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Physiological stress induces the metastasis marker AGR2 in breast cancer cells

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Abstract As an approach to understanding the factors that activate expression of tumor progression genes, the role of physiological stress in the activation of a panel of tumor cell markers was investigated. These studies identify the developmental gene product, anterior gradient 2 (AGR2) as a cancer cell marker specifically up-regulated in response to depletion of serum and oxygen. AGR2 has been identified as a tumor marker in primary and secondary cancer lesions, and as a marker for detection of circulating tumor cells (CTCs). Elevated levels of AGR2 are known to increase the metastatic potential of cancer cells, but conditions leading to increased expression of AGR2 are not well understood. The present results identify novel physiological parameters likely to contribute to *AGR2* induction in situ.

Keywords *AGR2* · hAG2 · *ESR1* · Osteopontin · *TFF3* · Circulating tumor cells · Breast cancer · Stress

Introduction

Detailed molecular characterization of aggressive tumor cells can lead to improved prognostic accuracy and therapeutic outcome [1]. We recently identified the human developmental gene product, anterior gradient 2 (AGR2), as a potential marker for detection of circulating tumor cells in the blood of patients with metastatic cancers [2]. *AGR2* has likewise been identified in primary and secondary cancer lesions in several recent studies aimed at cataloging genes involved in the generation and/or metastasis of tumors [3–7]. Human AGR2 is a homolog of the secreted *Xenopus laevis* protein (XAG-2). In *Xenopus*, XAG-2 is primarily involved in the induction and differentiation of the cement gland, as well as in the patterning of anterior neural tissues [8]. Over-expression of mammalian *AGR2* in benign nonmetastatic rat tumor cells increased the metastatic potential of these cells dramatically in vivo [6], linking up-regulation of *AGR2* with a propensity for tumor cells to undergo metastasis. However, the factors that lead to up-regulation of *AGR2* in tumor cells are poorly understood.

We have considered the role of physiological stress in the induction of cancer progression genes. The microenvironment of tumors often becomes heterogeneous and contains localized regions of harsh living conditions for normal and tumor cells alike [9]. Within the tumor mass, cells can gain an increased tumorigenic and/or metastatic

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potential if they can adapt to periods of serum depletion and hypoxia by expressing or activating gene products that promote survival, angiogenesis, motility, or invasion [10–13]. In an attempt to identify links between induction of potential tumor progression genes and specific parameters of physiological stress, breast cancer cells were subjected to conditions reflecting tumor cell-associated pathophysiological stress in the form of serum and oxygen depletion. The treated cells were examined for expression of a panel of previously identified tumor cell markers including *AGR2*. *AGR2* emerged in this screen as a gene sharply up-regulated in response to physiological stress. This induction pattern offers new insight into the potential role of *AGR2* in cancer cells, suggesting that *AGR2* contributes to the survival of cells undergoing physiological stress in the microenvironment of the tumor. The *AGR2* induction response could be blocked effectively by a synthetic inhibitor of ERK1/2, suggesting that the activation signal for *AGR2* induction during stress is mediated through an ERK1/2-dependent pathway.

Materials and methods

Cell culture, treatments, RNA isolation and cDNA synthesis

MDA-MB-231 cells were maintained in DMEM containing 10% FBS and 1% penicillin/streptomycin. Prior to treatment, cells were grown to 80–90% confluency under the conditions described above. For serum withdrawal experiments, cells were re-fed with serum free media and incubated in a standard cell culture incubator. For oxygen depletion experiments, cells were re-fed with media containing 10% FBS and placed into a hypoxia chamber (Billups Rothenberg). The air in the chamber was displaced with a low oxygen gas mixture of 1% oxygen, 5% carbon dioxide, and 94% nitrogen at a flow rate of 20 l min^{-1} for 10 min. The sealed chamber was then placed in a standard cell culture incubator and cells were supplied with fresh low oxygen gas mixture every 24 h. Simultaneous serum and oxygen depletion experiments were performed by combining the above treatments. Untreated cells were re-fed with media containing 10% FBS and incubated for the indicated times in a standard cell culture incubator. After treatment, cells were harvested, resuspended in 1 ml of Trizol reagent (Invitrogen, Carslbad, CA) and RNA was isolated according to manufacturer's protocol. Total RNA was treated with recombinant DNase I (Ambion, Austin, TX) and 1 μg of total RNA was placed into a 20 μl reverse transcription reaction containing SuperScriptII (Invitrogen, Carlsbad, CA). Reverse transcription reactions were incubated at 42°C for 1 h. cDNA was subsequently diluted to 200 μl using nuclease free water prior to quantitative PCR analysis. 10 μl of diluted cDNA was used in each quantitative real-time PCR reaction.

