparation of Human Renal Cytosol and Membrane Fractions—Frozen human kidney tissue was thawed on ice, minced, and then homogenized in 3 volumes of 20 mM potassium phosphate buffer, pH 6.5, containing 0.32 M sucrose, using a Polytron (Westbury, NY). The homogenate was centrifuged at 140,000 ×*g* for 60 min, and the supernatant and precipitate obtained were used as the cytosol and membrane fractions, respectively.

HPLC Analysis—Samples were subjected to HPLC on a reversed-phase column (μ Bondasphere, 5 μm, C18, 300 A, 3.9 × 150 mm), and eluted with a linear gradient formed between 10 and 60% acetonitrile containing 0.1% trifluoroacetic acid at the flow rate of 1 ml/min. Proteins were monitored by measuring the absorbance at 210 nm.

Analysis of N-Terminal Amino Acids—The purified protein obtained on HPLC was digested with *Achromobacter* protease I at 37°C overnight in 50 mM Tris-HCl, pH 9.0, containing 2 M urea, and then cleavage with 1% cyanogen bromide was performed in 70% formic acid at room temperature. The resulting peptides were separated by HPLC. The amino acid sequence was determined with a protein sequencer (Applied Biosystems model 473 A). Sequence homology was searched in the protein sequence database of the SWISS-PROT.

Cadmium-Saturation Assay for MT—The total amount of MT in tissues was determined by means of the cadmium saturation assay (*19*). The cytosol fraction was incubated with 0.2 μg of Cd^{2+} at room temperature for 10 min, and then bovine hemoglobin was added to the mixture for the binding of excess Cd^{2+}. The mixture was placed in boiling water for 1 min and centrifuged at 10,000 ×*g* for 3 min after cooling. The concentration of Cd^{2+} in the supernatant was determined with an atomic adsorption spectrophotometer (Shimadzu AA-6500).

Zinc Assay—Zinc was assayed by the method of Yamashita *et al.* (*20*). After the sample had been treated with trichloroacetic acid, 1.6 ml of 80 mM 5-Br-PAPS in 0.2 M carbonate buffer, pH 9.8, and 0.4 ml of 29 mM salicylaldoxime were added to 0.2 ml of the resulting supernatant, and then the reaction mixture was incubated for 10 min at room temperature. The absorbance at 560 nm was measured.

Assay for Peptidase Activity—Peptidase activity was determined fluorometrically with 4-aminocoumarin (AMC)–peptide conjugates as substrates. The reaction mixture containing 50 μl of 100 μM substrate, 30 μl of 100 mM Tris-HCl buffer, pH 8.0, and 20 μl of enzyme solution was incubated at 37°C for 20 min. The enzyme reaction was terminated by the addition of 2 ml of 1 M acetic acid. The fluorescence intensity was measured at excitation and emission wavelengths of 380 and 460 nm, respectively.

Western Blot Analysis—The purified AP-N from human prostate, and the membrane fractions from renal cancer and surrounding normal tissues were subjected to electrophoresis on an 8% SDS–polyacrylamide gel under reducing conditions, and then proteins resolved in the gel were transferred to a nitrocellulose membrane. The membrane was incubated with a polyclonal antibody against the purified AP-N from human prostate. The antigen-antibody complex was detected with a secondary antibody, goat anti-rabbit IgG conjugated with horseradish peroxidase, and visualized with 4-chloro-1-naphthol as a substrate of peroxidase.

Immunohistochemistry for MT and AP-N—Three μm sections were cut from representative paraffin-embedded samples, deparaffined in xylene, and then rehydrated in a graded alcohol series. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in methanol for 20 min. After extensive washing in tap water and rinsing in phosphate-buffered saline (PBS), the sections were incubated in normal horse serum for 30 min at room temperature to block non-specific binding. The sections were then incubated with anti-MT antibody E9 (Dako, Copenhagen, Denmark) or an anti–AP-N antibody at 4°C overnight. The antigen-antibody reaction was visualized by the avidin-biotin-complex technique using 3,3′-diaminobenzidine tetrahydrochloride as a substrate. The sections were counterstained with hematoxylin and examined by light microscopy.

