Altered levels of acid, basic, and neutral peptidase activity and expression in human clear cell renal cell carcinoma

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Submitted 27 April 2006; accepted in final form 15 September 2006

Varona A, Blanco L, López JI, Gil J, Agirregoitia E, Irazusta J, Larrinaga G. Altered levels of acid, basic, and neutral peptidase activity and expression in human clear cell renal cell carcinoma. Am J Physiol Renal Physiol 292: F780–F788, 2007. First published September 19, 2006; doi:10.1152/ajprenal.00148.2006.—Peptides play important roles in cell regulation and signaling in many tissues and are regulated by peptidases, most of which are highly expressed in the kidney. Several peptide convertases have a function in different tumor stages, and some have been clearly characterized as diagnostic and prognostic markers for solid tumors, including renal cancer; however, little is known about their in vivo role in kidney tumors. The present study compares the activity of a range of peptidases in human tumor samples and nontumor tissue obtained from clear cell renal cell carcinoma (CCRCC) patients. To cover the complete spectrum and subcellular distribution of peptide-converting activity, acid, neutral, basic, and omega activities were selected. CCRCC displays a selective and restricted pattern of peptidase activities. Puromycin-sensitive aminopeptidase activity in the tumor increases [tumor (t) = 10,775 vs. nontumor (n) = 7,635 units of peptidase (UP)/mg protein; P < 0.05], whereas aminopeptidase N decreases (t = 6,664 vs. n = 33,381 UP/mg protein; P < 0.001). Aminopeptidase B activity of the particulate fraction in tumors decreases (t = 2,399 vs. n = 13,536 UP/mg protein; P < 0.001) compared with nontumor tissues, and aspartyl-aminopeptidase activity decreases significantly in CCRCC (t = 137 vs. n = 223 UP/mg protein; P < 0.05). Soluble and particulate pyroglutamyl peptidase I activities, aminopeptidase A activity, and soluble aminopeptidase B activity do not vary in renal cancer. The relative expression for the aforementioned peptidases, assayed using quantitative RT-PCR, increases in CCRCC for aminopeptidases B (1.5-fold) and A (19-fold), aspartyl-aminopeptidase (3.9-fold), puromycin-sensitive aminopeptidase (2.5-fold), and pyroglutamyl peptidase I (7.6-fold). Only aminopeptidase N expression decreases in tumors (1.3-fold). This peptidase activity profile in the neoplastic kidney suggests a specific role for the studied convertases and the endogenous substrates involved remain under study.

Several cell surface ectopeptidases may have a key growth control and differentiation role in many cell systems (11, 19). They are known to play a part in neoplasia (36), and some have been clearly characterized as solid tumor markers (11): CD13/aminopeptidase N (APN) (EC 3.4.11.2), CD10/neutral endopeptidase (EC 3.4.24.11), and CD26/dipeptidyl peptidase IV (EC 3.4.14.5). However, their precise mechanism of action and the endogenous substrates involved remain under study.

Ectopeptidase-mediated peptide regulation is an endocrine or paracrine mechanism and a major system for physiological control of these signals. However, in vivo peptide control is also carried out by several convertases, which are capable of expression in both membrane-bound and soluble cell fractions and thus may also play a role in cancer (13).

The renal parenchyma shows a high content of several types of peptidase (36), particularly bestatin-sensitive peptidases (11, 36). APN (EC 3.4.11.2) and aminopeptidase A (APA; EC 3.4.11.7) are present in the brush-border membrane of renal tubules, where they take part in the luminal hydrolysis of the oligopeptides that are filtered across the glomerular membrane (11). Aminopeptidase B (APB; EC 3.4.11.6) is present on the luminal side of the endoplasmic reticulum (42), in the Golgi apparatus, and, together with APN, in the basolateral plasma membranes of renal cells, where it may be involved in negative feedback systems by facilitating the degradation of excessive bioactive peptides; however, its subcellular localization would appear to be mainly cytosolic (36). The expression of aspartyl aminopeptidase (Asp-AP; EC 3.4.11.21) and of puromycin-sensitive aminopeptidase (PSA; EC 3.4.11.14) is also cytosolic. The major expression of pyroglutamyl peptidase I (PGI; EC 3.4.19.3) is cytosolic (20), although its activity has also been detected in the particulate fraction. Soluble fraction proteases may be involved in regulating peptide autocrine and intracrine action mechanisms (12).

