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INTRODUCTION

Accumulating evidence supports an association between angiogenesis and the processes of tumor invasion and metastasis. In this way, several angiogenic factors and their receptors have been identified as important mediators of angiogenesis (1). We as well others have accumulated evidence suggesting that **HEREGULIN (HRG)**, a growth factor involved in the progression of breast cancer to a more malignant phenotype (2-4), also is a potent stimulator of *in vitro* growth of a special blood vessel cells found in the umbilical cord, called **HUVEC (Human Umbilical Vein Endothelial Cells)**¹. Hence, HRG pathway can enhance tumor neovascularization and this upregulation of angiogenesis may contribute to a more aggressive disease. Significantly, we have also demonstrated that the angiogenic inducer factor **CYR61 (Cysteine-rich angiogenic protein 61)** is a down-stream effector of HRG-induced breast cancer chemomigration and metastasis, probably through interactions with the $\alpha_v\beta_3$ integrin receptor. CYR61 stimulates chemotaxis in endothelial cells and induce neovascularization *in vivo* (5). Moreover, we have shown that CYR61 overexpression in tumor cells enhances tumorigenicity by increasing tumor size and vascularization (6). In this regard, we have determined previously that CYR61 gene expression is elevated in highly invasive and metastatic human breast cancer cells and tumor biopsies (5). Accordingly, CYR61 overexpression is correlated with more advance stage of malignancy in patients samples (7). Taken together, these findings prompted us to hypothesize that HRG (directly or indirectly through CYR61) is an important regulator of the vascular compartment in breast cancer with stimulating effects on tumor neovascularization which, in turn, promotes progression and dissemination of breast carcinoma.

We recently showed that in ovariectomized nude mice breast carcinoma cells secreting HRG promoted more vascularized tumors (8). We demonstrated that one of the mechanisms by which HRG achieved this aggressive phenotype was mediated *via* an increase in the expression of **Vascular Endothelial Growth Factor (VEGF)**, a key tumor angiogenic factor. In MCF-7/HRG-derived tumors, a great increase in VEGF expression was observed by immunohistochemistry staining with anti-VEGF antibody. These results were further confirmed by an ELISA assay, in which VEGF levels were assessed in the conditioned media collected from MCF-7/HRG cells. A 3- to 8-fold increase in VEGF expression was observed in the conditioned media from HRG-transfected cells. Of interest, in our experiments there was a positive correlation between the increase in the ability of the HRG transfectants to secrete VEGF and the levels of HRG expression. Consistent with this finding, HRG has been shown to selectively upregulate VEGF secretion in both cancer and HUVEC cells and stimulates *in vivo* angiogenesis (9). Nevertheless, some of the effects that were observed *in vivo* were probably mediated indirectly *via* the up-regulation of other genes in an autocrine/paracrine manner. For example, the expression of CYR61 was significantly up-regulated in the MCF-7/HRG-derived tumors.

Our results, together, suggest that this HRG-induced upregulation of angiogenesis may contribute to the more aggressive phenotype observed in the clinical setting in patients with HRG-overexpressing cancers. Thus, the initial goal of the proposed study was to clarify the role of both HRG and CYR61 in human breast cancer angiogenesis, opening new molecular avenues in the treatment of breast cancer by blocking its neovascularization with anti-HRG and/or anti-CYR61 compounds.

¹ Lupu R *et al.*, unpublished observations

BODY

Tasks 1, 2, and 3 in the proposed study were:

- **Task 1:** to establish the culture of endothelial cells from breast tumor endothelial cells and
- **Task 2 and 3:** to characterize the effects of HRG by itself or the effect of culture median from breast carcinoma cell lines overexpressing HRG on the growth and migration of endothelial cells.

Unfortunately, the original attempt to selectively isolate endothelial cells from fresh tumor mass was tested without much success. Although an escape strategy proposed in Task 2 was to use HUVEC cells to characterize the molecular mechanism(s) of action of HRG endothelial cells, our preliminary results strongly underscored the importance of HRG and CYR61 as new potential targets for breast cancer therapy. Since the inhibition of tumor neovascularization is a new therapeutic approach to breast cancer treatment (10), we believed more convenient to accomplish with the task 4, which was to evaluate HRG blockade as a potential and novel therapeutic tool.

We have demonstrated that HRG modulates drug sensitivity in human breast cancer cells (11). Interestingly, virtually every conventional cytotoxic anticancer drug has been "accidentally" discovered to have antiangiogenic effects in various *in vivo* models (12,13). However, it has been suggested that exploiting chemotherapeutic drugs, as anti-angiogenics is likely to be compromised by the high concentrations of pro-angiogenic survival/growth factors present in the tumor microenvironment. Accordingly, it has been recently demonstrated that some angiogenic factors, such as VEGF and bFGF (basic Fibroblast Growth Factor) significantly reduced the potency of chemotherapy against HUVEC cells (14). Because the pro-angiogenic abilities of HRG and CYR61, we hypothesized that HRG could also act directly -or indirectly through CYR61- as a survival factor for both tumor and endothelial cells modifying chemotherapy effectiveness. With this in mind, we first evaluated the impact of HRG expression in modulating breast cancer cell response to different classes of chemotherapeutic agents available for clinical use (Cisplatin, 5-Fluorouracil, and Paclitaxel -Taxol[®]). Next, HRG-negative MCF-7 cells engineered to overexpress CYR61 gene were also assessed for chemotherapy effectiveness.

The MTS assay was used to determine the relative sensitivities of MCF-7/HRG cells and MCF-CYR61 transfectants to Cisplatin, 5-Fluorouracil, and Paclitaxel (Taxol[®]). Briefly, cells were plated in 200 μ l of medium per well in 96-well plates at a density of 2000 cells per well. Following overnight incubation, increasing concentrations of the specified drugs were added and the plates were incubated until confluence of the control samples at day 7. Drugs were not renewed during the entire period of cell exposure. The cell viability effects from exposure of cells to drugs were analyzed generating concentration-effect curves as a plot of the fraction of unaffected (surviving) cells *versus* drug concentration. Dose response curves were plotted as percentages of the control cell absorbance, which were obtained from control wells treated with appropriate concentrations of DMSO (*v/v*) that were processed simultaneously. For each treatment, cell viability was evaluated as a percentage using the following equation: (A_{490} of treated sample/ A_{490} of untreated sample) x 100. IC_{50} values were designated for the concentrations of each drug required to decrease cell viability by 50%. As the percentage of control absorbance was considered to be the surviving fraction of cells, the IC_{50} values were defined as the concentration of drug that produced 50% reduction in control absorbance (by interpolation).