Statistical Analysis—The significance of differences between groups was calculated using Student's *t*-test.

RESULTS

Differences in the Protein Components of Human Renal Cancer and Surrounding Tissues—To investigate the differences in the protein components between human renal cancer and surrounding tissues, proteins in the cytosol fractions prepared from these tissues were analyzed by reversed-phase HPLC. A typical HPLC pattern is shown in Fig. 1. When the cytosol fraction from renal cancer tissue was compared with that from adjacent normal tissue, proteins designated as peaks 1 to 4, which were eluted at positions corresponding to retention times of 15 to 20 min, were found to disappear. The disappearance of these peaks was

observed for each of the 16 samples tested. To identify the proteins which disappeared in the renal cancer tissue, the N-terminal amino acid sequences of the proteins corresponding to peaks 1 to 4 described above were analyzed. The sequences of these proteins were identified as those of MT isoforms.

Since MTs were shown to decrease on HPLC analysis of renal cancer tissue, the amounts of MTs in cancer and surrounding tissues were determined. The concentration of MTs in surrounding tissues was significantly different from that in renal cancer tissues. The mean concentration in surrounding tissues was about 15 times higher than that in cancer tissues (Table I, $n = 16$, $p < 0.0001$). The MT level changes depending upon the amounts of heavy metals in tissues. Therefore, the levels of zinc, which is an essential heavy metal for the human body, were determined in renal cancer and surrounding tissues (Table I). The zinc content in surrounding tissues was significantly higher than that in cancer tissues ($n = 16$, $p < 0.0001$). In addition, we noted that the concentrations of MTs in renal cancer and surrounding tissues were closely related to the zinc levels in these tissues, respectively (Fig. 2).

To investigate the localization of MT in human kidney tissues, we performed immunohistochemical analysis. Representative micrographs illustrating the immunohistochemical localization of MT in human kidney tissues are presented in Fig. 3. In normal kidney tissues (adjacent to renal tumors) (A) the expression of MT was found in the epithelial cells of renal proximal tubules and was distributed predominantly in the cytoplasmic domain of epithelial cells. In contrast, MT was not detected in any renal cancer tissue (B) although pathological differences were observed in renal cancer and surrounding tissues. Obvious differences in the expression pattern for MT in these tissues were observed for each of the 34 sections examined.

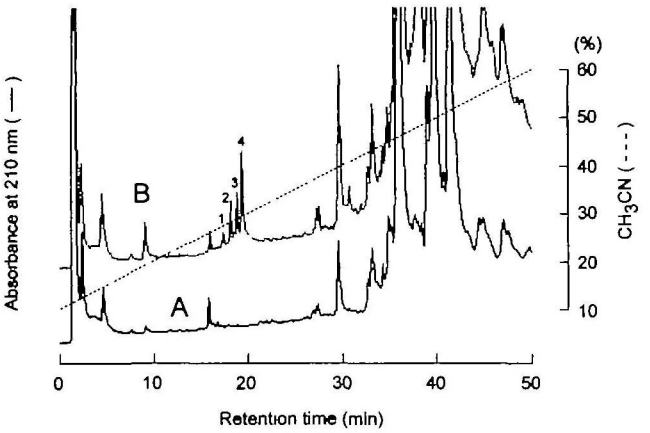

Fig. 1. **Reversed-phase HPLC of the cytosol fractions from renal cancer (A) and surrounding normal (B) tissues.** Cytosol fractions, which were prepared from renal cancer and surrounding normal tissues, were analyzed by HPLC on a reversed-phase column. Proteins were eluted with a gradient formed between 10 and 60% of acetonitrile containing 0.1% trifluoroacetic acid at the flow rate of 1.0 ml/min.