RENAL CELL CARCINOMA ACCOUNTS FOR ~3% OF ADULT MALIGNANCIES, WITH OVER 36,000 NEW CASES AND 12,000 DEATHS EXPECTED IN 2005. The clear cell type [clear cell renal cell carcinoma (CCRCC)] is by far the most frequent histological subtype, accounting for 70% of cases (25). It has been demonstrated that a wide variety of agents are involved in the development of renal cancer in experimental animals, but none of them has been proved to be an important etiological factor for renal cancer in humans (3). Furthermore, the pathways involved in renal cell carcinogenesis are numerous and not sufficiently well understood.

Bioactive peptides are regulated through specific “convertases,” which hydrolyze them (13). These peptide-converting enzymes were originally considered to be only involved in protein and peptide scavenging. However, recent studies have demonstrated that they are involved in several physiological functions (4, 33), including angiogenesis (44) and apoptosis (37).

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There is evidence to support the involvement of the afore-mentioned peptidases in renal cancer, inhibition of tumor cell invasion in vitro (35), and apoptosis onset in solid-cell tumor lines (8, 37). Apart from peptidases, which have been described as tumor markers, in recent years there has been increasing interest in the physiological relevance of APB in the development of tumors, as its increased pH dependence and ubiquitous presence argue in favor of APB involvement in a broad spectrum of important functions in superior animals in which some potential peptide substrates of this enzyme might play a central role (6). Most of these data come from the use of peptidase inhibitors (36) and make renal cell peptidases potential targets for anticancer therapy.

However, although peptide convertases seem to play a major role in cancer control, the involvement of peptidases in renal cancer has rarely been analyzed (14, 18, 26).

In this study, we investigated the relative expression and the soluble and particulate activities of acid (Asp-AP, APA), basic (APB), neutral (PSA, APN), and omega (PGI) peptidases in a CCRCC series. Acid, basic, neutral, and omega activities were selected to cover the complete spectrum of peptide-converting activity.

MATERIALS AND METHODS

The authors declare that all of the experiments carried out in this study comply with current Spanish and European Union laws.

Materials. All of the chemicals used were obtained from Sigma Chemical (St. Louis, MO).

Renal tissue specimens and sample storage. We analyzed renal tissue from 20 patients (15 male, 5 female, mean age 63 yr, range 38–78 yr). Patient consent and Hospital Ethics Committee approval were obtained a priori. Fresh tissue samples were obtained from surgical specimens from renal cancer patients. Tumor and nontumor (surrounding uninvolved tissue) areas were obtained for the study in all cases. Tissue samples were embedded in optimum cutting temperature, frozen in isopentane, and stored at −80°C until the enzyme assays were performed. In addition, selected tissue samples were formalin fixed and paraffin embedded for histopathological diagnosis following routine methods in the pathology laboratory. The 2002 TNM edition (40) and the Fuhrman’s system (10) were used for tumor staging and grading, respectively.

Sample preparation. Explanted tissue samples were homogenized in 10 mM Tris-HCl buffer, pH 7.4, for 30 s at 800 rpm using a Heidolph PZR 50 Selecta homogenizer and ultracentrifuged in a Centrifron T-2070 Kontron Instruments apparatus at 100,000 g for 35 min. The resulting supernatants were used to measure soluble enzyme activities and protein concentrations. To avoid contamination with soluble enzymes, the resulting pellets were washed three times by suspension in 10 mM Tris-HCl buffer, pH 7.4. Subsequently, pellets were homogenized in 10 mM Tris-HCl buffer, pH 7.4, and then centrifuged at low speed, 1,500 g, for 1 min to purify the nucleus from the samples. The supernatants thus obtained were used to determine particulate enzyme activities and protein concentrations. All steps were carried out at 4°C.

