Stroma Cells: A Novel Target of Herceptin Activity

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ABSTRACT

Purpose: Stroma cells play a relevant role in tumor development and progression. We investigated the activity of herceptin (HER), a humanized monoclonal antibody widely used for the treatment of HER2-overexpressing epithelial cancer, toward stroma cell lines L87/4 and L88/5.

Experimental design: We studied the antiproliferative potential of HER and role of human serum in HER activity. We also investigated the ability of HER to alter ancillary functions of L87/4 and L88/5, such as support to long-term hematopoiesis, growth factor production, breast cancer cell adhesion, and proliferation.

Results: Flow cytometry showed that HER2 membrane expression in L87/4 and L88/5 stroma cells was intermediate between the expression in HER2-negative/dim MCF-7 breast cancer cells and HER2-bright SK-BC3 breast cancer cells. HER2 gene amplification was not detected by fluorescence in situ hybridization in either stromal cell lines. HER significantly inhibited L87/4 and L88/5 proliferation. Mean ID_{50} were found to be 2000 and 1700 μg/ml for L87/4 and L88/5, respectively, after 3-day exposure and 800 μg/ml for both cell lines after 9-day exposure. The presence of 10% human serum in the culture increased HER inhibitory activity. IC_{50} of stroma cells was found to be intermediate between HER2-bright breast cancer cells (SK-BC3) and HER2-negative/dim breast cancer cells (MCF-7). The drug did not significantly affect the ability of stroma cells to support long-term hematopoiesis in the cobblestone area forming cell assay. In contrast, in coculture assay, MCF7 cells demonstrated a worse adhesion and growth capability on HER-treated stroma layers when compared with untreated stroma. Moreover, HER significantly reduced vascular endothelial growth factor production by L88/5 cells.

Conclusions: Our data support the novel finding that HER may have a relevant activity against stroma cells.

INTRODUCTION

HER, a humanized version of the anti-HER2 murine MoAb, is now widely used for the treatment of patients with HER2-overexpressing breast cancer. HER demonstrated antitumor activity as a single agent in patients with metastatic breast cancer. Moreover, improved survival and higher response rates have been achieved when HER was added to first-line combination chemotherapy with anthracycline/cyclophosphamide or paclitaxel. These results prompted large cooperative groups to design HER clinical trials in the adjuvant setting (1–3). An HER mechanism of action includes antagonizing the constitutive growth-signaling properties of the HER2 system, restoring E-cadherin expression levels, enlisting immune cells to attack and kill the tumor cells, augmenting chemotherapy-induced cytotoxicity (4), modulating the production and effect of pro- and antiangiogenic growth factors, and inducing normalization and regression of the tumor vascular network (5).

Stroma cells, together with extracellular matrix components, provide a microenvironment that is pivotal in tumor growth, invasion, and metastatic progression (6–8). A recent report has shown that stroma alterations precede the malignant conversion of epithelial cells (9). Moreover, using a three-dimensional cell-cell interaction model, Shekhar et al. demonstrated that tumor fibroblasts play an active role as a morphogenic and mitogenic inducer of epithelial cells. In these studies, further enhancement of tumor growth and progression required the establishment of active angiogenesis (10).

In the current study, we investigated the activity of HER toward human stroma cell lines L87/4 and L88/5 with regard to inhibition of cell proliferation, generation of growth factors, adhesion capability of MCF7 breast cancer cells to fibroblasts and proliferation, and stroma-mediated support of hematopoiesis.

MATERIALS AND METHODS

Cell Lines. L87/4 and L88/5 are two permanent SV40-transformed human stroma cell lines derived from the bone marrow of a hematologically normal male patient (11). MCF-7 (HER2 negative/dim) and SK-BC3 (overexpressing HER2 and HER2 bright by flow cytometry) are human breast cancer cell lines widely used for studies of interactions between mammary epithelial cells and the surrounding stroma (12–14). Cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum. Confluent monolayers were trypsinized and maintained by weekly passages at 1:10 to 1:20 dilution.

