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Construction and characterization of a new chimeric antibody against HER2

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Abstract

Aims

Immunotherapy with anti-HER2 antibody has shown promising results in patients with HER2-positive breast cancer. We have recently reported characterization of a mouse monoclonal antibody (mAb) against HER2, which binds to an epitope different from that recognized by Trastuzumab and specifically inhibits proliferation of tumor cells overexpressing HER2. In the present study we report chimerization of this antibody.

Materials and Methods

The immunoglobulin variable region heavy (VH) and light (VL) chain genes of 1T0 hybridoma cells were amplified and ligated to human gamma-1 and kappa constant region genes using Splice Overlap Extension (SOE) PCR. The chimeric antibody was subsequently expressed and characterized by ELISA, Western blot and flow cytometry.

Results

The purified chimeric antibody specifically binds to recombinant HER2 and HER2 overexpressing tumor cells and inhibited proliferation of these cells. The binding affinity of the chimeric mAb was comparable to the parental mouse mAb.

Conclusion

This chimeric anti-HER2 mAb is potentially a valuable tool for targeted immunotherapy.

Keywords: Chimeric antibody, breast cancer, HER2, monoclonal antibody
Introduction

The human proto-oncogen HER2, also known as ErbB2 is located on chromosome 17 and encodes a 185 kDa transmembrane glycoprotein that belongs to the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases [1]. HER2 acts as the preferred heterodimerization partner for other members of HER receptors (HER1/EGFR, HER3 and HER4) and triggers several downstream signaling cascades such as MAPK and PI3K/AKT pathways [2]. HER2 gene overexpression is found in a number of human malignancies including breast cancer, pancreatic adenocarcinoma, ovarian and colorectal cancers [3, 4]. Overexpression of HER2 correlates with tumor metastasis and poor prognosis. Approximately 30% of women with breast cancer have HER2 protein overexpression, which is associated with poor prognosis [5]. The oncogenic potential and accessibility of HER2 have made it a suitable target for cancer immunotherapy by monoclonal antibodies (mAbs). Trastuzumab (Herceptin, Genentech Inc., San Francisco Calif, USA) represents the first humanized mAb which was approved by the United States Food and Drug Administration (FDA) in 1998, for therapeutic use in patients with HER2-overexpressing breast cancer [6]. However, many patients do not respond and progress within 1 year of initiating Trastuzumab therapy, which could be due to the inefficiency of Trastuzumab to inhibit HER2 binding to other members of the HER family [7]. Combination of two mAbs recognizing two distinct epitopes on HER2 is an effective alternative strategy to overcome this resistance [8]. Pertuzumab is another humanized mAb that binds to an epitope on domain II of the extracellular region of HER2, different from the binding site of Trastuzumab on domain IV. Consequently, it potently blocks
ligand-activated signaling transduced from HER-2/HER-1 and HER-2/HER3 heterodimers. Antitumoral activity of Pertuzumab has been shown both in vitro and in vivo models [9]. Combination of Pertuzumab and Trastuzumab has recently demonstrated improved survival in patients with breast cancer [10]. Based on these findings, FDA has recently approved Pertuzumab in combination with Trastuzumab for patients with HER2-positive metastatic breast cancer [11]. Development of new HER2 specific mAbs may improve the therapeutic efficacy of the current anti-cancer treatment protocols. In the present study, we present data on chimerization of a new mouse mAb against HER2 [12], which binds to an epitope of HER2 different from that of Trastuzumab.
Materials and Methods

Cell lines
The 1T0 monoclonal antibody producing hybridoma was prepared as described previously [12]. It was grown in RPMI 1640 Medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco), 100 µg/mL streptomycin, and 100U/mL penicillin (Gibco) at 37°C in a humidified atmosphere of 5% CO₂. HER2-overexpressing human breast cancer cell line BT-474 and CHO-K1 were purchased from National Cell Bank of Iran (NCBI, Tehran, Iran) and cultured under similar conditions, with the exception of BT-474 culture medium which was also supplemented with 10 µg/mL insulin (Exir Co., Boroojerdi, Iran).

