Ph.D.THESIS

THE ROLE OF CD44 IN THE TRASTUZUMAB RESISTENCE BREAST CANCER CELL LINE

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DEBRECEN, 2008

INTRODUCTION

Activation of the ErbB family of receptor tyrosine kinases is involved in a range of human cancers. Transmembrane signaling mediated by ErbB proteins is stimulated by peptide growth factors and is blocked by monoclonal antibodies such as trastuzumab and pertuzumab. Trastuzumab is a recombinant, humanized anti-ErbB2 antibody, which is widely used for the treatment of breast cancer. Despite encouraging clinical results some cancers are primarily resistant to trastuzumab, and a majority of those initially responding become resistant during prolonged treatment. The mechanisms of trastuzumab resistance have not been fully understood but it is known that ErbB receptors exert their function in conjunction with non-ErbB proteins, e.g. CD44.

The EGFR receptor family

The EGFR receptor family has a four member: the epidermal growht factor receptor (EGFR/ErbB1/HER1), the ErbB2 (Her2/Neu), the ErbB3 (HER3) and the ErbB4 (HER4). Overexpression of ErbB2 has been unquestionably linked to adverse prognosis in breast cancer. ErbB2 is viewed as an non-autonomous, ligand-less, positive regulator of ErbB signaling, but its participation in non-ErbB protein-mediated signaling is attracting more and more interest as well. Among these, β-integrins, MUC-4 and CD44 are probably the best known candidates whose roles in cancer progression are well documented. ErbB2 is embedded into this network of signaling and accessory molecules and by its promiscuous association profile it promotes cancer progression.

The signal-transduction and dimerization of ErbB-s protein.

ErbB2 heterodimerizes with other members of the ErbB family of receptor tyrosine kinases (RTK) leading to enhanced ligand binding affinity, protection from lysosomal degradation and diversified signaling.

The role of ErbB2 in the breast cancer and the trastuzumab therapy

Being a membrane protein and a key member of survival and proliferation signaling pathways ErbB2 is the target of receptor-oriented antibody therapy. Trastuzumab (Herceptin), a humanized monoclonal anti-ErbB2 antibody, induces objective clinical responses in 40% of patients

as a single agent given as first-line treatment of ErbB2-overexpressing metastatic breast cancer. Although combination of trastuzumab with conventional chemotherapy increases response rates dramatically, the development of resistance seems currently inevitable. Direct action of trastuzumab on ErbB2 (e.g. ErbB2 down-regulation, inactivation of Akt, inhibition of metalloprotease-mediated shedding) and antibody-dependent cellular cytotoxicity (ADCC) have been invoked to explain the mechanism of action of trastuzumab. Despite intense investigations trastuzumab resistance remains enigmatic and unpredictable in the clinical setting. Production of EGF-like growth factors, loss of PTEN, masking of ErbB2 and impaired ADCC reaction have all been suggested as possible mechanisms.

Interaction between ErbB2 and CD44

CD44 is recognized as the major hyaluronan receptor having several, alternatively spliced isoforms varying in their physiological function. Binding of hyaluronan activates CD44-mediated signal transduction pathways via interactions between CD44, Grb2, Vav2 and ErbB2. CD44 is involved in the direct regulation of ErbB2 and multiple other RTKs. It has been suggested that ligation of CD44 by endogenous hyaluronan leads to the activation of the phosphatidylinositol 3-kinase (PI3K)-Akt survival pathway, and that displacement of endogenous hyaluronan by exogenous hyaluronan oligosaccharides disrupts the activation. CD44-mediated cytoskeletal rearrangements have been observed implying the involvement of CD44 in cellular adhesion, migration and invasion. CD44 is almost absent in normal human breast epithelial cells, emerges in benign and premalignant lesions, and is upregulated in carcinomas. However, a recent study suggested that CD44 opposes rather than promotes the spreading of breast carcinoma in mice. While the role of CD44 in breast cancer progression *in vivo* is not completely settled, the accumulation of hyaluronan around malignant cells or in adjacent stroma has been unambiguously shown to be an indicator of poor prognosis in breast cancer. In line with the findings on human tumours, hyaluronan synthesis facilitates the invasive growth of grafted tumour cells *in vivo*, and blocking hyaluronan interactions with its receptors, using soluble CD44 or hyaluronan oligomers, inhibits tumour cell growth in experimental animals. Besides creating signals to prevent apoptosis, hyaluronan is required for activation of the ErbB2-ErbB3 receptor leading to the formation of cardiac valves. Thus, there is mounting evidence that ErbB2, CD44 and hyaluronan are connected both in

physiological signaling as well as cancer pathogenesis through mechanisms largely unknown at present.

