Aminopeptidase Activities in Breast Cancer Tissue

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Background: Endopeptidases such as cathepsins help determine the prognosis of breast cancer (BC). However, little information is available about the role in BC of aminopeptidases (APs), which have been implicated in the metabolism of several local hormonal factors.

Methods: Using aminoacyl-β-naphthylamides as substrates, we measured fluorometrically alanyl-AP (AlaAP), arginyl-AP (ArgAP), cysteiny1-AP (CysAP), glutamyl-AP (GluAP), aspartyl-AP (AspAP), and prolylglutamyl-AP activities in their soluble and membrane-bound forms in surgically removed BC tissue from which we separated samples of neoplastic, adjacent tumoral, and unaffected surrounding tissue.

Results: Compared with unaffected tissue, neoplastic tissue had significantly higher activities of soluble alanyl-AP (553.9 ± 82.8 vs 1615.2 ± 183.0 pmol/mg protein; P < 0.001), arginyl-AP (372.4 ± 56.6 vs 1027.2 ± 143.5 pmol/mg protein; P < 0.001), and cysteinyl-AP (74.8 ± 10.0 vs 282.9 ± 37.2 pmol/mg protein; P < 0.001), and of membrane-bound arginyl-AP (457.7 ± 97.9 vs 886.6 ± 140.0 pmol/mg protein; P < 0.01). However, membrane-bound aspartyl-AP activity was significantly lower in neoplastic tissue (17.3 ± 1.4 vs 9.2 ± 1.2 pmol/mg protein; P < 0.05) and prolylglutamyl-AP activity was significantly lower in neoplastic and adjacent tissues (12.8 ± 0.9 vs 7.0 ± 1.2 and 8.0 ± 1.3 pmol/mg protein; P < 0.001 for both comparisons).

Conclusions: The present results document changes in AP activities in BC tissue. These changes may reflect the functional status of the AP substrates, which can be selectively activated or inhibited locally in the affected tissue as a result of specific conditions brought about by the tumor.

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Materials and Methods

Nine BC patients ranging in age from 47 to 63 years (mean, 54.7 years) participated in this study after giving their informed consent. All BCs were diagnosed as ductal infiltrating carcinomas. Tumor samples, always containing some contiguous healthy tissue, were obtained shortly after surgery.
after surgical removal, quickly separated into pieces of neoplastic tissue, nonnecrotic tissue removed from the edges of tumoral area (adjacent neoplastic tissue), and unaffected surrounding tissue, which was used as a control. Samples were quickly frozen in dry ice and stored at −80 °C until processed.

Tissue samples (mean weight, 300 mg) were homogenized in 10 volumes of 10 mmol/L Tris-HCl buffer (pH 7.4) and ultracentrifuged at 100 000 g for 30 min (4 °C) to obtain the soluble fraction. The resulting supernatants were used to measure soluble enzymatic activity and protein content, assayed in triplicate. To solubilize membrane proteins, the pellets were rehomogenized in Tris-HCl buffer (pH 7.4) containing 10 mL/L Triton X-100. After centrifugation (100 000 g for 30 min at 4 °C), the supernatants were used to measure membrane-bound activity and proteins, also in triplicate. To ensure complete recovery of activity, the detergent was removed from the medium by adding adsorbent polymeric Biobeads SM-2 (100 g/L) to the samples, which were then shaken for 2 h at 4 °C (9).

AlaAP, ArgAP, and CysAP were measured fluorometrically using alanyl-β-naphthylamide (AlaNNap), arginyl-β-naphthylamide (ArgNNap), and cystinyl-β-naphthylamide (CysNNap) as substrates, according to the modified method of Greenberg (10). Ten microliters of each supernatant was incubated for 30 min at 25 °C with 1 mL of the substrate solution (21.4 mg/L AlaNNap, 33.5 mg/L ArgNNap, or 56.3 mg/L CysNNap), 100 mg/L bovine serum albumin (BSA), and 100 mg/L diithiothreitol (DTT) in 50 mmol/L phosphate buffer, pH 7.4, for AlaAP and ArgAP and 50 mmol/L HCl-Tris buffer, pH 6, for CysAP.

pGluAP was measured in a fluorogenic assay using pyroglutamyl-β-naphthylamide (pGluNNap) as the substrate, according to the modified method of Schwabe and McDonald (11): 10 μL of each supernatant was incubated for 120 min at 37 °C with 1 mL of substrate solution (25.4 mg/L pGluNNap, 100 mg/L BSA, 100 mg/L DTT, and 378 mg/L EDTA in 50 mmol/L phosphate buffer, pH 7.4).