Quantitative PCR analysis

Where possible, primer sequences that amplified a product of about 100 bp within 300 bases of the 3′ end of the transcript were selected. Primer sequences used for analysis were:

AGR2 (5′TTGCTTGACAACATGAAGAAAGC3′; 5′TTTCAAGTCTCAAGGCCTGACA3′),

VEGF (5′AGATCCGCAGACGTGTAAATGTT3′; 5′TCGGCTTGTCACATCTGCAA3′),

S100A16 (5′TGATGTTCCTGCCAAATTCCT3′; 5′TGCTACCACTGCCACCAAGAG3′)

and *RPS27A* (5′TCGTGGTGGTGCTAAGAAAA3′; 5′TCTCGACGAAGGCGACTAAT3′).

All the primers were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA). Primer specificity was validated using BLASTN software (http://www.ncbi.nlm.nih.gov/BLAST/). Only primers showing unique identity to the target genes were considered. The AGR2 primers were also specifically compared with the sequence of the related gene AGR3, and show no significant homology. Quantitative real-time RT-PCR was performed using the SYBR Green PCR Master Mix and an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Reactions were run using the following cycling conditions (pre incubation at 94°C 10 min, followed by 45–50 cycles of 94°C for 15 s, 60°C for 1 min). Gene expression levels were determined using a standard calibration curve prepared from gene specific RT-PCR products with known concentrations. Gene expression levels between samples were normalized using the expression levels of the ribosomal protein *RPS27A* gene, which was shown recently to exhibit the least amount of variability in expression levels among different tissues [14]. PCR products were also assessed by dissociation curve analysis and agarose electrophoresis to verify the proper amplicon size and melting temperature.

Pathway inhibitors

SB202190 (p38 inhibitor), PD98059 (ERK1/2 inhibitor), SP600125 (JNK inhibitor) and Wortmannin (PI3K inhibitor) were a generous gift from Dr. Danny Dhanasekaran (Fels Institute for Cancer Research, Temple University School of Medicine, Philadelphia PA). At the start of each treatment, pathway inhibitors were resuspended in DMSO and administered at final concentrations of 10 μM, 50 μM, 50 μM, and 500 nM respectively. None of the compounds was cytotoxic at the selected concentrations.

Results

AGR2 expression is induced in conditions of serum and oxygen depletion

As an approach to understanding the conditions that activate potential tumor progression genes we investigated the role of physiological stress. During the multi-step process of carcinogenesis, tumor cells experience local decreases in blood supply resulting in sporadic regions deficient in serum-derived growth factors and oxygen. The presence of regions of pathophysiological stress in breast tumors in particular is well documented [15–17]. In order to mimic such conditions, the established breast cancer cell line MDA-MB-231 was subjected to conditions of serum depletion, alone or combined with oxygen depletion (culturing in 1% oxygen rather than the normal level of 20% oxygen). The physiological parameters of serum and oxygen depletion mimic the depletion of serum-derived growth factors and oxygen experienced by breast cancer cells when they are poorly vascularized in vivo [10, 18].

Expression of a panel of genes previously identified as tumor cell markers in cancer tissue or CTCs [e.g., 2] was probed by quantitative reverse transcription polymerase chain reaction (QRT-PCR) analysis of the stressed cells. The most striking result from this analysis was a marked induction of *AGR2*. In order to explore this further, the QRT-PCR analysis was repeated, and the cells were probed by Northern blotting as well. The quantitative RT-PCR results (Fig. 1A) indicate that *AGR2* is induced about 5-fold within 24 h following serum depletion. Oxygen depletion alone had little observable effect on *AGR2* expression, but did appear to enhance the inductive effects of serum depletion by several folds.

In order to verify that oxygen depletion was indeed occurring in these conditions, expression of vascular endothelial growth factor (*VEGF*) was monitored in parallel. The *VEGF*-encoded protein is a major pro-angiogenic molecule that has been well characterized for its role in suppressing the pro-apoptotic affects of serum depletion under hypoxia [10, 17, 19]. As expected, up-regulation of *VEGF* expression occurred in response to oxygen depletion alone (Fig. 1B). However, in contrast to the response seen with *AGR2*, there was virtually no induction of *VEGF* with serum depletion alone, and no enhancement of the response when both conditions were combined. The different patterns of induction observed between *AGR2* and *VEGF* in response to serum and oxygen depletion verify that both parameters were effective, and suggest that distinct pathways are involved in the regulation of the expression of each gene.