1. Increased Resistance to Cisplatin (CDDP) in MCF-7 Breast Cancer Cells Expressing the Full-Length HRG β -2 cDNA. MCF-7 cells transfected with the full length cDNA of HRG β -2 (MCF-7/HRG) were 11-fold

more resistant to CDDP-induced effects on cell viability at IC_{30} value compared to wild-type MCF-7 cells or MCF-7 cells harboring the empty vector only (MCF-7/pBabe cells). Moreover, high concentrations of CDDP ($> 30 \mu M$) had only a limited effect on the cell viability of MCF-7/HRG cells and the IC_{50} values in these cells remain to be determined (Figure 1. A.).

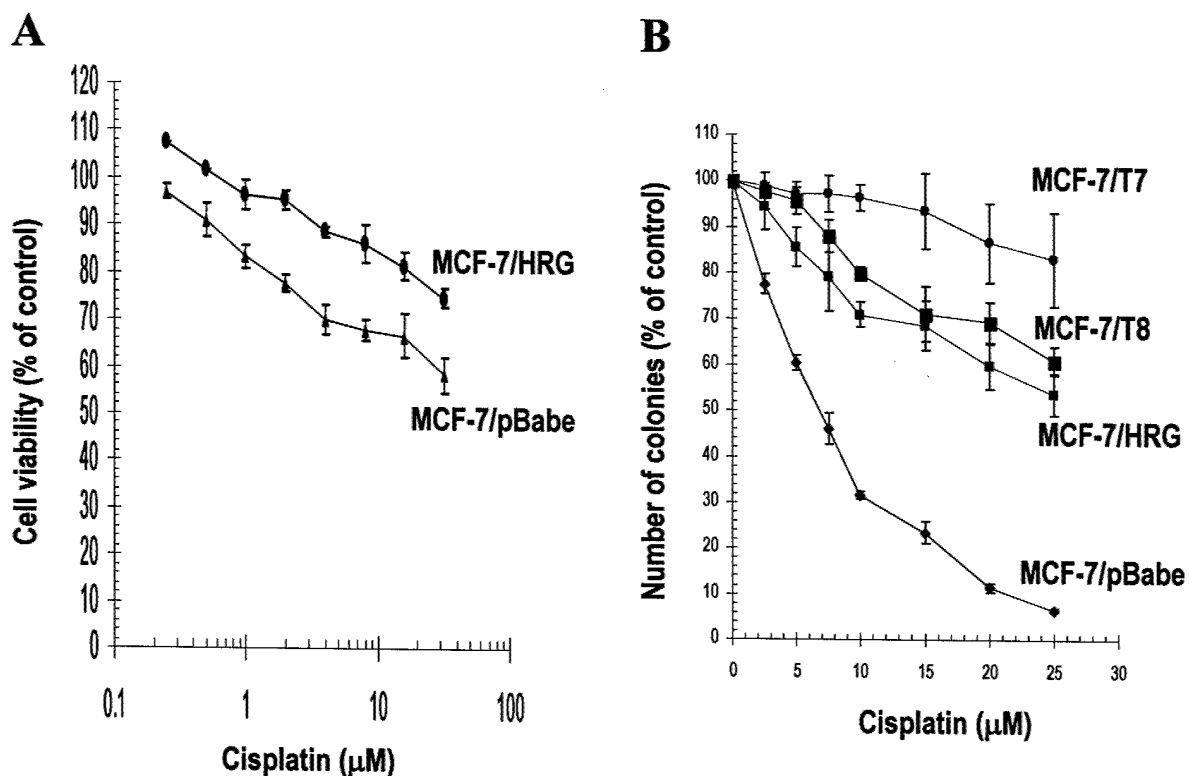


Figure 1. Effects of Cisplatin (CDDP) Exposure on the Cell Viability of MCF-7 Engineered to Overexpress HRG. *A.* Data represent the mean \pm S.D. (*bars*) of 4 or more independent experiments made in triplicate. *B.* For colony forming assays in soft agarose (anchorage-independent growth assays), a 1-ml top layer containing a single suspension of 2×10^4 cells-0.35% agarose, was added to a hard 1-ml bottom layer of 0.6% agarose-IMEM-10% FBS in 35-mm dishes. The top layer contained increasing concentrations of cisplatin. Dishes were incubated in a humidified 5% CO_2 incubator at $37^\circ C$, and colonies measuring ≥ 50 mm were counted after 10-14 days after staining with crystal violet using an image analyzer. Data represent the mean \pm S.D. (*bars*) of 2 independent experiments made in triplicate.

In a colony-forming assay, CDDP inhibited MCF-7/pBabe cells with an IC_{50} of $\sim 6 \mu M$. However, doses as high as $20 \mu M$ did not inhibit colony formation in MCF-7 cells infected or transfected with the full length HRG β -2 cDNA (MCF-7/HRG cells and MCF-7/T clones, respectively).

We next examined whether forced expression of CYR61 in MCF-7 cells modified CDDP effectiveness. Conversely to HRG overexpression, the ectopic expression of CYR61 alone in HRG-negative MCF-7 cells was unable to modulate the sensitivity of breast cancer cells to CDDP.

2. Increased Resistance to 5-Fluorouracil (5-FU) in MCF-7/HRG Breast Cancer Cells. The concentrations of 5-FU required to reduce cell viability by 50% in MCF-7/HRG cells ($IC_{50} = 16 \mu M$) were 5-fold higher than those in control cells ($IC_{50} = 3.4 \mu M$; Figure 2). No differences in 5-FU sensitivity were seen between MCF-7/CYR61 clones control cells.

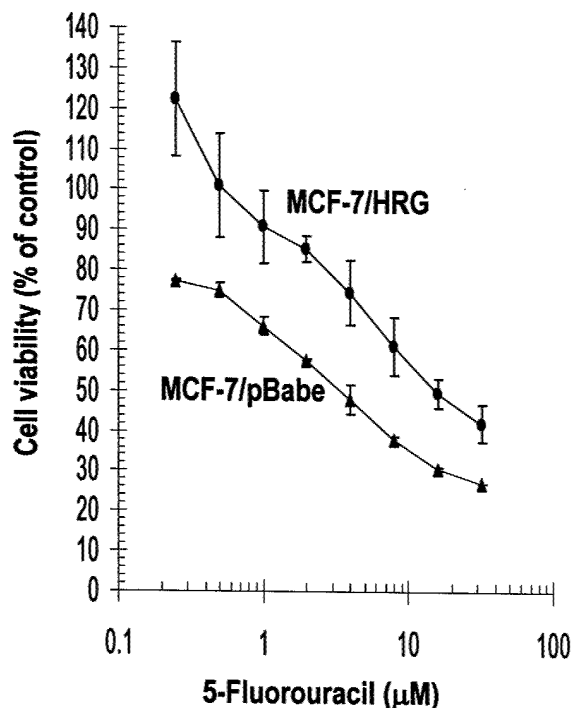


Figure 2. Effects of 5-Fluorouracil (5-FU) Exposure on the Cell Viability of MCF-7 Breast Cancer Cells Engineered to Overexpress HRG. Data represent the mean \pm S.D. (*bars*) of 4 or more independent experiments made in triplicate.

