ACE and ACE2 expression in normal and malignant skin lesions

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Abstract: The renin-angiotensin system (RAS) is known mainly as a regulator of cardiovascular homeostasis. However, it has also been shown to mediate processes such as proliferation, apoptosis, angiogenesis, and carcinogenesis. Nonmelanoma skin cancers (NMSC) — including basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) — are among the most common cancers. The aim of the present study was to determine the immunohistochemical expression of angiotensin-converting enzyme (ACE), angiotensin-converting enzyme 2 (ACE2), and Ki-67 antigen in archival samples of normal skin, actinic keratosis, and malignant skin lesions. Cytoplasmic-nuclear ACE immunoreactivity was observed in 99% of examined cases of both normal skin and cancers. Significantly higher ACE immunoreactivity occurred in normal skin, as compared with BCC and SCC (p < 0.01, p < 0.0001, respectively). Additionally, ACE immunoreactivity was also significantly higher in BCC, compared with SCC (p < 0.05). ACE2 immunoreactivity was noted in basal epidermal layers and in sebaceous gland cells in normal skin, though not in NMSC. These novel observations suggest that ACE and skin RAS may be involved in the pathogenesis of malignant skin lesions. (Folia Histochemica et Cytobiologica 2013, Vol. 51, No. 3, 232–238)

Key words: skin cancer, BCC, SCC, actinic keratosis, ACE, ACE2, immunohistochemistry

Introduction

The renin-angiotensin system (RAS) consists of systemic and local parts. The systemic RAS has been mainly perceived as an important regulator of cardiovascular homeostasis and as a key factor in the pathogenesis of hypertension and atherosclerosis. The local renin-angiotensin systems observed in various organs act mainly over a limited area [1]. The first element of RAS is liver-derived angiotensinogen, a glycoprotein cleaved by renin to generate decapeptide angiotensin I (Ang I) [1]. The angiotensin-converting enzyme (ACE) is a protease capable of cleaving the inactive Ang I to active octapeptide angiotensin II (Ang II), which is regarded as the most active regulator of the systemic RAS [1]. The actions of Ang II are mediated predominantly through its specific receptors, Ang II receptor type 1 (AT1R) and Ang II receptor type 2 (AT2R) [2]. Most of the known effects of Ang II — such as its stimulation of angiogenesis, cellular proliferation, inflammatory and antiapoptotic
responses — occur via AT1R [3, 4]. AT2R-mediated actions have been shown to oppose those elicited by AT1R [5, 6]. However, several lines of evidence suggest that signaling via AT2R may also be proangiogenic and proinflammatory [7]. Although Ang II is the most important effector of the RAS, there are also other products of aminopeptidase activity of ACE and ACE2, such as angiotensin III (Ang III), angiotensin IV (Ang IV), and angiotensin-(1-7) [(Ang-(1-7)], which all show potent biological activity [9–12]. Ang-(1-7) is an endogenous 7-amino-acid peptide hormone that exerts antiproliferative activity and counteracts the vasodilative and apoptotic properties of Ang II [13]. The specific effects of Ang-(1-7) are mediated by a recently identified receptor, the mas oncogene product (MAS) [14]. Ang-(1-7) may be formed from Ang I through cleavage of angiotensin-(1-9), or it may be generated directly from Ang II by the enzymatic activity of ACE2 [15]. ACE2, discovered almost a decade ago, is an ACE homologue and a zinc-metallopeptidase [15]. It has been suggested that ACE2 may oppose the effects of ACE on the organ and tissue levels through the generation of Ang-(1-7) by the local RAS [15]. The local RAS systems have been detected in various species and in diverse organs, such as the brain, the testes, the prostate, the pancreas, the adrenal gland, and the mammary glands [16–22]. The local RAS systems enable the generation of Ang II, and may therefore exert biological activity on an organ level. Recent studies suggest that, on a tissue level, the local RAS may influence cell proliferation and apoptosis, which are considered crucial in carcinogenesis [23, 24].

Evidence suggests that ACE may modify the actions of neuroendocrine peptides in the skin [25, 26]. It is noteworthy that proopiomelanocortin (POMC) — the neuroendocrine precursor protein — has also been found to be expressed in the innervating neurons, epidermal and dermal skin cells, and immune cells, such as the Langerhans cells of the skin [25–27]. Some studies have suggested that one of the fragmentation products of POMC is adrenocorticotropic hormone (ACTH), which, together with its structurally related peptides (ACTH_{7-39}, ACTH_{4-11}, ACTH_{1-10} and ACTH_{18-39}), has been shown to inhibit the activity of ACE in a noncompetitive way without being the substrate of the enzyme [28]. It has also been demonstrated that ACTH produced in the skin can be metabolized by ACE [27].