The shedding of CD44

The transmembrane form of CD44 is processed by consecutive cleavages by metalloproteases and γ-secretase generating a soluble extracellular domain and an intracellular domain, which is translocated to the nucleus and activates transcription. CD44 shedding is stimulated by hyaluronan oligosaccharides and EGF. The soluble extracellular domain of CD44 has important roles in its own right by disrupting endogenous CD44-hyaluronan interactions. The serum level of soluble CD44 has been shown to correlate with tumor burden and indicate poor prognosis and resistance to chemotherapy. In addition to their involvement in CD44 processing both metalloproteases and γ-secretase participate in the cleavage of ErbB4. More importantly, the metalloprotease-mediated shedding of ErbB2 extracellular domain is inhibited by trastuzumab. These findings imply an intimate relationship between CD44, ErbB proteins and membrane-associated proteolytic enzymes.

SPECIFIC AIMS

Our major goal was to investigate the potential role of CD44 in the trastuzumab-resistance.

- How is the expression level of CD44 on the trastuzumab resistant JIMT-1 cell line?
- Is it any connection between CD44 and ErbB2 on molecular level?
- Is it any role of the CD44-ErbB2 connection of the trastuzumab-resistance? What is the mechanism of this process?
- Has a role of CD44 in the ErbB2 internalization?
- Has any affect of CD44 to the proliferation of JIMT-1 cell?
- What is the mechanism of CD44 activation and what is the effect of hyaluronan, EGF and anti-ErbB antibodies to the CD44 mediated process?
- Is it any role of CD44 in the cell motility of JIMT-1 cell line?

MATERIALS AND METHODS

Cells. JIMT-1 cells were grown in F-12/ DMEM (1:1) supplemented with 10% FCS, 60 units/L insulin and antibiotics.[30] The SKBR-3 cell line was obtained from the American Type Culture Collection (Rockville, MD) and grown according to its specifications.

Antibodies. Trastuzumab (Herceptin) was purchased from Roche Ltd. (Budapest, Hungary). Ma2C4 was a generous gift from Genentech (South San Francisco, CA). Monoclonal antibodies against ErbB2 (ErbB2-76.5) and CD44 (Hermes-3) were produced from their hybridoma supernatants (ErbB2-76.5 obtained from Y. Yarden, Weizmann Institute of Science, Rehovot, Israel; Hermes-3 produced by the HB-9480 hybrodima obtained from ATCC) and purified using protein A affinity chromatography. Hermes-3 was kindly donated by Dr. Sirpa Jalkanen (University of Turku, Finland). Cy3- and Cy5-conjugated goat anti human IgG (H+L) Fab was obtained from Jackson ImmunoResearch Europe (Cambridgeshire, UK). Conjugation of primary antibodies with AlexaFluor (Molecular Probes, Eugene, OR), Cy3 and Cy5 (Amersham, Braunschweig, Germany) dyes was carried out according to the manufacturers' specifications.

Hyaluronan. Highly purified large molecular weight hyaluronan (HA-LMW) with an average molecular mass of 1.2×10^6 Da was donated by Genzyme (Cambridge, MA). Purified hyaluronan decasaccharides (HA10) were kindly provided by Seikagaku Corporation (Tokio, Japan).

Western blotting. Whole cell lysates were prepared in lysis buffer containing 20 mM Tris- HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM EGTA, 1 % Triton X-100, 1 Complete Mini (Roche, Mannheim, Germany) protease inhibitor cocktail tablet/10 mL, 1 mM Na_3VO_4, 1 mM PMSF, 10 mM NaF, 10 mM β-glycerol phosphate and 10 mM $Na_4P_2O_7$. Immunoprecipitation of ErbB2 and ErbB1 were carried out with specific antibodies

Flow cytometric measurement of cell numbers and receptor expression levels.