3. Increased Paclitaxel (Taxol[®]) Resistance in MCF-7/HRG cells and MCF-7/CYR61 transfectants. In anchorage-dependent cytotoxicity assays, paclitaxel exposure decreased cell viability in a dose-dependent fashion in both MCF-7/HRG and control cells. However, MCF-7/HRG cells were significantly more resistant to paclitaxel ($IC_{50} = 5 \text{ nM}$) than control cells ($IC_{50} = 0.38 \text{ nM}$; Figure 3). When the role of CYR61 overexpression in Taxol sensitivity was evaluated, control cells (MCF-7/V3-2) were also significantly more sensitive to the drug ($IC_{50} = 0.5 \text{ nM}$) than MCF-7/CYR61 transfected cells ($IC_{50} = 8 \text{ nM}$).

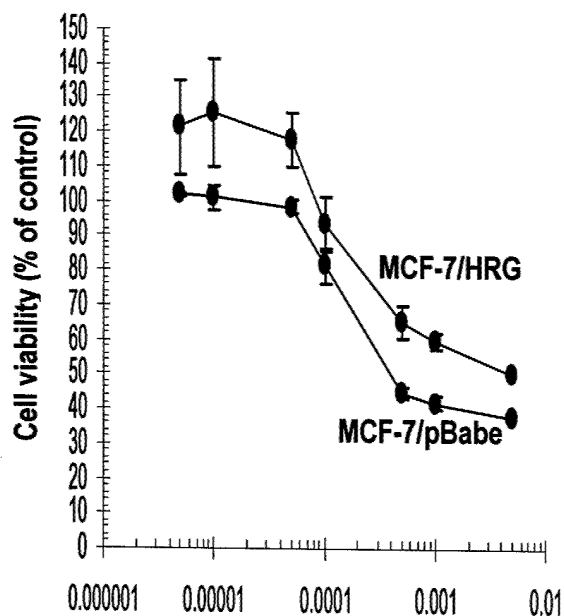


Figure 3. Effects of Paclitaxel (Taxol[®]) Exposure on the Cell Viability of MCF-7 Breast Cancer Cells Engineered to Overexpress HRG. Data represent the mean \pm S.D. (*bars*) of 4 or more independent experiments made in triplicate.

Anchorage-independent growth of cancer cells is thought to be one of the best *in vitro* correlated of the ability of cancer cells to grow *in vivo*. Thus, we performed colony-forming assays in soft agarose (anchorage-independent growth assays) to evaluate whether the CYR61-induced resistance to paclitaxel varied as a function of the anchorage culture conditions. Very low concentrations of paclitaxel dramatically decreased colony formation in semisolid agar of control cells ($IC_{50} = 0.12$ nM). In contrast, significant higher concentrations of paclitaxel were required to inhibit the anchorage-independent clonogenic growth of MCF-7/C2-6 ($IC_{50} = 0.98$ nM) and MCF-7/C2-9 ($IC_{50} = 0.22$ nM) CYR61 transfectants in soft agar. Thus, under anchorage-independent growth conditions, transfection of CYR61 increased paclitaxel resistance of MCF-7/C2-9 and MCF-7/C2-6 transfectants by 2- and 10-fold, respectively. This increase in paclitaxel resistance was less evident in anchorage-independent cytotoxicity assays (soft agar cultures) when MCF-7/HRG cells were used (data not shown).

3.1. CYR61 Overexpression Protects Breast Cancer Cells Against Paclitaxel-Induced Apoptosis. Apoptosis is the predominant mechanism of cytotoxicity induced by chemotherapeutic agents. Hence, the failure of cancer cells to activate apoptosis may lead to drug resistance. To investigate whether paclitaxel elicited cytotoxicity by induction of apoptosis, control cells (MCF-7/V3-2) and CYR61 transfected cells (MCF-7/C2-6 and MCF-7/C2-9 clones) treated with paclitaxel were examined for DNA fragmentation. Electrophoresis of DNA samples from control cells treated with 1 nM paclitaxel showed a weak nucleosomal ladder formation. Interestingly, DNA from MCF-7/CYR61 transfectants treated with the same concentration of paclitaxel (1 nM) displayed no signs of DNA degradation. To confirm that overexpression of CYR61 indeed protects cells against paclitaxel-induced apoptosis, we used a fluorometric TUNEL assay, which measure the fragmented DNA of apoptotic cells by catalytically incorporating fluorescein-12-dUTP at 3'-OH DNA ends using the enzyme Terminal Deoxynucleotidyl Transferase (TdT). TdT forms a polymeric tail using the principle of the TUNEL (TdT-mediated dUTP Nick-End Labeling) assay. In our experiments, the fluorescein-12-dUTP-labeled DNA was visualized directly by fluorescence microscopy. Paclitaxel exposure for 24 h resulted in a rapid induction of apoptosis in MCF-7/V3-2 cells. In contrast to control cells, the CYR61 derivatives MCF-7/C2-9 and MCF-7/C2-6 did not undergo significant apoptosis after paclitaxel exposure as assessed by TUNEL labeling.

Microtubule-active drugs, including paclitaxel, cause mitotic arrest, and this can result in apoptosis. Paclitaxel-induced apoptosis involves p53/p21^{WAF1/CIP1} pathway and it has been shown show that paclitaxel induces dose- and time-dependent accumulation of the p53 protein in MCF-7 human breast cancer cells (15). To dissect the molecular mechanisms underlying CYR61-induced cytoprotection against paclitaxel, we investigated whether induction of p53 can account for the difference in response of control cells and CYR61 transfectants treated with paclitaxel. Western blotting analyses demonstrated that paclitaxel exposure for 24 h induced a dose-dependent accumulation of p53 in control cells. Conversely, MCF-7/C2-6 CYR61 transfectants showed a profound reduced ability to induce p53 in response to paclitaxel-induced damage (not shown). Similar results were observed when MCF-7/C2-2 and MCF-7/C2-9 CYR61 transfectants were exposed to paclitaxel (data not shown).

