Human Homologue of Cement Gland Protein, a Novel Metastasis Inducer Associated with Breast Carcinomas

Dong Liu,¹ Philip S. Rudland,¹ D. Ross Sibson,³ Angela Platt-Higgins,² and Roger Barraclough²

¹Cancer Tissue Bank Research Centre and ²School of Biological Sciences, University of Liverpool, Liverpool, United Kingdom and ³Clatterbridge Cancer Research Trust, J.K. Douglas Laboratories, Wirral, United Kingdom

Abstract

A suppression subtractive cDNA library representing mRNAs expressed at a higher level in the malignant human breast cancer cell line, MCF-7, relative to a benign breast tumor-derived cell line, Huma 123, contained a cDNA, M36, which was expressed in estrogen receptor α (ERα)–positive breast carcinoma cell lines but not in cell lines from normal/benign/ERα-negative malignant breast lesions. M36 cDNA had an identical coding sequence to anterior gradient 2 (AGR2), the human homologue of the cement gland–specific gene (Xenopus laevis). Screening of breast tumor specimens using reverse transcription-PCR and immunocytochemistry with affinity-purified anti-AGR2 antibodies showed that the presence of AGR2 mRNA and protein were both statistically significantly associated previously with tumor progression (12). In this article, we report one novel cloned cDNA, M36, which matches identically the coding sequence of human anterior gradient 2 (AGR2) cDNA, the human homologue (previously hAG-2) of the Xenopus laevis cement gland–specific gene, XAG-2 (17). The XAG-2 gene product has developmental significance in Xenopus embryos (18). Here, we show that the human homologue of this developmentally associated protein is differentially expressed between benign and malignant human breast carcinoma specimens. Furthermore, its cDNA, when introduced into a benign, nonmetastatic, rat mammary cell line, confers a metastatic phenotype on benign nonmetastatic cells.

Materials and Methods

Cell lines and cell culture. The normal human mammary epithelial cell line, Huma 7, was subcloned from primary cultures of reduction mammoplasty specimens of normal breast tissue immortalized with SV40 (19). The benign human mammary epithelial cell line, Huma 123, and the derivative benign human mammary myoepithelial-like cell line, Huma 109 (15), were derived from HMT-3522, itself obtained from a primary cell culture of human benign breast disease displaying prominent epithelial hyperplasia (20). These cell lines, and the malignant human mammary epithelial cell lines derived from pleural effusions of breast cancer patients, MCF-7, T47D, ZR-75, and MDA-MB-231 (21), were cultured as described previously (12, 15, 19). The benign rat mammary epithelial cell line, Rama 37, was cultured as described previously (22). The transfected derivative cell lines were grown in medium containing 1 mg/mL genetin. All cells were passaged on reaching 70% confluency. Culture in medium depleted of steroid hormones was carried out as described previously (23, 24).

Subtractive hybridization and Northern hybridization screening. A suppression subtractive (11) library consisting of PCR products representing mRNAs expressed at a higher level in the malignant breast epithelial cell line, MCF-7, relative to a benign human breast-derived cell line, Huma 123, was constructed using a PCR-Select cDNA Subtraction kit (Clontech, Palo Alto, CA) as described previously (14). Reverse Northern screening of the subtracted cDNA library was carried out as described previously (12). Total cellular RNA was prepared using the guanidinium-isothiocyanate–cesium chloride method (25–27). Poly(A)-containing RNA was isolated from total RNA using the Fast Track mRNA isolation kit (Invitrogen, Groningen, the Netherlands). Northern hybridization procedures were done as described previously (12). cDNA probes were radioactively labeled to $1 \times 10^6$ dpm/µg DNA by random-primed DNA synthesis (28) using a labeling kit (Roche Molecular Biochemicals, Mannheim, Germany). The constitutive probe, 36B4, a cDNA to human acidic ribosomal phosphoprotein PO mRNA (29), was used to normalize RNA loading on the gel.

Requests for reprints: Roger Barraclough, Biosciences Building, University of Liverpool, Crown Street, Liverpool L69 7ZB, United Kingdom. Phone: +44-151-795-4469; Fax: +44-151-795-4406; E-mail: brb@liverpool.ac.uk.