Enzyme assays. Peptidase activities were measured by incubating samples with a saturating (0.125 mM) concentration of β-naphthylamide-derived substrates following the method described by Mantle (21). Alanine aminopeptidase activities (PSA, APN) were measured in triplicate using Ala-β-naphthylamide as a substrate. To discriminate between PSA and APN forms of total alanine aminopeptidase activity, incubations with the specific PSA inhibitor puromycin (40 μM) were performed in parallel. APB and acid aminopeptidase activities (Asp-AP, APA) were quantified with Arg-β-naphthylamide and Asp-β-naphthylamide substrates, respectively. Omega peptidase (PGI) activity was measured fluorimetrically using pGlu-β-naphthylamide as a substrate. These assays are based on the fluorescence of β-naphthylamide generated from the hydrolysis of the substrate by the enzyme. Reactions were initiated by adding 30–50 μl of sample to 1 ml of appropriate incubation mixture, depending on the enzyme and substrate analyzed as follows: PSA, APN, APB, and PGI activities (50 mM phosphate buffer, pH = 7.4) for PSA, APN, and PGI activities, pH = 6.5 for APB and 0.125 mM aminoacyl-β-naphthylamide); and Asp-AP/APA activities (50 mM Tris-HCl buffer, pH = 7.4, 1 mM MnCl2, and 0.125 mM aminoacyl-β-naphthylamide). After 30-min incubation at 37°C, 1 ml of 0.1 M sodium acetate buffer (pH 4.2) was added to the mixture to terminate the reaction. The released β-naphthylamide was determined by measuring the fluorescent intensity at 412 nm with excitation at 345 nm, in a Shimadzu RF-540 spectrophotometer. Blanks were used to determine background fluorescence. Relative fluorescence was converted into picomoles of product using a standard curve, constructed with increasing concentrations of β-naphthylamide.

Protein determination. Protein concentration was measured in triplicate by the method described by Bradford (5), using 1 mg/ml BSA as the calibrator. The results were recorded as units of peptidase (UP) per milligram of protein. One unit of peptidase activity is the amount of enzyme necessary to release 1 pmol of β-naphthylamide per minute. Fluorogenic assays were linear with respect to hydrolysis time and protein content.

Statistical analyses. Data were analyzed statistically using SPSS, version 10. An unpaired Student’s t-test was performed to detect differences between uninvolved tissues and tumors. Statistically significant differences were considered at P < 0.05.

Real-time quantitative PCR analysis. Quantitative RT-PCR for detecting PSA, APN, APB, Asp-AP, APA, and PGI mRNA was performed to determine the transcription levels of these peptidases. The RNA of tumor and nontumor tissue samples from six CCRCC patients was isolated with the RNaseasy Protect kit (Qiagen), including a DNase digestion step using an RNase-free DNase kit (Qiagen) to exclude possible contamination by genomic DNA.

The Taqman real-time PCR system is based on a three-primer method. Two of the primers are designed following the classical PCR concept (upper and lower primers). The third primer has a fluorescent group that, when included in the amplification, increases fluorescence emission. This system enhances amplification specificity (three primers are used) and avoids nonspecific double-stranded DNA detection, as the fluorescence is bound to one of the primers. cDNA was obtained using a Transcriptor reverse transcriptase kit (Roche). Intron spanning Taqman probes were designed following the Roche Universal Probe Library method (www.roche-applied-science.com). Amplifications were run in a 7900 Real-Time PCR System (Applied Biosystems). Each value was adjusted by using 18S RNA levels as reference.

The following primers were employed: human PSA: 5'-CAGTT-GAGGGATTGTGAGTTG-3' (upper primer), 5'-TGAAGGACGT-GGTGACTCT-3' (lower primer), probe: human no. 19; human APN: 5'-CATCCATCAGAGAGGCAGAC-3' (upper primer), 5'-TGCCT-GAAGAGATCGTCTCTTG-3' (lower primer), probe: human no. 18; human APB: 5'-ACCATGTACGCACAGAGG-3' (upper primer), 5'-CAGCTCTCAAGGCAGGTA-3' (lower primer), probe: human no. 22; human Asp-AP: 5'-AGTCCCTCTCTCCTCATCAG-3' (upper primer), 5'-TCCAAAAAATTTTACAGG-3' (lower primer), probe: human no. 64; human APA: 5'-CGGAGAACCAGCAGT-CAAG-3' (upper primer), 5'-GGGCTCATCAAAAAACAGGGA-3' (lower primer), probe: human no. 72; and human PGI: 5'-GGAGAAAG-CACAGTCCACAG-3' (upper primer), 5'-TGTAGCCCTTTGTTG- GTCCA-3' (lower primer), probe: human no. 47.
RESULTS

The peptidase activity detected in the soluble and membrane-bound subcellular fraction of CCRCC patients is shown in Table 1. Activity is recorded as picomoles of product per minute per milligram protein (UP/mg protein) and presented as means ± SE for n = 20.