AspAP was determined fluorometrically with AspNNap as the substrate, according to the method of Cheung and Cushman (12) modified as follows: 10 μL of each supernatant was incubated for 120 min at 37 °C with 1 mL of the substrate solution (25.8 mg/L AspNNap, 100 mg/L BSA, 100 mg/L DTT, and 394 mg/L MnCl₂ in 50 mmol/L Tris-HCl buffer, pH 7.4).

GluAP was also determined in a fluorometric assay using GluNNap as the substrate according to the method of Tobe et al. (13) modified as follows: 10 μL of each supernatant was incubated for 120 min at 37 °C with 1 mL of the substrate solution (27.2 mg/L GluNNap, 100 mg/L BSA, 100 mg/L DTT, and 5.55 g/L CaCl₂ in 50 mmol/L Tris-HCl, pH 7.4).

All reactions were stopped by the addition of 1 mL of 0.1 mol/L acetic buffer (pH 4.2). The amount of β-naphthylamine released as a result of enzymatic activity was measured fluorometrically at an emission wavelength of 412 nm with excitation at 345 nm. Proteins were quantified in triplicate by the method of Bradford (14), using BSA as the calibrator. Specific soluble and membrane-bound AP activities were expressed as picomoles of AlaNNap, ArgNNap, CysNNap, AspNNap, pGluNNap, or GluNNap hydrolyzed per minute per milligram of protein. Fluorogenic assays were linear with respect to time of hydrolysis and protein content.

**Statistical Analysis**

We used one-way ANOVA to analyze differences between groups. Posthoc comparisons were made using Duncan’s test; P values below 0.05 were considered significant.

**Results**

The mean ± SE activities of soluble and membrane-bound AP in the three samples of tissue studied are presented in Figs. 1 and 2.

The highest activities were observed for AlaAP, ArgAP, and CysAP, and the lowest for AspAP, GluAP, and pGluAP. In comparison with unaffected tissue, the results showed highly significant differences in specific soluble and membrane-bound AP activities, especially in neoplastic tissue. Whereas comparisons between adjacent tumoral and unaffected tissue showed no significant differences, in samples of neoplastic tissue, the soluble activities of AlaAP (P <0.001), ArgAP (P <0.001) and CysAP (P <0.001) were increased threefold, and membrane-bound ArgAP (P <0.01) was increased twofold. However, the membrane-bound AspAP activity was significantly lower in neoplastic tissue (P <0.05), and pGluAP activity was significantly lower in neoplastic and adjacent tissues (P <0.001).

**Discussion**

The etiology of BC remains unknown, and changes in hormonal status during its development are not totally understood. Proteases are particularly interesting in cancer research because they may play a part in the metastatic process (15). AP activity not only reflects tissue damage but also participates in the functional control of local factors that act through intracrine, autocrine, or paracrine mechanisms. Therefore, AP activity reflects the functional status of its peptidergic substrates. AlaAP, which exhibits broad substrate specificity, may hydrolyze bradykinins (4) and enkephalins (16), and may also act as an angiotensinase (17). ArgAP activity specifically hydrolyzes basic N-terminal residues from peptides and amino acid derivatives (7). Because of its exopeptidase activity, it has been implicated in the metabolism of met-enkephalin (18) and angiotensin (Ang) III (17); its endopeptidase activity is also thought to be involved in neurotensin metabolism (19). CysAP activity has been reported to hydrolyze oxytocin (OT) and vasopressin (VP) (20). AP A activity, including GluAP and AspAP, dis-
plays restricted specificity that may be responsible for
the cleavage of aspartic acid from Ang II to produce Ang III (7),
from Ang I to produce des-Asp3 Ang I (21), and from the
cholecystokinin octapeptide (22). pGluAP reportedly is
able to remove the NH2-terminal pyroglutamic acid resi-
due from peptides such as thyrotropin-releasing hormone
or gonadotropin-releasing hormone (GnRH) and artificial
substrates (23) (Table 1).