©2005 American Association for Cancer Research.
Coupled transcription and translation assay in vitro. Transcription and translation assays in vitro were carried out using a TNT T7/T3-coupled reticulocyte lysate system (Promega, Madison, WI) to produce a protein product labeled with [35S]methionine in vitro. DNA template (2 μg) was transcribed and translated in 50 μL containing 40 units RNAse inhibitor, 25 μL TNT rabbit reticulocyte lysate, 20 μg/ml L-[35S]methionine (~1,000 Ci/μmol at 10 μCi/ml), and 2 μL TNT reaction buffer. The mixture was incubated at 30°C for 90 minutes. The resulting [35S]-labeled proteins and radioactive standards were fractionated on urea-containing SDS, 15% (w/v) polyacrylamide gels (SDS-PAGE) with 6% (w/v) polyacrylamide stacking gels (30). The gels were stained with Coomassie blue, destained with 40% (v/v) methanol, 7% (v/v) acetic acid, dried under vacuum, and autoradiographed with Kodak X-Omat film (Eastman Kodak, Rochester, NY) at ~70°C for 3 to 10 days.

Production and purification of recombinant protein anterior gradient 2 and its antisera. The full-length M36 cDNA or one with the M36 signal sequence deleted was cloned into the expression vector, pET-16b (Novagen, Madison, WI), downstream of the His tag, to yield a recombinant cDNA construct designated pET-M36, which was first verified by automated DNA sequencing and then transformed into Escherichia coli BL21DE3 cells. Induction of recombinant protein was carried out by adding isopropyl-β-D-thiogalactopyranoside (1 mM/L) to the culture medium (A600 = 0.5) for 2 hours. Purification of recombinant AGR2 protein to a single band on SDS-PAGE gels was carried out using His-Bind resin (Novagen). The amino acid sequence of the purified recombinant AGR2 protein was confirmed by an automated sequencer. The production of rabbit anti-AGR2 serum was conducted by Eurogentec (Seraing, Belgium). The anti-AGR2 antibodies were affinity purified by their binding to antigen immobilized on a polyvinylidene difluoride (PVDF) membrane. Briefly, recombinant AGR2 (1 mg) was subjected to SDS-PAGE and electrotheropically transferred to a PVDF membrane and the part of the membrane containing the immobilized antigen was incubated with serum from a rabbit immunized with recombinant AGR2. Bound antibody was eluted with a 100 mM/L glycine buffer (pH 2.5) followed by neutralization with 1 mL of Tris buffer (pH 8.0).

Western blot analysis. Cells were grown to 70% to 80% confluence, washed twice with ice-cold PBS buffer, and lysed by lysis buffer (30 mM/L Tris-HCl (pH 7.5), 150 mM/L NaCl, 1% (v/v) NP40, 0.5% (w/v) sodium deoxycholate, 1 mM/L EDTA, 0.1% (w/v) SDS), and a protease inhibitor cocktail (Sigma, Poole, Dorset, United Kingdom) was added. The clarified lysates were collected by centrifugation at 12,000 × g for 20 minutes at 4°C. The protein concentration in the lysate was measured by Bio-Rad protein assay (Bio-Rad Laboratories, Hemel Hempstead, Herts, United Kingdom). Lysates containing equal amounts of total proteins were resolved by SDS-PAGE. The proteins were electrotransferred onto PVDF membranes using a Bio-Rad semidry transfer apparatus. The membranes were incubated with the affinity-purified, in-house rabbit polyclonal anti-AGR2 antibody. After washing and incubating with anti-rabbit horseradish peroxidase–conjugated IgG, the membranes were washed and detected by the Supersignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc., Perbio Science, Cambridge, Cambridgeshire, United Kingdom). The sections were visualized as a brown stain by incubating with 3,3′-diaminobenzidine (Sigma, Dorset, United Kingdom) and 0.075% (v/v) H2O2, counterstained with Mayer's hemalum, and mounted in dibutyl polyisryene xylene (Merck, Dorset, United Kingdom). All staining results were examined by three independent observers and scored as plus/minus using 5% of carcinoma cells staining as a cutoff. Photographs were carried out as described previously (33, 34).

Human breast specimens. Human breast specimens, normal specimens from reduction mammoplasties, benign fibroadenomas, and invasive ductal carcinoma of no special type were obtained from the Cancer Tissue Bank Research Centre (Liverpool, United Kingdom) with full and informed patient consent and with ethical approval. The carcinomas were subdivided into two groups based on immunocytochemical staining for ERs, a cutoff of 5% of the carcinoma cells stained by antibodies to ERs divided the negative from the positive group.