The abbreviations used are: HER, herceptin; HS, human serum; VEGF, vascular endothelial growth factor; EGF, epithelial growth factor; CAFC, cobblestone area-forming cell; bFGF, basic fibroblast growth factor; MNC, mononucleated cell; IL, interleukin; MoAb, monoclonal antibody.
Immunophenotyping of Cell Lines and Bone Marrow.

For flow cytometry evaluation, cell lines and fresh bone marrow cells were incubated with anti-HER2/neu, CD45, and CD10 MoAb (Becton Dickinson, Mountain View, CA) for 30 min at room temperature. After washing, cells were resuspended and analyzed with a FACScalibur flow cytometer (Becton Dickinson). Negative controls were stained as reported above with irrelevant MoAb (Becton Dickinson).

HER2/neu Gene Amplification. The number of HER2/neu gene copies was determined by using the PathVysion HER2 DNA kit (Vysis, Bergisch-Gladbach, Germany), which consists of two labeled DNA probes: (a) the HER2 probe that spans the entire HER2/neu gene; and (b) the CEP17 probe that hybridizes to the alpha satellite region located at the centromere region of chromosome 17. Cytospins with 50–100 × 10^3 L87/4 and L88/5 stroma cells were performed. Slides were pretreated by incubation in 2 × SSC for 20 min at 37°C and then fixed with serial ethanol dilutions (70, 85, and 100%). DNA and probes were simultaneously denatured at 80°C for 2 min. Hybridization was carried out overnight at 42°C. Two washes were performed at 72°C ± 1°C in 0.4 × SSC/0.3% NP40 for 2 min and 2 × SSC/0.1% NP40 for 1 min. Cytospin was counterstained with 4',6-diamidino-2-phenylindole. Signal evaluation was performed according to standard criteria.

HER Treatment of L87/4, L88/5, and Breast Cancer Cell Lines. A set of two preliminary experiments was performed to investigate the antiproliferative potential of HER and role of HS in HER activity. L87/4 and L88/5 stroma cells were incubated for 9 days at 37°C, 5% CO2, and fed weekly with fresh medium. After 5 weeks, wells with cobblestone areas > 6 cells were scored as positive using an inverted phase contrast microscope (15).

Coculture Experiments. HER-treated semiconfluent stroma layers were obtained in 6-well plates as described above.

RESULTS

HER2 Protein Expression and HER2/neu Gene Amplification. Flow cytometry showed membrane expression of HER2 protein in L87/4 (98%) and L88/5 (99%) cells. These results were intermediate between those of HER2-negative/dim MCF-7 cells and HER2-bright SK-BC3 cells (Fig. 2A). A bone marrow stroma cell population coexpressing HER2 and the stroma marker CD10 is shown in Fig. 2B. Fluorescence in situ hybridization analysis revealed no amplification of the HER2/neu gene in both stromal cell lines.

HER Inhibits Stroma Cell Proliferation. HER significantly inhibited L87/4 and L88/5 proliferation (Table 1). Both

On day 4, 100 MCF-7 epithelial cells/well were seeded. After 3 days, the medium was removed, and plates were washed twice with PBS, air dried, and stained with a commercially available APAAP-based kit for the detection of cytokeratin-positive cells (Epimet; Baxter, Planegg, Germany). The number and size of epithelial colonies were determined using an inverted phase contrast microscope.

Quantitative Determination of VEGF, bFGF, and EGF Concentrations. ELISA tests (R&D Systems, Inc., Minneapolis, MN) were performed on cell culture supernatants to quantify VEGF, bFGF, and EGF generation after 3-day exposure of stroma cells to HER ID_{50} concentration.

Data Analysis and Statistical Evaluations. Inhibition of cell growth was evaluated as the proportion of the mean number of cells growing under exposure to HER, compared with the mean number of cells in the control. Dose-response curves were produced using a standard software program (SAF). The ID_{50} of stroma cells was calculated from graphical analysis (Fig. 1). CAFC frequencies in the limiting dilution assay were determined using Poisson statistics (15, 16). Statistical comparisons were performed using the $t$ test and ANOVA, when data were normally distributed, and the nonparametric analyses of Spearman and Mann-Whitney, when data were not normally distributed. Differences with a $P < 0.05$ were considered as statistically significant.
cell lines demonstrated a similar sensitivity to the drug. Prolonged exposure was 2.5 and 2 times more toxic than 3-day exposure for L87/4 and L88/5, respectively.

