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# ENHANCED EXPRESSION OF ANGIOTENSIN II RECEPTOR SUBTYPES AND ANGIOTENSIN CONVERTING ENZYME IN MEDROXYPROGESTERONE-INDUCED MOUSE MAMMARY ADENOCARCINOMAS

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Angiotensin II receptors of the $AT_1$ subtype were very highly expressed in medroxyprogesterone-induced ductal adenocarcinomas of the mammary gland in BALB/c mice. $AT_1$ receptors are associated only to neoplastic epithelial cells. Lobular adenocarcinomas expressed very few $AT_1$ receptors and expressed $AT_2$ receptors only in areas corresponding to peritumoral connective tissue. Binding to angiotensin converting enzyme was present in all adenocarcinomas studied and was higher in ductal than in lobular adenocarcinomas. Normal mammary gland did not express either angiotensin II receptors or angiotensin converting enzyme. The present results are the first demonstration of angiotensin receptor subtypes and converting enzyme in mammary adenocarcinomas differentially expressed in tumors of ductal and lobular origin. Localization of receptor subtypes could be useful to study the differentiation of mammary cells during experimental mammary carcinogenesis in mice. 

Administration of medroxyprogesterone acetate (MPA) in large doses for long periods induces mammary tumors in mammals (1-8 ). In BALB/c mice, MPA administration resulted in a very high incidence of mammary tumors (1), which are metastatic cystic adenocarcinomas of the Dunn B type, preceded by ductal preneoplastic lesions (2). MPA dependent (MPA-D) and independent (MPA-I) lines have been developed by subcutaneous transplantation of primary MPA-induced tumors (3).

Polypeptide growth factors have been long implicated in the malignant transformation of breast epithelial cells (9). Some growth factors and their receptors, including insulin-like growth factor-I (IGF-I) (10), transforming growth factor-β-like polypeptides (11), and epidermal growth factor receptors (EGF) (12) have been recently detected in MPA-induced mammary adenocarcinomas in BALB/c mice. These results suggest a role for growth factors during mammary tumor progression.

On the basis of its ability to stimulate protein synthesis and cell growth, it has been suggested that Angiotensin II (Ang II) can act as a growth factor (13). The role of Ang II on growth is complex, and the peptide was proposed to modulate growth in concert with other promoters (14). Interleukin 1 and tumor necrosis factor potentiate Ang II effects on phospholipid turnover (15). Ang II may contribute to regulate the proliferation of malignant cells (16-17) and could also affect tumor growth by increasing tumor blood flow (18).

The localization and quantification of several components of the renin-angiotensin system could be achieved with quantitative autoradiography (19). Angiotensin-converting enzyme (ACE) (kininase II, EC 3.4.15.1), one of the enzymes responsible for Ang II formation, can be specifically identified using radiolabelled antagonists (20). Ang II receptors have been recently classified into two main subtypes, using newly developed selective ligands (21). The $AT_1$ receptors are selectively antagonized

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by losartan, and mediate most of the known actions of Ang II (22). Binding of Ang II to the $AT_2$ receptors is resistant to losartan, and is displaced by selective compounds such as CGP 42112 and PD 123177 (23).

Taking into account the possible role of Ang II as a growth factor, we were interested in studying the presence of elements of the renin-angiotensin system in MPA-induced mammary carcinomas. We localized and quantified both Ang II receptor subtypes and binding to ACE in these experimental mammary neoplasms.

## MATERIALS AND METHODS

Animals MPA-induced tumors in BALB/c mice and hormone dependent and independent tumor lines were obtained as previously described (1). Control female lactating BALB/c mice with 1 week-old pups, were purchased from Jackson Laboratories (Bar Harbour, ME ). Animals were killed by decapitation and the mammary tumors or control mammary glands were immediately dissected and frozen in isopentane on dry ice. Seven different tumors were studied. Five tumors were of ductal origin, (two were hormone-dependent (MPA-D) and three were hormone-independent (MPA-I)). Hormone-dependent tumors preferentially grow in MPA-treated animals, and very slowly in non-treated mice. Conversely hormone-independent tumors show a similar growth curve in MPA-treated or non-treated animals (3). Two lobular hormone-independent tumors were also studied (3). Tissues were kept frozen at -70°C until processed. Tissue sections, 16 μm thick, were cut in a cryostat at -17°C, thaw mounted on gelatin-coated glass slides and dried overnight in a desiccator at 4°C. Consecutive sections were cut for incubation to determine binding to Ang II receptor subtypes, to ACE, and for histological controls stained with hematoxylin-eosin. Sections at three different levels of the tumors were analyzed and the results combined for quantification in each tumor.