Nonmelanoma skin cancers (NMSC), including basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), are the most common skin cancers in the Caucasian population [29, 30]. SCC is less common than BCC, and accounts for 20% of all NMSC. It is related to a poorer prognosis due to the formation of metastases [31]. Actinic keratosis (AK) is regarded as the premalignant lesion of SCC that is induced mainly by exposure to UV radiation [32]. It is believed that AK transforms to carcinoma in situ, and subsequently to advanced SCC through a multistep cancerogenesis process [33].

Although ACE and ACE2 have been studied intensely in numerous human diseases, their expression in normal skin and its lesions has not yet been investigated. For this reason, the present study aimed to evaluate the immunoreactivity of ACE and ACE2 in normal skin, AK, SCC, and BCC, with regard to patients' clinical and pathological parameters.

Material and methods

Patients and tissue samples. The study was performed on archival paraffin blocks of 89 cases of skin lesions (16 cases of AK, 38 cases of SCC, and 35 cases of BCC) and 14 cases of normal skin obtained from patients treated at the Department and Clinic of Dermatology, Venereology, and Allergology in Wroclaw during the years 2005–2007. In the BCC cases, 9 (25.7%) came from females and 26 (74.3%) came from males. Twenty-three (65.7%) BCCs were from skin that was exposed to the sun, and the remaining 12 (34.3%) were diagnosed in normally occulted body localizations (legs, trunk). The study included only superficial (15 cases; 42.9%) and nodular (57.1%) BCC subtypes. The clinical and pathological data of the SCC patients examined are summarized in Tables I and II.

Immunohistochemical (IHC) method. Tissue samples were fixed in 10% buffered formalin, dehydrated, and embedded in paraffin. Paraffin sections, 6 µm thick, were stained with haematoxylin and eosin (H&E) to verify the diagnosis. ACE, ACE2 and Ki-67 IHC staining was performed on 4-µm-thick paraffin sections fixed on Superfrost Plus slides (Menzel Gläser, Braunschweig, Germany). Deparaffinization and antigen retrieval were performed in Target Retrieval Solution with pH 9 (ACE, ACE2) or pH 6 (Ki-67) at 97°C for 20 min using the PT Link platform (Dako, Glostrup, Denmark). Subsequently, the sections were washed in TBS (tris-buffered saline and incubated with primary antibodies directed against ACE (1:100, Santa Cruz Biotechnology, Santa Cruz, USA), ACE2 (1:100, Santa Cruz Biotechnology), and Ki-67 (1:100, MIB-1, Dako) in an Autostainer Link48 automated staining platform (Dako) for 20 min at room temperature. The slides were then washed in TBS and visualization was performed using the EnVision FLEX system (Dako) according to the manufacturer’s instructions.

Histopathological examination and analysis of IHC reactions. H&E sections were evaluated by two pathologists to
confirm the diagnosis and to assess the grade of tumor malignancy on Broders’ scale. The depth of skin infiltration was also established in the SCC cases.

All IHC reactions were evaluated by two pathologists under BX-41 light microscope (Olympus, Tokyo, Japan). The evaluation of ACE and ACE2 reaction intensities was appraised using the 12-point immunoreactive (IRS) scale of Remmele and Stegner, in which the percentage of positive cells (0: absence of cells with positive reaction; 1 point: 1–10% cells; 2 points: 11–50%; 3 points: 51–80%; 4 points: over 80% cells with positive reaction) and the intensity of the reaction (0: no reaction; 1: low intensity of the reaction product; 2: moderate intensity of the reaction color; 3: intense color of the reaction) are taken into account. The final score assigned was the multiplicative product of these two parameters [34]. The immunoreactivity of Ki-67 was analyzed utilizing a semiquantitative 5-point scale in which 0 denotes 0% positive nuclear stained cells, 1 represents 1–10%, 2 represents 11–25%, 3 represents 26–50%, and 4 indicates > 50% [35]. In cases where the scores differed, the results were discussed by the observers until a consensus was achieved.