Soluble and particulate alanine aminopeptidase activity (PSA and APN neutral activities, respectively) in the tumor and healthy tissue from human CCRCC kidney samples is shown in Fig. 1. In the soluble fraction of the tumors, PSA activity increased slightly (1.5-fold) but significantly compared with nontumor tissue [Fig. 1A; tumor (t) = 10,775 ± 1,162 vs. nontumor (n) = 7,635 ± 510 UP/mg protein; Student’s t-test, P < 0.05]. On the other hand, in the particulate fraction (Fig. 1B), APN activity decreased significantly (5-fold) in the tumor compared with surrounding uninvolved tissue (t = 6,664 ± 1,111 vs. n = 33,381 ± 2,323 UP/mg protein; Student’s t-test, P < 0.001).

In Fig. 2, we represent soluble and membrane-bound APB activities (basic activities) in tumor and unaffected human tissue from human kidney with CCRCC. No significant differences were found in soluble APB activity in the tumor compared with the nonpathological surrounding tissue (Fig. 2A; t = 5,308 ± 709 vs. n = 5,771 ± 703 UP/mg protein). However, the membrane-bound APB activity decreased significantly, more than fivefold, in tumor compared with healthy cells (Fig. 2B; t = 2,399 ± 421 vs. n = 13,536 ± 1,849 UP/mg protein; Student’s t-test, P < 0.001).

Soluble and membrane-bound acid aminopeptidase activities (Asp-AP/APA) in tumor and unaffected tissue of the human kidney with CCRCC are represented in Fig. 3. In contrast to neutral and basic activities, statistically significant differences in acid aminopeptidase activities were only detected between soluble fraction values (Fig. 3A; t = 137 ± 19 vs. n = 223 ± 25 UP/mg protein; Student’s t-test, P < 0.05). In this case, Asp-AP activity decreased twofold in the tumor compared with uninvolved tissue values. In the particulate fraction, APA activity did not change significantly (Fig. 3B; t = 616 ± 111 vs. n = 715 ± 86 UP/mg protein).

Finally, in Fig. 4, soluble and particulate PGI activities (omega activities) in tumor and healthy tissue from human CCRCC kidney are represented. Neither soluble PGI activity (Fig. 4A; t = 135 ± 29 vs. n = 128 ± 27 UP/mg protein) nor membrane-bound PGI activity (Fig. 4B; t = 165 ± 39 vs. n = 207 ± 68 UP/mg protein) were found to vary significantly in tumor compared with pathology-free tissue.

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Table 1. Peptidase activities in clear cell renal cell carcinoma

<table>
<thead>
<tr>
<th>Peptidase Type</th>
<th>Recommended Name/EC Number</th>
<th>Soluble Activity, UP/mg protein</th>
<th>Membrane-Bound Activity, UP/mg protein</th>
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<tr>
<td></td>
<td></td>
<td>Mean ± SE</td>
<td>P value</td>
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<tr>
<td>Neutral</td>
<td></td>
<td>Tumor 10,775±1,162</td>
<td>&lt;0.05</td>
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<td></td>
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<td>Nontumor 7,635±510</td>
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<td>Basic</td>
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<td>Tumor 5,308±709</td>
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<td></td>
<td></td>
<td>Nontumor 5,771±703</td>
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<tr>
<td>Acid</td>
<td></td>
<td>Tumor 137±19</td>
<td>&lt;0.05</td>
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<td></td>
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<td>Nontumor 120±27</td>
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On the left, peptidase activity types (neutral, acid, basic, or omega peptidase) and recommended names and EC nomenclatures are mentioned. On the right, we show the activity values of soluble and of membrane-bound fractions in tumor cells and nondiseased surrounding tissue recorded as picomoles of hydrolyzed aminocyl-β-naphthylamide per minute per milligram of total protein in the sample (units of peptidase (UP)/mg protein; means ± SE, n = 20). Significant statistical differences (P) between tumor tissue and the clearly uninvolved part of the pathological kidney are shown. NS, not significant.