Amplification, cloning and sequencing of antibody variable region genes
Variable regions of the heavy chain (VH) and light chain (VL) of 1T0 antibody were amplified by RT (reverse transcriptase)-PCR using RNA isolated from the hybridoma. In brief, total RNA was isolated with RNA Bee-RNA Isolation Reagent (AMS Biotechnology, UK) from 1×10⁷ murine hybridoma cells that secrete 1T0 antibody, and the corresponding cDNA was synthesized with avian myeloblastosis virus (AMV) reverse transcriptase using oligo dT as primer (Fermentas, Thermo Fisher Scientific Inc, USA). The VH gene was amplified using the degenerate primers mUIgVH-S and mUIgGHC-AS and the VL gene was amplified using the degenerate primers mUIgLkL-S and mlgkC-AS (Table 1). PCR reactions were performed in 25 µl volume, containing 1 µl of cDNA, 6 and 1µM of forward and reverse primers, respectively, 2 mM MgSO₄ concentration, 1u/µl Pfu DNA polymerase (Fermentas) and 10X reaction buffer. After 3 min denaturation at 94°C, the PCR reaction was followed by 45 cycles of 1 min at 92°C, 1 min at 52°C, 1 min at 72°C and a final 72°C for 10 min. To confirm the identity of PCR products, the VH
and VL genes of 1T0 were cloned into pGEM-T easy vector system (Promega, Madison, WI, USA) and sequenced.

**Isolation of human IgG1 and IgCκ constant region genes**

Similar to VH and VL, Cκ of human kappa chain and CH of human IgG1 were amplified by RT–PCR, using the RNA isolated from human peripheral blood mononuclear cell (PBMC). The Cκ was amplified using the primers Cκ-S and XhoICK-AS and the CH was amplified using the primers CH-S and BamhICH-AS (Table 1). PCR reactions were performed in 25 μl volume, containing 1 μl of cDNA, 1μM primers, 2 mM MgSo4, 1u/μl Pfu DNA polymerase (Fermentas) and 10X reaction buffer. After 3 min denaturation at 94ºC, the PCR reaction was continued by 30 cycles of 1 min at 92ºC, 1 min at 58ºC, 1.5 min at 72ºC and a final 10 min 72ºC. To confirm the validity of PCR products, the CH and CL genes were cloned into pGEM-T vector system (Promega) and sequenced.

**Construction of the mouse-human chimeric antibody expression vector using Splice Overlap Extension (SOE) PCR**

Splice overlap extension (SOE) PCR allows the fusion of two sequences of DNA without the use of restriction enzymes [13]. PCR products of VH and VL genes were modified to contain restriction sites, Kozak sequences and a leader sequence taken from the original cDNA of 1T0 hybridoma in the forward primers and 15bp complementary region of CH and CL in the reverse primers (Table 1), respectively. The VH, CH and VL, CL were amplified, extracted from gel and fused during PCR1, as the overlapping sequences. They were subsequently hybridized and extended to produce full-length chimeric VH-CH and VL-CL sequences. The VH-CH and VL-CL obtained from PCR1 were then amplified by external primers (VH-Sall-T-S, BamhICH-AS for VH-CH and Vk-kpn-T-S, Cκ-S for VL-CL) in a second round of PCR (PCR2). The PCR product of VH-CH was inserted into pBudCE4.1 (Invitrogen, Grand...
Island, NY, USA) at Sall/BamHI restriction sites to generate pBud-VH-CH, which contains the heavy chain sequence of mouse-human chimeric antibody. After sequencing and confirmation of this construct, PCR product of VL-CL was subsequently inserted into pBud-VH-CH at KpnI/XhoI sites to generate pBud-VH-CH-VL-CL (pBud-c-1T0), which contains the heavy and light-chain sequences of mouse-human chimeric antibody (Figure 1).