Statistical analysis. The obtained results were subjected to statistical analysis using Prism 5.0 software (GraphPad, CA, USA). The Mann-Whitney test was employed to compare ACE immunoreactivity in all the tested groups. Correlations between the analyzed markers were determined using the Spearman correlation test. Differences were considered significant when p < 0.05.

Results

ACE immunoreactivity was found in all cases of normal skin, AK, and SCC. Staining was observed in the cytoplasm and nuclei of cells of all epidermis layers, hair follicles, sebaceous glands, and cancer cells (Figure 1A–C, E). Similarly, almost all BCC cases (97.1%) were ACE-immunoreactive (Figure 1D). On the contrary, a weak ACE2 cytoplasmic reaction was seen only in the cells of the basal layer of the epidermis and sebaceous glands in normal skin, and not in AK or NMSC (Figure 1G–H). Nuclear Ki-67 antigen immunoreactivity was observed in all the analyzed cases of normal skin and the studied skin lesions (Figure 1F).

The highest ACE immunoreactivity was noted in normal skin (IRS 9.07 ± 2.62). The intensity of the reaction was lower in cases of AK (IRS 6.75 ± 3.00) and BCC (IRS 6.31 ± 3.47), and was lowest in the SCC cases (IRS 4.13 ± 2.46) (Figure 2). The ACE immunoreactivity was significantly lower in SCC (p < 0.0001) and BCC (p < 0.01) when compared with normal skin. ACE immunoreactivity was also significantly lower in the SCC cases, in comparison with AK (p < 0.01) and BCC (p < 0.05). It should be noted that an almost significant higher immunoreactivity of ACE was detected in normal skin, as compared with AK (p = 0.0516) (Figure 2).

No significant correlations were recorded between the immunoreactivity of ACE and Ki-67 antigen expression in all the studied cases and in particular lesions. No associations were noted between ACE immunoreactivity and the clinical or pathological data of the SCC and BCC patients.

Table I. Patient and tumor characteristics of the SCC cases

<table>
<thead>
<tr>
<th>Parameter</th>
<th>No. of cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>22 (57.9)</td>
</tr>
<tr>
<td>Female</td>
<td>16 (42.1)</td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
</tr>
<tr>
<td>Keratodes</td>
<td>31 (81.6)</td>
</tr>
<tr>
<td>Akeratodes</td>
<td>7 (18.4)</td>
</tr>
<tr>
<td>SCC on Broders’ scale</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>7 (18.4)</td>
</tr>
<tr>
<td>2</td>
<td>11 (28.9)</td>
</tr>
<tr>
<td>3</td>
<td>13 (34.3)</td>
</tr>
<tr>
<td>4</td>
<td>7 (18.4)</td>
</tr>
<tr>
<td>SCC depth of infiltration</td>
<td></td>
</tr>
<tr>
<td>Epidermis</td>
<td>8 (21.0)</td>
</tr>
<tr>
<td>Subcutaneous tissue</td>
<td>30 (89.0)</td>
</tr>
<tr>
<td>Density of inflammatory infiltration</td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>8 (21.0)</td>
</tr>
<tr>
<td>Moderate</td>
<td>22 (58.0)</td>
</tr>
<tr>
<td>Massive</td>
<td>8 (21.0)</td>
</tr>
<tr>
<td>Sun exposure</td>
<td></td>
</tr>
<tr>
<td>Exposed</td>
<td>35 (92.1)</td>
</tr>
<tr>
<td>Occulted</td>
<td>3 (7.9)</td>
</tr>
</tbody>
</table>

SD — standard deviation
Figure 1. Expression of ACE in: A. normal skin; B. actinic keratosis (AK); C. squamous cell carcinoma (SCC); D. basal cell carcinoma (BCC). E. A cytoplasmic-nuclear ACE immunoreactivity in normal skin keratinocytes. F. Nuclear Ki-67 antigen immunoreactivity in BCC cancer cells. Arrows point ACE2 immunoreactivity in basal epidermis layer (G), and sebaceous gland (H).
Discussion

In this immunohistochemical study, we documented a decrease in ACE immunoreactivity associated with the progression of carcinogenesis. The highest ACE immunoreactivity gradually decreased from high expression in normal skin along the carcinogenic pathway of SCC.