Binding Assays For quantification of Ang II receptor subtypes, the Ang II agonist [$^{125}$I]Sar$^1$-Ang II (Peninsula, Belmont, CA), iodinated by New England Nuclear, Wilmington, DE; specific activity 2200 Ci/mmol, was used as the ligand. Tissue sections were preincubated at 22°C for 15 min in 10 mM phosphate buffer, pH 7.4, containing 120 mM NaCl, 5 mM EDTA, 0.005% bacitracin (Sigma Chemical Co., St. Louis, MO) and 0.2 % proteinase-free bovine serum albumin (Sigma), followed by incubation at 22°C for 120 min in fresh buffer containing $5x10^{-10}$ M [$^{125}$I]Sar$^1$-Ang II (total binding). Consecutive tissue sections were incubated as above, with addition of $1 \times 10^{-5}$ M of the selective $AT_1$ antagonist losartan potassium (DuP 753, DuPont) to determine the number of $AT_1$ receptors, or with addition of $1 \times 10^{-5}$ M of the selective $AT_2$ displacer PD 123177 {1-(4-amino-3-methylphenyl)methyl-5-diphenyl-acetyl-4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid-2HCl, from Parke-Davis, Ann Arbor, MI} to determine the number of $AT_2$ receptors. Non-specific binding was determined in consecutive sections incubated as above in the presence of $5 \times 10^{-6}$ M unlabeled Ang II. After incubation, the sections were washed 4 times, for 1 min each, in fresh ice-cold 50 mM Tris-HCl buffer, pH 7.6, followed by a wash for 30 sec in distilled water at 0°C.

For quantification of ACE binding, we used the radiolabelled specific ACE inhibitor [$^{125}$I]-351A as a ligand (specific activity 2,200 Ci/mmol, iodinated by New England Nuclear). Consecutive tissue sections were preincubated for 30 min at room temperature in 50 mM Tris buffer, pH 7.4, containing 100 mM NaCl. Sections to be later incubated for nonspecific binding were preincubated in the same buffer containing $1 \times 10^{-7}$ M MK 521. After preincubation, the sections were incubated with 2 nM [$^{125}$I]-351A in the same buffer, for 2 hr, at room temperature. Non-specific binding was determined in the presence of $2 \times 10^{-6}$ M MK 521. After incubation the sections were washed 4 times, 1 min each, in ice-cold 50 mM Tris-HCl buffer, pH 7.4, followed by one wash, for 30 s, in cold distilled water (20).

Quantitative Autoradiography Sections were dried after washing and exposed to Hyperfilm-[$^3$H] (Amersham Corporation, Arlington Heights, IL) along with 16 μm sections of [$^{125}$I]-labeled Micro-scale standards (Amersham). Binding was quantified as described, using computerized microdensitometry (24).

## RESULTS

Autoradiography revealed very high Ang II binding in all five ductal tumors (Tumors 1-5, Figure 1; table 1). The whole tumoral mass in each section was quantified for [$^{125}$]Sar$^1$Ang II binding. In consecutive sections, the binding was completely inhibited by the $AT_1$ selective blocker losartan, but not by the $AT_2$ competitor PD 123177. This indicated that the signal represented exclusively $AT_1$ receptors. Binding was confined, in all cases, to the neoplastic epithelial tissue, and was not detected within the stroma of the tumor (Figures 1 and 3). Ang II binding was very high in both MPA-I and MPA-D ductal tumors (Table 1).

