Switching Off HER-2/neu in a Tetracycline-Controlled Mouse Tumor Model Leads to Apoptosis and Tumor-Size-Dependent Remission


Institute of Toxicology [I. B., S. Ge., C. K. H., F. O.], Departments of Radiology [A. H., J. H., W. G. S., M. T.], Hematology [U. W., B. S.], and Gynecology [B. T.], University of Mainz, 55131 Mainz; Baxter Oncology GmbH, 60314 Frankfurt [S. Gi.]; Atlanta Pharma, 78467 Konstanz [T. B.]; Zenaturis, 60314 Frankfurt [S. B.]; Urological Clinic and Polyclinic [W. B.]; Children’s Hospital [C. S., D. P., T. T., B. Z.], and Institute of Pathology [H. A. L.], University of Mainz, 55131 Mainz; Institute of Legal Medicine and Rudolf-Boehm Institute of Pharmacology and Toxicology, Center for Toxicology, University of Leipzig, 04107 Leipzig [J. G. H.], Germany

ABSTRACT

Overexpression of the receptor tyrosine kinase HER-2/neu is associated with poor prognosis in patients with breast and ovarian cancer. Recent excitement has surrounded the therapeutic effects of HER-2-blocking therapy strategies and has rekindled interest on the molecular mechanisms of HER-2/neu in tumor biology. To study the role of HER-2/neu overexpression in vivo, we used a murine fibroblast cell line (NIH3T3-her2) conditionally expressing human HER-2/neu under control of a tetracycline-responsive promoter. Expression of HER-2 could be down-regulated below detection limit (>625-fold dilution) by exposure of NIH3T3-her2 cells to anhydrotetracycline (ATc). Subcutaneous injection of NIH3T3-her2 cells into nude mice resulted in rapid tumor growth. Mice with mean tumor volumes of 0.2, 0.8, 1.9 and 14.9 cm³ were treated daily with 10 mg/kg ATc to switch off HER-2/neu expression, producing reductions in tumor size of 100, 98.1, 81.4, and 74.2%, respectively, by 7 days after onset of ATc administration (P = 0.005, Kruskal–Wallis test). Different long-term effects of HER-2 down-regulation were observed when mice with small (0.2 cm³; n = 7), intermediate (0.8–1.2 cm³; n = 10) and large (≥1.9 cm³; n = 11) tumors received ATc for up to 40 days. Complete remission was observed for 100, 40, and 18% of the small-, intermediate-, and large-sized tumors, respectively (P = 0.003). However, after 20–45 days of ATc administration, recurrent tumor growth was observed for all mice, even in those with previous complete remissions. The time periods for which mean tumor volume could be suppressed to volumes <0.1 cm³ under ATc administration were 34, 22, 8, and 0 days for tumors with initial volumes of 0.2, 0.8, 1.9 and 14.9 cm³, respectively (P = 0.005, Kruskal–Wallis test). Interestingly, HER-2 remained below the detection limit in recurrent tumor tissue, suggesting that initially HER-2-dependent tumor cells switched to HER-2 independence. The “second hits” leading to HER-2-independent tumor growth have not yet been identified. The rapid regression of tumors after down-regulation of HER-2 was explained by two independent mechanisms: (a) a block in cell cycle progression, as evidenced by a decrease in Ki-67 antigen expression from 40% before ATc treatment to 8.3% after 7 days of ATc treatment; and (b) induction of apoptosis as demonstrated by caspase-3 activation and by the terminal deoxynucleotidyltransferase (TdT)-mediated nick end labeling assay (TUNEL). In conclusion, we have shown that switching off HER-2 may disturb the sensitive balance between cell proliferation and cell death, leading to apoptosis and tumor remission. Tumor remission was dependent on the volume of the tumors before down-regulation of HER-2/neu.

INTRODUCTION

The epidermal growth factor receptors (HER/erbB) constitute a family of four members involved in cell cycle control and cell differ-
In vitro Inhibition of HER-2/neu Expression

NIH3T3-her2 cells were harvested from 90% confluent dishes in dishes and plated at a density of 10^5 cells/dish on 75-cm^2 flasks. For down-regulation of HER-2/neu, NIH3T3-her2 cells were incubated with 10 ng/ml ATc (Acros Chimica, Geel, Belgium) for 0 (controls), 1, 3, and 7 days. ATc was added 24 h after plating of cells. For all four incubation periods with ATc, cells were cultured for 8 days and harvested simultaneously. The design of the study was to start incubations with ATc at different time points but to harvest all dishes simultaneously. For the immunohistochemical analysis, cells were cultured on SUPERFROST PLUS slides (Menzel-Gläser, Braunschweig, Germany). The slides were air dried overnight and stored at −20°C until immunohistochemical analysis.