Cellular localization and immunocytochemistry. The localization of 4 μm tissue sections was determined after staining with H&E. Immunocytochemical staining for vimentin, skeletal muscle actin, myoglobin, and ERs was carried out as described previously (33, 34). Immunocytochemical staining for AGR2 was done with affinity-purified AGR2 antibodies with or without prior incubation with 0.1 mg/ml recombinant AGR2 protein either for sections of human specimens [1:500 dilution in PBS buffer containing 2% (w/v) bovine serum albumin incubated at room temperature for 2 hours] or for sections of rat specimens [1:200 dilution in PBS buffer containing 0.5% (w/v) bovine serum albumin incubated at room temperature overnight]. The bound antibodies were detected using biotinylated donkey anti-rabbit serum followed by ABC complex/horseradish peroxidase kit (DAKO Ltd., Cambridge, United Kingdom). The sections were visualized as a brown stain by incubating with 3,3′-diaminobenzidine (Sigma, Dorset, United Kingdom) and 0.075% (v/v) H2O2, counterstained with Mayer's hemalum, and mounted in dibutyl polyisryene xylene (Merck, Dorset, United Kingdom). All staining results were examined by three independent observers and scored as plus/minus using 5% of carcinoma cells staining as a cutoff. Photography was carried out as described previously (33).

Cell adhesion assays. The cells were grown to 70% to 80% confluence, washed twice with PBS, trypsinized, and counted using a Coulter counter (Beckman-Coulter UK Ltd., High Wycombe, Buckinghamshire, United Kingdom). The cells were resuspended at 2 × 106 cells/ml and counted again to check the concentration before adding 1 mL cells to each well of a 24-well plate. After incubation for 30 minutes at 37°C, the cells were washed thrice with PBS buffer to remove any cells in suspension, and
Results

Overexpressed M36 cDNA in the malignant human breast cancer cell lines corresponds to anterior gradient 2 mRNA. A suppression subtractive library was constructed containing cDNAs expressed at a higher level in the malignant mammary cell line, MCF-7, than in the cell line, Huma 123, derived from a benign mammary lesion (Materials and Methods). Four cloned cDNAs (M36, M40, M202, and M234) of 174 cloned cDNAs sequenced each exhibited 100% identity to the coding sequence of human cDNA AGR2 (Genbank accession no. NM_006408). AGR2 is the human homologue of the X. laevis AGR2 (XAG-2) gene, which is expressed by the cement gland of the developing Xenopus embryo (18). The nucleotide sequences for M36 or M202 cDNA contained an open reading frame of 175 amino acids that was identical in amino acid sequence to that of AGR2 protein (Genbank accession no. NP_006399). In a T7/T3 RNA polymerase–coupled transcription/translation system, clones, M36 and M202, but not empty vector, yielded a single 35S-labeled primary translation product of 20 kDa on urea-containing SDS-PAGE (data not shown), a size that corresponds to the 20 kDa derived from the amino acid sequence.

Quantitative reverse Northern hybridizations using as probes double-stranded mixed cDNAs showed that the level of AGR2 mRNA was >15-fold higher in the RNA from MCF-7 cells than in that from the Huma 123 cells (data not shown). Northern hybridization experiments showed that the M36 probe hybridized to a major band of RNA with a molecular size of 0.9 kb (mean of three independent experiments) corresponding to the AGR2 mRNA and to an additional faint band at 1.6 kb in all the positive lanes (Fig. 1). The 1.6-kb band corresponds in size to the recently updated 1.7-kb variant mRNA of AGR2 containing a longer untranscribed 3′-end (Genbank accession no. NM-006408). The AGR2 mRNA was present in all three ERα-positive breast cancer cell lines tested, MCF-7, T47D, and ZR-75, but undetectable in the ERα-negative MDA-MB-231 breast cancer cell line and in the SV40-immortalized normal human breast cell line, Huma 7, the benign human breast tumor cell line, Huma 123, and its myoepithelial-like convertant cell line, Huma 109 (Fig. 1). The same distribution was found for the AGR2 protein using Western blotting with the AGR2 antibody (see Materials and Methods; Fig. 1), the signal being abolished by prior incubation of the antibody with 0.1 mg/mL recombinant AGR2 (data not shown). These results suggest that the expression of AGR2 mRNA and protein correlates with the presence of ERα at least in these cell lines.

AGR2 mRNA was present at a 7.3 ± 0.2-fold (mean ± SD of three independent experiments) higher level in MCF-7 cells grown in the presence of estrogen than in cells grown in estrogen-depleted conditions, whereas the level of a previously described, estrogen-responsive mRNA, that of pS2 (35), was increased only...
3.4 ± 0.1-fold. In these quantitative results, mRNA levels were normalized with respect to 36B4 mRNA, a mRNA that is not dependent on the presence of estrogen and its receptor for its production (29).