PCR1 reactions were performed in 20 µl volume, containing 1 µl of cDNA extracted from VH and CH or VL and CL, 2 mM MgSo4 concentration, 1u/µl Pfu DNA polymerase (Fermentas) and 10X reaction buffer. After 3 min denaturation at 94ºC, the PCR reaction was followed by 5 cycles of 1 min at 92ºC, 1 min at 58ºC, 1.5 min at 72ºC. The temperature was hold on 92ºC for 3min and after addition of external primers in 5µl volume containing 10X reaction buffer, PCR2 reaction was performed by 35 cycles of 1 min at 92ºC, 1 min at 58ºC, 1.5 min at 72ºC and a final cycle at 72ºC for 10 min. To confirm the identity of PCR products, the amplified VH-CH and VL-CL genes were cloned into pGEM-T vector (Promega) and sequenced.
Table 1: Sequences of PCR primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Amplified genes</th>
<th>Sequence</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>mUIgVH-S</td>
<td>VH</td>
<td>CAGGTSMARCTGCAGSAGTGCWGG</td>
<td>348 bp</td>
</tr>
<tr>
<td>mUlgGHC-AS</td>
<td>VH</td>
<td>AGGAGCCAAGTGGATACAGATGG</td>
<td></td>
</tr>
<tr>
<td>mUIgVκL-S</td>
<td>Vκ</td>
<td>GAACTTCTGCCACATCTCTCTCTCTCCC</td>
<td>321 bp</td>
</tr>
<tr>
<td>mIgκC-AS</td>
<td>Vκ</td>
<td>TGGTGGAAGATGGATACAG</td>
<td></td>
</tr>
<tr>
<td>Cx-S</td>
<td>Cx</td>
<td>ACTGTGCTGCTGCACCATCTCTCTCTCTCCC</td>
<td>318 bp</td>
</tr>
<tr>
<td>XholCx-AS</td>
<td>Cx (SOE PCR)</td>
<td>CTCCAGCTACACCTCTCCCCCTGTTGAAGCTCTTTGACACGGCGA</td>
<td></td>
</tr>
<tr>
<td>CH-S</td>
<td>Cy1</td>
<td>GCCCACCACAAAGGGCCATCCGTC</td>
<td>990 bp</td>
</tr>
<tr>
<td>BamHICh-AS</td>
<td>Cy1 (SOE PCR)</td>
<td>GGAATCCTCATTACCCTGGAGAAGGGAGGCGTCTTT</td>
<td></td>
</tr>
<tr>
<td>Vκ-kpn-T-S</td>
<td>VκT0 (SOE PCR)</td>
<td>GGATCCGCCACCATGGAGTTTACAGCCAGTTTCTCTTCTTG</td>
<td>381 bp</td>
</tr>
<tr>
<td>Jκ-T-AS</td>
<td>VκT0 (SOE PCR)</td>
<td>AAGCTTTTTTATTCAGCTGGTCCCCCTCCGAAGCTG</td>
<td></td>
</tr>
<tr>
<td>VH-Sali-T-S</td>
<td>VHT0 (SOE PCR)</td>
<td>GTCACTGCACTCCATGGACTTGGTGCTACGCTT</td>
<td>405 bp</td>
</tr>
<tr>
<td>JH-T-AS</td>
<td>VHT0 (SOE PCR)</td>
<td>GCCCTTTGAGGAAGGGTGGTGGGTGGTGGTGACGAGG</td>
<td></td>
</tr>
</tbody>
</table>

(In degenerate primers, R=A or G, S=C or G, K=G or T, M=A or C, Y=C or T, W=A or T, H= A, T or C, N= A, T, C or G).

Transfection of chimeric antibody and establishment of stable transfected cell lines

To develop a stable transfectant expressing chimeric 1T0 antibody (c-1T0), CHO cells were grown to 80% confluency in 12-well culture plates. pBud-c-1T0 construct was prepared using Plasmid Maxiprep (Qiagen, Stockholm, Sweden) and then, CHO-K1 cells were transfected with 6 µg DNA of pBud-c-1T0 construct in combination with 6 µL JetPEI transfection reagent (Polyplus-transfection, New York, NY) according to the manufacturer’s recommendations. After 48 h culture, transient expression of c-1T0 was assessed by ELISA. To establish stable transfectant, cells were subsequently selected using 1mg/ml of Zeocin (Gibco, Grand Island, NY, USA) within a minimum of two weeks.
Screening of chimeric antibody production by ELISA

Chimeric antibody activity was detected using an indirect ELISA method [12]. In brief, a 96-well ELISA plate (Maxisorp, Nunc, Roskilde, Denmark) was coated with 0.5 µg/mL recombinant extracellular part of HER2 (eBioscience Inc., San Diego, USA) in PBS and incubated 1.5 h at 37°C and blocked with PBS supplemented with 0.05% Tween (Sigma, St Louis, MO, USA) and 3% non-fat skim milk. Fifty microliters of supernatants of transfected CHO cells were added at 37°C for 1.5 h. Trastuzumab was used as positive control. After washing with PBS-Tween, horseradish peroxidase (HRP)-conjugated rabbit anti-human Ig (prepared in our lab) was added and plate incubated for 1 h at 37°C. After further washing, the reaction was revealed with 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma). Sulfuric acid was added to stop the reaction and the optical density (OD) was measured by a multiscan ELISA reader (Organon Teknika, Turnhout, Belgium) at 450 nm.