Determination of Tumor Growth

We injected 7 × 10^6 NIH3T3-her2 cells s.c. into the dorsal skin of 3–4 week-old male nude mice (cd nu /nu−: Charles River, Sulzfeld, Germany). Animals were housed under specific pathogen-free conditions. Eight to 10 days after injection of the NIH3T3-her2 cells, small tumors with a mean diameter of 0.5 cm became visible. The tumor diameter was measured with a caliper rule. The maximum and minimum diameters of the tumor were determined. The mean value of the maximum and minimum diameters was defined as the mean diameter. For Western blot analysis and immunohistochemistry, tumors were treated for 1, 3, and 7 days with 10 mg ATc/kg of body weight. Thereafter, mice were sacrificed by cervical dislocation. Tumors were isolated, shock-frozen in liquid nitrogen-cooled 2-methylbutane, and stored at −80°C. Mice not receiving ATc therapy were used as negative controls. Each time an ATc-treated tumor was harvested, a time-matched control tumor was harvested simultaneously. Control tumors continually increased in size to a mean diameter of 3.5 cm at which time mice were sacrificed. Tumor volume (V) was calculated by the formula: V = a · b · h/2, where a represents the minimum and b the maximum tumor diameter. Complete remission was achieved when absolutely no tumor volume was visible macroscopically. The treatment protocols, including the groups of mice with different tumor volumes, are shown in Table 1. To study the possibility of achieving a complete remission, we tested additional mice, giving 7, 10, and 11 mice with initial tumor volumes of 0.2, 0.8–1.2, and ≥1.9 cm^3, respectively.

MRI

MRI measurements were performed as described by Hast et al. (23) with use of a 1.5-Tesla whole-body MR-System (Magnetom Experimental; Siemens, Erlangen, Germany). Mice were anesthetized by s.c. injection of 61.5 mg/kg ketamine (Ketamine–ratiopharm 50%; Ratiopharm, Ulm, Germany) and 2.3 mg/kg xylazine (Rompun 2%; Bayer, Leverkusen, Germany). After a bolus injection of saline-diluted contrast agent (Magnevist; Schering AG, Berlin, Germany) into the tail vein, T1-weighted sequences were performed.

Western Blot Analysis

Preparation of Cell Extracts. Cell cultures were scraped from culture dishes, washed twice in PBS, and resuspended in solubilization buffer. Tumor

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Table 1: Treatment protocols of nude mice after induction of subcutaneously growing NIH3T3-her2 tumors

<table>
<thead>
<tr>
<th>Therapy group no.</th>
<th>Mean (±SD) tumor volume at onset of ATc administration (cm^3)</th>
<th>Time between injection of NIH3T3-her2 cells and onset of ATc administration (days)</th>
<th>No. of daily doses of 10 mg/kg ATc</th>
<th>No. of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1.2 ± 0.2</td>
<td>16–20</td>
<td>7</td>
<td>Controls: n = 6</td>
</tr>
<tr>
<td>II</td>
<td>3.9 ± 0.4</td>
<td>21–25</td>
<td>7</td>
<td>Controls: n = 3</td>
</tr>
<tr>
<td>III</td>
<td>0.2 ± 0.03</td>
<td>8–12</td>
<td>37</td>
<td>Treated mice: n = 6</td>
</tr>
<tr>
<td>IV</td>
<td>0.8 ± 0.2</td>
<td>14–16</td>
<td>40</td>
<td>Controls: n = 5</td>
</tr>
<tr>
<td>V</td>
<td>1.9 ± 0.4</td>
<td>18–22</td>
<td>37</td>
<td>Treated mice: n = 5</td>
</tr>
<tr>
<td>VI</td>
<td>14.9 ± 0.2</td>
<td>29–31</td>
<td>30</td>
<td>Treated mice: n = 2</td>
</tr>
</tbody>
</table>
HER-2/neu membrane proteins, a lysis buffer was used, containing 25 mM Tris-phosphate, 2 mM EDTA, 2 mM DTT, 10% glycerol, and 1% Triton-X-100 (pH 8). For solubilization of nuclear proteins such as caspase-3, a CHAPS cell extraction buffer was used, containing 20 mM PIPES (adjusted with NaOH to pH 7.2; Amersham Life Science, Cleveland, OH), 0.1% CHAPS, 10 mM DTT, 100 mM NaCl, 1 mM EDTA, and 10% sucrose. Both solubilization buffers were supplemented with 1% of a commercially available protease inhibitor cocktail (Sigma) containing 4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64), bestatin, leupeptin, and aprotinin.