Quantitative determination of receptor expression levels was carried out on a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) using Qifikit (DakoCytomation, Glostrup, Denmark) according to the manufacturer's instructions. In proliferation assays cells were counted with a FacsArray flow cytometer (Becton Dickinson).

Xenograft tumours. The severe combined immunodeficiency (SCID) C.B-17 scid/scid mouse population originated from the laboratory of Fox Chase Cancer Center, Philadelphia, PA, and were housed in a pathogen-free environment. Trastuzumab was administered at a dose of 5 μg/g by

weekly intraperitoneal (i.p.) injection. Control mice received weekly i.p. injection of 100 µl physiologic saline. Animals were euthanized by CO_2 inhalation. The experiments were done with the approval of the ethical committee of the University of Debrecen.

4-methylumbelliferone treatment. 4-methylumbelliferone (4-MU) (Sigma, Budapest, Hungary), an inhibitor of hyaluronan synthase, was suspended in 1% arabic gum and administered orally at a dose of 3 mg/g body weight twice daily. For *in vitro* experiments 4- MU was dissolved in PBS and added to the culture medium at a concentration of 1 mM.

Immunohistochemistry. Subcutaneous tumours were removed from anesthetized mice The slides of xenograft were labeled with a saturating concentration (10-20 µg/ml) of fluorophore-conjugated antibodies in 100 µl PBS containing 1% BSA (PBS-BSA) overnight on ice. For labelling of hyaluronan tissue sections were treated with endogenous biotin blocking kit (Molecular Probes) followed by labelling with 5 µg/mlbiotinylated HABC (hyaluronan binding complex) at 4 ^{o}C overnight. Prior to staining with fluorescein-avidin the samples were washed five times in PBS-BSA.

Confocal microscopy. A Zeiss LSM 510 confocal laser-scanning microscope (Carl Zeiss AG, Göttingen, Germany) was used to image samples. AlexaFluor488 was excited at 488 nm and detected between 505-530 nm. Cy3 and AlexaFluor546 were excited with the 543 nm line of a green He-Ne laser, and emission was measured between 560-615 nm. Cy5 and AlexaFluor647 were excited with the 633 nm line of a red He-Ne laser, and their emissions were measured over 650 nm. Fluorescence images were taken as 1-µm optical sections using a 63x (NA=1.4) oil immersion objective.

Image analysis. Confocal microscopic images were analyzed with the DipImage toolbox (Delft University of Technology, Delft, The Netherlands) under Matlab (Mathworks Inc., Natick, MA).
The cell membrane was identified by a manually-seeded watershed algorithm using a customwritten interactive algorithm implemented in Dipimage/Matlab.
Colocalization between two different fluorescent labels was calculated according to Pearson's formula:

Fluorescence resonance energy transfer (FRET). Flow cytometric FRET measurements were performed on a FACSVantage SE instrument with DiVa option (Becton Dickinson) equipped with three lasers emitting at 488, 532 and 633 nm. A detailed description of the method has been published elsewhere.

Internalization of trastuzumab. siRNA-transfected and control cells were incubated with 20μg/ml AlexaFluor647-labeled trastuzumab at 37°C. The samples were treated with acid strip buffer (0.5 M NaCl, 0.1 M glycine, pH 2.5) for 3 minutes on ice followed by washing and resuspension in PBS. Cells were analyzed by flow cytometry, and the internalized fraction of trastuzumab was calculated by dividing the mean fluorescence intensity of the acid-stripped sample with that of the non-acid-treated control.

RNA interference (RNAi). Small interfering RNA (siRNA) against human CD44 were designed and synthesized by Dharmacon (Chicago, IL) using the SMARTselection rules. siRNA transfection of JIMT-1 was carried out with the Nucleofector device of Amaxa (Cologne, Germany) according to the manufacturer's specifications. The optimal electroporation conditions (solution V, program T-20) were selected using GFP plasmid transfection.

Detection of CD44 ectodomain shedding by ELISA. JIMT-1 cells were grown to confluency in 24-well plates. The culture medium was removed, and the cells were washed twice with PBS followed by adding 200 μl of Hank's buffer supplemented with 1 mg/ml BSA. After inducing CD44 ectodomain shedding by various treatments the supernatant was collected and centrifuged at 400 *g* for 10 min to get rid of cells detached from the plate. The concentration of shed CD44 ectodomain was measured from undiluted supernatants with a commercially available Instant-ELISA kit (Bender MedSystems, Vienna, Austria) according to the manufacturer's specifications.