Structural characterization of chimeric antibody by ELISA and SDS-PAGE

Stable transfected cells producing c-1T0 were adapted to serum free medium (EX-CELL™ Sp2/0, Sigma, St Louis, MO, USA). Supernatant of c-1T0 was purified using a 1 ml HiTrap Protein G HP column (Amersham Biosciences, New Jersey, USA). The culture supernatant (1 litre) was passed through the column and the column was subsequently washed thoroughly with PBS. Bound recombinant chimeric antibody was eluted by 50 ml of elution buffer (0.1 M glycine/HCl, pH 2.7; flow rate 1 ml/min). The pH of eluted fraction was immediately normalized using 1 M Tris/HCl, pH 9.0 buffer. For verification of c-1T0, we examined presence of human IgG1 and IgCk in c-1T0 antibody by ELISA method. Briefly, a 96-well ELISA plate was coated with 5 µg/mL mouse monoclonal antibody against human IgG (8a4, kindly provided by Professor Roy Jefferis) in PBS. SPG purified c-1T0 in different
concentrations were added at 37°C for 1.5 h. Herceptin was used as positive control. After washing, horseradish peroxidase (HRP)-conjugated rabbit anti-human Ig and sheep anti-human IgCκ was added separately for assessment of human IgG1 and IgCκ and the plate was incubated for 1 h at 37°C. After further washings, the reaction was revealed with TMB substrate. Sulfuric acid was added to stop the reaction and ODs were measured as mentioned above.

The structure of c-1T0 antibody was analysed by SDS-PAGE [14]. Briefly, 500 ng of SPG purified c-1T0 antibody was separated on 10% SDS-PAGE under reducing and non-reducing conditions and visualized with silver staining. Herceptin was used as a positive control. After electrophoretic separation, the gel was washed three times with distilled water and incubated for 30 min with 50 ml of fixing solution (Methanol 50 ml, acetic acid 10 ml and 40 ml H2O). Three times washing was repeated and the gel was incubated 1 min with 50 ml of 0.2g/lit solution of sodium thiosulphate. After further washings, the gel was incubated 25 min with 50 ml of silver nitrate solution (20%). The gel was subsequently washed and developed with a solution containing sodium carbonate (30g/lit), 100µl of formaldehyde (37-41%) and 4 ml sodium thiosulphate (0.2g/lit). Finally, citric acid (3%) was added to stop the reaction.

Analysis of specific binding of chimeric antibody to rHER2 by Western blot technique

Western blot was employed to compare specific binding of mouse 1T0 and c-1T0 to rHER2. Fifty nanogram of rHER2 was separated on 12% SDS-PAGE and transferred to PVDF membrane (Roche Diagnostics, Mannheim, Germany). After blocking of membrane with blocking buffer (PBS-Tween-20 containing 5% non-fat skim milk) overnight at 4°C and washing three times with washing buffer (PBS-Tween-20) for 15min, mouse 1T0 and c-1T0 antibodies were added at 10 µg/mL in blocking buffer at room temperature for 1.5 h while shaking. Ten
µg/mL of Herceptin was used as a positive control. Washing steps were repeated and HRP-
conjugated sheep anti-mouse immunoglobulin (prepared in our lab) for mouse 1T0 and HRP-
conjugated sheep anti-human Ig (prepared in our lab) for c-1T0 and Herceptin were added at
room temperature for 1.5 h on shaker. After washing, PVDF membrane was treated with ECL
(Amersham Biosciences, New Jersey, USA) and the bands were visualized on Kodak X-ray
film (Eastman Kodak, Rochester, NY, USA).

Affinity constant determination by ELISA

An ELISA-based method was used to define the binding affinity of mouse 1T0 and c-1T0 [15].
Briefly, wells of a microtiter ELISA plate were coated with several concentrations (2-0.031
µg/mL) of recombinant extracellular part of HER2. After blocking with 0.05% Tween (Sigma)
and 3% non-fat skim milk, serial concentrations of mouse 1T0 (10-0.15 µg/mL) and c-1T0 (5-
0.07 µg/mL) in blocking buffer were added into coated wells and incubated at 37ºC for 1.5 h.
Washing was repeated and wells incubating with HRP-conjugated sheep anti-mouse Ig and
sheep anti-human Ig (prepared in our lab) for 1.5 h at 37ºC. After the final wash step, TMB
substrate solution was added followed by stopping solution and ODs were measured. Sigmoidal
curves of ODs versus the logarithm of antibody concentrations were constructed. The antibody
concentration giving 50% of the maximum absorbance value ([Ab]t) at a particular antigen
coating concentration was chosen for the affinity measurement using the formula $K_{aff} = 1/2(2$
$[Ab]_0 - [Ab]_t)$. $[Ab]_0$ and $[Ab]_t$ represent the antibody concentrations resulting in 50% of the
maximum absorbance value at two consecutive concentrations of coated antigen where $[Ag] =$
$2[Ag_0]$. The mean of such calculations for three non-overlapping antigen concentrations was
taken as the final $K_{aff}$ value.