Currently, there are no other reports showing the expression of RAS elements in normal skin or malignant skin lesions. In different organs, such as the pancreas, the local RAS system is autonomous from the activity of the systemic RAS and may exert differential biological effects, such as stimulation of angiogenesis, prevention of chemotherapy toxicity in pancreatic ductal adenocarcinoma, or initiation and propagation of acute pancreatitis [36]. Similarly to our own observations, Larrinaga et al. indicated the special role of intrarenal RAS (iRAS) in renal cancer. In this study, the activity and immunoreactivity of both ACE and ACE2 was downregulated in renal cancer cells, as compared with adjacent normal kidney tissue [37]. The authors speculated about the possibility of using these enzymes as prognostic and diagnostic factors, although this hypothesis remains to be clarified [37]. The role of RAS in cancer pathogenesis has also been supported by the study of Dolley-Hitze et al., which was performed on renal clear-cell cancer and recognized the expression of AT1R and AT2R as negative prognostic and predictive factors [38]. Thus, on the basis of our findings of the lower immunoreactivity of ACE in more malignant skin lesions as compared with the premalignant AK and normal skin, one could hypothesize a tumor-suppressing role of ACE.

Many lines of evidence suggest that the use of ACE inhibitors (ACEi) and angiotensin receptor blockers (ARB) — both commonly used drugs in antihypertensive therapy — might be preventative in cancer development through the inhibition of systemic and local RAS activity [39–44]. Patients with diagnosed urothelial tumor of the upper urinary tract treated with RAS inhibitors due to hypertension were characterized by longer event-free survival in comparison with patients treated with other antihypertensive drugs [39]. Similar observations were made by Sugimoto et al. for *H. pylori* related gastric cancer [40]. Several studies concerning the incidence of NMSC in patients treated with RAS inhibitors have confirmed the protective role of ACEi and angiotensin receptor blockers (ARBs) by showing the lower incidence of SCC and BCC in these patients’ cohorts, as compared with patients treated with other antihypertensive drugs or without antihypertensive therapy [41–44].

*In vitro* investigations and studies on animal models have confirmed the protective role of ACEi in tumor progression [45, 46]. Yoshiji et al. have shown that ACEi can suppress cell proliferation and vascular endothelial growth factor (VEGF) expression in hepatocellular carcinoma and angiogenesis [47]. In addition, *in vitro* investigations on triple negative breast cancer cell lines (lacking estrogen, progesterone, and HER2 receptors) and on invasive ductal breast carcinoma tissues have demonstrated the inductive role of ACE and AT1R in the process of breast cancer angiogenesis [48–50].

Although the population-based studies revealed that RAS inhibitors may be protective against NMSC [41, 42], in this study we found decreasing ACE expression with increasing malignant potential of the NMSC. Our results may indicate that ACE expression can act as a tumor suppressor and that the protective role of RAS inhibitors might be caused by the inhibition of the systemic RAS and the generation of Ang-(1-7) by ACE2 [15]. Higher levels of Ang-(1-7) could lead to increased apoptosis, decreased proliferation, and angiogenesis.

Moreover, local ACE expression *via* synthesis of Ang II could also downregulate the expression of Klotho protein, which has been shown to act as suppressor gene [51]. In a mouse model of glomerulonephritis, Klotho had a nephroprotective effect by abrogating oxidative stress [52]. In addition, mice characterized by overexpression of this protein show prolonged lifespan, in contrast to mice with knockdown of its expression [51, 53]. Hypothetically, a decrease in ACE expression could result in Klotho overexpression in affected keratinocytes and in cancer cells increasing their survival capacities. However, this hypothesis requires further research.

Our research has shown that ACE2 immunoreactivity is present only in the basal cell layer of normal...
epidermis and sebaceous glands, and not in AK and NMSC. This may support the results of population studies regarding the administration of RAS inhibitors and the systemic role of Ang-(1-7) [13, 44].

This study failed to identify any associations with patients’ clinical and pathological data, since ACE immunoreactivity did not vary with patients’ age, lesion localization, invasive potential, extent of immunohistochemical response, or cell proliferation measured by expression of the Ki-67 antigen. Similarly to Bieniek et al., we did not find any associations between Ki-67 immunoreactivity and patients’ sex or BCC histological subtype [54].

Our results of decreased expression of ACE in malignant skin cancers may indicate the involvement of ACE in the pathogenesis of SCC. ACE might also be involved in the pathogenesis of BCC, since its immunoreactivity was significantly lower in this tumor in comparison with normal skin. Future studies are necessary to further elucidate the hypothetic tumor-suppressing role of ACE.

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