AP activity not only reflects tissue damage but is also
indicative of local hormonal status. In fact, the changes
observed in the present study are not unidirectional; some
activities increased, whereas others decreased. These re-
results illustrate the functional complexity of local factors,
which are selectively activated or inhibited by the tumoral
process in the affected tissue.

The present results demonstrate a highly significant
increase in soluble AlaAP activity (AP M) in neoplastic
tissue. The CD13 molecule has been reported as identical
to AlaAP (24), and immunocytochemical findings have
shown that it is present on healthy breast epithelium and
in breast carcinomas (25). This enzymatic activity is also
increased in the serum of BC patients and has been
proposed as a marker for cancer (26, 27). These findings,
especially the study of Dixon et al. (25) in BC specimens,
are in agreement with our present results.

Several AP activities (AlaAP, ArgAP, and AspAP),
which were significantly modified in the material we
analyzed, possess angiotensinase activity. Ang III is pro-
duced from Ang II by AP A or A-like activity. Ang III is
also produced from Ang I through the production of
des-Asp1-Ang I, which is further converted to Ang III by
the action of angiotensin-converting enzyme. GluAP has
been ruled out as the particulate enzyme that degrades
Ang I to des-Asp1-Ang I; another enzyme (AspAP) with
higher affinity for AspNNap than GluNNap must be
responsible (21). Ang III is further converted to Ang IV by

Soluble

Fig. 1. Soluble AP activities in BC tissue.
Samples were obtained in the same patient from neoplastic tissue (Tumoral), nonnecrotic tissue removed from the edges of the tumoral area (Adjacent), and unaffected
surrounding tissue (Control). Values represent mean ± SE of specific AP activities expressed as picomoles of the corresponding aminoacyl-β-naphthylamide hydrolyzed
per minute per milligram of protein. ***; P < 0.001 vs control and adjacent tissue.
AP B or AP M. In accordance with this scheme, our findings of a decrease in AP A activity and an increase in AP M and AP B activities suggest that the metabolism of Ang II to Ang III is slow, whereas the metabolism of Ang III to Ang IV is rapid. As a result, Ang II action predominates. Ang II has been demonstrated to stimulate cellular growth, proliferation, and differentiation as well as angiogenesis (28). In this connection, the expression of the Ang II type 1 receptor has been demonstrated in healthy and diseased human breast tissues (29). Ang II stimulates hypertropic growth of vascular smooth muscle cells. Accompanying this growth is the induction of the expres-

![Membrane-bound AP activities in BC tissue.](image)

**Fig. 2.** Membrane-bound AP activities in BC tissue.

Samples were obtained in the same patient from neoplastic tissue (Tumoral), nonnecrotic tissue removed from the edges of the tumoral area (Adjacent), and unaffected surrounding tissue (Control). Values represent mean ± SE of specific AP activities expressed as picomoles of the corresponding aminoacyl-β-naphthylamide hydrolyzed per minute per milligram of protein. *+*, $P<0.05$ vs control and adjacent tissue for AspAP; ***, $P<0.01$ vs control and adjacent tissue for ArgAP; ***, $P<0.001$ vs control for pGluAP.

### Table 1. Peptide hydrolysis by APs.\(^a\)

<table>
<thead>
<tr>
<th>AP</th>
<th>EC number</th>
<th>Other names</th>
<th>Peptide substrate</th>
<th>Arylamide substrate</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>GluAP</td>
<td>3.4.11.7</td>
<td>AP A</td>
<td>Ang II, Ang I, CCK(^b)</td>
<td>GluNap, AspNap</td>
<td>(7, 22)</td>
</tr>
<tr>
<td>AspAP</td>
<td>3.4.11.6</td>
<td>Angiotensinase A</td>
<td>Ang II, Ang I, CCK</td>
<td>AspNap, GluNap</td>
<td>(12, 21, 22, 33)</td>
</tr>
<tr>
<td>ArgAP</td>
<td>3.4.11.6</td>
<td>AP B, AP MI</td>
<td>Ang III, enkephalins, neurotensin</td>
<td>ArgNap, LysNap</td>
<td>(17–19)</td>
</tr>
<tr>
<td>AlaAP</td>
<td>3.4.11.2</td>
<td>AP M, AP N, CD13</td>
<td>Enkephalins, bradykinin, Ang III</td>
<td>AlaNap, LeuNap</td>
<td>(4, 16, 17, 24)</td>
</tr>
<tr>
<td>CysAP</td>
<td>3.4.11.3</td>
<td>Oxytocinase</td>
<td>OT, VP</td>
<td>CysNap</td>
<td>(20)</td>
</tr>
<tr>
<td>pGluAP</td>
<td>3.4.11.8</td>
<td>Pyrollidonecarboxylate peptidase</td>
<td>TRH, GnRH, neurotensin, bombesin</td>
<td>pGluNap</td>
<td>(23)</td>
</tr>
</tbody>
</table>