Analysis of cell surface binding of chimeric antibody by flow cytometry
Indirect staining at surface membrane level was performed on BT-474 cells (National Cell Bank of Iran, Tehran, Iran). After trypsinization, $10^6$ cells were harvested, washed two time with washing buffer (PBS, 0.1% NaN3), and incubated with 100 µL of 10 µg/mL of mouse 1T0 and c-1T0 antibodies as primary antibodies at 4°C for 1 h. Mouse IgG1 mAb and human IgG of irrelevant specificity (produced in our lab) were included as negative controls. After incubation and washing process, cells were incubated with FITC-conjugated sheep anti-mouse Ig and sheep anti-human Ig (prepared in our lab) at 4°C for 1 h. The cells were finally scanned by a flow cytometer (Partec, Nuremberg, Germany). Flomax flow cytometry analysis software (Partec) was used to analyse the data.

**Assessment of tumor growth inhibition by XTT assay**

For tumor growth inhibition experiments, BT-474 cells were seeded in 96-well flat-bottom tissue culture plates (30000 cells/well) in serum-containing RPMI-1640 medium (Gibco, Grand Island, NY, USA). The cells were treated with different concentrations of mouse 1T0, c-1T0 and Herceptin (10, 2 and 0.2 µg/ml) for 16h at 37°C in a humidified atmosphere of 5% CO2. After incubation, the RPMI medium was exchanged with serum free medium containing XTT solution (Roche, Indianapolis, IN) for 16h at 37°C as recommended by the manufacturer. After incubation with XTT, microtiter plates were read by an ELISA reader (Organon Teknika, Turnhout, Belgium) at 450 nm with the reference wavelength of 690 nm. Controls included background (cells only) and Herceptin. All experiments were performed in triplicate. The following formula was used to estimate the tumor growth inhibition rate induced by anti-HER2 antibodies:

\[
\text{Tumor growth inhibition (\%) = \left[ \frac{\text{OD without antibody} - \text{OD with antibody}}{\text{OD without antibody}} \right] \times 100}
\]
Assessment of tumor proliferation inhibition by radioactive thymidine incorporation assay

The antiproliferative activity of mouse 1T0 and c-1T0 was tested on HER2-overexpressing cell line BT-474. BT-474 cells were seeded in 96-well flat-bottom tissue culture plates and were allowed to recover and adhere overnight. Antibodies were added to wells at different concentrations of mouse 1T0, c-1T0 and Herceptin (10, 2 and 0.2 µg/ml) for 16h at 37°C in a humidified atmosphere of 5% CO2. After incubation, 3H-thymidine (PerkinElmer, Boston, USA) was added at 0.5 µCi per well for 8 h. Cultures were then harvested and transferred to scintillation fluid for measurement of 3H-thymidine incorporation by a beta counter (Wallac 1410 Liquid Scintillation Counter, Pharmacia, Sweden). Controls included background (cells only) and Herceptin. All experiments were performed in triplicate. The following formula was used to estimate the proliferation inhibition rate:

Proliferation inhibition (%) = \[
\frac{[(\text{CPM without antibody}-\text{CPM with antibody})/\text{CPM without antibody}] \times 100}
\]
Results

Amplification of the VH and VL genes of mouse 1T0 mAb

The VH and VL genes were amplified using specific primers designed for the leader sequences of VH and VL genes (Figure 2, A and B). The ORF of the VH region of the mAb 1T0 is 405 bp in length, encoding a 135-aa polypeptide and the ORF of VL is 381 bp in length, encoding a 127-aa polypeptide, including the leader peptides. Both VH and VL genes have a signal leader sequence on their N-terminal region, encoding 19- and 20-aa polypeptides, respectively. The Cκ and CΥ1 were amplified with specific primers from cDNA of normal human PBMC encoding 107- and 330-aa polypeptides, respectively (Figure 2, C and D). Finally, the VLT0-Cκ and VHT0-Cγ1 segments (Figure 2, E and F) were linked to each other by SOE PCR technique as described in the Materials and Methods.

Expression of chimeric c-1T0 antibody in CHO cells

The c-1T0 construct was transfected in CHO cells by JetPEI transfection reagent and culture supernatants were collected to assess chimeric antibody production by antigen specific indirect ELISA. After selection in Zeocin and four rounds of subcloning, a stable transfected cell line (c17) that produces high levels of chimeric antibody was selected. Based on the results obtained from the antigen specific ELISA using recombinant extracellular region of HER2 as the coating antigen and different concentrations of Herceptin as the standard protein, 960 ng/ml of chimeric antibody was detected in serum free medium (Figure 3, A and B).