3.2. Wortmannin, a Blocker of the Phosphatidylinositol 3'-Kinase (PI3'-Kinase) Activity, Suppresses Paclitaxel Resistance in MCF-7/CYR61 transfectants. Simultaneously to paclitaxel resistance, CYR61 overexpression also resulted in a cross-resistance to Wortmannin and LY294002, two pharmacological inhibitors of the PI3'-kinase activity (data not shown). To evaluate whether active PI3'-kinase activity did promote cellular resistance of CYR61 transfectants, cell viability was determined after incubating control cells and MCF-7/CYR61 transfectants with paclitaxel in the presence or absence of sub-optimal doses (*i.e.*, the

concentrations were themselves non toxic) of Wortmannin. For control cells, addition of 5 μ M Wortmannin did not alter significantly paclitaxel-induced effects on cell viability (data not shown).

In order to examine the effect of exposure schedule on Wortmannin-induced sensitization to paclitaxel, the effects of a 24 h pre-exposure to sub-optimal doses of Wortmannin (non toxic concentrations) on the sensitivity of control and CYR61 transfected cells were examined. Similarly to what happened with the simultaneous schedule, 5 μ M Wortmannin pre-exposure yielded a significant decrease in the paclitaxel IC₅₀ values of CYR61 transfectants. Conversely, pre-treatment with Wortmannin for 24 h prior paclitaxel exposure produced no significant enhancement of paclitaxel sensitivity in control cells (data not shown).

3.3. Effects of Blocking $\alpha_v\beta_3$ Integrin Using Arginine-Glycine-Aspartate (RGD)-based "peptidomimetics" on CYR61-induced Paclitaxel Resistance. It has been shown that CYR61 is an angiogenic ligand for $\alpha_v\beta_3$ integrin receptor (16). We have previously reported that a functional blocking antibody against $\alpha_v\beta_3$ is capable of blocking HRG induction of the aggressive phenotypes of the breast cancer cells (5). Thus, we have proposed that CYR61 can mediate tumor growth and angiogenesis of breast cancer cells in either autocrine or paracrine manner through its binding to the $\alpha_v\beta_3$ integrin receptor. Here, we decided to examine whether CYR61-induced paclitaxel resistance was associated with an increased integrin signaling.

First, we examined the ability of $\alpha_v\beta_3$ integrin signaling to promote cell survival in MCF-7 cells overexpressing HRG or CYR61. Our results showed that RGD-based "peptidomimetics" highly selective for $\alpha_v\beta_3$ integrin (SC68448, S-247, and S-196, kindly provided by Dr. Daviv Griggs at Pharmacia Corporation) have the ability to decrease cell viability in both control and MCF-7/HRG cells. Interestingly, the degree of cell viability reduction in the presence of increasing concentrations of $\alpha_v\beta_3$ integrin antagonists was significantly much greater in MCF-7/HRG cells than in wild-type MCF-7 cells (data not shown). A more profound inhibitory effect on cell viability was induced by several $\alpha_v\beta_3$ integrin antagonists in CYR61 transfectants, in contrast to control cells. No significant effects were observed when RGD-based "peptidomimetics" directed against other integrins, such as II_bIII_a, were used (data not shown).

3.4. Overexpression of HRG and/or CYR61 Increases the Levels of the Integrin Receptor $\alpha_v\beta_3$ in MCF-7 cells. Since the degree of cell viability inhibition in the presence of peptidomimetic antagonists of the integrin $\alpha_v\beta_3$ was much greater in MCF-7/HRG cells and MCF-7/CYR61 transfectants in comparison to control cells, this could reflect that each cell line possesses a different complement of integrin receptors, varying in both type and number. To investigate whether expression of HRG and/or CYR61 affects the levels of the integrin receptor $\alpha_v\beta_3$ integrin, the cellular distribution of $\alpha_v\beta_3$ was analyzed by immunofluorescence. Use of a monoclonal anti- $\alpha_v\beta_3$ antibody demonstrated that, in fact, MCF-7/HRG (data not shown) and MCF-7/CYR61 (data not shown) cells stained positively for $\alpha_v\beta_3$, whereas no significant staining was observed in control cells. Although direct quantitative interpretation of immunofluorescence is not possible, comparison of the intensity of the $\alpha_v\beta_3$ dots observed with the $\alpha_v\beta_3$ -specific antibody revealed an obvious and highly reproducible difference between MCF-7/HRG cells, MCF-7/CYR61 transfectants, and control cells.

3.5. CYR61 Overexpression Increases the Levels of Focal Adhesion Kinase in MCF-7 cells. It has been shown that integrin signaling can mediate survival signaling in many types of normal cells against different apoptotic stimuli, such as serum withdrawal (17) and cell damage induced by chemotherapeutic agents (18). Among the signaling molecules involved in integrin-mediated cell survival is the focal adhesion kinase (FAK), which becomes activated following integrin ligation and may in turn activate downstream survival pathways

such as those composed of PI3'-kinase and the serine/threonine kinase AKT (19, 20). In the present study, we provided evidence that CYR61-induced paclitaxel resistance was dependent on the PI3'-kinase/AKT survival pathway. Moreover, our results further demonstrated that $\alpha_v\beta_3$ integrin-mediated signaling was an important survival input in CYR61-induced cell chemoresistance. We finally asked whether a signaling mediator for this CYR61-induced survival system was FAK. Likewise, we observed that CYR61 overexpression caused a significant increase in the expression of FAK in MCF-7 human breast cancer cells (Figure 4-5).



Figure 4. Expression of Focal Adhesion Kinase (FAK) in MCF-7 human breast cancer cells engineered to overexpress CYR61. Lane 1: MCF-7/V3-2, lane 2: MCF-7/C2-6, and lane 3: MCF-7/C2-9.