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Fig. 1. Soluble puromycin-sensitive aminopeptidase (PSA; A) and particulate aminopeptidase N (APN; B) activities in kidney samples from 20 patients with clear cell renal cell carcinoma. Columns compare neoplastic tissue (tumor) with clearly uninvolved (nontumor) surrounding tissue. Values represent means ± SE of peptidase activities recorded as picomoles of alanyl-β-naphthylamide hydrolyzed per minute (UP) per milligram of protein. *P < 0.05, ***P < 0.001.
With respect to the transcription of the aforementioned peptidases, the relative expression of PSA, APN, APB, Asp-AP, APA, and PGI in the tumor and nontumor tissue from human CCRCC kidney samples \( (n/11005) \) is shown in Fig. 5. Only APN/CD13 expression decreased (1.3-fold) in the tumor compared with the surrounding unaffected tissue, which correlated with the results in enzyme activity (Fig. 5B). The changes observed in PSA expression also correlated with this peptidase activity; thus mRNA for PSA in the tumor was 2.5-fold higher than mRNA for PSA in nontumor cells (Fig. 5A). The expression levels for APB (Fig. 5C), Asp-AP (Fig. 5D), APA (Fig. 5E), and PGI (Fig. 5F) in CCRCC for tumor vs. nontumor tissue increased 1.5-, 3.9-, 19-, and 7.6-fold, respectively.

**DISCUSSION**

Among the factors involved in the etiopathogenesis of CCRCC, a major point of interest in cancer research concerns proteases, which are involved in metastasis (31), and particularly peptidases, which would appear to play a role, not only in metastatic processes but also in tumor cell growth and differentiation-related pathways (8, 36).

Our results demonstrate that peptidase activities are selectively modified by the tumoral process in affected tissue. Thus, in the soluble fraction of tumors, PSA activity increased slightly, and, conversely, Asp-AP decreased significantly. In soluble APB and PGI activities, no significant differences were found between tumor and nonaffected tissue. Membrane-bound CCRCC fractions showed significant decreases in APN and APB activities. However, particulate PGI activity and APA activity did not vary significantly.

These differences are unlikely to be due to general changes in proteolysis, since the profile of the changes observed is not the same for each enzyme or subcellular fraction analyzed.

However, when RT-PCR assays were performed to elucidate whether changes in peptidase activities were due to alterations in protein transcription, only relative PSA and APN expressions correlated positively with the results obtained for their own activities. Thus relative PSA expression increased 2.5-fold (tumor vs. nontumor), and, in contrast, APN expression decreased 1.3-fold, in the same way as PSA and APN activities varied in CCRCC. Relative APB and Asp-AP expressions increased, conversely to the decreases in these activities, and overexpression was also observed for APA and PGI activities, which did not change significantly when they were assayed.

In the case of relative PSA and APN expression, the results coincide with those obtained for enzymatic activities, suggesting that PSA and APN protein levels are, in fact, up- and downregulated, respectively. The downregulation observed for APN/CD13 expression concurs with studies involving particulate APN in specific roles in renal cancer (11, 14). It has been reported that the expressions of APN and APA seem to vary in an antiparallel way in renal cell carcinomas (18). In light of
these results, interactive regulation between APN and APA has been proposed, and we observed antiparallel expressions for APN and APA that could be partially due to this potential mechanism of genetic regulation. In support of this hypothesis, there is evidence that bestatin, which inhibits APN but not APA, causes enhancing expression of the latter enzyme (18). Regarding the upregulation of PSA in CCRCC, some authors have proposed that overexpression of certain soluble peptidases in tumor cells might play a role in the induction and maintenance of genetic instability in malignant cells (41). For example, upregulation of tripeptidyl-peptidase II induces accelerated growth and resistance to apoptosis in the human kidney (41). In this sense, our results extend this potential role to the cytosolic PSA of renal cells. Moreover, as discussed further below, PSA could be involved in antiapoptotic mechanisms in solid tumors (37, 38), as we obtained data (2.5-fold PSA overexpression in CCRCC) that coincide with the proposal of resistance to apoptosis in tumors caused by upregulation of cytosolic peptidases.