TABLE I. **Concentrations of MT and zinc in renal cancer and surrounding tissues.**

	Cancer surrounding tissues	Cancer tissues
MT (μg/mg protein)	126.3 ± 66.0	8.5 ± 10.9*
Zinc (nmol/mg protein)	17.6 ± 5.3	7.6 ± 4.9*

The values represent the means ± SD for 16 samples. *Significantly different from in cancer surrounding tissues ($p < 0.0001$).

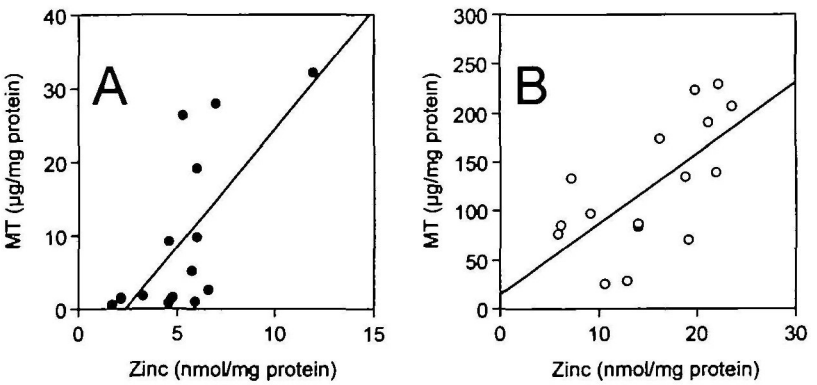

Fig. 2. **Relationship between the zinc and MT concentrations in human renal cancer (A) and surrounding (B) tissues.** (A) $y = 3.242x - 7.856$, $r = 0.706$, $n = 16$, (B) $y = 7.171x + 15.475$, $r = 0.660$, $n = 16$.

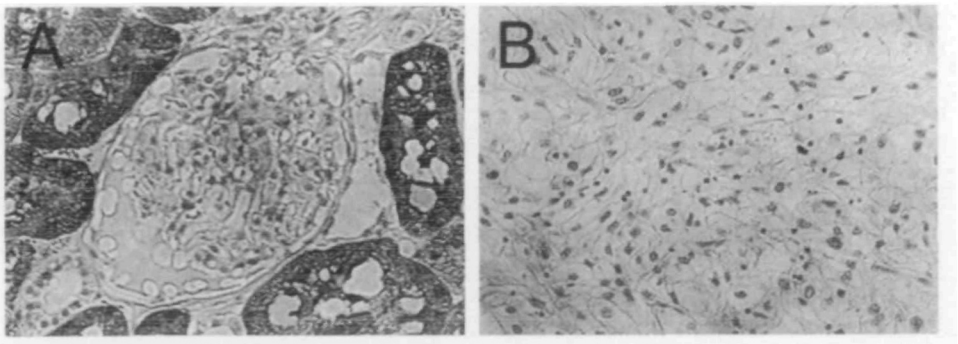

Fig. 3. **Immunohistochemical staining for MT in human kidney tissues.** Sections of surrounding normal (A) and cancer (B) tissues were stained for MT. (Original magnification ×200).

Differences of Peptidase Expression in the Membrane Fractions of Human Renal Cancer and Surrounding Tissues—AP-N and dipeptidyl peptidase IV (DPP-IV) are known to be abundant in human renal proximal tubules (*9*). The activities of these peptidases were investigated in the membrane fractions of renal cancer and surrounding tissues using peptidyl-MCA derivatives as substrates. Although no significant difference in DPP-IV activity for Gly-Pro-MCA as a substrate was observed in renal cancer and surrounding tissues, the activities of aminopeptidases (APs) toward Met-MCA, Ala-MCA, and Leu-MCA as substrates decreased significantly, *i.e.* about 10%, in cancer tissues compared with in adjacent normal tissues ($n = 7, p < 0.0001$). However, these activities do not represent the AP-N activity because Met-MCA, Ala-MCA, and Leu-MCA used as substrates are not specific substrates for AP-N. To detect specific AP-N expression in the membrane fractions of these tissues, we also performed Western blot analysis, using a specific polyclonal antibody against the purified AP-N. As shown in Fig. 4, the AP-N level in the membrane fraction of cancer tissues was lower than in that of adjacent normal tissues ($n = 3$).