Identification of anterior gradient 2 mRNA and protein in human breast tumor specimens. The occurrence of AGR2 mRNA in human benign breast lesions and malignant breast carcinomas was examined by reverse transcription-PCR (RT-PCR; Fig. 2). Using 25 cycles of PCR, only 3 of 9 (33%) normal and 13 of 25 (52%) benign samples were positive for AGR2 mRNA (positivity defined as a single PCR band of 354 bp), whereas 44 of 56 (79%) breast carcinoma samples were positive for AGR2 mRNA (Table 1). This proportion was significantly different from the normal and benign specimens ($P = 0.0029$, Fisher’s exact test). Moreover, 31 of 34 (91%) ERα-positive carcinoma specimens yielded a strong PCR product, whereas only 13 of 22 (59%) ERα-negative carcinomas were positive for AGR2 mRNA, values that were also significantly different ($P = 0.007$, Fisher’s exact test). These results show that AGR2 mRNA is dependent on the presence of ERα in the majority of breast carcinoma specimens as well as in the breast carcinoma cell lines.

The affinity-purified AGR2 antibodies (Materials and Methods) were used to stain immunocytochemically histologic sections of human breast specimens. The epithelial cells of human normal breast tissue and benign lesions either were stained modestly for AGR2 protein or were unstained (Fig. 3A and B), but the epithelial cells of ERα-positive breast carcinomas were stained strongly for AGR2 protein (Fig. 3C). The positive staining for AGR2 protein was completely abolished by prior incubation of the antibodies with recombinant AGR2 protein (Fig. 3D). The immunocytochemical staining for AGR2 protein showed a granular appearance, reminiscent of secretory granules (Fig. 3E). There was little or no staining for AGR2 in >50% of ERα-negative breast carcinoma specimens (Fig. 3F). Overall, AGR2 protein immunocytochemical positivity (defined as >5% of epithelial cells staining) was found in only 2 of 5 (40%) normal specimens and 7 of 15 (47%) benign breast tumor specimens, but 33 of 44 (75%) breast carcinoma specimens were positive for AGR2 protein. This proportion was significantly different from normal and benign specimens ($P = 0.025$, Fisher’s exact test). Twenty-six of 29 (90%) ERα-positive specimens were positively stained, whereas only 7 of 15 (47%) ERα-negative carcinomas were positive for AGR2 protein, significantly different from the ERα-positive carcinomas ($P = 0.0033$, Fisher’s exact test). These experiments showed quantitative results for AGR2 protein that were similar to those obtained for mRNA by RT-PCR.

Identification of anterior gradient 2 as a novel metastasis inducer. The full-length M36 (AGR2) cDNA was inserted into the multiple cloning site of the mammalian expression vector pcDNA3, downstream of the cytomegalovirus promoter, to yield a recombinant cDNA construct designated pcDNA-M36. The pcDNA-M36 expression construct, or the same amount of empty pcDNA vector as a negative control (pcDNA), was transfected into the benign rat mammary epithelial cell line, Rama 37 (Materials and Methods). Following selection in geneticin, single colonies from the Rama 37 pcDNA-M36 transfectants were picked and expanded along with two independent pools from the same transfectants. Using PCR or Southern hybridization, RT-PCR, or Northern hybridization, AGR2 sequences were shown to be present in the DNA and RNA from the transfectant pools and clones but absent from the Rama 37 cells and the Rama 37 cells transfected with empty vector summarized in Table 2.

The incidences of primary tumors and metastases produced by AGR2 transfection are shown in Table 2 along with statistical analyses. A single s.c. injection of cells transfected with the empty

![Figure 2](https://www.aacrjournals.org/figs/65/9/3799/fig2.jpg)  
**Figure 2.** Identification of AGR2 mRNA from human breast tumor specimens using RT-PCR. RNA from human breast carcinoma specimens (lanes 1-28) was amplified by RT-PCR using primers specific for AGR2 cDNA (A) or human glyceraldehyde-3-phosphate dehydrogenase cDNA (B) to yield PCR products of 354 and 452 bp, respectively. The ERα status of specimens was recorded (+ or -) as described in Materials and Methods. The resulting RT-PCR products were subjected to agarose gel electrophoresis and stained with ethidium bromide as described in Materials and Methods. Lanes M, DNA molecular weight markers.
vector yielded primary tumors in the mammary glands with a mean latent period of 49 ± 11 days and with a tumor incidence of 50% (Table 2). This incidence was not significantly different from that obtained with Rama 37 cells (Table 2). None of the tumor-bearing rats exhibited lung metastases, and this was confirmed by subsequent histologic examination of selected tissues, including lung and lymph nodes. However, the mean latent period for palpable primary tumors of the pcDNA