Western Blotting. Total cellular proteins (25 μg for analysis of HER-2 and 40 μg for analysis of caspase-3) were mixed with sample buffer according to the protocol of Laemmli (25) and resolved on a 10% (HER-2) or 15% (caspase-3) SDS–polyacrylamide gel by electrophoresis. Thereafter, proteins were electrotransferred to Poly Screen polyvinylidene difluoride transfer membranes (NEN Life Science, Boston, MA). The membranes were blocked with PBS-Tween (PBS + 0.1% Tween 20) containing 10% Roti-Block (Roth, Karlsruhe, Germany) for 1 h and then incubated with the anti-HER-2/neu or anti-caspase-3 antibodies for 2 h or, as a loading control, with the anti β-actin antibody for 30 min at room temperature. After washing, the membranes were incubated with the secondary antibody, horseradish peroxidase-conjugated antimouse immunoglobulin for 20 min or antirabbit immunoglobulin for 40 min at room temperature. After a final wash, proteins were visualized with a chemiluminescence detection system (Western Lightning Chemiluminescence Reagent Plus; Perkin-Elmer Life Science, Boston, MA) with subsequent exposure to Kodak X-OMAT films. The expression levels were quantified by densitometric analysis using Scion Imaging software (Scion Image β 4.02 for Windows 98). The MagicMark Western Standard from Invitrogen GmbH (Karlsruhe, Germany) served as an internal protein standard. For repeated staining, individual membranes were stripped with a buffer containing 0.76% Tris base, 2% SDS, and 0.7% 2-mercaptoethanol adjusted with HCl to pH 6.8 for 1 h at 50°C. The SDS–polyacrylamide gel and the membrane (after the last staining with antibodies) were also stained with Coomassie blue to confirm that equal amounts of proteins had been applied to each lane.

Origin and Dilution of Antibodies. The monoclonal antibody against human HER-2/neu (185-kDa protein) was obtained from Quartett (Berlin, Germany) and used at a dilution of 1:270. The monoclonal antibody against mouse β-actin (42-kDa protein; Sigma) was used at a dilution of 1:2000. The antimouse caspase-3 antibody (Cell Signaling; Biolabs, Frankfurt, Germany), detects the full length caspase-3 (32–35 kDa) and the cleaved large fragment (17–20 kDa) of caspase-3. The anti-caspase-3 antibody was diluted 1:500. The secondary antibody, peroxidase-linked antirabbit, was obtained from Cell Signaling (Biolabs) and used in a dilution of 1:1500. Peroxidase-linked antimouse antibody (Sigma) was used at a dilution of 1:5000 to detect β-actin and at 1:50,000 to detect HER-2. Primary and secondary antibodies were all diluted in PBS-Tween containing 10% Roti-Block.

Immunohistochemistry

Preparation of Cryosections. Using a cryotome (CM 3000 Cryostat; Leica Instruments GmbH, Nussloch, Germany), we produced 5-μm-thick cryosections and transferred them to SUPERFROST PLUS slides. After air drying overnight, slides were stored at −20°C until use.

Immunohistochemical Detection of HER-2/neu. The frozen slides were thawed to room temperature, fixed in cold (−20°C) acetone for 10 min, and rinsed for 10 min in TBS [50 mM Tris, 150 mM NaCl (pH 7.4–7.5)]. To block endogenous peroxidase, slides were rinsed in methanol containing 8.5% H2O2 for 30 min at room temperature and washed three times in TBS for 5 min each. Subsequently, nonspecific binding of immunoglobulin was blocked by the addition of 3% BSA in TBS for 1 h at room temperature. The primary monoclonal antibody against HER-2/neu (rabbit antihuman HER-2, a ready-to-use solution; DAKO, Hamburg, Germany) was incubated for 30 min at room temperature. For detection of the primary antibody, we used a commercially available detection kit (ABC-kit, XHCO2; Dianova, Hamburg, Germany). The slides were washed three times in TBS and incubated with biotinylated goat antirabbit antibodies (diluted 1:50) for 30 min at room temperature (Dianova). After slides were rinsed in TBS, the ABC-Reagent (avidin and biotinylated horseradish peroxidase) was added for 30 min at room temperature. The slides were then washed again with TBS. The 3,3′-diaminobenzidine solution (0.6% 3,3′-diaminobenzidine–0.3% H2O2 in H2O) was added for 5 min. The slides were then rinsed for 10 min under tap water. Counterstaining was performed in hematoxylin solution (1:10 dilution in distilled water; Merck, Darmstadt, Germany) for 1.5 min, after which the slides were again rinsed for 10 min under tap water. To obtain stronger contrast of the hematoxylin stain, slides were placed in bluing reagent (70% ethanol containing 1.5% NH4OH) for 1 min followed by rinsing in tap water. The tissue was dehydrated by incubation with increasing alcohol concentrations (70, 96, 100, and 100% isopropanol for 10 s each). After being rinsed in Rotihistol (Roth, Karlsruhe, Germany), the slides were covered with Entellan Neu (Merck). All slides were processed in one session. Two slides obtained from the same tumor were used as positive and negative controls. The negative control was prepared by use of TBS in place of the primary antibody.