Immunohistochemical analysis was also performed to investigate the localization of AP-N in human kidney tissues. As shown in Fig. 5A, the expression of AP-N in normal kidney tissues (adjacent to renal tumors) was observed in the epithelial cells of renal proximal tubules. At this magnification, it is clear that AP-N expression was concentrated in the apical membrane of luminal epithelial cells, with a very low level of staining in the cytoplasm. In contrast, AP-N was not detected in any renal cancer tissue (B). Obvious differences in the expression pattern for AP-N in these tissues were also observed for each of the 34 sections examined.

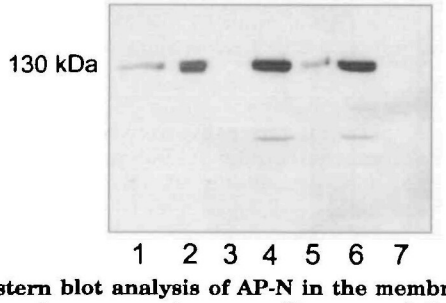

Fig. 4. **Western blot analysis of AP-N in the membrane fractions of renal cancer and surrounding normal tissues.** One microgram of the purified AP-N (lane 1) and 60 μg of the membrane fraction of renal cancer (lanes 3, 5, and 7) or surrounding normal (lanes 2, 4, and 6) tissue were separated by electrophoresis on an 8% SDS–polyacrylamide gel. After the proteins in the gel had been transferred to a nitrocellulose membrane by electroblotting, AP-N was probed with the antibody against the purified AP-N from human prostate.

DISCUSSION

The present study demonstrates that the MT and zinc levels in human renal cancer tissues are markedly decreased as compared with those in surrounding normal tissues. MT is known to be induced by exposure to heavy metal cations, stress, surgery, and tumors, and is found in various tissues of different animal species. This protein participates in the absorption, metabolism, transport, and storage of essential metals such as zinc and copper. MT contributes to the detoxification of heavy metal cations in the human body since heavy metal cations bound with MT do not exert toxicity in cells. Although zinc is an essential element for humans, many animal experiments have demonstrated that an increase in the zinc level is necessary during tumor growth. The levels of MT and zinc in liver were reported to increase during experimental carcinogenesis (*21*). Jin *et al.* (*22*) have also reported that overexpression of MT was observed in invasive ductal breast cancer and that the zinc levels in breast cancer tissues were twice as high as those in benign breast tissues.

Although several studies have demonstrated that MT expression increases in cancer tissue, the precise role of MT in tumor cell growth remains obscure. The concentration of zinc was reported to increase in human malignant disease with concomitent overexpression of MT (*23*). In contrast, Zaichick *et al.* (*24*) reported that the zinc level markedly decreased in human prostatic adenocarcinomas as compared with in normal tissue. The present study also demonstrates that the zinc levels in renal cancer tissues significantly decrease as compared with in surrounding normal tissues, implying that the zinc levels decrease with carcinogenesis in renal tissues. The decrease of MT in renal cancer tissues was observed to be greater than that of zinc. These findings suggest that the lower zinc levels in the renal cancer tissues cause the reduction of the amount of MT (Fig. 2). Since Gunshin *et al.* (*25*) recently reported and cloned the divalent cation transporter which probably imported zinc into cells, this decrease in the zinc level may seem to be based on a disorder of zinc import in the cancer tissues. It is unclear from the results of this study whether the import system of zinc was damaged or not. Although Saika *et al.* (*26*) have also investigated the expression of MT in