### Table 1. Identification of AGR2 mRNA and protein in breast specimens using RT-PCR and immunocytochemistry

<table>
<thead>
<tr>
<th>Clinical specimens</th>
<th>RT-PCR screening of specimens</th>
<th>Immunocytochemical screening of specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. positive*</td>
<td>No. negative*</td>
</tr>
<tr>
<td>Normal</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Benign</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>Total</td>
<td>16</td>
<td>18</td>
</tr>
<tr>
<td>ERα-positive carcinomas</td>
<td>31</td>
<td>3</td>
</tr>
<tr>
<td>ERα-negative carcinomas</td>
<td>13 ‡</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td>44†</td>
<td>12</td>
</tr>
</tbody>
</table>

*Positive RT-PCR is defined as a single band of molecular weight 354 bp for AGR2 on the gel; negative RT-PCR is defined as no clear band on the gel, all have a band of 452 bp for control GPDH on the gel.
† Positive immunocytochemistry is defined as >5% of epithelial cells staining; negative immunocytochemistry is defined as <5% of epithelial cells staining.
‡ P = 0.007, statistically significantly different from ERα positive (Fisher’s exact test).
§ P = 0.0029, statistically significantly different from normal and benign specimens (Fisher’s exact test).
† P = 0.0033, statistically significantly different from ERα positive (Fisher’s exact test).
* P = 0.0025, statistically significantly different from benign specimens (Fisher’s exact test).

Figure 3. Immunocytochemical staining of human breast specimens for AGR2. Histologic sections showing a normal human breast containing ducts and lobules (A), a benign fibroadenoma (B), an ERα-positive (C-E), and an ERα-negative (F) invasive ductal carcinomas stained immunocytochemically by affinity-purified antibodies to recombinant AGR2 protein (A-C, E, and F) or by antibodies preincubated with 0.1 mg/mL recombinant AGR2 protein (D) as described in Materials and Methods. For A-D and F, bar (shown on A), 50 μm; for E, bar, 20 μm.
The incidences of primary tumors for the AGR2-transfected clones and pools in the mammary glands ranged from 30% to 64% of injected rats (Table 2), none were statistically different from pcDNA-vector-transfected cells (Table 2), Rama 37 cells first test (Table 2), and all but one not significantly different from Rama 37 second test (Table 2). However, 92% and 86% of rats injected with pool 1 and pool 2 cells, respectively, and 77% to 82% of rats injected with the three clones of AGR2-transfected cells developed either gross metastases in the lungs, which were visible at necropsy, or micrometastases evident on subsequent histologic examination (Table 2). These values for the incidences of metastases were significantly different from the control group of nonmetastatic cells with an expression vector containing AGR2.

### Table 2. Incidence of tumors and metastases produced by M36 cDNA transfected cells

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Presence or absence of AGR2 mRNA</th>
<th>Primary tumors</th>
<th>Metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rama 37 1st test</td>
<td>Negative</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Rama 37 2nd test</td>
<td>Negative</td>
<td>31 ± 7</td>
<td>15 ± 5</td>
</tr>
<tr>
<td>pcDNA vector</td>
<td>Negative</td>
<td>49 ± 11</td>
<td>0.0002</td>
</tr>
<tr>
<td>Pool 1</td>
<td>Positive</td>
<td>38 ± 18</td>
<td>0.46</td>
</tr>
<tr>
<td>Pool 2</td>
<td>Positive</td>
<td>54 ± 15</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Clone 1</td>
<td>Positive</td>
<td>81 ± 6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Clone 2</td>
<td>Positive</td>
<td>37 ± 14</td>
<td>0.39</td>
</tr>
<tr>
<td>Clone 3</td>
<td>Positive</td>
<td>46 ± 22</td>
<td>0.014</td>
</tr>
</tbody>
</table>

NOTE: Fifteen percent of injected rats were omitted from the analysis due to the presence of ascites or ulceration. N/A, not applicable.

*Mean time of appearance of primary tumors (P values < 0.05, significantly different from Rama 37, second test).
†Mean growth period from primary tumor appearance to culling (P values <0.05, significantly different from Rama 37, second test).
‡Mean weight of primary tumor (all AGR2 transfectants not significantly different from Rama 37, second test).
§Incidence of primary tumors (% (no. rats with tumors/no. rats injected), all transfectants not significantly different from Rama 37, first test, and all but one transfectant not significantly different from Rama 37, second test.
∥Incidence of metastases: % (no. rats with metastases/no. rats with tumors), all AGR2 transfectants significantly different from Rama 37, first test, Rama 37, second test, and pcDNA vector transfectant.