Immunohistochemical Detection of Ki-67. In addition to the staining with the antihuman HER-2 antibody, the same protocol was also performed with an antimouse Ki-67 antibody (Ki-67 rabbit antirabbit; Dianova, Hamburg, Germany) diluted 1:50 in TBS containing 5% FCS.

TUNEL Assay. For detection of DNA strand breaks in individual cells by light microscopy, a commercially available kit (In Situ Cell Death Detection Kit; POD; Roche Diagnostics GmbH, Mannheim, Germany) was used. A modified TUNEL protocol was performed. The frozen slides were fixed directly with 1% paraformaldehyde (pH 7) for 30 min at room temperature (26) and washed with TBS for 10 min. Thereafter, slides were rinsed in TBS containing 1% Triton-X-100 (diluted 0.1% sodium citrate for 2 min at 4°C). For the following staining procedure, the same protocol was used as described for immunohistochemical detection of HER-2/neu. The TUNEL reaction mixture and the converter–POD solution were prepared according to the manufacturer’s instructions.

Evaluation of Immunohistochemical Slides. Using the antihuman HER-2/neu antibody, the antimouse Ki-67 antibody, and the TUNEL reagent, we determined the percentage of HER-2/neu–, Ki-67–, and TUNEL-positive tumor cells in relation to all tumor cells. For this purpose, five representative areas with vital tumor cells or tissue were randomly selected. Evaluation was performed with a Nikon Optiphot microscope with ×400 magnification. The percentage of HER-2/neu (cytoplasmic and membrane)-positive cells that were also positive for Ki-67 and TUNEL (nuclear) staining was determined independently by two experienced investigators (J. G. H. and I. B. S.). Mean values for all five areas were calculated. In all cases the values obtained by both investigators differed by <10%.

Flow Cytometry. We used 1 × 10^6 NIH3T3-her2 cells for staining with the mouse antihuman monoclonal antibody directed against the extracellular domain (Ab-5; Oncogene, Darmstadt, Germany) and the mouse antihuman monoclonal antibody directed against the intracellular, COOH-terminal domain of human HER-2/neu (Ab-3; Oncogene). Staining with the monoclonal antibody directed against rat HER-2/neu (Ab-4; Oncogene) served as negative control. Cells were incubated with the respective antibodies for 20 min at 4°C and washed twice before FITC-conjugated antimouse IgG (DTAF; Dianova) was added as a secondary antibody. After samples were washed with PBS, they were analyzed by flow cytometry (Coulter EPICS XL; Beckman Coulter, Krefeld, Germany), using the Expo 32 software (Beckman Coulter). Before incubation with Ab-3, directed against the intracellular domain of HER-2, cells were permeabilized with the IntraPrep Permeabilization Reagent (ImmunoTech, Marseilles, France). The results are expressed as mean specific fluorescence intensities.

All chemicals and reagents not specifically mentioned were obtained from Sigma-Aldrich (Schnelldorf, Germany).

Statistical Analysis. For statistical analysis of possible differences in the occurrence of complete remission of tumors with different volumes, we used the χ2 test (two-sided). The Kruskal–Wallis test (two-sided) was applied to test for (a) differences in...
the extent of tumor remission between groups of mice with different tumor volumes at the onset of ATc administration, and (b) differences in the time periods for which mean tumor volumes could be suppressed to volumes $\leq 0.1$ cm$^3$ between groups of mice with different tumor volumes at the onset of ATc administration.

RESULTS

Conditional Expression of HER-2/neu in Vitro. Human HER-2 was conditionally expressed in NIH3T3 cells as described by Baasner et al. (21). The TET-OFF system based on a tetracycline-controlled transactivator protein, tTA (22), was used (Fig. 1B). The resulting cell line, named NIH3T3-her2, was incubated with ATc (10 ng/ml) for 1, 3, 7, and 9 days. A clear decrease in HER-2/neu expression was observed during incubation with ATc as evidenced by Western blot analysis (Fig. 2A). Results similar to those shown in Fig. 2A were obtained in three independent experiments. The resulting mean (±SD) HER-2 expression values obtained by densitometric analysis were 50.8 ± 12.3%, 24.0 ± 15.3%, 3.9 ± 2.3%, and 0% of the values for untreated cells after 1, 3, 7, and 9 days incubation with ATc, respectively (Fig. 2D). In similar experiments, ATc-mediated down-regulation of HER-2/neu was examined by immunohistochemistry. The number of HER-2-positive tumor cells was determined in three independent experiments and expressed as a percentage of all tumor cells (Fig. 2E). Mean values (±SD) were 83.9 ± 11.3%, 35.8 ± 6.0%, 11.5 ± 11.2%, and 3.97 ± 3.97% of the values for untreated cells after 1, 3, 7, and 9 days incubation with ATc, respectively (Fig. 2E). Similarly, flow-activated cell-sorting analysis showed a clear down-regulation of HER-2 during incubation with ATc (Fig. 2F). Antibodies against the extracellular (Ab-5) and the intracellular (Ab-3) domains of HER-2 were used. For both the extra- and intracellular domains, clear down-regulation was observed during incubation with ATc. Interestingly, the HER-2/neu down-regulation was more pronounced for the extracellular compared with the intracellular domain, whereas an antibody directed against the extracellular domain of rat HER-2 (Ab-4), serving as a negative control, was not affected by ATc (Fig. 2F).