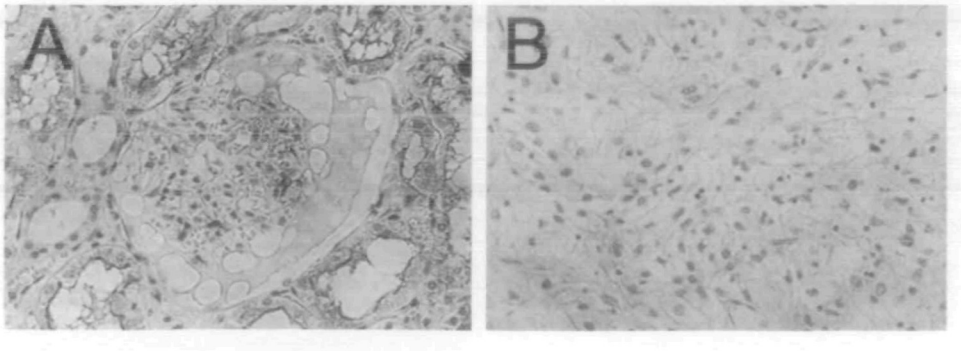

Fig. 5. **Immunohistochemical staining for AP-N in human kidney tissues.** Sections of surrounding normal (A) and cancer (B) tissues were stained for AP-N. (Original magnification ×200).

renal cell carcinomas, their conclusion was more ambiguous than the results of the present study.

Aminopeptidase A (AP-A)/gp160 is known to be a differentiation-related kidney glycoprotein which is expressed on the surface of epithelial cells of the glomerulus and proximal tubule cells of the human nephron (*27*). Nanus *et al.* (*28*) reported that alterations of the AP-A protein, including loss of protein expression or enzymatic activity, occurred in ~20% of primary clear cell renal cancers. In this study, however, we demonstrated that the activities of APs were significantly decreased in cancer tissues compared with in adjacent normal tissues, suggesting decreases in other AP(s) except AP-A. Therefore, we speculated that changes in the levels of other AP caused significant differences in the activities of APs. To identify the AP that decreased in renal cancer tissue, we performed Western blot analysis and immunohistochemistry, using a specific antibody against the purified AP-N. AP-N is well known to be highly expressed on the renal proximal tubules (*9*), while the precise relation of AP-N to renal cancer remains unclear. In human renal cancer tissues, the expression of AP-N was observed to decrease markedly as compared with in normal surrounding tissues. The expression of AP-N is also known to increase in acute myeloblastic leukemia and in some cases of acute lymphoblastic leukemia. Bogenrieder *et al.* (*29*) reported that AP-N was strongly expressed in benign prostatic epithelial cells, but not in primary and metastatic prostate cancer. Moreover, it has been reported that the expression of AP-N in breast and colonic carcinomas is not related to the stage of the cancer (*10*, *11*). Since AP-N is a zinc-dependent exopeptidase, zinc is necessary for its enzymatic activity. In tumor progression, AP-N participates in growth (*16*) and metastasis (*17*, *18*), suggesting that there is a close relationship between AP-N expression and the progression of tumor cells. However, the amount of AP-N in renal cancer tissues has been demonstrated to decrease accompanying a decrease in the zinc level in this study (Fig. 4). The expression of AP-N may be related to the zinc levels in renal cancer tissues because zinc is necessary for the enzymatic activity of AP-N.

In conclusion, we have shown here that in human renal cancer tissues MT, zinc and AP-N are markedly and consistently decreased as compared with in normal tissues surrounding tumors, suggesting that they do not participate in renal tumor progression. Moreover, our results strongly suggest that the immunohistochemical detection of MT and AP-N could provide useful information as a pathological diagnostic tool for classifying renal cancer or surrounding tissues.

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