The role of angiotensin II in the regulation of breast cancer cell adhesion and invasion

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Abstract

As breast cancer remains the most common cause of cancer death in women, there is a continuing need not only to further characterise the processes of cancer progression, but also to improve accuracy of prognostic markers. Breast epithelial cells express components of the renin angiotensin system and studies suggest that these may be altered in disease progression. In addition, altered integrin expression correlates with lymph node metastasis. The aim of this study was to investigate the relationship between angiotensin II (AII) and integrins in breast tissue and, in particular, their role in breast cancer cell metastasis. Using in vitro assays, AII (10^{-6} M)-treated MCF-7 and T47D breast cancer cells both show reduced adhesion to extracellular matrix proteins collagen-, fibronectin- and laminin-coated wells (P<0.001) and reduced invasion through collagen-, fibronectin- and laminin-coated membranes (P<0.05). This action was inhibited by co-treatment with the angiotensin type 1 receptor (AT1R) antagonist losartan (10^{-5} M). The addition of the AT2R inhibitor PD123319 (10^{-5} M) to AII-treated cells had no significant effect. Semi-quantitative reverse transcriptase-PCR and western blotting revealed that cells treated with AII (10^{-6} M) expressed lower levels of both integrin α3 and β1. Using specific inhibitors, this was shown to occur through protein kinase C signalling. These data suggest that AII reduces cell adhesion and invasion through the type 1 receptor and that this effect may be due to reduced expression of integrins, in particular α3 and β1.

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Introduction

All processes of cell adhesion, migration and invasion are mediated through cell surface receptors that contribute to the metastatic process (Ivaska & Heino 2000, Bogenrieder & Herlyn 2003). Molecules that mediate these processes include transmembrane glycoproteins that determine both cell–cell binding and the interaction between cells and extracellular matrix (ECM) proteins. One major class of such adhesion receptors are the integrins, which are heterodimers of α and β subunits (Humphries 1990, van der Flier & Sonnenberg 2001, Hynes 2002). The role played by cell surface molecules, such as the integrins, in cancer cell adhesion and invasion is well documented (Mizejewski 1999, Putz et al. 1999, Bogenrieder & Herlyn 2003) although their regulation is incompletely understood. Loss of specific integrin molecules has been shown to be of particular importance in breast cancer disease progression, and generally integrin expression is reduced in breast cancer samples compared with non-cancerous tissue (Zutter et al. 1990, Koukoulis et al. 1991, Pignatelli et al. 1991, Gui et al. 1997). Altered integrin expression is also correlated with nodal positivity and, in particular, α3 and β1 integrins may have specific important roles (Morini et al. 2000). Furthermore, a β1 specific functional antibody inhibits invasion of the breast cancer cells through matrix proteins such as laminin, collagen and fibronectin in in vitro assays (Berry et al. 2003).

In common with many other tissue types, there now appears to be a localised renin angiotensin system (RAS) in the breast, and breast epithelial cells express the angiotensin type 1 receptor (AT1R) (Inwang et al. 1997, De Paepe et al. 2001). In situ hybridisation revealed that prorenin mRNA transcription was invariably distributed in a band of connective tissue cells and myoepithelial cells, completely surrounding the AT1R-containing ductal epithelial cells in normal tissue and in in situ lobular carcinoma. This band of prorenin gene-transcribing cells was disrupted and
attenuated in later stage invasive tumours, and in some of these, it could not be detected at all (Tahmasebi et al. 1998). These data suggest that a local tissue RAS is present in breast tissue and is potentially able to supply angiotensin (AII) to receptors in ductal epithelial cells. This mechanism is disrupted in cancer (Tahmasebi et al. 1998). From this, it is possible to infer that AII and the tissue RAS may have an important role in the development of cancer.

All is known to stimulate breast cell proliferation (Greco et al. 2002, Muscella et al. 2003), but it may have other activities. One possibility is that it may be involved in breast disease progression as suggested by preliminary studies, in which it was demonstrated that treatment of breast cancer cells with AII reduced breast cancer cell invasion through a simple matrix composed of collagen type IV. The regulation of integrin expression clearly requires elucidation. Since the local generation of AII may be important in tissue organisation in the breast (Tahmasebi et al. 1998), it is possible to speculate that tissue RASs may be involved. This paper examines the action of AII on cell adhesion and invasion and the expression of integrin subtypes.