Structural characterization of c-1T0 antibody
Transfected CHO cells were maintained in a serum-free culture medium. The chimeric antibody was purified from the culture supernatant by affinity chromatography using SPG column. The purified chimeric antibody was analysed by SDS-PAGE under non-reducing and reducing conditions. Silver staining of SDS-PAGE gel (Figure 4) shows monomeric (~150 kDa) form of the chimeric antibody under non-reducing condition (c-1T0, lane 1). The monomeric light (~25 kDa) and heavy chains (~50 kDa) were detected under reducing conditions (c-1T0, lane 2). The parental mouse 1T0 mAb gave a similar pattern under non-reducing and reducing conditions.

Western blot analysis revealed that the chimeric c-1T0 and the parental mouse 1T0 mAbs react with the non-reduced recombinant extracellular HER2 protein (Figure 5). Lack of reactivity with the reduced HER2 protein indicates recognition of a conformational epitope by our mAb. A similar pattern of reactivity was observed for Trastuzumab, which was used as a control.

**Affinity constant determination**

The binding affinity of the chimeric antibody was determined by an ELISA method as described in the Materials and Methods. Based on the binding curves obtained for the chimeric and mouse parental mAbs (Figure 6, A and B), the mean $K_{\text{aff}}$ of mouse 1T0 and c-1T0 were $0.6 \times 10^9$ and $1.3 \times 10^9$, respectively.

**Assessment of cell binding activity by flow cytometry**

In order to determine the binding reactivity of c-1T0 to the HER2 overexpressing cells, we performed flow cytometric analysis using c-1T0 and mouse 1T0 as first layer and sheep-anti human-FITC and sheep-anti mouse-FITC as second layer,
respectively. In parallel to Trastuzumab as positive control, c-1T0 showed positive
reactivity and detected HER2 on surface of BT-474 cells similar to the parental
mouse 1T0 antibody (Figure 7).

Tumor cell growth inhibition by c-1T0 chimeric mAb

A colorimetric (XTT) assay was performed to assess the effect of c-1T0 on growth of
BT-474 cell line. The growth inhibition rate of triplicate wells was determined and percent
of inhibition was calculated according to the formula described in the Materials and
Methods. Accordingly, c-1T0 induced a dose dependent growth inhibition, similar to the
parental mouse 1T0 mAb in the BT-474 tumor cell line (Figure 8).

Tumor proliferation inhibition by c-1T0 chimeric mAb

The ability of c-1T0 to inhibit tumor cell proliferation was assessed in vitro in parallel to
the parental mouse 1T0 mAb by radioactive labeled thymidine assay. The stimulation
index of triplicate wells was determined and percent of inhibition of cell proliferation was
calculated for mouse 1T0 and c-1T0 (Figure 9). Both mAbs induced a similar dose
dependent pattern of inhibition.
Discussion

Monoclonal antibodies are a part of the biological drugs that represent a growing segment of the pharmaceutical industry. Approximately 26 mAbs have so far been approved by FDA and over 200 mAbs are still awaiting approval [16]. An early success of mouse mAb for therapeutic purposes provoked a response similar to serum sickness of antiserum therapy [17]. When a mouse mAb is multiply injected to a patient, the human anti-mouse antibody response (HAMA) is induced [18]. Chimerization is one approach to reduce the immunogenicity of therapeutic mouse mAb for human treatment. In 1984, Boulianne et al. [19] and Morrison et al. [20] produced chimeric antibodies by joining the mouse variable domains to human constant domains. Although chimerization reduces HAMA response of murine antibodies, human anti-chimeric antibody (HACA) response could be created because of immunogenic epitopes in the mouse variable regions. Despite their potential immunogenicity, chimeric antibodies have been widely used for immunotherapy of cancers. One of the most widely used therapeutic chimeric antibodies is Rituximab. If these antibodies prove to be effective in vivo then their humanization might be considered later, particularly if they are found to be highly immunogenic in human. Another approach to further reduce the immunogenicity of murine mAb is humanization in which all framework regions (FWR) residues that are not essential for antigen binding are replaced with human FWR counterpart sequences [21].
HER2 gene overexpression has been found in a number of human malignancies [22] and is a proven therapeutic target. In 1990, Fendly and coworkers [23] produced mAbs directed against extracellular domain of HER2. Two of these antibodies, 4D5 and 2C4 were shown to inhibit growth of breast cancer cells both in vitro and in vivo [24]. These mouse mAbs were chimerized and subsequently humanized and designated as Trastuzumab and Pertuzumab [25, 26]. Trastuzumab is a humanized mAb that binds to the extracellular domain IV of HER2, and induces down-regulation of the PI3K/Akt pathway. Treatment with Trastuzumab has proven to be effective in management of HER2-amplified/overexpressing tumors [27]. Nevertheless, resistance to therapy is a serious challenge [28]. The majority of metastatic breast cancer patients who initially respond to Trastuzumab begin to demonstrate disease progression within one year [29]. Newly generated mAbs with specificity to novel epitopes on extracellular domain of HER2 [9, 12, 30] might be able to enhance anti-cancer activity. Synergistic effect of some mAbs with Trastuzumab has been demonstrated in HER2 overexpressing breast cancer xenograft models [8, 31]. Among these antibodies, FDA has approved Pertuzumab (Perjeta-Genentech) in combination therapy with Trastuzumab [11]. Pertuzumab is another humanized mAb that binds to domain II and efficiently inhibits dimerization of HER2 [32].