Transfected was statistically different from Rama 37 (Table 2). Rats injected with the two independent pools or three independent clones of pcDNA-M36-transfected, AGR2-expressing cells yielded tumors with a range of mean latent periods to palpability (Table 2). All of these were longer, some significantly longer than Rama 37 cells (Table 2), as were the growth periods between tumor appearance and culling (Table 2). There was no relationship between latent period and growth period for the individual clones and pools. The weights of tumors at culling for any of the pools and clones tested were not significantly different from Rama 37 cells (Table 2).

The incidences of primary tumors for the AGR2-transfected clones and pools in the mammary glands ranged from 30% to 64% of injected rats (Table 2), none were statistically different from pcDNA-vector-transfected cells (Table 2), Rama 37 cells first test (Table 2), and all but one not significantly different from Rama 37 second test (Table 2). However, 92% and 86% of rats injected with pool 1 and pool 2 cells, respectively, and 77% to 82% of rats injected with the three clones of AGR2-transfected cells developed either gross metastases in the lungs, which were visible at necropsy, or micrometastases evident on subsequent histologic examination (Table 2). These values for the incidences of metastases were significantly different from the control group of pcDNA empty vector-transfected Rama 37 cells and two separate groups of animals injected with Rama 37 cells (all P < 0.0003, Fisher’s exact test), in which no lung macrometastasis or micrometastasis was found. There was no significant correlation between metastatic potential and the length of time the tumors took to grow following detection, nor any significant relationship when latent period plus tumor growth period was plotted against metastatic potential (least squares regression analysis of a fit of the points to a straight line yielded probabilities in the range 0.12-0.55), ruling out the possibility that metastasis arose due to a longer period of tumor growth. The results show that transfection of the nonmetastatic cells with an expression vector containing AGR2 induces metastasis in two pools and three separate clones of cells.

**Histology and immunocytochemistry of tumors produced by transfected cells.** Some of the primary tumors from rats injected with the AGR2-expressing Rama 37 cells transfected with pcDNA-M36 were composed of cuboidal cells, many forming cords that were surrounded by neoplastic spindle cells, whereas others consisted predominantly of neoplastic spindle cells. Many tumors showed central necrotic cores. In many primary tumors arising from cells transfected with the pcDNA-M36 construct, extensive numbers of blood vessels were seen. Some tumor cells had breached the surrounding connective tissue capsules and had invaded the adjacent host skeletal muscle (Fig. 4A). In general, the histology of the metastases was the same as that of the primary tumor. Both cannonball metastases and tumor cells penetrating the surrounding lung tissue were evident (Fig. 4B). The primary tumors and metastases were also extensively stained by antibodies to vimentin (Fig. 4B), and in that case, tumor cells in endothelial cell–lined spaces, possibly lymphatics (Fig. 4C) and in blood vessels (data not shown), were also observed. The primary tumor cells and
lung metastases also exhibited staining for milk fat globule membrane antigen (data not shown) and by pan-keratin antibodies (Fig. 4D) and by peanut lectin (data not shown). Differentiation of tumor cells to skeletal muscle–like elements was common in both the primary tumor and its metastases, sometimes forming large multinucleate cells. These skeletal muscle–like elements were immunocytochemically stained by antisera to skeletal muscle actin (data not shown) and to myoglobin (Fig. 4E). Skeletal muscle

Figure 4. Histology and immunocytochemical staining of primary rat tumors and metastases produced by M36 cDNA transfectants. Histologic sections of tissues from animals injected with Rama 37 cells transfected with the pcDNA3-M36 construct were stained with H&E or immunocytochemically stained. Carcinoma cells (T) of a primary tumor in the mammary gland locally invading muscle tissue (M), visualized by H&E staining (A). A cannonball metastasis (M) in the lung stained by antibodies to vimentin (L, B). Metastatic tumor cells in the lungs (large arrow) possibly in a lymphatic space adjacent to a blood vessel (small arrow) stained by antibodies to vimentin (C). Tumor cells in a lung metastasis stained by antibodies to pan-keratin (D). Skeletal muscle elements (arrow) within a lung metastasis stained by antibodies to myoglobin (E). Histologic sections of primary tumors from animals injected with Rama 37 cells transfected with Rama 37 cells transfected with vector containing M36 cDNA (G-I) were stained with affinity-purified antibodies to recombinant AGR2 protein (F-H) or with antibodies preincubated with 0.1 mg/mL recombinant AGR2 protein (I) as described in Materials and Methods. Bar, 50 μm (A), 200 μm (B), and 20 μm (C); (D-I) as (A).
elements were not found in any of the primary tumors arising from cells transfected with the control pcDNA vector.