Materials and methods

Antibodies

Anti-α3 integrin polyclonal antibody was from Biogenesis (Poole, Dorset, UK) and anti-β1 integrin monoclonal antibody was from Abcam (Cambridge, UK). Anti-mouse IgG peroxidase and anti-rabbit IgG peroxidase were both from Amersham.

Cell culture

MCF-7, T47D breast cancer cells and NIH 3T3 fibroblast cells were obtained from European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK). Cell culture and all other reagents were obtained from Sigma unless otherwise stated.

MCF-7 and T47D cells were grown in 75 cm² tissue culture flasks with Modified Eagle’s Medium or Dulbecco’s Modified Eagle’s Medium (DMEM) respectively, supplemented with l-Gln (4 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), sodium pyruvate (2 mM), essential amino acids and 5% fetal bovine serum in a humidified 95% air/5% CO₂ environment at 37 °C. Prior to experimentation, cells were grown in serum-free medium for 24 h. Cells were then washed using Hank’s balanced salt solution and harvested using 0.05% (w/v) trypsin in 0.02% (w/v) EDTA for 1 min at 37 °C and cells were recovered by centrifugation at 900 g for 10 min. Cells were counted and the viability was assessed using the Trypan Blue exclusion assay (Freshney 1994).

Experimental cells were grown for a further 48 h with either the addition of serum-free medium (vehicle) or AII (10⁻⁶ M) alone or a combination of AII (10⁻⁵ M) with the AT1R antagonist losartan (10⁻⁵ M), a kind gift from Merck (Rahway, NJ, USA) or the AT2R antagonist, PD123319 (10⁻³ M). All dilutions were made in serum-free medium. These cells were then used for cell migration or invasion assays or to provide RNA or protein for reverse transcriptase (RT)-PCR and immunoblotting studies.

Preparation of conditioned medium

NIH 3T3 cells were grown to confluence in DMEM supplemented as above. The medium was then replaced with serum-free DMEM containing 50 μg/ml ascorbic acid for 24 h. This medium was decanted and centrifuged to remove any cells in suspension, and used immediately after preparation as a chemotactic agent.

Cell adhesion

ECM protein collagen type IV (50 μg/well), laminin (62.5 μg/well) and fibronectin (50 μg/well) were coated in 96-well tissue culture plates (Dejana et al. 1987). Prior to use, all wells were rehydrated with Bovine serum albumin (BSA) solution (100 μg/ml) for 2 h, which was then removed. Cells were added to each well (500 cells per 200 μl unsupplemented DMEM). The plates were incubated at 37 °C in a humidified 95% air/5% CO₂ environment for 1 h. After incubation, medium was removed and wells were washed with unsupplemented DMEM to remove unattached cells. Remaining cells adhering to the matrix proteins were stained with Diff-Quik from Dade Behring AG (Diédingen, Switzerland) and counted.

Cell invasion

Modified Boyden chambers (Falk et al. 1980) were used. MCF-7 and T47D cells were added to inserts (8 μm pore) coated with either collagen type IV (50 μg/insert), laminin (62.5 μg/insert) or fibronectin (50 μg/insert) (Terranova et al. 1986, Albini et al. 1987) and rehydrated for 2 h with BSA solution (100 μg/ml) prior to use. The amount of protein used to coat the inserts was defined as the amount of matrix protein required to
block the migration of non-invasive 3T3 cells over a 24-h period. Cells (10 000 per 300 μl of unsupplemented DMEM) were added to the inserts, which were placed into companion plates filled with 900 μl conditioned NIH 3T3 cell medium. The plates were incubated at 37 °C in a humidified 95% air/5% CO₂ environment. After 24 h, the cells on the upper surface of the insert were completely removed by scrubbing with cotton buds in unsupplemented DMEM. The invasive cells attached to the lower surface of the filter were stained with Diff-Quik and counted.

