Uptake of radiolabelled herceptin by experimental mammary adenocarcinoma

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Abstract

BACKGROUND: The aim of this study was to examine the biodistribution of 131I-herceptin in C3H/Bi mice with transplantable mammary adenocarcinoma with a high frequency of C-erbB2 receptor expression.

MATERIAL AND METHODS: Mice C3H/Bi with subcutaneously transplanted mammary adenocarcinoma were used as animal model to study the interaction between C-erbB2 receptor and herceptin, a humanized anti-C-erbB2 monoclonal antibody. The expression of the gene encoding C-erbB2 receptor in the tumours was studied by the RT-PCR technique.

RESULTS: Expression of this gene was found in 66% of the studied cases. Similarly, the presence of the C-erbB2 receptor in 77% of the tumours was detected by a Western blot analysis with the use of herceptin. Biodistribution experiments of iodine-labelled herceptin in mice C3H/Bi revealed its maximal accumulation in the tumours at 48 hours since the i.v. injection (7% ID/g). The tumour/muscle radioactivity ratio reached its highest value (above 20) also at 48 hours after the injection.

CONCLUSIONS: C3H/Bi mice with this adenocarcinoma may be a good experimental model to study herceptin, or its fragments, labelled with different radionuclids for preliminary evaluation of their usefulness in the therapeutic and diagnostic aspects of breast cancer.

Key words: herceptin, C-erbB2, receptor binding, breast cancer
studies. All experiments on living animals were carried out according to the protocols approved by the local ethical committee.

**Tumour implantation**

The mammary tumour bearing C3H/Bi mice were anaesthetized and killed by cervical dislocation. The tumours were removed and, after washing with cold and sterile 0.9% NaCl, sliced into 2–3 mm pieces and homogenized (1.9 w/v). Tumour cell suspension was filtered through aseptic gauze and then injected subcutaneously (150 μl) into three different areas of the dorsal region of healthy mice lightly anaesthetized with ketobutal (pentobarbital sodium salt, 60 mg/kg body weight, intraperitoneal injection).

**Tumour membrane preparation**

The homogenate was prepared from tumours after discarding the necrotic fragments. The tissue was homogenized in an ice-cold 0.01 M Tris-HCl buffer, pH 8.0 containing 0.005 M phenylmethanesulfonyl fluoride (PMSF) in ratio 1 : 9 and then centrifuged at 20000 rpm for 20 min at 4°C. The supernatant was centrifuged at 25000 rpm for 30 min at 4°C. The pellet was resuspended in the same buffer and spun again at 25000 rpm. The pellet was then dissolved in 0.01 M Tris-HCl buffer, pH 8.0 containing 0.15 mM NaCl, 1 mM EGTA and 0.005 M PMSF and protein concentration was measured by Lowry’s method using bovine serum albumin as a standard.

**Electrophoresis of membrane proteins**

Electrophoresis was performed in 7.5% polyacrylamide gel under reduced conditions (SDS-PAGE). Aliquots of membrane proteins (20 μl, about 50 μg of protein) were mixed with the same volume of sample buffer (0.0625 M Tris-HCl, pH 6.8; 4% SDS; 50% glycerol; 20% β-mercaptoethanol and 0.25% bromophenol blue), heated for 1 min at 95°C and run on SDS-PAGE gels. The separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane.

**Immunoblotting**

Proteins transferred to a PVDF membrane [12] were blocked by incubation with a 5% milk solution in TTBS (0.1 M Tris-HCl, pH 7.4 containing 0.9% NaCl and 0.1% Tween 20) for 2 h at room temperature. Then, the herceptin (Hoffmann-La Roche, Germany) at 1:100 dilution in a 5% milk solution in TTBS was added and incubated with PVDF for 1 h at room temperature with constant shaking. After three 15 min washes in TTBS, the PVDF membranes were incubated for 1 h at room temperature with a secondary antibody (goat anti human biotynylated IgG kit, Vector Laboratories, USA) at 1:300 dilution. After being washed three times in TTBS, the PVDF membranes were incubated in ABC reagent (Vector Laboratories) for 30 min in the dark and developed with 0.06% diaminobenzidine and 0.009% H2O2 in 0.05 M Tris-HCl buffer, pH 7.5. When the colour had developed, the membranes were washed extensively with distilled water.