Genes analyzed in parallel with *AGR2* that showed no induction in response to either serum or oxygen depletion included the negative control β-actin (*ACTB*) (data not

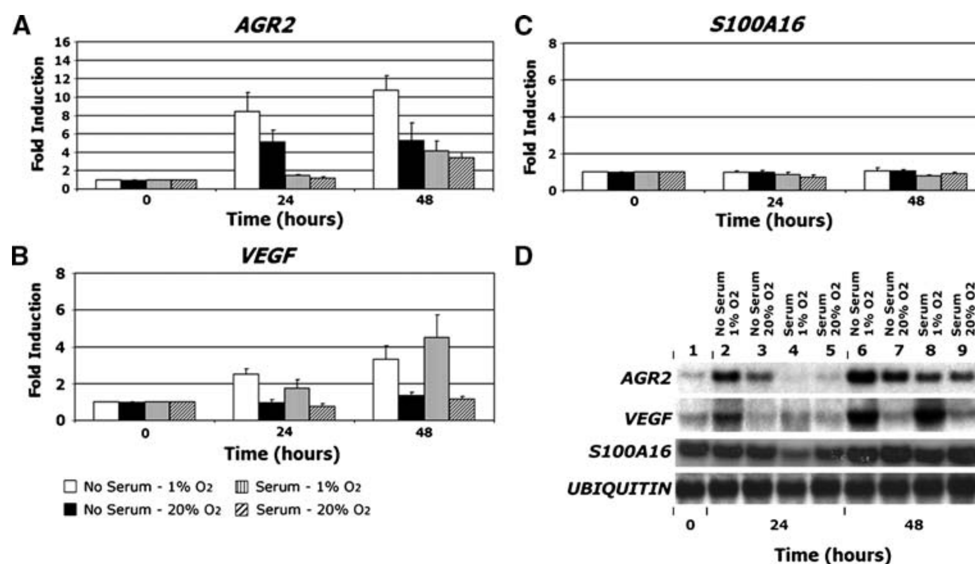

Fig. 1 Upregulation of *AGR2* expression in response to serum and oxygen depletion. (**A–C**) Expression of *AGR2* (**A**), *VEGF* (**B**) and *S100A16* (**C**) was measured by quantitative real-time PCR in MDA-MB-231 cells following their exposure to indicated treatments for the specified period of time. Expression values are presented relative to those of untreated cells at time 0. The values and the error bars represent, respectively, the average and the standard error of the mean from three independent experiments. The scale of panel A is adjusted to accommodate *AGR2* induction levels. (**D**) Expression of *AGR2, VEGF, S100A16* and Ubiquitin was measured by Northern blotting of total RNA prepared from MDA-MB-231 cells following their exposure to indicated treatments for the specified period of time. The migration positions of the resulting bands were calculated relative to the positions of the 18S and 28S rRNAs. The signals revealed by the *AGR2*, *VEGF*, and *S100A16* probes ran at about 0.9, 3.7, and 1.2 kb, respectively, consistent with the expected sizes

Table 1 Expression of *AGR2* and *ESR1* in response to serum and oxygen depletion in MDA-MB-231 cells[a]

	AGR2	*ESR1*	*TFF3*	*SPP1*
T = 0	6,456.6	3.4	3,341.6	3440.1
24 h: No Serum in 1% O_2	74,656.4	3.1	12,918.3	4085.2
24 h: No Serum in 20% O_2	45,718.2	2.7	13,234.7	3,740.7
24 h: Normal serum in 1% O_2	9,740.5	2.2	4,049.3	3,157.0
24 h: Normal serum in 20% O_2	9,719.1	1.8	2,923.4	3,890.4
48 h: No Serum in 1% O_2	89,505.1	4.0	22,781.4	13,818.1
48 h: No Serum in 20% O_2	58,241.4	2.4	20,922.9	5,769.0
48 h: Normal serum in 1% O_2	40,731.3	3.5	6,002.3	4,184.7
48 h: Normal serum in 20% O_2	24,913.1	2.5	5,795.3	3,164.2