Conditional Expression of HER-2/neu in Vivo. NIH3T3-her2 cells were injected s.c. into nude mice. After 16–20 days s.c.-growing tumors reached a volume of 0.9–1.4 cm$^3$. Mice were then treated with daily s.c. injections of 10 mg ATc/kg of body weight for 1, 3, and 7 days. Western blot analysis showed a marked decrease in HER-2 expression as early as 24 h after the first injection of ATc (Fig. 3A). After 3 days, HER-2 was no longer detectable by Western blotting. Mean (±SD) values after densitometric analysis were 18.2 ± 6.9% below detection limit.
after 1 day and 0% after 3 and 7 days under administration of ATc compared with time-matched control tumors (Fig. 3D). Thus the decrease in HER-2 expression after exposure to ATc was even faster in vivo than in the in vitro experiments. Mice not receiving ATc were used as negative controls. When ATc-treated tumors were harvested after 1, 3, and 7 days, time-matched control tumors were harvested simultaneously. Thus, the three controls shown in Fig. 3A are of incremental ages matching those of the tumors treated with ATc for 1, 3, and 7 days. Obviously, HER-2 expression increased with tumor growth (Fig. 3A, Lanes 1–3). In similar experiments, the ATc-mediated decrease in HER-2 expression was examined by immunohistochemistry. A clear decrease in HER-2 expression was observed during administration of ATc. After 7 days, HER-2 was no longer detectable. Mean (±SD) values for the HER-2-positive cells were 62.5 ± 3.6%, 2.8 ± 2.3%, and 0% on days 1, 3, and 7 of ATc administration, respectively (Fig. 3E).

**Down-Regulation of HER-2/neu Leads to Tumor Remission.** To develop a treatment model, nude mice were injected s.c. with 7 × 10^6 NIH3T3-her2 cells. Eight to 10 days after injection, small tumors became visible (Fig. 4). Tumors were allowed to reach different volumes (Table 1). Mice were then randomized into ATc-treated and control groups. The ATc-treated group received daily s.c. injections of 10 mg ATc/kg of body weight. The day of the first administration of ATc was defined as day 0. Representative examples of one ATc-treated mouse from group I and one control mouse are shown in Fig. 4A. Tumor remission under ATc treatment (Fig. 4B, IV–VI) as well as further tumor growth in the control mouse (Fig. 4B, VII–IX) were also visualized by MRI using gadolinium–diethylenetriaminepentaacetic acid as a contrast agent. In groups I and II, mice with mean tumor volumes of 1.2 and 3.9 cm^3 were treated with 7 subsequent daily doses of ATc. Strong remission was observed for both groups (Fig. 4, C and D). In contrast, a continuous increase was seen for control tumors. A longer follow-up of groups I and II was not possible because the tumors were subsequently isolated for analysis of HER-2 expression, proliferation, and apoptosis.

**Tumor Remission Depends on Initial Tumor Volume.** To examine whether the tumor volume at the onset of ATc administration was relevant for tumor remission, we examined four groups of mice with tumors 0.2, 0.8, 1.9, and 14.9 cm^3 in volume. Mice were treated with up to 40 subsequent daily doses of 10 mg/kg ATc (Fig. 4, E–H). The results show that the extent and duration of tumor remission depended on the initial tumor volume. The 0.2-, 0.8-, 1.9-, and 14.9-cm^3 tumors showed 100, 98.1, 81.4, and 74.2% reductions in growth (Fig. 3). In similar experiments, the ATc-mediated decrease in HER-2 expression was even faster in vivo than in the in vitro experiments. Mice not receiving ATc were used as negative controls. When ATc-treated tumors were harvested after 1, 3, and 7 days, time-matched control tumors were harvested simultaneously. Thus, the three controls shown in Fig. 3A are of incremental ages matching those of the tumors treated with ATc for 1, 3, and 7 days. Obviously, HER-2 expression increased with tumor growth (Fig. 3A, Lanes 1–3). In similar experiments, the ATc-mediated decrease in HER-2 expression was examined by immunohistochemistry. A clear decrease in HER-2 expression was observed during administration of ATc. After 7 days, HER-2 was no longer detectable. Mean (±SD) values for the HER-2-positive cells were 62.5 ± 3.6%, 2.8 ± 2.3%, and 0% on days 1, 3, and 7 of ATc administration, respectively (Fig. 3E).