In vitro scratch assay JIMT-1 cells were cultured on chambered coverslips, and a cell-free stripe was generated by scratching a confluent cell layer with a micropipette tip [46]. A ~1 mm-wide cell-free scratch was reproducibly created. Cells were treated with different agents for 24h, and they were imaged at the beginning and at the end of the experiment with a low magnification objective using the transmission channel of Zeiss LSM 510 microscope. In order to take images of identical fields at the beginning and at the end of the experiment the x-y coordinates displayed by the microscope were used. The pixel width is saved in the image header by the microscope. The scratch width in these calibrated images was analyzed by ImageJ.

RESULTS

CD44 is overexpressed on trastuzumab resistant JIMT-1 cells and associated with ErbB2.

We compared the expression levels of CD44 in trastuzumab resistant and sensitive breast cancer cell lines in order to reveal the possible roles of CD44 in trastuzumab resistance. Flow cytometric data showed a ~35-times higher expression level of CD44 in trastuzumab resistant JIMT-1 cells (2.3±0.3 million/cell) than in SKBR-3 (65000±5000/cell), their trastuzumab-sensitive counterpart. Since CD44 has been shown to interact with ErbB2 in ovarian cancer, we investigated the potential interaction between them. Analysis of confocal microscopic images yielded cross-correlation coefficients of 0.612 and 0.602 between CD44 and ErbB2 on JIMT-1 and SKBR-3 cells, respectively. The crosscorrelation coefficient between two different antibodies against ErbB2 was used as a positive control. The fact that the cross-correlation coefficient of the positive control was not substantially different from that between CD44 and ErbB2 implies a strong colocalization between these molecules on the micrometer scale. Normalized flow cytometric fluorescence resonance energy transfer (FRET) values of 16±3% and 10±3% for the association of CD44 and ErbB2 in JIMT-1 and SKBR-3, respectively, show that these molecules are associated at the molecular level as well, since FRET values above 5% are considered to imply significant association. The above biophysical data demonstrating association between ErbB2 and CD44 in JIMT-1 cells were reinforced by molecular biological methods. ErbB2 co-immunoprecipitated with CD44, and its tyrosine phosphorylation was increased by large molecular weight hyaluronan, whereas hyaluronan decasaccharide inhibited ErbB2 tyrosine phosphorylation (. Neither slow, nor large molecular weight hyaluronan modified the activation state of ErbB1 .

CD44 expression correlates with trastuzumab internalization in JIMT-1 xenografts.

In order to study the possible role of CD44 overexpression in trastuzumab resistance sevenweek old female SCID mice were inoculated by JIMT-1 cells with a single subcutaneous injection. Mice received trastuzumab or physiologic saline weekly starting immediately after tumour injection. Mice were treated with trastuzumab for 9 weeks, then by physiologic saline for another 6 weeks before sacrificing. Tumour sections were triple-stained against ErbB2, trastuzumab and CD44. The watershed algorithm was used to segment the images. The seeds for the watershed algorithm were placed inside cells positive for CD44 and ErbB2. Since mouse stromal cells express

neither CD44 nor ErbB2, this approach ensured that the analysis was restricted to JIMT-1 cells. We observed a lack of tight correlation between trastuzumab binding and ErbB2 expression in these mice whose trastuzumab therapy had been suspended for 6 weeks. The trastuzumab/ErbB2 ratio was inversely correlated with CD44 expression, i.e. cells in which the relative binding of trastuzumab to ErbB2 was low displayed high CD44 expression. The lack of strict correlation between trastuzumab binding and ErbB2 expression could be caused by local heterogeneities in trastuzumab binding or internalization. We excluded the role of trastuzumab binding by investigating tumour sections from mice sacrificed immediately after 15 weeks of trastuzumab treatment and showing a tight correlation between trastuzumab binding and ErbB2 expression. Unfortunately, direct quantitation of trastuzumab internalization by measuring fluorescence intensity inside cells was not possible due to the low signal-tonoise ratio. Therefore, we used an alternative approach by analyzing the correlation between CD44 and ErbB2 expressions before, during and after trastuzumab treatment, and found that they show a positive correlation in control mice and in mice in which trastuzumab treatment has been stopped. Strikingly, CD44 and ErbB2 expression levels were inversely correlated with each other during trastuzumab treatment, i.e. pixels with high CD44 expression displayed low ErbB2 intensity and vica versa. This finding can be rationalized by assuming that ErbB2 is internalized and down-regulated more efficiently in cells with high CD44 expression.