RT-PCR

Oligonucleotide primers of α3, β1 integrins and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were custom synthesized by MWG-Biotech AG (Ebersberg, Germany). RT-PCR reagents were obtained from Promega. Primer pairs for α3 (forward 5'-TGG GCA GAT GGA TGT GGA TGA GAA-3', reverse 5'-GAT GAT GAT GGG GCG GAG TTT GTC-3') and β1 (forward 5'-AAT GGG AAC AAC GAG GTC ATG GTT-3', reverse 5'-TTG TGG GAT TTG AAC GAG CAG TAC-3') integrins were derived from published material (Orr & Kamen 1994). Total RNA was isolated from MCF-7 and T47D cell cultures using Tri-reagent (Helena Biosciences, Sunderland, UK) according to the manufacturer’s protocol. One microgram of total RNA was then reverse transcribed using Moloney-Murine Leukemia Virus reverse transcriptase. Two microlitres of the resultant cDNA were then incubated in a reaction mixture (50 μl) containing PCR buffer (10 mM Tris–HCl, 50 mM KCl, 0.1% Triton X-100), 10 mM DNTPs, integrin or GAPDH forward primer (1 μM), integrin or GAPDH reverse primer (1 μM), 25 mM MgCl₂, 100 U Taq polymerase and diethylpyrocarbonate water. PCR amplification was carried out over 25 cycles for integrin primers (1 min at 94 °C, 1 min at 60 °C, 2 min at 72 °C and 1 cycle of 10 min at 72 °C) and 20 cycles for GAPDH primer pairs (1 min at 94 °C, 1 min at 60 °C, 2 min at 72 °C and 1 cycle of 10 min at 72 °C). PCR products were separated using 1.2% agarose gels, stained with ethidium bromide, visualised under UV, and analysed by densitometry using Gel Doc system (UVP Ltd, Cambridge, UK). These cycling conditions were established empirically to give linear increase in product intensity proportional to the amount of template.

SDS gel electrophoresis

Cells were sonicated for 2×30 s pulses at 4 °C. The sonicate was centrifuged at 5000 g. The supernatant was removed and further centrifuged at 100 000 g for 60 min at 4 °C. The pellet was resuspended in membrane buffer (Tris–HCl 50 mM, NaCl 100 mM, MgCl₂ 6 mM). Membrane protein (50 μg) was solubilised using PBS containing 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate and 0.1% (w/v) SDS with 10 μg/ml each of the protease inhibitors, aprotinin, leupeptin, soya bean trypsin inhibitor, for 1 h at 4 °C. Solubilised proteins were separated on non-reduced 8% SDS-PAGE gels at 200 V according to the method of Laemmli (1970). Separated proteins were transferred on to a nitrocellulose membrane overnight at 30 V. Protein was assayed according to the method of Lowry et al. (1951).

Immunoblotting

Nitrocellulose membranes, with transferred proteins, were blocked with 5% BSA (Tris buffered saline, 0.1% Tween-20) for 1 h at room temperature. Membranes were incubated with primary antibody at room temperature for 1 h, washed (3×10 min) with washing buffer (TBS, 0.1% Tween-20), followed by incubation with anti-rabbit IgG peroxidase (1:2000 v/v) or anti-mouse IgG peroxidase (1:2000 v/v) as appropriate for 1 h at room temperature, then washed (3×10 min) with washing buffer. Specific bands were visualised using enhanced chemiluminescence materials from Amersham and analysed by densitometry (Gel Doc System, UVP Ltd, Cambridge, UK).

Results

Cell adhesion

Treatment of both MCF-7 (Fig. 1a) and T47D (Fig. 1b) cells with AII (10⁻⁶ M) for 48 h causes concentration-dependent reduction in adhesion to collagen type IV-coated inserts. Further examination of these AII treated cells showed that MCF-7 (Fig. 2a) and T47D (Fig. 2b) cell adhesion was also reduced when using inserts coated with fibronectin (50 μg/insert) or laminin (62.5 μg/insert). BSA-coated (62.5 μg/insert) inserts were used as controls (Fig. 2a) and showed no activity. In order to determine which AII receptor subtype produced this effect, receptor-specific antagonists were used. The significant inhibition by AII of MCF-7 and T47D cell adhesion to collagen was attenuated by incubation with the AT1 receptor antagonist losartan (10⁻⁵ M), but not by the AT2 receptor antagonist PD123319 (10⁻⁵ M) (Fig. 2a and b).
Cell invasion