**RNA extraction**

RNA was isolated from mammary adenocarcinomas by Total RNA Prep Plus Minicolumn Kit (A&A Biotechnology, Poland) based on RNA isolation method developed earlier [13]. The isolated RNA had an A260/280 ratio of 1.6–1.8.

**RT-PCR**

cDNA was synthesized by a RevertAid™ cDNA Synthesis Kit (Fermentas, Lithuania). A reaction mixture (total volume 20 μl) containing 1 ml of total RNA (3 μg), 1 ml of oligo(dT)18 primer (0.5 μg) and 8 ml of deionized nuclease free water was prepared. The mixture was spun down and incubated at 70°C for 5 min, then chilled on ice and the following components were added: 4 μl of 5 X reaction buffer, 1 μl of ribonuclease inhibitor (20 U/μl) and 4 μl of 10 mM of dNTPs mix. The mixture was incubated at 37°C for 5 min and 1 μl of RevertAid MuLV reverse transcriptase (200 U/μl) was added. The mixture was incubated at 42°C for 60 min. The reaction was stopped by heating at 70°C for 10 min. Sets of primers for c-erbB2 (5’-CTCTGACGTCCATCATCCTC-3’, 5’-CG-GATCTCCTGCTGCCTCGGT-3’) were planned on the basis of article by Schwartz et al [14]. PCR was carried out as follows: denaturation (94°C, 1 min), annealing (55°C, 1 min) and extension (72°C, 2 min) for 30 cycles. As a reference the primers for β-actine gene (5’-TGTATGCTCTGGTGTCCTGCCAC-3’, 5’ACAGAG-TACCTGCGCTCAGGAG-3’) were used [15].

The amplification of the c-erbB2 receptor and β-actine genes was performed in the same tube (multiplex PCR, MPCR) as follows: denaturation (94°C, 30 s); annealing (60°C, 30 s); extension (72°C, 30 s) during 35 cycles. The PCR products were separated by electrophoresis in 2% agarose gel.

**Radiolabelling of herceptin**

The labelling of herceptin with [131I] was carried out using the chloramine T method [16]. Labelled herceptin was purified from free radioiodine with the use of a molecular-sieving column (PD-10) and filtered through a 0.22 mm filter.

**Biodistribution**

Biodistribution studies were carried out on C3H/Bi mice weighing about 30 g, which had developed three tumours with a weight from 0.3 to 0.6 g. Tumour bearing mice were injected in the tail vein (i.v.) with approximately 0.3 μCi (11 kBq) of 131I-herceptin (specific activity c.a. 3.0 MBq/μg of herceptin). The radioactivity in various organs of the mice was determined at intervals of 0.5, 2, 6, 24, 48, 72, 96, 120 and 144 h. For each interval four mice were used. After anaesthetizing, the blood was obtained by heart puncture. All tissue samples were removed, washed in cold PBS, weighed and their radioactivity was measured in a gamma counter. The results were calculated and expressed as a percentage of the injected dose per one gram of tissue (%ID/g) or a percentage of the injected dose (%ID) for radioactivity eliminated with urine.

**Results**

C3H/Bi mice loaded with transplantable mammary tumours, histologically classified as adenomacarcinomas [17], were used in the experiments. The investigation of c-erbB2 gene expression by the RT-PCR technique was conducted on 18 randomly selected tumours. Twelve of them (66%) showed the presence of c-erbB2 gene
expression (98 bp fragment). In the same experiment, reference \( \beta\)-actine gene was amplified (368 bp) as a positive control of PCR reaction and its expression was stated in all analysed tumours (Fig. 1).

The presence of the investigated receptor in the adenocarcinoma was also confirmed by a Western blot analysis with the use of herceptin as a monoclonal anti-C-erbB2 antibody. For this purpose, the adenocarcinoma cell membranes were subjected to polyacrylamide gel electrophoresis with sodium dodecyl sulfate. Electrophoretically separated proteins were transferred onto a PVDF membrane and analyzed by the Western blot technique, which revealed the presence of C-erbB2 receptor in 14 (77%) out of 18 analyzed tumours (Fig. 2).