RNA interference (RNAi)-mediated suppression of CD44 expression inhibits trastuzumab internalization.

The *in vivo* results supporting the role of CD44 in trastuzumab-mediated ErbB2 internalization were tested by *in vitro* RNAi experiments. Two siRNAs against CD44 elicited efficient and specific inhibition of CD44 expression in JIMT-1 supported by the lack of effect of an irrelevant siRNA on CD44 expression, and the lack of CD44 siRNA-induced suppression of the expression of an irrelevant protein. Quantitative flow cytometric analysis confirmed that CD44 siRNAs inhibited trastuzumab internalization by ~50%.

4-methylumbelliferone (4-MU) synergizes with trastuzumab in inhibiting the growth of JIMT-1 xenografts.

We showed that CD44 plays a role in trastuzumab internalization both *in vivo* and *in vitro*. However, the contribution of trastuzumab-mediated ErbB2 internalization to the mechanism of action of the antibody has been repeatedly questioned. Therefore, we directly tested whether the CD44-hyaluronan pathway can influence the therapeutic efficacy of trastuzumab. Mice injected with JIMT-1 xenografts were treated with 4-MU (an inhibitor of hyaluronan synthase) or trastuzumab, or both starting immediately after tumour inoculation. 4-MU combined with trastuzumab was significantly more effective in inhibiting the growth of JIMT-1 xenografts than trastuzumab alone. 4-MU did not have any effect on tumour growth on its own, although it reduced the amount of hyaluronan in JIMT-1 tumour tissue by ~40%. In addition, CD44 expression was increased by 4-MU probably due to its decreased hyaluronan-mediated down-regulation. 4-MU downregulated ErbB2 to the same extent as trastuzumab. We attribute this finding to the fact that 4-MU increased the CD44 level of cells which was shown to correlate with increased ErbB2 internalization. Not only did 4-MU synergize with trastuzumab in slowing tumour growth, but it also enhanced trastuzumab-mediated down-regulation of ErbB2. Since we assumed that 4-MU may unmask the trastuzumab-binding epitope on ErbB2, it was necessary to show that hyaluronan colocalized with the plasma membrane. Quantitative analysis of the images showed a shift on the trastuzumab-ErbB2 contour plot towards higher trastuzumab binding upon 4-MU treatment. Next, we tested the effect of *in vitro* 4-MU treatment on trastuzumab binding. We found that 4-MU treatment enhanced the amount of bound trastuzumab relative to the intensity of ErbB2. The 4-MU-induced changes in the trastuzumab/ErbB2 ratio present convincing evidence for the *in vitro* and *in vivo* masking effect of hyaluronan on the trastuzumab binding epitope.

CD44 is essential for the proliferation of JIMT-1 cells *in vitro*.

Since CD44 is overexpressed by JIMT-1 cells and it is involved in regulating trastuzumab-mediated ErbB2 internalization, we investigated whether the inhibition of CD44 expression alters the effect oftrastuzumab *in vitro*. As previously shown JIMT-1 cells were resistant to trastuzumab *invitro*, but their proliferation was inhibited by siRNA-mediated suppression of CD44 expression. However, no additive effect between trastuzumab and CD44 siRNA was detected. SKBR-3 cells

responded to trastuzumab treatment, but in agreement with their low CD44 expression failed to show any response to siRNA-mediated suppression of CD44 expression.

Hyaluronan oligosaccharide, EGF and heregulin induce shedding and internalization of CD44

Hyaluronan fragments and EGF have been shown to enhance CD44 cleavage. We wanted to test whether these agents and heregulin are effective in the trastuzumab resistant JIMT-1 cell line. We found that CD44 shedding was significantly stimulated by hyaluronan oligosaccharide, EGF and heregulin. We also followed the fate of the intracellular domain of CD44 in confocal microscopic experiments. Hyaluronan oligosaccharide, EGF and heregulin induced a striking increase in CD44 endocytosis as evidenced by the appearance of vesicles positive for CD44. We quantitated CD44 internalization by separately measuring the fluorescence intensities in the intracellular space and in the cell membrane. This analysis confirmed that all three of the agents significantly increased CD44 endocytosis. Intramembrane cleavage of CD44 generates a truncated protein binding only the anti-CD44cyto antibody. The fact that the anti-CD44cyto and Hermes-3 signals show considerable overlap implies that both full length and cleaved CD44 accumulate in the intracellular space.