We have recently generated a panel of mouse mAb directed against HER2 which recognize epitopes distinct from Trastuzumab [12]. Two of these mAbs (1T0 and 2A8) were later found to significantly inhibit the proliferation of HER2-expressing tumor cell line, BT-474, dose-dependently (manuscript in preparation). In the present
study, we presented data on chimerization of one of these mAbs, 1T0. The VH and VL genes were successfully amplified and integrated to human IgG1 and Cκ by SOE PCR. Liu and colleagues [33] generated a mouse/human chimeric mAb against HER2 and assessed its structural and biological activities. They amplified the VH and VL genes of the mouse mAb from genomic DNA of the hybridoma clone. Luo and coworker [34] isolated Fab genes of a mouse mAb from cDNA of hybridoma cell line and constructed a mouse/human chimeric mAb. Either DNA sequencing or ELISA could be used to show that the expression vector of chimeric antibody is constructed successfully [34, 35]. The results of ELISA in this work showed that the transfected CHO cells produce mouse/human chimeric mAb (c-1T0).

Using an antigen based indirect ELISA as well as immunoblotting techniques we demonstrated the HER2 binding activity of the chimeric c-1T0 antibody. The immunoblot results showed that similar to the parental mouse 1T0 mAb, c-1T0 recognizes a conformational epitope on extracellular domain of HER2 (Figure 5). The results obtained by flow cytometry indicate that c-1T0 binds to native HER2 expressed on the surface of tumor cells as efficiently as the mouse counterpart and Trastuzumab (Figure 7). These findings suggest that this antibody could be used to target tumor cells. Interestingly, the affinity constant of the chimeric antibody was slightly higher than the parental mouse 1T0 mAb. The improved binding activity of our chimeric antibody might be due to the higher flexibility of the hinge region of human IgG1 as compared to mouse IgG1. A similar mouse/human chimeric IgG1 mAb with specificity for Cryptococcus neoformans and a higher binding affinity compared to the parental mouse IgG1 mAb has previously been reported [36].
The in vitro biological activity of our chimeric antibody was assessed by incorporation of radioactive thymidine and XTT techniques (Figure 8 and 9). The results indicated that c-1T0 inhibits the proliferation of BT-474 cells dose dependently similar to Trastuzumab and the mouse 1T0. However, despite the overall similarity, c-1T0 displayed a better inhibitory response in the XTT assay, but not the thymidine incorporation assay, which could be due to the differences of the assay systems employed in this study. The XTT assay measures the metabolic activity of the growing cells, whereas the thymidine incorporation assay measures the DNA synthesis status of proliferating cells. Furthermore, Trastuzumab failed to inhibit cell growth and proliferation at low concentration (2.5ug/ml) in both assay systems, implying functional limitation of this mAb at low concentrations.

The mechanisms of anticancer activity of c-1T0 are not completely known, but taking into consideration the similar in vitro tumor growth inhibitory activity of c-1T0 and Trastuzumab, several mechanisms could be proposed including: (a) downregulation of total levels of HER2 on the cell surface [37], (b) blocking cleavage of the extracellular domain of HER2 and thereby preventing formation of the constitutively active membrane-bound 95-kDa HER2 protein called p95HER2 [28], (c) induction of cell cycle arrest by p27kip1 and inhibition of cdk2 activity [38] and (d) blocking of the dimerization of HER2 with HER3.

Considering the synergistic anti-tumor effect induced by combination of two different mAbs with different epitope specificities, such as Trastuzumab and Pertuzumab [8] and the fact that c-1T0 recognizes an epitope of HER2 different from that recognized by Trastuzumab, our mAb might display a synergistic anti-tumor effect in
combination with Trastuzumab or Pertuzumab. We are currently investigating the biological activity of c-1T0 mAb alone and in combination with Trastuzumab antibody in vivo in nude mice implanted with breast tumor cells to assess its potential implication for immunotherapy of HER2-expressing malignancies. Further in vivo investigations are also required to assess functional activities of c-1T0 mAb mediated by the host T lymphocytes and NK cells, such as antibody-dependent cell cytotoxicity.