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To determine whether the chance to achieve complete remissions depended on initial tumor volume, we gave daily injections of ATc to additional mice with tumors 0.2 cm^3 (n = 7), 0.8–1.2 cm^3 (n = 10), and ≥1.9 cm^3 (n = 11) in volume (Table 2). Complete remissions were achieved in 100, 40, and 18% of the mice with small (<0.2 cm^3), intermediate–(0.8–1.2 cm^3), and large-sized (≥1.9 cm^3) tumors (P = 0.003, χ^2 test; Table 2). Thus, tumor remission clearly depended on initial tumor size at the onset of ATc administration.

**Remission Rate**

Remission rate was observed in all mice, even in those with previous complete remissions. The time periods for which complete remission could be achieved were 80 ± 1 cm^3 under ATc administration was 34 ± 2 ± 8, and 0 days for tumors with an initial tumor volume of 0.2, 0.8, 1.9, and 14.9 cm^3, respectively (Fig. 4, E–H). Thus, clear differences depending on initial tumor volumes were obtained (P = 0.005, Kruskal–Wallis test). Thereafter, tumors continued to grow. The recurrent tumors were analyzed for HER-2/neu expression. In all seven recurrent tumors analyzed, HER-2 ex-
Fig. 4. Tumor remission after down-regulation of HER-2. A, tumor growth in two representative nude mice. I—III show an ATc-treated mouse (daily s.c. injections of 10 mg ATc/kg) on the left and a control mouse on the right. B, the same tumors were also visualized by MRI. IV—VI show tumor remission during ATc administration, whereas VII—IX show further tumor growth in the control mouse. Pictures were taken on days 0 (I, IV, and VII), 4 (II, V, and VIII), and 6 (III, VI, and IX) of ATc administration. The day of the first administration of ATc was defined as day 0. C, ATc treatment of tumors with a mean volume of 1.2 cm$^3$ (group I). Tumors were induced by s.c. injection of $7 \times 10^6$ NIH3T3-her2 cells in nude mice. One group of mice ($n = 6$) received seven daily injections of 10 mg ATc/kg of body weight as soon as the mean tumor volume reached 1.2 cm$^3$. The second group of mice ($n = 6$) served as controls. Data are mean (SD; bars) values of six mice. D, ATc treatment of tumors with a volume of 3.9 cm$^3$ (group II). Experiments were performed similarly to those described in the legend for C. E, ATc treatment of tumors with a volume of 0.2 cm$^3$ (note the different scale of the Y axis). Experiments were performed as described in the legend for C, with the exception that 37 subsequent daily doses of ATc were given. F, ATc treatment of tumors with a volume of 0.8 cm$^3$. Experiments were performed as described in the legend for C, with the exception that 40 subsequent daily doses of ATc were given. G, ATc treatment of tumors with a volume of 1.9 cm$^3$. Experiments were performed as described in the legend for C, with the exception that 40 subsequent daily doses of ATc were given. H, ATc treatment of tumors with a volume of 14.9 cm$^3$ (note the different scale of the Y axis). Thirty subsequent daily doses were given. The Western blot (inset) was performed with tumor tissue obtained at day 30. Lane 1, tissue from one of these recurrent tumors (negative); Lane 2, NIH3T3-her2 cells as positive controls; Lanes 3 and 6, molecular weight marker; Lanes 4 and 5, loading control for Lanes 1 and 2 using anti-β-actin staining.
The chance for complete remissions of NIH3T3-her2 tumors in nude mice after switching off of HER-2 expression depended on the tumor volume at the onset of ATc administration. A clear difference in the frequency of complete remissions was observed among the three groups of mice with different tumor volumes ($P = 0.003$, $\chi^2$ test).

Table 2. Number of complete remissions of 0.2, 0.8–1.2, and $\geq 1.9 \text{ cm}^3$ NIH3T3-her2 tumors in nude mice.