The effect of pertuzumab and cetuximab on EGF- and heregulin-induced CD44 cleavage

Cetuximab and pertuzumab block EGF receptor and ErbB2-mediated signaling, respectively, by different mechanisms. We tested whether these receptor-blocking antibodies inhibit EGF- and heregulin-induced CD44 cleavage. ELISA experiments showed that pertuzumab significantly inhibited CD44 shedding, CD44 internalization and intramembrane cleavage induced by EGF and heregulin.

The effect of trastuzumab on heregulin and hyaluronan oligosaccharide-induced CD44 cleavage in vitro

The mechanism of action of trastuzumab and reasons for trastuzumab resistance are multifactorial, and often determined by ErbB-related signaling pathways. Here we examined whether trastuzumab exerts any effect on CD44 shedding which was shown to be regulated by ErbB family members. Heregulin-induced CD44 shedding, endocytosis and intramembrane cleavage were all inhibited by trastuzumab pretreatment. Somewhat surprisingly, trastuzumab almost completely abolished the hyaluronan oligosaccharide-evoked effect on CD44 shedding, internalization and cleavage implying that ErbB2 is incorporated into the membrane protein complex in which CD44-mediated signaling and cleavage of CD44 take place.

The effect of trastuzumab treatment on CD44 shedding in JIMT-1 xenografts in vivo

Since *in vitro* experiments suggested that trastuzumab inhibited both heregulin- and hyaluronan-mediated CD44 cleavage, we examined whether it has any effect *in vivo* on CD44 shedding from JIMT-1 xenograft tumors. Mice were injected with 4×10^6 JIMT-1 subcutaneously. Trastuzumab-treated animals were given 5 μg/g trastuzumab weekly starting on the day of tumor inoculation, while control mice were treated with physiological saline. Since trastuzumab significantly retarded tumor growth, blood samples taken from control mice on week 5 were compared to blood samples of trastuzumab-treated animals collected on weeks 9-11, since the mean tumor size in both cases was ~400 mm^3. Trastuzumab significantly decreased the CD44 concentration in the blood ($p<0.05$, Fig. 4). Since the applied ELISA assay is specific for human CD44, which could get to the mouse circulation only from JIMT-1 tumor cells, the results strongly

suggest that trastuzumab inhibited CD44 shedding from the tumor cells. We also compared the effect of trastuzumab on large tumors with a mean volume of ~850 mm^3. These blood samples were collected on weeks 7-9 and 13-14 from control and trastuzumab-treated mice, respectively. The effect of trastuzumab on CD44 shedding from large JIMT-1 xenografts was negligible.

The effects of anti-ErbB antibodies on hyaluronan oligosaccharide, EGF and heregulin-induced cell motility

Although CD44 shedding has been convincingly linked to cell motility, we wanted to examine if changes observed in CD44 cleavege correlate with cell locomotion in our experiments. *In vitro* scratch assays showed that migration of JIMT-1 cells was significantly increased by hyaluronan oligosaccharide, EGF and heregulin. The motogenic effects of hyaluronan and heregulin were inhibited by trastuzumab. Similarly, the effect of EGF was sensitive to cetuximab, and pertuzumab blocked both EGF- and heregulin-induced effects on cell motility. All effects proved to be statistically significant . In summary, the effect of every treatment on CD44 shedding and internalization was perfectly mirrored by its effect on cell motility.

The influence of hyaluronan oligosaccharide on the rate of trastuzumab internalization

Since the results presented above strongly imply that ErbB receptors influence CD44 cleavage and internalization, we tested whether a reciprocal effect of CD44 on trastuzumab internalization is detectable. The rate of trastuzumab internalization was significantly higher in JIMT-1 cells pretreated with hyaluronan oligosaccharide than in control cells. We conclude that CD44 and ErbB receptors mutually affect each other's internalization rate in JIMT-1 cells.