Herceptin in SDS-PAGE with 2-mercaptoethanol migrates as two bands; one typical for heavy chains of IgG (about 50 kDa) and the second for light chains (about 24 kDa). Both heavy and light chains were radioactive after herceptin iodination, as determined by electrophoresis followed by autoradiography (data not shown).

Accumulation of herceptin in mouse organs (%ID/g of tissue), as well as its elimination with urine (%ID), have been summarized in Table 1. The highest herceptin concentration in adenocarcinoma was observed 48 h after administration (about 7% ID/g). After this time, a decreasing tendency was observed. However, a high herceptin concentration was noticed in the blood. The percentage of dose per gram of blood was at its highest between 2 and 48 h after the injection. The ratio of tumour to muscle radioactivity reached the highest value (about 20) 48 h after injection. The tumour to blood ratio was at its highest level (2.5) 96 h after the administration of herceptin (Fig. 3).

**Discussion**

C-erbB2 gene is over expressed in at least one quarter of human breast cancers [4, 5] and correlates with a poor clinical outcome in women with node-positive and node-negative disease [5, 18]. To target this receptor, monoclonal antibodies directed against C-erbB2 were developed [19]. In preclinical studies these antibodies inhibited the growth of C-erbB2-overexpressing human breast tumour cells and sensitised them to tumour necrosis factor and to cis-diaminedichloroplatinum [20–22].

Taking into consideration the data showing the clinical usefulness of herceptin, we have analysed its biodistribution in mice loaded with transplantable mammary adenocarcinoma with a high frequency of C-erbB2 receptor expression. We first determined, by the RT-PCR technique, the c-erbB2 gene expression in the tumours. In further studies, herceptin was used as antibody for C-erbB2 receptor detection on the cell membranes prepared from the above-mentioned tumours. The presence of c-erbB2 ampli-

con and protein product of this gene in tumour tissue was stated in about 70% of the analyzed cases. However, the frequency of this expression on protein level is higher than at the mRNA level. The specificity of the RT-PCR method is therefore higher than immunoblotting analysis. The recognition of the mouse C-erbB2 receptor by herceptin may suggest that both human and mouse receptors share some antigenic determinants.

In the biodistribution studies, herceptin was well concentrated in the tumours (Tab. 1). The highest accumulation of this antibody in the tumours was observed 48 h after its injection. The ratio of radioactivity in tumour and muscle tissues showed a growing tendency with time and its highest value (about 20) was also observed after 48 hours. Taking into account that biodistribution studies were performed on a randomly selected group of tumour bearing mice, and that receptor expression was detected in about 70% of tumours, we might expect the herceptin uptake to be higher by 20–30%, if the experiment was conducted only on mice with c-erbB2/C-erbB2 positive tumours.

Unfortunately, also 48 hours after the administration of herceptin, a high concentration of this protein in the blood was noticed. The slow elimination of this antibody from the blood may be partially connected with the probable presence in the sera of the soluble extracellular domain of the C-erbB2 receptor. Such a domain of this receptor was observed in the sera of breast cancer patients undergoing herceptin treatment [23, 24]. The presence in the blood of extracellular fragments responsible for ligand binding was also documented for other membrane receptors [25, 26].
One of the main problems for antibody-mediated cancer therapy with radiolabeled antibodies is their slow delivery from the circulation to the target cancer cells [27–29]. Slow tumour-delivery of labelled herceptin was also observed in our study. This may be a source of toxic effects on bone marrow as well as on some organs.

In the case of herceptin, it was suggested that radiolabels are preferable over radioiodine. A scintigraphic image could be obtained as early as 3 h after injection of 111In-labeled herceptin. The accumulation of 111In-herceptin in the WIBC-9 tumors was significantly higher than that of the 125I-labeled herceptin [30, 31].

To enhance perfusion into cancerous tissue, small variants of antibodies were chemically or genetically engineered. A study conducted on Rhesus monkeys showed that full-length herceptin did not penetrate the inner limiting membrane of the retina. In contrast, the Fab antibody fragment diffused through the neutral retina to the retinal pigment epithelial layer [32]. It is also possible that the labelled Fab herceptin fragment will penetrate the C-erbB2 over expressing tumours much more easily than the full-length antibody.