Figure 3 shows the results of an experiment to determine the correct amount of matrix protein required to block cell migration. It can be clearly seen that increasing the concentrations of collagen coating reduced the number of non-invasive 3T3 cells able to migrate through the membrane, and when the membrane is coated with 50 \( \mu \)g, the cell migration is blocked and thus cell invasion can be measured. Similar analyses were performed for both laminin and fibronectin (data not shown). Both MCF-7 and T47D cell invasion through collagen-coated inserts was reduced by pretreatment with AII in a concentration-related manner. Similar results were obtained with fibronectin- or laminin-coated inserts (Fig. 4a and b). Simultaneous pretreatment with losartan abolished the effect of AII on cell invasion through collagen, whereas PD123319 had no effect (Fig. 4a).

\( \alpha_3 \) and \( \beta_1 \) integrin expression

In order to determine how these effects on cell migration and invasion are regulated, the expression of two integrin molecules, \( \alpha_3 \) and \( \beta_1 \), which have been implicated in breast disease progression, were investigated. These integrins were analysed by RT-PCR and immunoblotting after 48-h incubation with AII (10\( ^{-6} \) M). Both \( \alpha_3 \) and \( \beta_1 \) mRNA and protein expression were reduced by AII.
This action of AII was inhibited by co-incubation with losartan (10^{-5} M), but not by PD123319 (10^{-5} M).

Cellular signalling

To examine the signal transduction pathway by which AII exerts its effects, cells were incubated in the presence of Ro 31-8220 (5 \times 10^{-6} M), a protein kinase C (PKC) inhibitor. There was a down regulation of integrins \( \alpha_3 \) and \( \beta_1 \) protein by AII, but this was blocked by the addition of Ro 31-8220 (Fig. 6), suggesting a key role for PKC.

Discussion

Many reports have suggested a relationship between integrin expression and the degree of malignancy in breast cancer (Zutter et al. 1990, Koukoulis et al. 1991, Pignatelli et al. 1991, Gui et al. 1997, De Paepe et al. 2001). Although it is likely that the interactions between different members of the integrin family and other cell surface receptors are complex, previous investigations both in the patients and in the cell lines have suggested that \( \alpha_3 \) and \( \beta_1 \) subtypes may play critical roles (Gui et al. 1997, Morini et al. 2000, Berry et al. 2003).

Previous studies have also shown that a tissue localised RAS may be present in both normal and diseased breast tissue, bringing the possibility of localised production and actions of AII. That breast function may be regulated by AII is supported by the evidence clearly showing the presence of both AT1 and AT2 receptors in both normal and diseased breast tissue (Inwang et al. 1997, De Paepe et al. 2001). It is possible that, in normal tissue, AII regulates various epithelial functions, including cell growth and/or development, as well as electrolyte exchange (Norris et al. 1991, Rosenthal 1993, Schutz et al. 1996, Wang & Giebisch 1996, Leung et al. 1997, Muscella et al. 2003).

The present study emphasises that in addition, AII has roles in cell–substrate adhesion and invasion processes that in cancer may be associated with metastasis.