Conversely, when the whole tumoral mass was quantified in the two lobular adenocarcinomas studied (tumors 6 and 7) [$^{125}$I]Sar$^1$Ang II binding of the $AT_1$ subtype was very low (Table 1 and Figure 2). Instead, high Ang II binding was only localized

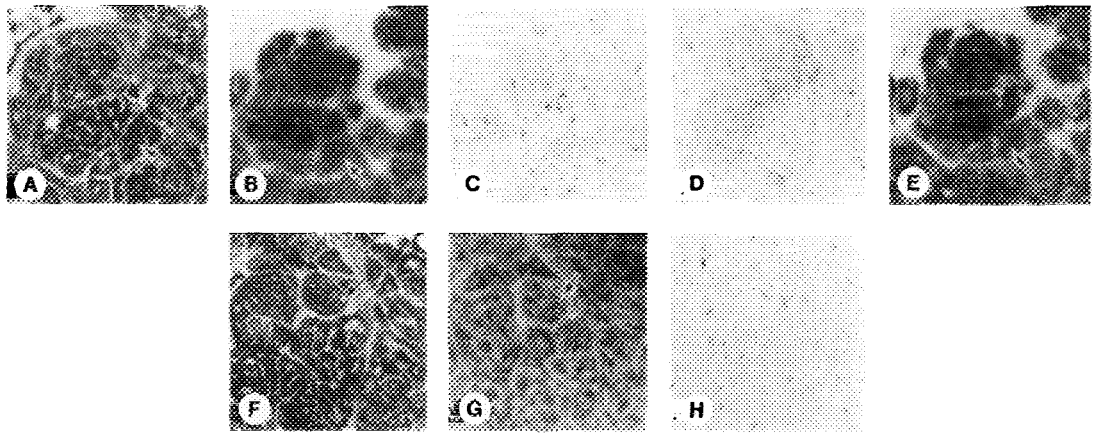


FIGURE 1. Comparative autoradiography of [$^{125}$I]Sar$^1$Ang II (upper row) and angiotensin converting enzyme (ACE)(lower row) distribution to a ductal tumor (tumor 3). A,F: sections stained with hematoxylin and eosin. B to H: adjacent sections to those in A. B: binding after incubation with $5x10^{-10}$ M [$^{125}$I]Sar$^1$Ang II (total binding). C: binding in the presence of $5x10^{-5}$ M of Ang II (non specific). D: binding in the presence of $5x10^{-5}$ M of losartan (AT2 receptors). E: binding in the presence of $1x10^{-5}$ M of PD123177 ($AT_1$ receptors). G: binding in the presence of $2x10^{-9}$ M of [$^{125}$I]-351A (total binding). H: binding in the presence of $1x10^{-6}$ M of MK 521 (non specific). Arrows point to the epithelial tumoral(neoplastic) tissue (13x).

TABLE 1. Autoradiographic quantification of [$^{125}$I]Sar$^1$ANG II binding to consecutive sections

| | Specific binding (fmol/mg protein) | +PD123177 $10^{-5}$M $AT_1$ (fmol/mg protein) | +Losartan $10^{-5}$M $AT_2$ (fmol/mg protein) |
|---|---|---|---|
| Ductal tumors (whole section) | | | |
| Tumor 1: (MPA-D) | 274 | 280 | ND |
| Tumor 2: (MPA-D) | 602 | 595 | ND |
| Tumor 3: (MPA-I) | 402 | 442 | ND |
| Tumor 4: (MPA-I) | 414 | 403 | ND |
| Tumor 5: (MPA-I) | 602 | 567 | ND |
| Lobular tumors | | | |
| a. whole section | | | |
| Tumor 6: (MPA-I) | ND | ND | ND |
| Tumor 7: (MPA-I) | ND | ND | ND |
| b. peripheral connective tissue | | | |
| Tumor 6: (MPA-I) | 54 | ND | 46 |
| Tumor 7: (MPA-I) | 50 | ND | 29 |
| Control Mammary Gland | ND | ND | ND |

ND: not detectable.