<table>
<thead>
<tr>
<th>Tumor size ($\text{cm}^3$)</th>
<th>Total no. of mice</th>
<th>No. of mice with complete tumor remission</th>
<th>No. of mice with complete tumor remission as controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>7</td>
<td>7</td>
<td>0</td>
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<tr>
<td>0.8–1.2</td>
<td>10</td>
<td>4</td>
<td>6</td>
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<tr>
<td>$\geq 1.9$</td>
<td>11</td>
<td>2</td>
<td>18</td>
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DISCUSSION

Overexpression of the HER-2 oncogene in mammary tissue of transgenic mice leads to breast cancer (6). HER-2/neu amplification and/or overexpression in human breast and ovarian cancer is associated with tumor progression and adverse prognosis (12). Trastuzumab
TUMOR REMISSION AFTER DOWN-REGULATION OF HER-2/neu

(Hereceptin), a humanized monoclonal antibody directed against HER-2, provides clinical benefits for patients diagnosed with advanced breast cancer that overexpress the HER-2 protein (27, 28). Several mechanisms contribute to the beneficial effects of trastuzumab. Trastuzumab acts as an antiangiogenic substance that leads to regression of the vasculature in experimental human breast tumors in mice (29). Several lines of evidence suggest that HER-2 down-regulation might induce apoptosis in tumor cells (16, 30–36). In these studies, down-regulation of HER-2 was achieved by Hereceptin (16), adenoviral type 5 E1A (32, 35, 36), phosphorothioate antisense oligodeoxyribonucleotides (34), hammerhead ribozymes (31), or by pharmacological antagonists of the HER-2 signal transduction pathway (30, 33). However, the effect of HER-2/neu overexpression on apoptosis is controversial because some investigators reported that, in contrast to the above reports, HER-2/neu overexpression is associated with enhanced apoptosis (37–39).

To investigate the cellular functions of HER-2/neu, we developed in vitro and in vivo models of conditional human HER-2/neu expression that used the TET-OFF system, based on a tetracycline-controlled transactivator protein, tTA (21, 22). In vitro, expression of HER-2 could be down-regulated below the detection limit by incubation of tumor cells with ATc. To examine the influence of HER-2 in vivo, we injected NIH3T3-her2 cells s.c. into nude mice. Small tumors were already visible after 8–10 days and continuously increased in size, reaching mean diameters of 3.0 cm after 30 days after injection. In contrast, wild-type NIH3T3 cells and NIH3T3 cells injected with the empty vector did not form tumors up to day 25.

Tumors induced after subcutaneous injection of NIH3T3-her2 cells were reversible by treatment of mice with ATc. To examine whether tumor remission depends on the initial tumor volume before down-regulation of ATc, we treated four groups of mice with very early (0.2 cm³), early (0.8 cm³), intermediate (1.9 cm³), and advanced (14.9 cm³) tumors with ATc for up to 40 consecutive days. Our results indicated that tumor remission does depend on the initial tumor volume. Seven days after the onset of HER-2 down-regulation, tumor size was reduced by 100, 98.1, 81.4, and 74.2% for tumors with initial volumes of 0.2, 0.8, 1.9, and 14.9 cm³, respectively. The time periods for which mean tumor volume could be suppressed to volumes <0.1 cm³ under ATc administration were 34, 22, 8, and 0 days in the same groups of mice. The probability of achieving complete remissions also depended on initial tumor volume at the onset of ATc administration: complete remission could be reached as a consequence of HER-2 down-regulation in 100, 40, and 18% of mice with small (0.2 cm³), intermediate (0.8–1.2 cm³), and large (>1.9 cm³) tumors, respectively. However, for all tumors, even for those that apparently underwent complete remission, recurrent tumor growth occurred within 45 days despite ATc administration. Western blot analysis demonstrated that growth of all recurrent tumors was HER-2 independent. Limited dilution experiments showed that HER-2 is at least 625-fold down-regulated in the recurrent, HER-2-independent tumors compared with primary tumors. We speculate that additional genetic events, such as new mutations, must have occurred, causing HER-2-independent tumor growth. It will be of high importance to identify these new mutations (second hits) because this may lead to new strategies for improving HER-2/neu-blocking tumor therapy. Obviously, for advanced tumors the probability of additional hits causing HER-2-independent growth is higher compared with small tumors.

Our results show that the efficacy of HER-2-blocking therapy strategies depends on tumor size. To our knowledge no clinical study has been performed to date for Hereceptin efficacy in different stages of disease. However, our data may encourage the development of HER-2-targeted therapeutic strategies as first-line therapies for small tumors rather than the limiting of this treatment modality to advanced node-positive disease. The clinical implication of this observation seems obvious; Hereceptin can be expected to be most efficient for treatment of tumors with low volumes and low staging. For tumors with a relatively high residual tumor volume after surgery, e.g., many
node-positive breast carcinomas or recurrent tumors, the effect of Herceptin can be expected to cause a temporary reduction in tumor volume but no long-term remission.