[a] Expression was measured by quantitative real-time PCR in MDA-MB-231 cells following exposure to the indicated treatments for the specified period of time. The numbers represent copy number per 50 ng total cDNA equivalent

shown) and the tumor marker *S100A16*, which was examined further for comparison. Calcium binding proteins of the S100 family play central roles in many intra- and extra-cellular processes. *S100A16* is prevalently expressed in breast cancer derived CTCs [2], and up-regulation has been observed in many tumors, suggesting a central cellular function related to malignant transformation [20]. QRT-PCR analysis of *S100A16* expression in parallel samples revealed no significant induction in response to either serum or oxygen depletion (Fig. 1C), emphasizing that not all tumor markers are induced by these particular stresses. The induction patterns observed for *AGR2*, *VEGF* and *S100A16* by QRT-PCR analysis were also apparent in Northern blot analysis (Fig. 1D).

AGR2 induction in response to physiological stress is independent of estrogen receptor (ESR1) expression

Expression of *AGR2* has been strongly correlated with expression of the estrogen receptor gene *ESR1* in human breast cancer cell lines and clinical tissue samples [6, 21–23]. *ESR1* expression is strongly repressed in the MDA-MB-231 tumor cell line, apparently as a result of promoter methylation [24]. Expression of *ESR1* was analyzed in parallel with *AGR2* induction during physiological stress. No induction of *ESR1* was apparent in conditions that induced a sharp increase of *AGR2* expression (Table 1). Thus, although *AGR2* and *ESR1* expression occur together in the majority of breast tumors, induction of *AGR2* is not dependent on expression of *ESR1*, and can be induced independently in conditions of physiological stress. Induction of AGR2 was also monitored in the estrogen receptor negative cell line BT20, where a qualitatively similar response of about 8-fold induction was seen after 48 h in conditions of no serum and low oxygen (data not shown). Two additional tumor markers in the panel showed a response to serum and oxygen deprivation. Trefoil factor family 3 (*TFF3/ITF*) and osteopontin (*SPP1*) were induced about 7-fold and 4-fold, respectively (Table 1). Both of these proteins are known to be induced in response to tissue injury and are linked with more aggressive behavior in cancer cells [25, 26], supporting a model in which induction of AGR2 is part of a cell survival response.

Induction of *AGR2* expression in response to serum and oxygen depletion is reduced by the ERK1/2 inhibitor PD98059

Understanding the upstream signaling pathways involved in regulating *AGR2* expression could prove important for the future development of targeted breast cancer therapies. In an effort to distinguish pathways involved in the regulation of *AGR2* expression, the serum and oxygen depletion treatments were repeated in the presence of inhibitors specific for the ERK1/2 (PD98059), JNK (SP600125), p38 (SB202190) and PI3K (wortmannin) signaling pathways. Of the inhibitors tested, only PD98059 was associated with an appreciable block to AGR2 induction (Fig. 2A). This finding suggests that a MAPK/ERK-dependent pathway is involved in regulation of *AGR2* expression during pathophysiological stress. *VEGF* and *S100A16* expression were not significantly affected by any of the inhibitor molecules (Fig. 2B and C), again arguing that separable induction pathways regulate the responses of *AGR2* and *VEGF* under the conditions assayed. Similar results for each gene were observed by Northern blot analysis (Fig. 2D).

Discussion

Although hypovascular conditions are toxic to most cells, it is becoming clear that such conditions can promote the growth of tumor cells [27]. Indeed, pathophysiological