\(^{a}\) McDonald et al. (7) and Checler (39).

\(^{b}\) CCK, cholecystokinin; LysNap, l-lysyl-β-naphthylamide; LeuNap, l-leucyl-β-naphthylamide; TRH, thyrotropin-releasing hormone.
sion of growth-related protooncogenes as well as the synthesis of several autocrine growth factors. An imbalance in the signals activated by Ang II may produce abnormal vascular growth (30). The angiotensin-converting enzyme inhibitor captopril inhibits proliferation of human ductal carcinoma cells in culture (31); in addition, angiotensin-converting enzyme and enkephalinase activities have been detected in human breast cyst fluid (32). Therefore, modified local angiotensinase activity, reported for the first time in this study, may also reflect changes in the renin-angiotensin system in neoplastic cells and hence changes in the functions in which this system is involved. Our results, together with earlier findings, support that the renin-angiotensin system may be involved in normal and abnormal breast tissue function. This is important in view of the broad current knowledge of the renin-angiotensin system and the possibilities for pharmacological intervention at several levels of this system.

The present results show different profiles for GluAP and AspAP. Previous evidence supports the existence of two enzymes with presumably different roles in the regulation of susceptible substrates (8, 12, 21). At present, GluAP, which removes the NH$_2$-terminal Asp residue, is considered responsible for the rapid metabolism of Ang II (7). In addition, AspAP, also acts rapidly on the NH$_2$-terminal aspartic acid residues of Ang analogs, which points to a physiological role in Ang metabolism (7). For this reason we use the expression “aminopeptidase A activity” here to refer to the activity that separates NH$_2$-terminal acid residues (Asp or Glu), keeping in mind that these residues may be released through the action of at least two different enzymes, GluAP and AspAP (8). In this connection, Wilk et al. (33) have recently purified from rabbit brain cytosol an AspAP with preference for NH$_2$-terminal Asp residues but which is distinct from GluAP.

The highly significant increase in CysAP activity in tumoral tissue is particularly interesting in view of its role in the hydrolysis of OT and VP (20). Although VP is not detected in healthy breast tissue, immunohistochemical methods have revealed the presence of VP in neoplastic cells. Moreover, OT and OT-associated human neurophysin have been reported to be common in cells of healthy breast tissue but are rarely or never detected in BC (34). In addition, OT inhibits proliferation of human BC cell lines (35) and thus may play a role in preventing this disease (36). Our results confirm the involvement of these peptides and suggest a major role for CysAP in the development of BC. However, because leucine aminopeptidase is able to cleave OT (37), this enzyme may also be involved in BC.

The highly significant decrease in membrane-bound pGluAP activity in tumoral and adjacent tissue suggests that modifications in this enzyme or its putative substrates also play a major role in BC pathogenesis. In this connection, GnRH receptors and GnRH mRNA have been found in breast tissue, raising the possibility of a local role for GnRH in the human mammary gland (38). The fact that of the 12 activities measured, only membrane-bound pGluAP activity was significantly modified in tumoral and adjacent tissue suggests a role for this activity in tumor progression.

In conclusion, the present results document changes in AP activities in BC tissue; we interpret these changes to reflect the functional status of their peptidergic substrates. Our findings emphasize the importance of local intracrine, autocrine, and paracrine hormonal factors in the pathogenesis of BC and suggest that they play a role in the tumoral process.

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References

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