Although there was no immunocytochemical staining for AGR2 protein in the histologic sections of primary tumors arising from animals injected with Rama 37 cells transfected with empty pcDNA plasmid vector (Fig. 4F), both carcinoma cells of primary tumors (Fig. 4G) and metastases in the lungs (Fig. 4H) produced from animals injected with the AGR2-expressing Rama 37 cells transfected with pcDNA-M36 were stained positively by the affinity-purified antibodies directed against recombinant AGR2 protein. The immunocytochemical staining by the antibodies to AGR2 was blocked completely by their prior incubation with recombinant AGR2 protein (Fig. 4I).

Enhanced adhesion is associated with the anterior gradient 2–transfected cells. The AGR2 transfecteds did not exhibit any significantly different growth rates in vitro compared with vector-transfected or parental Rama 37 cells (P = 1.0, Student’s t test), nor any altered invasive potential as measured by their behavior in an invasion assay using Matrigel-coated Transwell chambers (P = 1.0, Student’s t test). In contrast, a statistically significant increase in the rate of cellular adhesion to a plastic substratum (Fig. 5) was shown by all the AGR2 cDNA-transfected pools and transfected clones of cells examined relative to parental Rama 37 cells (P range, 0.009 to <0.0001, Student’s t test) and by all pools and two of three clones relative to Rama 37 cells transfected with empty vector (P range, 0.0074–0.001, Student’s t test). Although there was a broad correlation between the metastatic and the adhesive potential of the different transfectants, the similar levels of AGR2 in the pcDNA-M36-transfected cells precluded determining any significant relationship between AGR2 expression and adhesive/metastatic potential of the individual pools/clones of cells. To further identify the mechanism by which AGR2 affects adhesion, coating of the tissue culture dishes with either 2 or 20 μg of recombinant AGR2 protein lacking a signal peptide increased the rate of attachment of the AGR2-negative Rama 37 cells or Rama 37 cells transfected with empty vector but had little effect on the rate of attachment of the AGR2-transfected cell lines or pools (Fig. 5).

Discussion

A subtracted library of cloned cDNAs expressed at a higher level in the malignant human mammary cell line, MCF-7, relative to a benign cell line, Huma 123, derived from benign breast disease, yielded among others, a cDNA, M36, identical to the coding sequence of human AGR2, the anterior gradient 2 homologue (36), produced by the cement gland of the developing X. laevis embryo (18). Furthermore, the size of the primary translation products of M36 and the AGR2 protein were identical and the proposed product additionally exhibited 91% identity to the first 175 amino acids of the mouse gob-4 gene, which is expressed in intestinal mucus-secreting goblet cells (37). Transfection of M36 cDNA in an expression vector into a benign rat mammary cell line, Rama 37, induced a metastatic phenotype in vivo when the cells were injected into the mammary fat pads of syngeneic rats, whereas similar transfection of empty vector failed to induce a metastatic phenotype. The nonmetastatic Rama 37 cells have been shown previously to be converted to a metastatic phenotype by genes encoding proteins S100A4 (7), osteopontin (8), and cutaneous fatty acid-binding protein (38) but not by oncogenes, such as Ha-ras or DNA virus-transforming genes (39). Elevated levels of immunoreactive S100A4 (9) or osteopontin (10) in the breast cancers of patients have been shown to correlate with markedly reduced patient survival, whereas cutaneous fatty acid-binding protein occurs preferentially in malignant as opposed to benign prostatic lesions (38). Use of the Rama 37 cell line has thus identified previously cloned genes whose products are biologically relevant to the metastatic process in human cancer, and our present results suggest strongly that AGR2 may also be involved in metastasis. Human AGR2 was reported previously to be expressed in the ER-positive breast cancer cell line, MCF-7, and not in the ER-negative cell line, MDA-MB-231 (17), but this is the first report of its direct involvement with metastasis and/or malignancy. The apparent inconsistency of the involvement of a strongly ER-dependent gene/gene product being associated with the process of metastasis of breast tumor cells is supported by the observation that in a group of 225 tamoxifen-treated patients with ER-positive breast cancers, those with AGR2 in their breast cancer cells exhibited a statistically significantly poorer survival than those without AGR2 in their cancer cells. In contrast, the similarly treated 126 patients with ER-negative breast cancers showed no such relationship.4

---

4 Innes et al., in preparation.
The AGR2-induced tumors in the experimental rats differed somewhat from those induced by S100A4. Whereas S100A4 produced primarily cannon ball metastases in the lungs/lymph nodes (7), AGR2 induced both cannon ball metastases and micrometastases in the lungs, similar to those observed previously for osteopontin (8). The presence of AGR2-induced micrometastases in blood, and possibly in lymphatic vessels, suggests that AGR2 may induce metastasis to the lungs by both blood-borne and lymphatic routes.