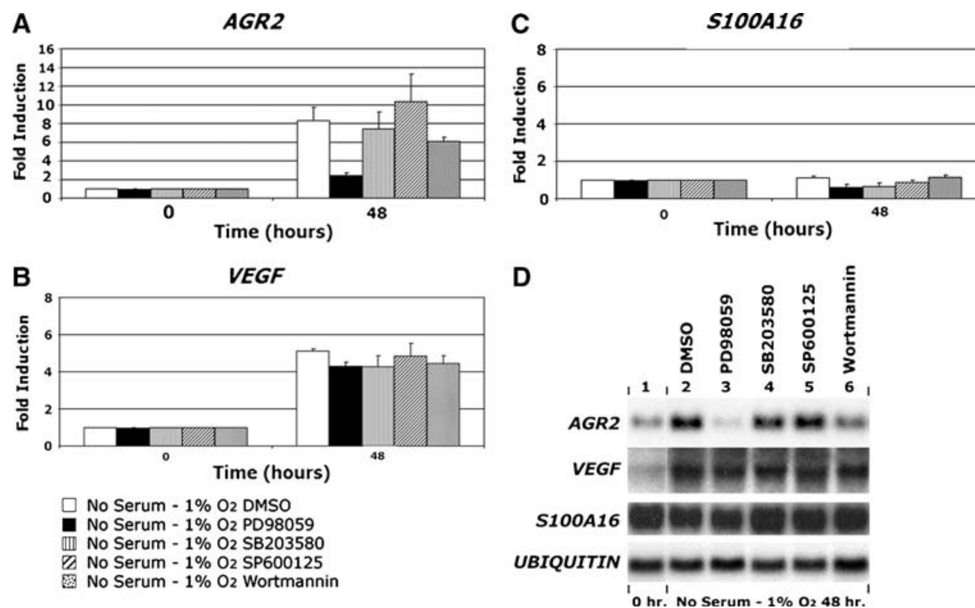

Fig. 2 Inhibitor PD98059 of ERK1/2 reduces induction of *AGR2* expression in response to serum starvation and hypoxia. (**A**–**C**) Expression of *AGR2* (**A**), *VEGF* (**B**) and *S100A16* (**C**) was measured by quantitative real-time PCR in MDA-MB-231 cells following their exposure to serum and oxygen depletion for 48 h in the presence of indicated inhibitors of specific signal transduction pathways. Expression values are presented relative to that of cells treated with vehicle DMSO at 0 h. The values and the error bars represent, respectively, the average and the standard error of the mean from three independent experiments. The scale of panel A is adjusted to accommodate *AGR2* induction levels. (**D**) Expression of *AGR2, VEGF*, *S100A16* and Ubiquitin was measured by Northern blotting of total RNA prepared from MDA-MB-231 cells following their exposure to serum and oxygen depletion for 48 h in the presence of indicated inhibitors of specific signal transduction pathways. Agreement between the positions of the resulting signals and the expected size of the respective mRNAs was confirmed as indicated in the legend to Fig. 1

stress within the tumor microenvironment can select for aggressive tumor cells by activating transcription of genes contributing to survival, proliferation, tissue invasion, and angiogenesis [11, 12, 17, 28, 29]. The sharp induction of *AGR2* expression in conditions of serum depletion and low oxygen, suggests that AGR2 plays a role in cell survival during periods of stress. This conclusion is concordant with a recent observation that AGR2 inhibits p53 tumor suppressor mediated effects in response to UV exposure [5]. Interestingly, a structural relationship involving putative functional domains was reported recently between AGR2 and proteins of the disulfide isomerase family of molecular chaperones [30]. The implication that AGR2 might function as a molecular chaperone, in combination with its sharp induction in response to physiological stress, suggests that AGR2 may play a role in clearing cells of misfolded proteins accumulated during periods of stress.

AGR2 expression is usually associated with *ESR1*-positive breast tumor cells, and is induced by estrogen in breast cells (and by androgen in prostate cells) in a receptor-dependent manner [7, 31]. However, results described here show that physiological stress leads to induction of *AGR2* in breast cancer cells independently of *ESR1* expression. This helps to explain the observation that a percentage (at least 20%) of clinical breast cancer specimens are *AGR2* positive and *ESR1* negative [22], and supports a view that the potential role of AGR2 in breast carcinogenesis and/or metastasis can be enacted independently of hormone activity.

AGR2 induction also showed a response pattern distinguishable from that of the hypoxia response gene *VEGF*. In addition, induction of *AGR2* expression in response to physiological stress was reduced by the ERK1/2 inhibitor PD98059, suggesting that an ERK1/2-dependent pathway participates in the regulation of *AGR2* expression. The MAPK/ERK pathway has been implicated previously in an anti-apoptotic response during similar cell treatments [19, 32, 33].

Although the precise function of AGR2 and the mechanisms of its regulation remain to be elucidated, the studies reported here suggest a model in which AGR2 contributes to the survival of metastatic and primary breast cancer cells in the pathophysiological environment of the growing tumor, and reveal a novel mode of *AGR2* regulation, independent of the estrogen receptor status of the cancer cell. A better understanding of *AGR2* function and the parameters that regulate its expression could help in the development of targeted therapies aimed at blocking breast cancer metastasis.

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