Executive Summary

The oncogenic potential and accessibility of HER2 have made it a suitable target for cancer immunotherapy by monoclonal antibodies. This study describes chimerization and characterization of a new mouse mAb (1T0) against HER2, which binds to an epitope of HER2 different from that of Trastuzumab. The chimeric antibody was expressed in eukaryotic cells and characterized by ELISA, SDS-PAGE and flow cytometry. Tumor cell growth and proliferation inhibition were assessed by XTT and thymidine incorporation assays, respectively. c-1T0 recognized a conformational epitope within the extracellular domain of HER2 distinct from Trastuzumab and displayed a binding affinity comparable to the parental mouse mAb.
The purified chimeric mAb induced a dose dependent cell growth and proliferation inhibition similar to the parental mouse mAb in HER2 overexpressing BT-474 tumor cell line.

Our chimeric mAb with specificity to a novel epitope on extracellular domain of HER2 is potentially a suitable tool for targeted immunotherapy of HER2 overexpressing malignancies.
Figure 1:

Map of pBudCE4.1 expression vector containing the mouse-human chimeric antibody (c-1T0). Light chain sequences were introduced in XhoI and KpnI sites after P\textsubscript{EF-1\alpha} promoter and heavy chain sequences were introduced in SalI and BamHI sites after P\textsubscript{CMV} promoter.
Figure 2:
PCR amplification of VH-CH and VL-CL genes for construction of the chimeric antibody. Mouse variable region heavy (VH) and light (VL) chain genes (A and B) and human constant region heavy chain of IgG1 (Cy1) and kappa light chains (Ck) (C and D) were amplified from cDNA of the mouse hybridoma 1T0 clone and cDNA of human PBMC, respectively. Vκ-Ck (E) and VH-Cy1 (F) fragments were amplified by SOE PCR as described in
Materials and Methods. The PCR products were run in 1% agarose gel. A 100bp Plus DNA ladder (A,C,D and F) (Sinaclone, Iran) and DNA molecular weight marker IX (B and E) (Roche, Germany) were used.

**Figure 3:**

A

Measurement of chimeric anti-HER2 mAb in culture supernatant of transfected cells. Titration of Trastuzumab (A) and c-1T0 culture supernatant (B) on recombinant extracellular region of HER2 is shown. Supernatant of Mock transfected CHO cell line was used as a control (dot line).
**Figure 4:**

SDS-PAGE electrophoresis pattern of chimeric antibody. SPG purified c-1T0 and mouse 1T0 IgG preparations were separated on 10% gel in non-reducing (1 and 3) and reducing (2 and 4) conditions. MW: molecular weight ladder (Sinaclon, Iran).
Western-blot analysis of chimeric antibody. Non-reduced (NR) and reduced (R) forms of rHER2 extracellular protein was separated on 10% SDS-PAGE gel and transferred onto nitrocellulose membrane. The membrane was blotted with c-1T0 (Lanes 3 and 6), mouse 1T0 (Lanes 2 and 5) and Trastuzumab (Lanes 1 and 4) and then visualized by ECL, as described in Materials and Methods.
Figure 6:

Experimental dose-response curves for mouse 1T0 (A) and chimeric c-1T0 (B) monoclonal antibodies at three different concentrations of recombinant extracellular HER2 protein.

Figure 7:

Detection of binding activity of chimeric c-1T0 antibody to HER2-expressing BT-474 cells by flow cytometry. BT-474 were harvested and stained with mouse 1T0 and chimeric c-1T0. Irrelevant mouse mAb (mlgG), irrelevant human IgG (hlgG) and Trastuzumab were used with...
the same concentration as negative and positive controls, respectively. Figures represent percent of positive cells.
Assessment of tumor growth inhibition activity of chimeric antibody by XTT assay. Serial concentrations of c-1T0 antibody were added to BT-474 cells. Cells were then incubated with XTT and OD was measured. Percent of inhibition was measured as described in Materials and Methods. Serial concentrations of mouse 1T0 and Trastuzumab were employed as controls.
Assessment of tumor proliferation inhibition activity of chimeric antibody by radioactive thymidine incorporation assay. Serial concentrations of antibody were added to BT-474 cells. Cells were then incubated with 3H thymidine and radioactive thymidine incorporation was subsequently measured by a beta-counter. Percent of proliferation inhibition was calculated as described in Materials and Methods.

* An interesting review on the mechanisms contributing to resistance to HER2 and EGFR antibody-based therapies.


** The combination of Pertuzumab and Trastuzumab induces remission in patients who had experienced progression during prior trastuzumab therapy.


** A cocktail of pertuzumab, trastuzumab and docetaxel significantly prolonged progression-free survival, with no increase in cardiac toxic effects