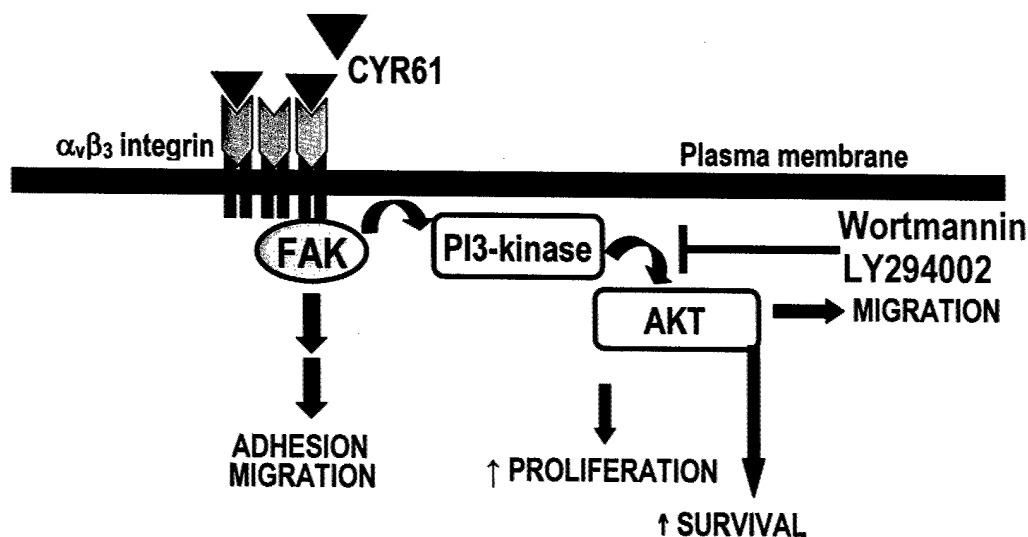


Figure 5. CYR61-induced Activation of the $\alpha_v\beta_3$ -FAK-PI3'kinase-AKT Pro-Survival Signaling as a Hypothetical Model for the Role of CYR61 in Breast Cancer Chemoresistance to Paclitaxel. Up-regulation of CYR61 may drive chemoresistance by inducing proliferative and/or survival input via $\alpha_v\beta_3$ integrin and FAK, which might integrate signals from CYR61 to the PI3'kinase/AKT kinase survival pathway.

In this study we provide new data for additional understanding of HRG-induced breast cancer response to chemotherapeutic drugs. We have previously demonstrated that HRG-transfected cells showed a marked increase in sensitivity to the topoisomerase II inhibitors doxorubicin and etoposide (11). Conversely, based on our current data, it seems that HRG overexpressing breast cancer cells are more resistant to CDDP, 5-FU, and paclitaxel. The potential application of this finding for therapy is the use of HRG as a new marker for drug response. If breast cancer tumors with HRG overexpression are more sensitive to drugs such as doxorubicin and etoposide, then drug regimens including topoisomerase II inhibitors as major active components could be chosen as opposed to other regimens based on alkylators (like cisplatin) or antimetabolites (like 5-Fluorouracil).

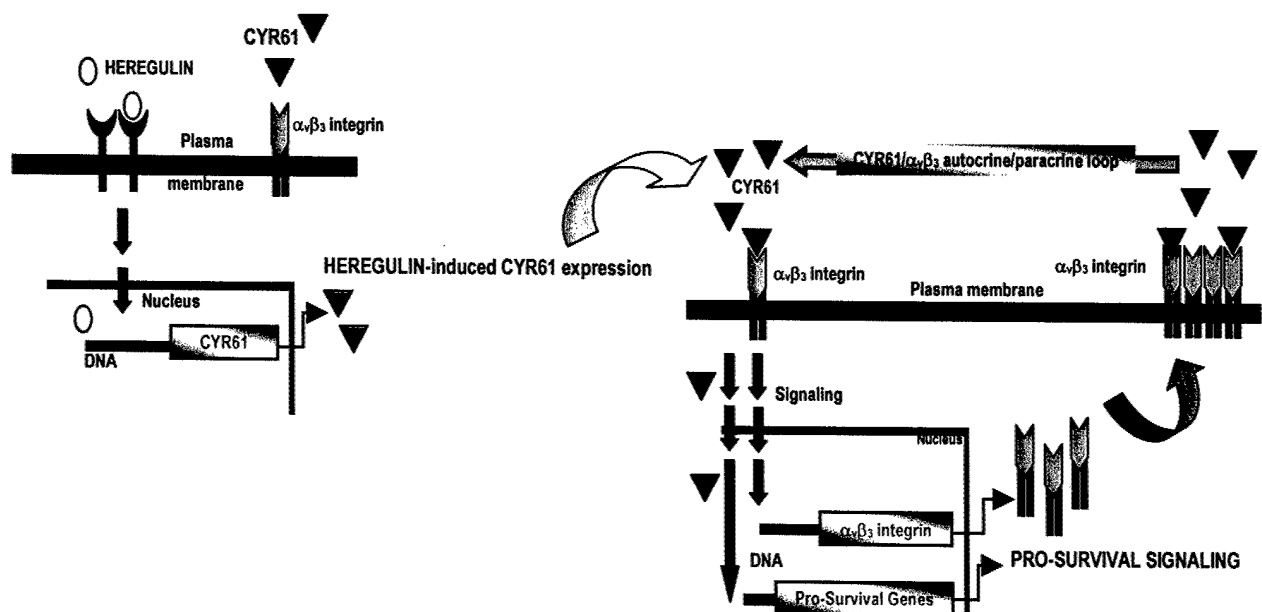
Interestingly, HRG expression rendered breast cancer cells more resistant to paclitaxel, a promoter of microtubule polymerization commonly used in the treatment of advanced or metastatic breast cancer that also possesses an antiangiogenic property associated with a down-regulation of Vascular Endothelial Growth Factor (21). When HRG-negative breast cancer cells were engineered to express CYR61, an angiogenic factor that acts as a downstream effector of HRG-induced metastatic phenotype, we did not see any change in doxorubicin, cisplatin, or 5-Fluorouracil effectiveness.

Downstream of CYR61, the differential ability of the PI3'-kinase inhibitors Wortmannin and LY2904002 to synergistically increase paclitaxel effectiveness in CYR61 transfectants but not in control cells strongly suggests that the activation of PI3'-kinase pathway could operate decreasing paclitaxel sensitivity in CYR61 overexpressing human breast cancer cells. In this regard, we have recently demonstrated that CYR61 induces tumor formation and tumor vascularization *in vivo*, events mediated through the activation of the serine/threonine kinase protein kinase B/AKT, a key regulator of cell survival protecting cells from apoptosis (22). It is likely then to suggest that CYR61-induced paclitaxel resistance may be mediated by activation of PI3'-Kinase and its target, AKT. In this way, several studies have recently indicated that alterations in the PI3'-kinase/AKT signal transduction pathway can modulate sensitivity to cancer chemotherapy (23). The absence of the classical DNA laddering pattern of apoptotic death and the diminished terminal Deoxynucleotidyl transferase-mediated dUTP nick-end labeling ending after paclitaxel exposure in CYR61 transfectants can indicate that resistance could be due to the presence of CYR61 expression and the inability of paclitaxel to induce apoptosis in the same cell. Moreover, our experimental observations evidenced that expression of CYR61 inhibited the function of wild-type p53 in MCF-7 human breast cancer cells.