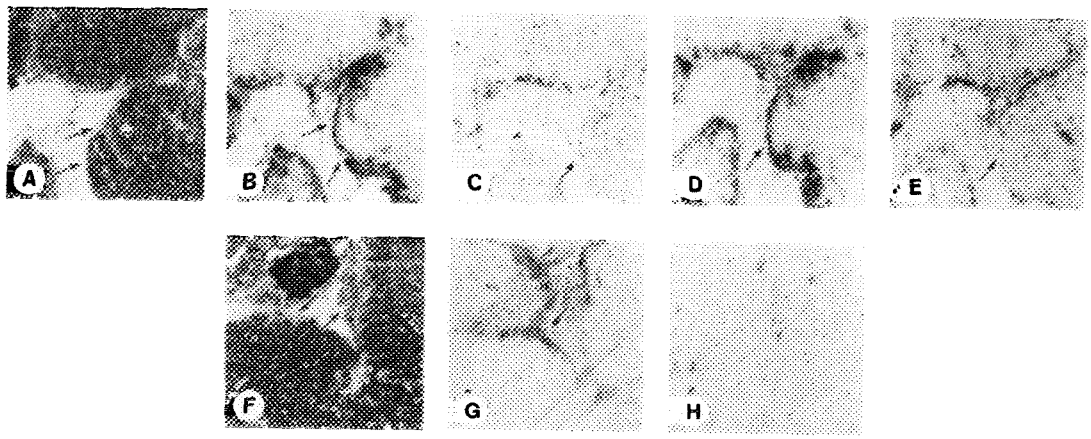


FIGURE 2. Comparative autoradiography of [$^{125}$I]Sar$^1$Ang II (upper row) and angiotensin converting enzyme (ACE)(lower row) distribution to a lobular tumor (tumor 6). A,F: sections stained with hematoxylin and eosin. B to H: adjacent sections to those in A. B: binding after incubation with $5x10^{-10}$ M [$^{125}$I]Sar$^1$Ang II (total binding). C: binding in the presence of $5x10^{-5}$ M of Ang II (non specific). D: binding in the presence of $5x10^{-5}$ M of losartan (AT2 receptors). E: binding in the presence of $1x10^{-5}$ M of PD123177 (AT$_1$ receptors). G: binding in the presence of $2x10^{-9}$ M of [$^{125}$I]-351A (total binding). H: binding in the presence of $1x10^{-6}$ M of MK 521 (non specific). Arrows point to the peripheral tissue (13x).

to small areas of stromal peripheral tissue of the tumors, corresponding to peritumoral connective tissue. In these areas, binding was mainly displaced by PD 123177, indicating a predominance of $AT_2$ receptors (Figure 2 and Table 1). Since a patchy distribution was observed, sections at different level of the lobular tumors showed different receptors concentration. Table 1 shows the values obtained for tumor 6 at the sections showed in Figure 2.

The expression of ACE, determined by means of binding to the radiolabelled inhibitor 351A, corresponds to the Ang II receptor expression. In ductal tumors, the signal was higher in the epithelial tumor tissue than in the surrounding stroma and was

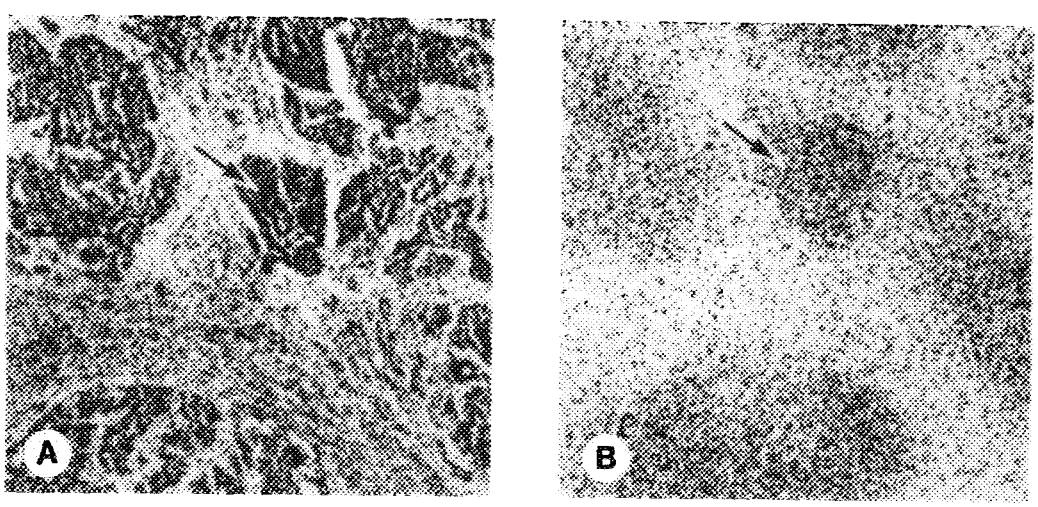


FIGURE 3. Specific localization of [$^{125}$I]Sar$^1$Ang II in a ductal tumor (number 3)(70x). A: section stained with hematoxylin and eosin. B: [$^{125}$I]Sar$^1$Ang II binding. Arrows point to the neoplastic tissue.