MCF-7 and T47D cells are good models for examining the role of AII in integrin expression, since they have previously been shown to express several integrins, including \( \alpha_3 \) and \( \beta_1 \), and both AT1 and AT2 receptors (Gui et al. 1997, Inwang et al. 1997). These systems are functionally linked and AII was here shown to decrease both cell–matrix adhesion and cell invasion through basement membrane proteins, exemplified in Figs 1–4 by results obtained with collagen, laminin and fibronectin. That this is associated with changes in cell–surface matrix recognition proteins is shown by the concomitant reduction in \( \alpha_3 \) and \( \beta_1 \) integrin gene transcription and protein expression. Interestingly, the laminin-binding integrin, \( \alpha_3 \beta_1 \), has been found to be highly expressed in squamous cell carcinomas and has been shown to be required for matrix metalloproteinase 9 expression and secretion (Iyer et al. 2005). Also, earlier studies undertaken using a monoclonal antibody raised to \( \beta_1 \) confirms that loss of \( \beta_1 \) activity in breast cancer cells does indeed inhibit cell invasion (Berry et al. 2003). Therefore, taken together, the results demonstrate that AII opposes changes in adhesion, invasion and integrin expression that in cancer are associated with the metastatic potential. However, these effects may be tissue specific. The AT1R expressing ovarian cell line, SKOV-3, was shown, in contrast, to have a complicated response to AII such that at 10^{-8} M, these cells had an increased invasive potential which returned to control levels in the presence of AII at 10^{-6} M (Iyer et al. 2005). Since ovarian cells normally express predominantly AT2R (Suganuma et al. 2005), this
**Figure 5** AII inhibits integrin \( \alpha_3 \) and \( \beta_1 \) mRNA and protein expression. Representative gels of semi-quantitative RT-PCR (a, b) and immunoblotting (c, d) and densitometric values, using RNA and protein extracted from MCF-7 cells treated for 48 h for (a, c) integrin \( \alpha_3 \) and (b, d) integrin \( \beta_1 \). Treatment with AII reduces \( \alpha_3 \) and \( \beta_1 \) mRNA and protein expression which, is inhibited by the AT1R antagonist, losartan, but not by the AT2R antagonist, PD123319 \((n=3, ^* P<0.05, ^{** *} P<0.001\) ANOVA, t-test).
effect may be a consequence of the abnormal expression of the type 1 receptor in this tissue type.

Using receptor-specific antagonists, the invasive and adhesive effects observed in breast cancer cells have been shown to be mediated via the AT1 receptor and more specifically through PKC signalling. This is in agreement with the data of Muscella et al. (2003), who recently showed, using subtype-specific inhibitors, that AII stimulation of breast cancer cell proliferation also involved activation of PKC and, more specifically, protein kinase-zeta. These data suggest that breast tumours may become metastatic in part through changes in AII availability and activity through the AT1 receptor subtype.

In previous studies examining the tissue RAS in the breast, Tahmasebi and co-workers (1998) showed by using in situ hybridisation that (pro)renin mRNA is transcribed in the myoepithelium of normal breast ducts, and in the stromal fibroblasts that surround them and in invasive breast cancer (pro)renin expression was markedly reduced. Since AT1 receptors are by far most abundant in the epithelial cells lining the ducts (Inwang et al. 1997, De Paepe et al. 2001), this suggests that AII may play an important role in the regulation of epithelial cell function. Such functions, from evidence in other epithelia, may conceivably include the regulation of water and electrolyte flux across the epithelial surface, and also cell proliferation, differentiation and tissue modelling (Norris et al. 1991, Rosenthal 1993, Schutz et al. 1996, Wang & Giebisch 1996, Leung et al. 1997, Muscella et al. 2003). In the breast tissue, these functions are, therefore, likely to be closely linked to the local generation of AII as a paracrine agent.

In cancer, it is evident that this close linkage breaks down and, in invasive carcinoma, the renin transcribing cells become fewer and lose their close association with the epithelial cells (Tahmasebi et al. 1998). De Paepe et al. (2001) have also shown that AT1R, which is highly expressed in both hyperplasia and in situ carcinoma cells, disappears is reduced in invasive carcinoma. Thus, the loss of local production of AII along with expression of its receptor in invasive tumours could result in increased migratory potential of invasive cells. Yang et al. (2005) have also suggested that in other tissues, AII has a role to play in epidermal growth factor (EGF)-regulated cell proliferation, this could clearly have an implication in later stage breast cancer when oestrogen receptor expression is reduced, while EGFR expression increases in the EGFR expressing aggressive cancers that tend to occur in younger women (Quintela et al. 2005). However, it is not clear whether this occurs through the AT1 or AT2 receptor and, indeed, there is currently no data on co-expression of ATR and EGFR in breast cancer tissue. It is possible to postulate, therefore, that local production of AII plays a key role in controlling and opposing the metastatic process and either loss of its production or the lack of expression of its receptor is in part responsible for the increased metastatic activity.

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