On the other hand, the lack of positive correlations between relative expression and enzymatic activity for APB, Asp-AP, APA, and PGI suggests specific cellular factors leading to inhibition and/or mutation mechanisms for these proteins in CCRCC. In this sense, overexpression mechanisms of aberrant and/or nonfunctional enzymes and proteins in tumor cells have been commonly described (26). In particular, the Nanus group has reported strong expression of APA and parallel decreases for this enzyme activity in CCRCC (26); they have also proposed several potential mechanisms to explain these data, such as inhibition of catalytic activity through cellular factors or point mutation in the zinc binding motif of the protein (26, 27). Since our data suggest that APA, which overexpresses in CCRCC, is a nonfunctional protein and/or its catalytic activity is decreased by the cellular environment in the tumor, our study coincides with that of the aforementioned group.

One of the most interesting findings of this study is the marked decreases in particulate activity of APN (5-fold) and APB (practically 6-fold) in neoplastic tissue compared with their membrane-bound activities in nontumor tissue. These results are consistent with other studies that involve particulate APN (11, 14) and APB (35) in specific roles in renal cancer. Many of these works describe decreases in APN expression in pathological tissue (11, 14) and so concur with our results.

The role of APN activity in cancer has classically been linked to tumor progression, angiogenesis, and invasiveness (29, 31, 34). However, our observations suggest a role linked to an additional factor other than progression, as in CCRCC APN activity, is strongly suppressed in cancer tissue but not in the normal tissue surrounding tumors. With respect to an additional role in cancer, several groups have described peptidase involvement in tumor cell growth and differentiation. For example, when peptidase inhibitors are incubated in vitro with COS-7 or with HL-60 cells, cell cycle arrest takes place at the G2/M phase border (8), suggesting peptidase involvement in cell cycle checkpoint regulation (43). As the results of the present work suggest a role for particulate APN, not only in progression or invasiveness but also in other primary tumor mechanisms, our data further support the hypothesis of in vivo peptidase involvement in cancer growth and/or differentiation. This point was first discussed by Ishii et al. (14), and our results coincide with theirs.

Apoptosis onset in tumor cells has frequently been proposed as one of the nonprogressive or invasive roles of peptidases in cancer (37, 38, 43). For example, peptidase inhibitor bestatin is involved in apoptosis onset in solid tumor cell lines (37). The apoptotic effect of bestatin would not appear to be caused through inhibition of APN, since other more specific APN inhibitors and antibodies against this peptidase have no effect (37, 38). The target of the bestatin effect may be a peptidase with high intracellular expression that is also sensitive to this inhibitor. Due to its sensitivity to bestatin and to puromycin, another apoptosis-inducing peptidase inhibitor in cultured cells, Sekine et al. (37, 38) propose cytosolic PSA as a likely target. The significant increase observed (1.5-fold) in PSA activity in tumors compared with nontumor tissue coincides with this group’s proposal. Peptidases described as being involved in solid tumor processes are usually cell surface protein markers (CD13, CD10, CD26, CD143, gp160), and intracellular forms are rarely involved. In consequence, most of the peptidase-controlled hormonal mechanisms that are usually involved in renal cancer are paracrine mechanisms. The peptide control alterations through cytosolic PSA obtained by us in CCRCC suggest that, not only paracrine mechanisms, but also potential intracrine mechanisms might be considered as suitable targets in renal cancer research. Several authors besides our group have already pointed toward specific roles, not only for cell surface peptidases but also for intracellular forms (12, 19, 37).
The potential role for membrane-bound APB activity in CCRCC is more difficult to elucidate due to a lack of previous studies. However, this peptidase is indirectly involved in the aforementioned apoptosis mechanisms. As described, the target for the apoptotic effect of bestatin and puromycin may be an intracellular peptidase sensitive to both inhibitors (37, 38). Since the chemical properties referred to converge in APB (42), basic aminopeptidase could also be a suitable target.