Since the disruption of microtubule structure by antimicrotubule agents results in induction of tumor suppressor gene p53 and the inhibitor of cyclin-dependent kinases p21^{WAF1/CIP1} (15) our data indicate that CYR61 overexpression might protect breast cancer cells from paclitaxel-induced apoptosis by enhancing PI3'-Kinase/AKT pro-survival signaling and inhibiting p53 pro-apoptotic functions. Nevertheless, overexpression and activation of erbB-2, an HRG-activated receptor, is a major mechanism through which human breast cancer cells acquire paclitaxel resistance (24, 25). Interestingly, immunoblotting assays revealed that CYR61-transfected cells have not an increase in the erbB-2 expression and phosphorylation (data no shown). Hence, paclitaxel resistance was no related to the activation of the erbB-2 receptor in CYR61 transfectants.

Our results also suggest that signaling through $\alpha_v\beta_3$ integrin allows for the maintenance of the cell viability of MCF-7/HRG and MCF-7/CYR61 cells treated with paclitaxel. Integrin signaling is involved in a variety of cellular activities, including cell proliferation, migration, and survival, as well as angiogenesis and tumor progression. Thus, we have previously shown that the functional $\alpha_v\beta_3$ integrin is required for maintaining the invasive capacity of HRG-expressing cells, and that the aggressive phenotypes induced by HRG are mediated, in part if not entirely, by CYR61 and its receptor $\alpha_v\beta_3$ integrin (5). Recently, the tumor cell microenvironment has been found to have a significant bearing on the survival of tumor cells following exposure to a wide variety of anti-neoplastic agents, prior to the acquisition of known drug resistance mechanisms. Of interest, integrin signaling has recently been shown to modulate cancer cell responses to chemotherapeutic agents. Specifically, interactions between cell surface integrins and extracellular matrix components have been shown to be responsible for this phenomenon of innate drug resistance, which we have termed Cell Adhesion Mediated Drug Resistance, or CAM-DR (26). Thus, signal transduction pathways initiated by integrin ligation may be potential bridge points for inhibiting cell survival during cytotoxic drug exposure.

HRG-induced Up-regulation of CYR61 May Predispose Breast Tumor Epithelial Cells Toward Continued Dysregulated Proliferation and Chemoresistance. The functional blocking of $\alpha_v\beta_3$ integrin in CYR61 transfectants induces cytotoxicity, suggesting that CYR61/ $\alpha_v\beta_3$ integrin-induced signaling is involved in breast cancer cell survival. Since CYR61 overexpression by itself activates the expression of the CYR61 receptor integrin $\alpha_v\beta_3$, up-regulation of CYR61 in human breast cancer may coordinate a chemoresistance phenotype in an autocrine/paracrine manner. This is schematically represented:



Recently, it has been established that angiogenic factors such as VEGF and bFGF significantly reduced the potency of chemotherapy against HUVEC cells (14). This cytoprotection to drug toxicity was found to be PI3'-kinase-dependent and was recapitulated in the absence of VEGF by overexpressing the dominant-active form of AKT. Interestingly, the potency of VEGF as a chemoprotectant was more pronounced with drugs that interfered with microtubule dynamics, such as paclitaxel, than those that damage DNA. Interestingly, paclitaxel also possesses an antiangiogenic property associated, at least in part, with a down-regulation of VEGF (21). In this context, our current data suggest that the up-regulation of HRG and/or its downstream effector CYR61 significantly impaired paclitaxel-induced cytotoxicity. Our observations also suggest that overexpression of CYR61 may inhibit the pro-apoptotic function of wild-type p53 by inhibiting its up-regulation. It has been demonstrated that an effective recruitment of AKT by appropriate survival signals may lead to inhibition of p53-dependent apoptosis.

In summary, exploiting chemotherapeutic drugs as anti-angiogenic is likely to be compromised by the high concentrations of pro-angiogenic survival/growth factors present in the tumor microenvironment. In this context, our current data provide new evidences about the role of HRG and its downstream effector CYR61, two pro-angiogenic factors, as survival factors protecting human breast cancer cells from paclitaxel-induced damage. We suggest that targeting HRG- and/or CYR61-induced angiogenic and survival pathways may prevent vessel growth simultaneously improving the efficacy of paclitaxel-based chemotherapy in breast cancer. The next Tasks in these project will be to assess the studies in athymic nude mice to determine whether the taxol

resistance occurs also in vivo and to assess whether the compounds targeted to block angiogenesis could be used clinically to reverse taxol resistance.

KEY RESEARCH ACCOMPLISHMENTS

- Conversely to HEREGULIN-induced sensitization to doxorubicin and etoposide (two topoisomerase II inhibitors, see ref. 11), Heregulin overexpression decreases cisplatin (an alkylating agent), 5-Fluorouracil (an antimetabolite drug) and Paclitaxel (an anti-mitotic drug) *in vitro* effectiveness against human breast cancer cells.
- The angiogenic factor CYR61, a down-stream effector of Heregulin-induced breast cancer progression.
- CYR61 overexpression induces up-regulation of $\alpha_v\beta_3$ integrin and Focal Adhesion Kinase (FAK) expression, which might integrate signals from CYR61 to the PI3'kinase/AKT kinase survival pathway.

REPORTABLE OUTCOMES

ABSTRACTS/PRESENTATIONS

Javier A. Menendez, Inderjit Mehmi, Ella Atlas, Miaw-Sheue Tsai, and Ruth Lupu. *The angiogenic factor CYR61, a downstream effector of Heregulin, protects breast cancer cells from paclitaxel-induced cell death through integrin $\alpha_v\beta_3$.* Abstract for the 14th EORTC-NCI-AACR SYMPOSIUM on "MOLECULAR TARGETS AND CANCER THERAPEUTICS", Frankfurt, Germany, 2002 (Abstract #366).

Javier A. Menendez, Inderjit Mehmi, Ella Atlas, Miaw-Sheue Tsai, David Griggs, and Ruth Lupu Abstract for the 7th INTERNATIONAL MEETING ON MOLECULAR ONCOLOGY, Crete, Greece, 2002 (Abstract #438).

4th ANNUAL LYNN SAGE BREAST CANCER SYMPOSIUM, Chicago, IL, 2002

Title: The angiogenic factor CYR61, a downstream effector of Heregulin, protects breast cancer cells from paclitaxel-induced cell death through integrin $\alpha_v\beta_3$. Javier A. Menendez, Inderjit Mehmi, Ella Atlas, and Ruth Lupu.