The Xenopus AGR2 protein is a product of the mucin-producing cement gland, which is the first ectodermal organ to appear in the developing Xenopus embryo (18). AGR2 is up-regulated in experimentally dorsalisized embryos and down-regulated in experimentally ventralized embryos (18). Although the precise role of AGR2 is presently unknown, its injection into early cleavage stage embryos results in enhanced cement gland development (18), and ectopically produced AGR2 not only can signal dorsoanterior ectodermal fate but also can induce neural markers in embryo cells, suggesting that extracellular AGR2 is able to alter the differentiation potential of its target cells (18). It is not yet clear whether AGR2 has similar activities in human development or whether such activities are related to its metastasis-inducing properties. However, in the transfection experiments, some of the cells containing the AGR2 (M36) construct exhibited muscle, and not neuronal, patterns of differentiation. The reason for this pattern of differentiation being evident in the Rama 37 cells transfected with AGR2 is not known; however, this pattern of differentiation has been described before in derivative cells of mouse oocytes (18). In the present experiments, a granular cytoplasmic appearance of immunocytochemical staining for AGR2 observed in some human carcinoma specimens suggests that AGR2 might be secreted by some carcinoma cells. Pilot experiments using an AGR2 COOH-terminal GFP fusion cDNA5 have shown that the fusion protein is secreted at least in HeLa cells, strongly suggesting that the AGR2 secretory signal is active.

To identify a possible mechanism for AGR2-induced metastasis, the effect of added recombinant AGR2 protein on Rama 37 cells was tested. AGR2 protein lacking the signal sequence up to a concentration of at least 24 μmol/L6 and pcDNA-M36 transfection failed to affect the growth rate of Rama 37 cells in culture. These results support the lack of a consistent effect of AGR2 on the latent period of tumor formation in vivo (Table 2). Furthermore, pcDNA-M36-transfected cell pools and clones did not show any enhanced invasive ability through Matrigel compared with parental and empty vector-transfected Rama 37 cells. However, the pcDNA-M36 transfectants exhibited an increased adhesive potential to a plastic substratum. The fact that AGR2 contains an active secretory signal is consistent with the punctate staining pattern for AGR2 observed in human breast cancers at higher magnification (Fig. 3E) and suggests that AGR2 functions extracellularly. An extracellular mechanism of AGR2 is shown by the observation that extracellularly added AGR2 enhances the rate of attachment of two AGR2-negative cell lines to that observed with five independent AGR2-producing cell clones and pools but had no effect on the AGR2-producing cell clones and pools. Although the mechanistic link between AGR2-induced increased adhesion to plastic and metastasis is not yet known, another well-characterized metastasis-inducing (8, 10) secreted protein, osteopontin (41), has also been shown to increase the adhesion of cells (42), including Rama 37 cells, to plastic substrates (43). Taken together, these results strongly suggest that AGR2 might also cause metastasis by enhancing this adhesive property of the Rama 37 cells.

In summary, this article describes the first demonstration of metastasis-inducing properties of the developmentally important protein, AGR2. The presence of detectable AGR2 mRNA and protein above a threshold in breast carcinoma cells significantly correlates with carcinoma in preference to benign/normal tissue, and ERα-positive in preference to ERα-negative carcinomas, suggesting that the metastasis-inducing properties of AGR2 may contribute, in some way, toward the malignant progression of some ERα-positive breast cancers. Identification of the receptor for AGR2 will provide the means to identify the signaling pathways that link the enhancement of cell attachment to the process of metastasis.

Acknowledgments


Grant support: Clatterbridge Cancer Research Trust studentship and UK Committee of Vice Chancellors and Principals ORS scholarship award (D. Liu), Cancer and Polio Research Fund, and North West Cancer Research Fund grant CR532.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Joe Carroll, Barry Cotterill and Karen Collard for excellent technical assistance.

References


8. Oates AJ, Barracklough R, Rudland PS. The identification of osteopontin as a metastasis-related gene

---

5 D. Liu, P.S. Rudland, R. Barracklough, unpublished data.
6 D. Liu, P.S. Rudland, R. Barracklough, unpublished results.
Human Homologue of Cement Gland Protein, a Novel Metastasis Inducer Associated with Breast Carcinomas

Dong Liu, Philip S. Rudland, D. Ross Sibson, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/65/9/3796

Cited articles
This article cites 38 articles, 11 of which you can access for free at:
http://cancerres.aacrjournals.org/content/65/9/3796.full#ref-list-1

Citing articles
This article has been cited by 20 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/65/9/3796.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/65/9/3796.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.