Fig. 5. PSA (A), APN (B), APB (C), Asp-AP (D), APA (E), and PGI (F) expression (in fold-number) of each individual tumor or nontumor sample relative to one of the normal tissues (n = 6 for each condition). Boxes represent mean (solid line) ± SD of quantified mRNA recorded as relative units of gene expression.
There is further evidence to support this potential role for APB as an apoptosis-mediated antitumor factor. For example, APB is involved in the conversion of angiotensin (ANG) III to ANG IV (1, 23), which seems to be capable of preventing cells from apoptosis (17). Unfortunately, the downregulation observed in particulate APB activity would appear to be contrary to this antitumor effect through apoptosis. The results of other non-CCRCC studies carried out, in breast cancer for example (23), are contrary to ours. However, the hypothesis of a role for APB in apoptosis-mediated antitumor effects in CCRCC cannot be ruled out at present. In fact, contradictory results between our work and other studies illustrate the functional complexity of carcinogenic mechanisms, which involve selective activation or inhibition of different processes and local factors by different tumoral phenotypes. Another controversial point in our results was the membrane-bound localization of the APB decreases we observed; however, the recent description of a novel puromycin-sensitive arginine aminopeptidase in intracellular particulate fractions (endoplasmic reticulum) of human cells supports our hypothesis (42). This study (42) partially supports the possibility of APB involvement in antitumorogenic apoptosis-mediated effects of bestatin.

We also found inhibition of soluble acid aminopeptidase activity in CCRCC to be particularly high (50% of activity in the unininvolved tissue). The implication of acid aminopeptidases in different types of renal cancer has been studied by several authors (11, 18, 26). Our results in Asp-AP activities coincide with these data, particularly with Nanus et al. (26), who reported loss of protein expression and enzymatic activity in ~20% of primary clear cell renal cancers. There is evidence to support the existence of two different acid peptidases, both involved in the degradation of peptides with NH2-terminal aspartate, but with different chemical profiles, cell distribution, and functions (23, 45). In support of this evidence of two different forms of aspartate-peptide acid convertases, in this work we obtained different profiles of acid aminopeptidase activity in soluble and in particulate fractions. Most research on the role of acid aminopeptidases in both renal and nonrenal cancer involves the membrane-bound form (APA) and not cytosolic Asp-AP (11, 18, 23, 26). APA has been associated with the gp160 kidney differentiation antigen, which is highly expressed on the surface of the normal nephron (27) and, which, on the contrary, seems to be absent in 30–40% of renal cancers, such as CCRCC (26). Partially as a result of this, studies on the potential involvement of soluble acid aminopeptidase activity in CCRCC are rare. The data obtained by us extend the role of acid aminopeptidase activity in renal cancer to include cytosolic forms. As in the case of the significant differences we have described in PSA, this alteration in cytosolic Asp-AP in CCRCC suggests an intracrine action for this former enzyme in renal cancer and is consistent with other authors who have described intracrine mechanisms in the control of several peptides (such as ANGs) involved in angiogenesis, tumor cell growth, and proliferative disorders (2, 32, 37).