The rapid remission of tumors after switching off of HER-2 expression was more than we had expected, considering the relatively large sizes of tumors at the beginning of ATc administration (0.2, 0.8, 1.9, 1.2, 3.9, or 14.9 cm³). We therefore examined the mechanisms responsible for tumor remission. During 7 days of ATc administration, the percentage of Ki-67-positive cells decreased from ~40% to 8.3%. Although such a block in cell cycle progression could explain the lack of additional tumor growth, it cannot account for the observed tumor remission. We therefore analyzed apoptosis by two independent assays, the TUNEL assay and Western blot analysis, to detect the inactive and the active forms of caspase-3. Both assays demonstrated an induction of apoptosis as a consequence of ATc-mediated down-regulation of HER-2. The TUNEL assay showed an increase in TUNEL-positive cells up to day 3 of ATc administration, whereas the inactive form of caspase-3 decreased up to day 7. A clear increase in

Fig. 7. Down-regulation of HER-2/neu induces apoptosis. A, apoptosis as evidenced by the TUNEL assay in NIH3T3-her2 cells in vitro during incubation with 10 ng/ml ATc. Representative examples of NIH3T3-her2 cells after 1, 3, and 7 days of incubation with ATc. B, mean (SD; bars) values for four independent in vitro experiments are shown. C, apoptosis as evidenced by the TUNEL assay in tumors in nude mice under therapy with ATc. Representative examples of a control tumor and tumors after 1, 3, and 7 days of therapy with ATc are shown. D, mean (SD; bars) values from one representative in vivo study are shown. Two independent experiments were performed. E, apoptosis as evidenced by Western blot analysis of caspase-3 in tumors in nude mice under therapy with ATC. The anti-caspase-3 antibody detects the inactive zymogen (32–35 kDa) and the active cleaved large fragment of caspase-3 (17–20 kDa). The Western blot shown is a representative of three independent experiments. F, loading control with anti-β-actin staining. G, Coomassie-stained membrane.
the active form of caspase-3 was seen as early as 24 h after initiation of ATc treatment. However, after 3 and 7 days of ATc administration, the active form of caspase-3 decreased dramatically. Most probably, this can be explained by further degradation of active caspase-3. This seems plausible because a strong remission of tumor volume occurred after 3 and 7 days of ATc administration. In addition, histological analysis of tumors treated for 3 and 7 days with ATc showed a strong decrease in viable cells with intact nuclei and a strong increase in dead tumor mass (Fig. 6C).

Our data suggest that antagonization of HER-2 may not only stop proliferation but also induce apoptosis and tumor remission. However, it must be kept in mind that this scenario may be observed only under highly specific experimental circumstances. In human tumors, several oncogenes and tumor suppressor genes are dysregulated in concert (40–42). In contrast, in the present tumor model, the tumorigenic property of NIH3T3-her2 cell-derived tumors is based on overexpression of a single oncogene, her-2. Interestingly, tumors in our NIH3T3-her2 model were reversible, possibly because only a single oncogene was dysregulated. Reversibility was rapidly lost in large NIH3T3-her2 tumors with a mean volume of 14.9 cm³ as well as in some smaller tumors, but after longer latency periods. Probably, activation of other oncogenes or inactivation of tumor suppressor genes led to HER-2-independent tumor growth. We are aware of the fact that our model is artificial. Nevertheless, it offers the opportunity to study the direct consequences and mechanisms attributable to a single oncogene.

At present, no experimentally proven molecular mechanism can explain why down-regulation of HER-2 not only stops further proliferation but also leads to induction of apoptosis and tumor remission. One can speculate that the extreme overexpression of HER-2 may constitute a strong anti-apoptotic signal, mediated by AKT kinase or by nuclear factor-κB (Fig. 1A; Refs. 8, 43). In response to the anti-apoptotic signaling, cells might up-regulate some pro-apoptotic pathways that will be without major consequences attributable to the strong anti-apoptotic signaling. When the anti-apoptotic signaling mediated by HER-2 is suddenly switched off by ATc, the sensitive balance between cell survival and cell death may be disturbed, and the compensatory up-regulated proapoptotic mechanisms, which probably cannot be down-regulated fast enough, will dominate and lead to cell death.

Our results show once again that overexpressed oncogenes represent an attractive therapeutic target and that their down-regulation can disturb the delicate balance between pro- and anti-apoptotic mechanisms and may be of therapeutic benefit. Tumor remission as a consequence of oncogene down-regulation has previously been shown for mutated H-RAS (44). In that study, a TET-regulated H-RASV12G transgenic mouse carrying valine instead of glycine in position 12 of H-RAS was generated. The TET-ON system was used (22) for expressing H-RASV12G in s.c. tumors in mice. Anticancer Res., 28: 38–44, 2008.

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Switching Off HER-2/neu in a Tetracycline-Controlled Mouse Tumor Model Leads to Apoptosis and Tumor-Size-Dependent Remission

Ilka B. Schiffer, Susanne Gebhard, Carolin K. Heimerdinger, et al.


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