The presence of 10% HS in the culture significantly increased HER inhibitory activity: 12.5% increase (range 8–18.1) for L87/4 (P = 0.008) and 14.3% increase (range 10.1–18) for L88/5 (P = 0.002) after 3-day exposure and 19.6% increase (range 16–22.4) and 22.9% increase (range 18.6–25.5) for L87/4 (P < 0.001) and L88/5 (P < 0.001), respectively, when stroma cells were exposed to HER for 9 days. As indicated in Table 1, IC_{50} of stroma cells was found to be intermediate between HER2-bright breast cancer cells (SK-BC3) and HER2-negative/dim breast cancer cells (MCF-7).

**Stroma-mediated Support of Hematopoiesis.** HER did not affect the ancillary function of stroma cells in supporting long-term culture-initiating cells frequency of human umbilical cord blood or bone marrow samples in CAFC assay.

### Table 1

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<th>Mean ID_{50} and range (µg/ml) of HER after 3- and 9-day exposure</th>
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<tr>
<td></td>
<td>3-day exposure (n = 3)</td>
</tr>
<tr>
<td>L87/4</td>
<td>2000 (1900–2100)</td>
</tr>
<tr>
<td>L88/5</td>
<td>1700 (1600–2000)</td>
</tr>
<tr>
<td>SK-BC3</td>
<td>1450 (1250–1600)</td>
</tr>
<tr>
<td>MCF-7</td>
<td>2850 (2600–3050)</td>
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**Fig. 2** A, membrane expression of HER2 protein evaluated by flow cytometry on L87/4 and L88/5 stroma cells and MCF-7 and SK-BC3 breast cancer cell lines. Arrows, the median channel of fluorescence. B, flow cytometry evaluation of HER2 protein expression in a stromal (CD10 positive) bone marrow cell population (right panel) in a breast cancer patient. The percentage of HER2- and CD10-positive cells is indicated. Left panel, gate used to exclude dead cells, platelets, and debris; central panel, negative controls.
Fig. 4  A and B, clusters of MCF7 breast cancer cells on L88/5 stroma layers. MCF7 breast cancer cells were stained with an APAAP-based kit for the detection of cytoskeleton cytokeratins. A, larger cluster; B, smaller cluster.
long-term hematopoiesis; CAFC frequencies in human umbilical cord blood or bone marrow samples were similar when MNCs were seeded on stroma cell layers exposed to HER or on those not exposed to the drug (Fig. 3). The mean CAFC number was 2904 (range 1136–5118) versus 2921 (range 1992–4593) in the control setting for L87/4 and 2574 (range 1087–5374) versus 2363 (range 879–4331) in the control setting for L88/5.

**Coculture Experiments.** After immunostaining, cytokeratin-positive MCF7 single cells or clusters were easily scored under an inverted phase contrast microscope (Fig. 4, A and B). As reported in Table 2, HER significantly inhibited the ability of MCF7 breast cancer cells to adhere to stroma layers and give rise to clusters (P < 0.02). About 52% of the breast cancer cells which adhered to stroma layers gave rise to clusters ≥ 3 cells. The mean number of clusters scored on stroma layers is reported in Table 3 (P < 0.01 for HER-treated stroma cells versus controls). Cluster size was similar in both coculture setting, the mean number of epithelial cells being 6 ± 4 for L87/4 and 7 ± 5 for L88/5 in HER untreated coculture and 6 ± 4 for L87/4 and 5 ± 3 for L88/5 in HER-treated cocultures.

**Growth Factor Production.** Culture in the presence of HER significantly decreased VEGF production by L88/5 cells (P = 0.006; Fig. 5). HER also reduced VEGF production by L87/4 cells, but this trend did not statistically reach significance (P = 0.18). bFGF and EGF production in both L87/4 and L88/5 cells were not affected by culture in the presence of HER.