The precise mechanism of action for peptidases in CCRCC remains unclear. Several groups have reported that these convertases may modulate the activity of peptide growth factors and their access to adjacent cells by converting pro-peptides to their active forms or inactivating small bioactive peptides (11, 31). Moreover, accumulating data suggest that peptidase activity on bioactive peptides converts these active molecules into fragments with retained but modified biological effects (13). Not only do changes in peptidase activities reflect tissue damage in cancer, but also the functional status of their peptidergic substrates, due to their involvement in the control of these local factors that act through intracrine, autocrine, or paracrine mechanisms (23). Since peptidases are broad-spectrum enzymes capable of catalyzing the hydrolysis of many different peptides (13), it is difficult to correlate the alterations in peptidase activities observed in CCRCC with specific peptide levels. Nevertheless, most of the peptidase activities we analyzed are described as ANG-degrading proteins (13). Furthermore, it is now clear that not only ANG II, the active fragment formed from the biologically inactive peptide ANG I, but also several of the metabolites from ANG I and ANG II are formed with retained but modified biological activity. Thus sequential removal of single amino acid residues from the NH2-terminal of ANG I and ANG II, which takes place through aminopeptidases, results in modified active peptides ANG III and ANG IV, respectively (7). Asp-AP and APA can delete Asp1-Arg2 residue from both ANG I and ANG II (1, 24, 39); PSA and APN can also remove this residue from ANG I and furthermore Arg2-Val3 from ANG III (24), and it has recently been reported that APB processes ANG peptides in peripheral organs (30). Therefore, all the peptidase activities we have described as significantly modified in CCRCC samples compared with adjacent unaffected tissue (Asp-AP, APA, PSA, and APN) would appear to participate in the ANG metabolism at different levels of the pathway. In fact, PSA and APB activities are sensitive to both bestatine and puromycin peptidase inhibitors (17, 36, 37, 38, 42), two antibiotics that seem to cause apoptosis-mediated prevention of tumor growth (36, 37, 38), and the ANG metabolism has been suggested as one of the signal pathways causing this antitumor effect of peptidase inhibitors (17). With respect to the potential nature of this probable peptidase-mediated alteration in the ANG signal pathway in CCRCC, the results of the present study (decreases in most peptidases) suggest that the metabolism of ANG II to ANG III and the conversion of ANG III to ANG IV are slow, and, as a result, the predominant action could be due to ANG II. A very similar alteration of the ANG pathway was first described by Martinez et al. (23) in breast cancer, and our results extend these findings to CCRCC. In support of the hypothesis that ANG II is the signal of the renin-ANG system, which predominates in the role of this hormone mechanism in human cancer, there are several studies that report that the octapeptide referred to stimulates cell growth, proliferation, differentiation, angiogenesis, and both the expression of growth-related protooncogenes and the synthesis of autocrine growth factors (9, 13, 15, 23). The decreases observed in peptidase activities provide further support for this hypothesis. Moreover, our results for APB, PSA, and Asp-AP activities, which have been described mainly as intracellular enzymes (42, 43, 45), coincide with those of other authors who recently proposed the involvement of an intracrine renin-ANG system in several growth disorders (2, 32). These intracrine renin-ANG system mechanisms have been widely involved in cardiac pathophysiology (32), apparently due to an intracrine action of ANG II (2). Due to the predominant ANG II action observed in CCRCC, data from this work also extend the intracrine growth effects of ANG II to renal cancer. This is
important for the development of new renal cancer therapies and makes ANG II action modulators a pharmacological possibility worth considering. All of these aspects make renal peptidases, not cell surface forms alone but also cytosolic proteins, suitable targets for anticancer therapy using specific inhibitors. However, the suitability of peptidase inhibitors as anti-oncogenic drugs remains controversial, due to their potential nephrotoxicity (22). In addition, the effects of these inhibitors on macrophage aminopeptidases could also be a risk in using this therapy (28). On the other hand, broad spectrum inhibitors, such as bestatin, seem to exhibit low toxicity to cultured cells, intact animals, and humans (36). In this sense, bestatin has been used in Japan as an immunomodulator and a reinforcement to anticancer therapy (35, 38) under the trade-name Ubenimex (Nippon Kayaku, Tokyo, Japan). Further studies and clinical trials need to be carried out to elucidate the benefits or risks involved in using peptidase inhibitors as antitumor drugs.

In summary, we conclude that CCRCC shows a selective and restricted pattern of peptidase activities and expression, which suggests a specific role for these proteases in renal cancer pathogenesis. An imbalance in the renin-ANG system in the kidney may be partially responsible for CCRCC. A deeper understanding of the functions of tumor peptidases, not only cell surface forms but also intracellular proteins, in controlling the level of ANGs and their effects on cell neighbors could be helpful in gaining insight into the paracrine, intracrine, and autocrine mechanisms underlying CCRCC etiogenesis.

ACKNOWLEDGMENTS

We thank Ekaiz Agirregoitia (University of the Basque Country) and Arkaiz Carracedo (Universidad Complutense de Madrid) for substantial contribution to this study. Likewise, the authors thank the agency O+iiker (Basque Health Research Institute) from the Spanish Basque Foundation for Health Innovation and Research (http://www.bioef.org) for having improved the English in this manuscript.

GRANTS

This work was supported by a grant from the Jesús Gangoiti-Barrera Foundation.

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