DISCUSSION

In the current work we investigated JIMT-1, a cell line showing *in vitro* resistance to trastuzumab, and found a high level of CD44 overexpression. We showed previously that the *in vivo* trastuzumab resistance of the cell line is partial, and development of complete trastuzumab resistance takes 5-10 weeks. We suggested that masking of ErbB2 may be the culprit. Given the suggested association of CD44 with ErbB2 we asked whether CD44 plays any significant role in the survival of JIMT-1 during trastuzumab therapy. Using siRNA-mediated suppression of CD44 expression we showed that CD44 is necessary for trastuzumab-induced internalization of ErbB2 and for the survival of JIMT-1 cells *in vitro.* 4-methylumbelliferone (4-MU), a hyaluronan synthase inhibitor, has been shown to increase the efficiency of chemotherapy.[31] We reasoned that hyaluronan may play a role in masking of cell surface ErbB2. We show that *in vitro* and *in vivo* treatment with 4-MU decreased the pericellular hyaluronan concentration in JIMT-1 xenografts accompanied by increased binding of trastuzumab to ErbB2. 4-MU acted synergistically with trastuzumab in inhibiting the progression of JIMT-1 tumours. Elucidation of the role of CD44 overexpression in trastuzumab resistant cell lines may help understand the causes of therapeutic failures in patients with this type of breast cancer.

Our results in connection with CD44 shedding suggest that ErbB1, ErbB2 and CD44 are clustered to form a functional unit in the cell membrane in which they mutually influence eachother's cleavage and endocytosis rates. The interaction between them is not limited to the regulation of proteolysis and internalization, but involves potentiation of signaling processes as well. The motogenic, but not the mitogenic activity of EGF depends on the activation of CD44. Hyaluronan has been shown to be required for heart valve mesenchyme formation induced by ErbB2 and ErbB3. These observations also support our model in which the complex of CD44 and ErbB proteins are assumed to be involved in the regulation of cell motility. From the standpoint of a clinician the functional interaction between CD44 and ErbB proteins has the potential to modify the therapeutic efficacy of ErbB1- and ErbB2-directed therapies suggesting that the interaction, or merely the expression level of CD44 is taken into consideration when patients are selected for antibody therapy. Much work needs to be done before these experimental observations will make their way to clinical practice.

THE THESIS IS BASED ON THE FOLLOWING PUBLICATIONS:

1. **Pályi-Krekk Zs**, Barok M**.**, Isola J., Tammi M., Szöllősi J., Nagy P.: Hyaluronan-induced masking of ErbB2 and CD44-enhanced trastuzumab internalization in trastuzumab resistant breast cancer. European Journal of Cancer, 2007 Sep 30. **IF: 4,167**

2. **Pályi-Krekk Zs**, Barok M., Kovács T., Saya H., Nagano O., Szöllősi J., Nagy P.: EGFR and ErbB2 are functionally coupled to CD44 and regulate CD44 shedding. *In press* Cancer Letter **IF: 3,277**

OTHER PUBLICATIONS:

1. Barok M., Isola J., **Pályi-Krekk Zs**., Nagy P., Juhász I., Vereb Gy., Kauraniemi P., Kapanen A., Tanner T., Vereb Gy., Szöllősi J.: Trastuzumab causes ADCC-mediated growth-inhibition of submacroscopic JIMT-1 breast cancer xenografts despite intrinsic drug resistance. Molecular Cancer Therapeutics, 6 (2007): 2065-2072. **IF: 5,131**

2. Fazekas Z, Petrás M, Fábián A, **Pályi-Krekk Z,** Nagy P, Damjanovich S, Vereb G, Szöllősi J. Two-sided fluorescence resonance energy transfer for assessing molecular interactions of up to three distinct species in confocal microscopy. Cytometry A. 2007 Nov 28; **IF: 3,293**

3. Rákosy Zs., Vizkeleti L., Ecsedi S., Voko Z., Bégány A, Barok M., **Krekk Zs**., Gallai M., Szentirmay Z., Ádány R., Balázs M.: EGFR gene copy number alterations in primary cutaneous malignant melanomas are associated with poor prognosis. International Journal of Cancer 2007 Oct 15; 121(8):1729-37. **IF: 4,693**

4.

The impact factor of own poblication: 7,444

The impact factor of other publication: 13,117

Total: 20,561