CONCLUSIONS

Overall, our results support the potential therapeutic use of HRG as a marker for drug response in human breast cancer. Moreover, although the exact mechanism(s) by which HRG promotes paclitaxel resistance in breast cancer cells is still unknown, it is tempting to postulate that CYR61-induced activation of $\alpha_v\beta_3$ -FAK-PI3' kinase-AKT pro-survival signaling could be a new erbB-2-independent pathway involved in this phenotype. We suggest that new anti-HRG and/or anti-CYR61 strategies may prevent vessel growth simultaneously rendering tumor cells more sensitive to paclitaxel-based chemotherapy in breast cancer. Moreover, current and future antagonists of specific integrin heterodimers, such those used in this study directed against $\alpha_v\beta_3$, may have the potential to sensitize tumor cells when used in combination with standard chemotherapy regimens.

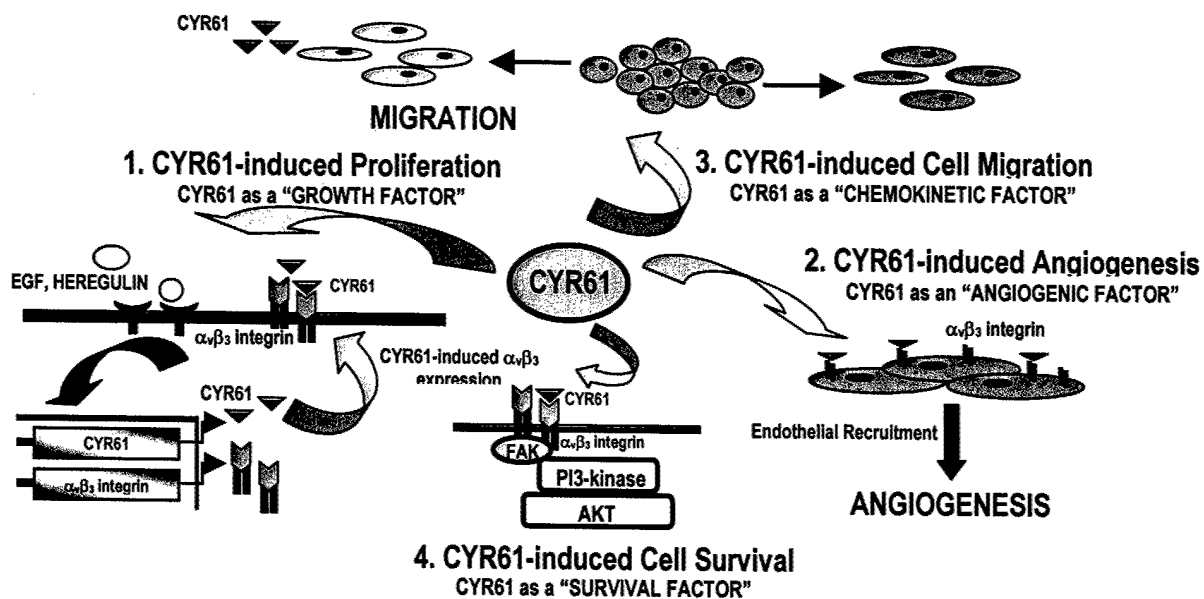


Figure 7. A New Hypothetical Model for the Role of CYR61 in Breast Tumorigenesis and Progression. Given that the angiogenic factor CYR61 is also a growth-regulator, it is hypothesized that up-regulation of CYR61 in tumor epithelial cells by HRG may drive breast tumorigenesis and progression in several concerted modes by::

- 1.) Promoting tumor cell proliferation in an autocrine/paracrine fashion either augmenting growth factor bioactivity or emitting proliferative signals via $\alpha_v\beta_3$ integrin receptor,
- 2.) Regulating endothelial recruitment tumor neovascularization in a paracrine fashion through an $\alpha_v\beta_3$ -dependent mechanism,
- 3.) Coordinating tumor epithelial cell migration as a chemokinetic factor, and
- 4.) Increasing chemoresistance either activating a $\alpha_v\beta_3$ -FAK-PI3'kinase-AKT kinase pro-survival signaling or blocking p53 pro-apoptotic functions.

ACKNOWLEDGMENTS

After this first year training period of the DOD BCRP post-doctoral traineeship I have developed and mastered several essential techniques in biochemistry, cell biology, and molecular biology, which is evident in the research accomplishments described above. In addition, it has offered me tremendous opportunities to establish interesting collaborations with other researchers in the field of human breast cancer. With the support from DOD, the proposed subject shall enhance our understanding at both the cellular and molecular levels of breast cancer progression, and could result in new molecular anticancer therapies based on blockade of HRG, CYR61, and/or the integrin receptor $\alpha_v\beta_3$.

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APPENDICES

14th EORTC-NCI-AACR SYMPOSIUM on "MOLECULAR TARGETS AND CANCER THERAPEUTICS", Frankfurt, Germany, 2002

Abstract number: 366

Title: The angiogenic factor CYR61, a downstream effector of Heregulin, protects breast cancer cells from paclitaxel-induced cell death through integrin $\alpha_v\beta_3$. Javier A. Menendez, Inderjit Mehmi, Ella Atlas, Miaw-Sheue Tsai, and Ruth Lupu.

