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Hyaluronan-induced masking of ErbB2 and CD44-enhanced trastuzumab internalisation in trastuzumab resistant breast cancer

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ABSTRACT

Although trastuzumab, a recombinant humanised anti-ErbB2 antibody, is widely used in the treatment of breast cancer, neither its mechanism of action, nor the factors leading to resistance are fully understood. We have previously shown that antibody-dependent cellular cytotoxicity is pivotal in the *in vivo* effect of trastuzumab against JIMT-1, a cell line showing *in vitro* resistance to the antibody, and suggested that masking of the trastuzumab-binding epitope by MUC-4, a cell surface mucin, took place. Here, we further explored the role of masking of ErbB2 in connection with CD44 expression and synthesis of its ligand, hyaluronan. We show that high expression of CD44 observed in JIMT-1 cells correlates with ErbB2 downregulation *in vivo*, while siRNA-mediated inhibition of CD44 expression leads to decreased rate of trastuzumab internalisation and low cell proliferation *in vitro*. An inhibitor of hyaluronan synthesis, 4-methylumbelliferon (4-MU) significantly reduced the hyaluronan level of JIMT-1 cells both *in vivo* and *in vitro* leading to enhanced binding of trastuzumab to ErbB2 and increased ErbB2 down-regulation. Furthermore, the inhibitory effect of trastuzumab on the growth of JIMT-1 xenografts was significantly increased by 4-MU treatment. Our results point to the importance of the CD44-hyaluronan pathway in the escape of tumour cells from receptor-oriented therapy.

1. Introduction

Overexpression of ErbB2 has been unquestionably linked to adverse prognosis in breast cancer.[1] ErbB2 heterodimerises with other members of the ErbB family of receptor tyrosine kinases (RTK) leading to enhanced ligand binding affinity, protection from lysosomal degradation and diversified signalling.[2] ErbB2 is viewed as an non-autonomous, ligandless, positive regulator of ErbB signalling,[2] but its participation in non-ErbB protein-mediated signalling is attracting more and more interest as well. Among these, β-integrins,[3] MUC-4[4] and CD44[5] are probably the best known candidates whose

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roles in cancer progression are well documented.[3,6,7] ErbB2 is embedded into this network of signalling and accessory molecules and by its promiscuous association profile it promotes cancer progression.[8] CD44 is recognised as the major hyaluronan receptor having several, alternatively spliced isoforms varying in their physiological function. [9] Binding of hyaluronan activates CD44-mediated signal transduction pathways via interactions between CD44, Grb2, Vav2 and ErbB2.[10] CD44 is involved in the direct regulation of ErbB2[11] and multiple other RTKs.[12] It has been suggested that ligation of CD44 by endogenous hyaluronan leads to the activation of the phosphatidylinositol 3-kinase (PI3K)-Akt survival pathway,[13] and that displacement of endogenous hyaluronan by exogenous hyaluronan oligosaccharides disrupts the activation.[12] CD44-mediated cytoskeletal rearrangements have been observed[6] implying the involvement of CD44 in cellular adhesion, migration and invasion.[14] CD44 is almost absent in normal human breast epithelial cells, emerges in benign and premalignant lesions, and is upregulated in carcinomas.[15] However, a recent study suggested that CD44 opposes rather than promotes the spreading of breast carcinoma in mice.[16]

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.[17] In line with the findings on human tumours, hyaluronan synthesis facilitates the invasive growth of grafted tumour cells *in vivo*,[18] and blocking hyaluronan interactions with its receptors, using soluble CD44 or hyaluronan oligomers, inhibits tumour cell growth in experimental animals.[19] Besides creating signals to prevent apoptosis,[13] hyaluronan is required for activation of the ErbB2-ErbB3 receptor leading to the formation of cardiac valves.[20] Thus, there is mounting evidence that ErbB2, CD44 and hyaluronan are connected both in physiological signalling and cancer pathogenesis through mechanisms largely unknown at present.[5,11]

Being a membrane protein and a key member of survival and proliferation signalling pathways, ErbB2 is the target of receptor-oriented antibody therapy.[21] Trastuzumab (Herceptin), a humanised 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.[22] Although combination of trastuzumab with conventional chemotherapy increases response rates dramatically, the development of resistance seems currently inevitable.[23] Direct action of trastuzumab on ErbB2 (e.g. ErbB2 down-regulation, inactivation of Akt, inhibition of metalloprotease-mediated shedding)[24] and antibody-dependent cellular cytotoxicity (ADCC)[25,26] 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,[27] loss of PTEN,[28] masking of ErbB2[4] and impaired ADCC reaction[29] have all been suggested as possible mechanisms.

In the current paper we investigated JIMT-1, a cell line showing *in vitro* resistance to trastuzumab,[30] 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.[26] We suggested that masking of ErbB2 may be the culprit.[4] Given the suggested association of CD44 with ErbB2[11] 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 internalisation 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.[32] We show that *in vitro* and *in vivo* treatment with 4-MU decreased the pericellular hyaluronan concentration around JIMT-1 cells 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.

2. Materials and methods

2.1. 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.

2.2. Antibodies

Trastuzumab (Herceptin) was purchased from Roche Ltd. (Budapest, Hungary). Mab 2C4 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 also 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.

2.3. 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).

2.4. 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,