TABLE 2. Autoradiographic quantification of Angiotensin-converting enzyme binding to the whole tumoral section

| | ACE binding (fmol/mg protein) |
|---|---|
| Ductal tumors (whole section) | |
| Tumor 1 : (MPA-D) | 422.8 |
| Tumor 2 : (MPA-D) | 27.8 |
| Tumor 3 : (MPA-I) | 33.3 |
| Tumor 4 : (MPA-I) | 69.3 |
| Tumor 5 : (MPD-I) | 186.0 |
| Lobular tumors (whole section) | |
| Tumor 6 : (MPA-I) | 5.2 |
| Tumor 7 : (MPA-I) | 3.7 |
| Control Mammary gland | ND |

ND: not detectable.

similar in MPA-D and MPA-I tumors (Figure 1 and Table 2). In lobular adenocarcinomas, binding was very low in the whole tumoral mass (Table 2), but a higher binding (21 fmol/mg protein) was associated with the peripheral connective tissue, corresponding to the localization of $AT_2$ receptors (Figure 2). ACE binding was higher in ductal tumors than in those of lobular origin (Table 2).

In sections of mammary gland obtained from control lactating female BALB/c mice, both Ang II and ACE binding were very low, and below the level of detection of our method.

## DISCUSSION

The present results demonstrate that Ang II $AT_1$ receptors and ACE are highly expressed in both MPA-D and MPA-I in vivo transplants of ductal MPA-induced mammary adenocarcinomas in female BALB/c mice. The distribution and number of the Ang II receptor subtypes correlates with the histogenesis of the tumors. Adenocarcinomas of ductal origin express large numbers of $AT_1$ receptors, which are associated with neoplastic epithelial cells. Conversely, $AT_1$ receptor expression is very low in lobular tumors. Instead, lobular tumors express mainly $AT_2$ receptors in the peripheral connective tissue. Both the expression of Ang II receptors and ACE appears to be a phenomenon related to the tumoral nature of the tissue, since neither is expressed in mammary glands from normal, lactating rats.

The precise cellular localization of ANG II receptors and ACE could not be determined in this study due to limitations in the power of resolution of our technique. However, the expression of ACE binding which follows the pattern and the relative number of the Ang II receptor subtypes, suggests the local formation of Ang II within the tumor tissue.

Ang II has been proposed to play a role in growth (13-14), in organogenesis (26), and in carcinogenesis(15-17). For example, some neuroblastoma cells contain all components of the renin-angiotensin system and ACE inhibitors reduce the growth

of one human neuroblastoma cell line (16). Another angiotensin-forming enzyme, renin, is present in glioblastoma multiforme and in other highly vascularized tumors, and on this basis it was suggested that Ang II is able to stimulate tumor vascularization (17). Ang II has been proposed to selectively increase tumor blood flow (18). Besides, inhibition of Ang II formation with ACE inhibitors has been shown increase survival of rats implanted with adenocarcinomas (14).

Several growth factors have been demonstrated to be present on MPA-induced tumors, such as EGF, TGF-β, IGF-I and IGF-II and no correlation with hormone dependence has been found (3,10-12). It is noteworthy that the expression of Ang II receptors or ACE like that of IGF-I, does not correlate with the hormone dependence of the tumor. IGF-I mRNA was expressed in the stromal but not in the epithelial cells of mammary tumors (25). Based on this selective localization, it has been postulated that interactions between malignant epithelial cells and their surrounding stroma may play an important role in the growth regulation of breast cancer by IGF-I (25). Our findings of the coexistence of Ang II receptors and ACE in similar areas supports a possible paracrine or autocrine function for Ang II, as it has been suggested for the IGF-I (25). Further studies are necessary to address a correlation between the expression of growth factors and Ang II receptors in these tumors as well as with cell growth.

The present results are the first demonstration of angiotensin receptor subtypes and converting enzyme in mammary adenocarcinomas. The differential localization and the presence of subtypes of Ang II receptors and ACE in tumors of different origin may indicate diverse roles for Ang II and are not inconsistent with the possibility that Ang II could play a autocrine-paracrine role as a growth factor in MPA-induced mouse adenocarcinomas.

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