Herceptin, in the case of C-erbB2 over expressing metastatic breast cancer patients, may serve as a vector for radiomunodiagnostic or radioimmunotherapy [7, 33]. In the present study it was shown that the mammary adenocarcinoma is characterized by relatively high C-erbB2 receptor expression on protein, as well as mRNA levels. C3H/Bi mice with this adenocarcinoma may be a good experimental model to study herceptin, or its fragments, labelled with different radionuclides for preliminary evaluation of their usefulness in the therapeutic and diagnostic aspects of breast cancer.

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References


Table 1. Biodistribution of 111In-herceptin in mammary adenocarcinoma bearing mice (%ID/g of tissue or blood and %ID eliminated with urine, mean ± SD, n = 4, i.v.)

<table>
<thead>
<tr>
<th>Time</th>
<th>30 min</th>
<th>2 h</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>96 h</th>
<th>120 h</th>
<th>144 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>0.2 ± 0.1</td>
<td>25.1 ± 1.4</td>
<td>24.0 ± 2.5</td>
<td>24.7 ± 1.4</td>
<td>6.1 ± 0.3</td>
<td>1.6 ± 0.12</td>
<td>9.7 ± 0.5</td>
<td>7.4 ± 0.5</td>
</tr>
<tr>
<td>Lung</td>
<td>0.01 ± 0.01</td>
<td>2.2 ± 0.4</td>
<td>2.5 ± 0.1</td>
<td>2.5 ± 0.15</td>
<td>1.3 ± 0.15</td>
<td>0.5 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>1.4 ± 0.16</td>
</tr>
<tr>
<td>Liver</td>
<td>0.07 ± 0.01</td>
<td>3.7 ± 0.35</td>
<td>1.1 ± 0.05</td>
<td>2.4 ± 0.18</td>
<td>1.2 ± 0.1</td>
<td>1.3 ± 0.14</td>
<td>2.4 ± 0.25</td>
<td>5.3 ± 0.5</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.06 ± 0.02</td>
<td>1.6 ± 0.1</td>
<td>0.35 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>0.8 ± 0.2</td>
<td>0.7 ± 0.08</td>
<td>0.7 ± 0.05</td>
<td>0.1 ± 0.06</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.11 ± 0.01</td>
<td>2.2 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>2.1 ± 0.1</td>
<td>0.8 ± 0.2</td>
<td>0.2 ± 0.02</td>
<td>0.4 ± 0.14</td>
<td>1.3 ± 0.16</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.34 ± 0.02</td>
<td>3.4 ± 0.4</td>
<td>0.6 ± 0.1</td>
<td>2.7 ± 0.2</td>
<td>1.4 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>3.0 ± 0.3</td>
<td>3.8 ± 0.2</td>
</tr>
<tr>
<td>Intestine</td>
<td>0.09 ± 0.06</td>
<td>4.9 ± 0.4</td>
<td>1.8 ± 0.1</td>
<td>2.0 ± 0.05</td>
<td>0.75 ± 0.2</td>
<td>0.6 ± 0.2</td>
<td>1.5 ± 0.13</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>Tumour</td>
<td>0.15 ± 0.01</td>
<td>2.1 ± 0.15</td>
<td>5.6 ± 0.4</td>
<td>7.0 ± 0.16</td>
<td>5.8 ± 0.35</td>
<td>4.0 ± 0.1</td>
<td>3.1 ± 0.2</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.025 ± 0.04</td>
<td>0.17 ± 0.02</td>
<td>0.4 ± 0.1</td>
<td>0.35 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.65 ± 0.1</td>
<td>0.6 ± 0.08</td>
<td>0.3 ± 0.08</td>
</tr>
<tr>
<td>Brain</td>
<td>0.13 ± 0.01</td>
<td>0.35 ± 0.02</td>
<td>0.4 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.2 ± 0.05</td>
<td>0.15 ± 0.06</td>
<td>0.42 ± 0.1</td>
<td>0.15 ± 0.06</td>
</tr>
<tr>
<td>Urine (total)</td>
<td>1.2 ± 0.1</td>
<td>0.5 ± 0.3</td>
<td>4.1 ± 0.2</td>
<td>10.1 ± 1.3</td>
<td>15.0 ± 1.35</td>
<td>25.1 ± 0.18</td>
<td>34.3 ± 2.8</td>
<td>13.4 ± 1.6</td>
</tr>
</tbody>
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