Abstract: Heregulin (HRG) expression promotes breast cancer progression, antiestrogen resistance, and metastasis. We recently established the angiogenic inducer cysteine-rich angiogenic protein 61 (CYR61) is a down-stream effector of HRG-induced breast cancer chemomigration and metastasis, probably through interactions with the $\alpha_v\beta_3$ integrin receptor. Both HRG and CYR61 enhance tumor neovascularization, and this upregulation of angiogenesis may contribute to a more aggressive disease. Moreover, chemotherapy effectiveness could be compromised by the high concentrations of pro-angiogenic survival/growth factors present in the tumor microenvironment. Since we previously demonstrated that HRG expression is related to doxorubicin (DOX) efficacy we envisioned that, in addition to their role as pro-angiogenic factors, HRG and/or CYR61 can also act as survival factors modifying breast cancer chemosensitivity. To address this question, we first evaluated the impact of HRG expression in modulating breast cancer response to anticancer drugs which are available for clinical use. MCF-7 cells transfected with the full-length HRG cDNA (MCF-7/HRG) were assessed for cisplatin (CDDP), 5-Fluorouracil (5-FU), and paclitaxel (PTX) sensitivity. MCF-7/HRG cells were significantly more resistant to CDDP as compared to control cells. A weaker but significant increase in 5-FU resistance was observed in MCF-7/HRG cells. Also, MCF-7/HRG became more resistant to PTX. Next, HRG-negative MCF-7 cells engineered to overexpress CYR61 gene were also assessed for chemotherapy effectiveness. MCF-7/CYR61 transfectants and control cells were equisensitive to DOX, CDDP and 5-FU. However, CYR61 overexpression resulted in PTX resistance levels similar to those found in MCF-7/HRG cells. Functional blocking of the integrin $\alpha_v\beta_3$, which was found to be overexpressed in MCF-7/HRG and MCF-7/CYR61 cells, induced a profound inhibitory effect on cell viability against MCF-7/HRG and MCF-7/CYR61 cells but not in control cells. Of note, sub-optimal doses of RGD peptidomimetics directed against integrin $\alpha_v\beta_3$, which were based on CYR61 structure, synergistically reversed the PTX-resistance in MCF-7/HRG and MCF-7/CYR61 cells and additively modulated PTX activity in control cells. PTX resistance in MCF-7/CYR61 cells was also reversed by the pharmacological inhibition of the phosphatidylinositol 3-kinase (PI3 kinase) activity, whereas PTX toxicity was not modified after PI3 kinase inhibition in control cells. DNA from CYR61 transfectants treated with PTX displayed no signs of the classical DNA laddering pattern of apoptotic death. Inhibition of PTX-induced apoptosis in CYR61 transfectants was also demonstrated by TUNEL assay. Moreover, MCF-7/CYR61 cells were unable to induce p53 expression in response to PTX-induced damage. Although the exact mechanism(s) by which HRG promotes PTX resistance is still unknown, it is tempting to postulate that the angiogenic factor CYR61 -a downstream effector of HRG- might protect breast cancer cells from PTX-induced apoptosis by enhancing $\alpha_v\beta_3$ -PI3 kinase pro-survival signaling and inhibiting p53 pro-apoptotic functions. In summary, this is the first report evidencing an association between increased CYR61 expression and breast cancer resistance to PTX. We suggest that new anti-HRG, anti-integrin $\alpha_v\beta_3$, and/or anti-CYR61 strategies may prevent vessel growth simultaneously rendering tumor cells more sensitive to PTX-based chemotherapy in breast cancer.

7th INTERNATIONAL MTG ON MOLECULAR ONCOLOGY, Crete, Greece, 2002

Title: Overexpression of the angiogenic factor CYR61 protects human breast cancer cells from Taxol-induced cell death: Involvement of the $\alpha_v\beta_3$ /Focal Adhesion Kinase/Phosphatidylinositol 3'-kinase/AKT Kinase Pathway Javier A. Menendez^{1,2}, Inderjit Mehmi¹, Ella Atlas^{1,2}, Miaw-Sheue Tsai³, David Griggs⁴, and Ruth Lupu^{1,2} Department of Medicine, Evanston Northwestern Healthcare Research Institute, ²Northwestern University, Medical School (Evanston, Illinois), ³Life Sciences Division, Ernest Orlando Berkeley National Laboratory (Berkeley, California), ⁴Discovery Oncology Research, Pharmacia Corp. (St. Louis, Missouri)

Abstract

We have previously shown that the angiogenic inducer cysteine-rich angiogenic protein 61 (CYR61), a member of the CCN protein family, plays a functional role in heregulin (HRG)-induced breast cancer progression possibly through its interaction with the $\alpha_v\beta_3$ receptor. Both HRG and CYR61 enhance tumor neovascularization, and this angiogenesis up-regulation may contribute to a more aggressive disease. In this investigation, we examined for the first time if CYR61 can also act as a survival factor modifying breast cancer chemosensitivity. To determine the function of CYR61 in this context, we first evaluated the impact of HRG expression in modulating breast cancer response to anticancer drugs that are available for clinical use. MCF-7 cells infected with the full-length HRG cDNA (MCF-7/HRG) were assessed for cisplatin (CDDP), 5-Fluorouracil (5-FU), and paclitaxel (Taxol) sensitivity. MCF-7/HRG cells were significantly more resistant to CDDP as compared to control cells. A weaker but significant increase in 5-FU resistance was observed in MCF-7/HRG cells. Also, MCF-7/HRG became more resistant to Taxol. Next, HRG-negative MCF-7 cells engineered to overexpress CYR61 gene (MCF-7/CYR61) were assessed for chemotherapy effectiveness. MCF-7/CYR61 transfectants and control cells were equisensitive to DOX, CDDP and 5-FU. Interestingly, CYR61 overexpression resulted in resistance levels to Taxol higher to those found in MCF-7/HRG cells in both anchorage-dependent and anchorage-independent assays. Simultaneously, we observed a cross-resistance to wortmannin and LY294002, two pharmacological inhibitors of the phosphatidylinositol 3'-kinase (PI3k) activity. In addition, the expression of the integrin receptor $\alpha_v\beta_3$ was markedly increased in MCF-7/CYR61 cells. Also, CYR61 transfection induced an up-regulation of the focal adhesion kinase (FAK) expression, which is integrating signals from integrins to the PI3k/AKT kinase survival pathway. These observations suggested that CYR61-induced Taxol resistance in MCF-7 breast cancer cells was mediated, at least in part, *via* activation of the $\alpha_v\beta_3$ /FAK/PI3k/AKT kinase signaling. Likewise, functional blocking of the integrin $\alpha_v\beta_3$ using arginine-glycine-aspartate (RGD) peptidomimetics induced a marked decrease in the cell viability of MCF-7/CYR61 cells but not in control cells. Moreover, under culture conditions inhibiting $\alpha_v\beta_3$ signaling, Taxol resistance was completely prevented in MCF-7/CYR61 cells. Further, the protective effect of CYR61 against Taxol-induced cytotoxicity was abolished in the presence of pharmacological inhibitors of PI3k activity. When Taxol-treated cells were examined for apoptosis-related parameters, no signs of the classical DNA laddering formation were observed in MCF-7/CYR61 cells compared to control cells. Accordingly, CYR61 overexpression induced a dramatic decrease in the number of TUNEL-positive cells after Taxol exposure. Furthermore, MCF-7/CYR61 cells showed a reduced ability to induce p53 expression in response to Taxol-induced damage, suggesting that CYR61 could suppress Taxol-induced apoptosis by inhibiting the function of wild-type p53 in breast cancer cells. In summary, our results establish a novel role for CYR61 in determining protection of human breast cancer cells against Taxol-induced apoptosis through the activation of the $\alpha_v\beta_3$ /FAK/PI3k/AKT kinase pro-survival signal transduction pathway. New strategies directed against CYR61-induced signaling may render tumor cells more sensitive to Taxol, a microtubule targeting chemotherapeutic agent which is the drug of choice in the treatment of metastatic human breast cancer.

4th ANNUAL LYNN SAGE BREAST CANCER SYMPOSIUM, Chicago, IL, 2002

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