**DISCUSSION**

Stroma cells might be a relevant target of antineoplastic therapy. This target is of particular interest in breast cancer where reciprocal cell–cell interactions between epithelial and stroma cells seem to play a crucial role (6–10). We evaluated the potential activity of HER toward human stroma cells L87/4 and L88/5, two permanent SV40-transformed human stroma cell lines derived from the bone marrow of a hematologically normal male patient. Both cell lines exhibit a fibroblastoid morphology, confirmed by the expression of CD10 and CD13 and the lack of expression of hematopoietic markers. L87/4 cells also express the macrophage marker CD68. Both cell lines produce a variety of hematopoietic growth factors, including IL-1β, IL-6, IL-7, IL-8, IL-11, stem cell factor, leukemia inhibitory factor, macrophage colony-stimulating factor, granulocyte colony-stimulating factor, granulocyte macrophage colony-stimulating factor, macrophage inflammatory protein-1α, transforming growth factor-β, and tumor necrosis factor-α. L87/4 have a more potent cytokine producer than L88/5. Both cell lines are highly radiotolerant and able to function as feeder cells, supporting long-term culture of hematopoietic progenitors (11, 17). Both cell lines express HER2 protein on their surface, but they lack HER2/neu gene amplification. Despite the absence of HER2 overexpression, HER demonstrated a relevant antiproliferative activity toward L87/4 and L88/5. This effect was increased by the presence of HS. This finding confirms that, as suggested in preclinical studies performed with breast cancer cell lines, HER activity is in part mediated by complement activation (4, 18).

After exposure to HER, L87/4 and L88/5 retained their capability of supporting long-term hematopoiesis; a similar frequency of CAFC was observed for MNCs seeded on treated or untreated stroma layers. On the other hand, HER inhibited VEGF production by one stroma cell line and interaction with MCF7 breast cancer cells by both cell lines. In a recent study, Izumi et al. (5) reported that HER induces normalization and regression of tumor vasculature in an experimental human breast tumor that overexpresses HER2 in mice. They also observed that the expression of proangiogenic factors, such as VEGF, transforming growth factor-α, angiopoietin-1, and plasminogen activator inhibitor-1, was reduced by HER treatment in vitro but not in vivo; these authors hypothesized that host cells may produce compensatory growth factors. In our experience, HER inhibited VEGF production by L88/5 stroma cells. This result confirms the active role of HER in modulating the production of angiogenic factors and underlines the importance of investigating how our findings can be translated to the in vivo situation.

HER significantly affected the ability of MCF7 breast cancer cells both to adhere to stroma layers, which is a prerequisite for growth, and give rise to clusters. This finding could be important with regard to the ability of breast cancer cells to generate metastases in the bone marrow. About 70% of patients with advanced breast cancer have metastases in the bone, and for approximately half of these patients, bone is the only site of metastases. Bone marrow microenvironment seems an appropriate niche for homing, growth, and development of disseminated micrometastatic cells. Moreover, Brooks et al. (19) observed that bone marrow stroma provides a preferential substratum for adherence of epithelial cells from malignant tissue compared with mammary fibroblasts, even if it does not represent a superior growth environment.

IC_{50} of stroma cells was found to be higher than concentrations found in HER-treated cancer patients. However, this finding is likely to reflect difficulties in generating highly sensitive in vitro models of HER-mediated cytotoxic and antiproliferative effects. In fact, it should be remembered that also in other studies (20, 21), the HER concentration needed to obtain in vitro antibody-dependent cellular cytotoxicity was similarly higher than HER levels observed in treated patients.

Taken together, our data support the novel finding that HER may have a target other than tumor cells. Thus, the antitumor activity of this drug could be attributable both to the direct effect on tumor cells, as well as to its ability to modulate the microenvironment production of growth factors and extracellular matrix components, thereby promoting a favorable stromal niche that favors cancer cell survival and invasion.
cellular matrix molecules, and establishment of heterotypic cell-cell contact.

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