Human Tumor Growth Suppression by Apoptosis Induced with Anti-ErbB-2 Chimeric Monoclonal Antibody

Shigeru Sasaki,1 Masayuki Tsujisaki,1 Tsuneharu Jinnohara,1 Tadao Ishida,1 Masuo Sekiya,1 Masaaki Adachi,1 Shuji Takahashi,2 Yuji Hinoda1 and Kohzoh Imai1

1First Department of Internal Medicine and 2First Department of Pathology, Sapporo Medical University, S-1 W-16, Chuo-ku, Sapporo 060

We established an anti-ErbB-2 mouse-human chimeric monoclonal antibody (MoAb), CH401, which was able to kill cancer cells overexpressing the ErbB-2 protein in vitro. The analysis of the killing mechanism indicated that MoAb CH401 might be the first anti-ErbB-2 mouse-human chimeric MoAb which can induce the apoptosis of cancer cells, since morphological changes and DNA fragmentation were recognized in MoAb CH401-treated cells. The ErbB-2 receptor appears to have two opposing functions: acting as a receptor both for a growth factor and for an apoptotic factor. Our results indicate that MoAb CH401 treatment may prove to be very useful for cancer therapy.

Key words: ErbB-2 — Apoptosis — G1 arrest — Chimeric monoclonal antibody — Growth factor receptor

The human erbB-2 gene product, which encodes a growth factor receptor with intrinsic tyrosine kinase activity, is expressed in a variety of adenocarcinomas.1–5 Many anti-ErbB-2 monoclonal antibodies (MoAbs) have been developed.6–8 ErbB-2 protein has the following advantages as a target molecule: [1] it is a receptor-type molecule expressed on the cancer cell surface; [2] the expression level in normal adult human tissues is very low; [3] the shedding level of this antigen as well as the incidence of positivity for circulating ErbB-2 antigen in gastrointestinal malignant diseases is very low. Accordingly, the blocking effect of circulating antigen on the function of anti-ErbB-2 MoAb should be negligible.9 Overexpression on cancer cells and extracellular accessibility of the ErbB-2 protein enable it to be a potential target, so anti-ErbB-2 MoAbs seem promising for immunotherapy.

Four anti-ErbB-2 mouse MoAbs have been established in our laboratory and characterized.9 The epitopes recognized by these MoAbs are in an extracellular domain within amino acids 292–370 of the human c-erbB-2 molecule. In vitro anti-tumor activity by these MoAbs has been measured and one of them has been selected and chimerized. A mouse-human chimeric MoAb, CH401, for ErbB-2 protein was established by a procedure using a heavy chain loss mouse mutant hybridoma and a human immunoglobulin expression vector, as described elsewhere.9, 10 Here we show that the anti-tumor activity of this MoAb is due to the induction of apoptosis of cancer cells.

MATERIALS AND METHODS

MoAbs The anti-ErbB-2 mouse MoAbs E907 and E401, and the mouse-human chimeric MoAb CH401, each of which recognizes an epitope existing within amino acids 292–370 of the extracellular domain of the human ErbB-2 receptor,9, 10 were used. These antibodies were of the IgG1 subclass. We developed a mouse-human chimeric form (CH401) of mouse MoAb E401.10 The affinities of mouse MoAb E401 and chimeric MoAb CH401 were similar. The specificity of mouse MoAb E401 and that of chimeric MoAb CH401 have also been confirmed to be the same.10 The epitopes recognized by mouse MoAbs E907 and E401 are different.8, 10 The mouse-human chimeric anti-intercellular adhesion molecule-1 (ICAM-1) MoAb chHA58 of class IgG1 was used as a control.11

Cells and cell culture SV22 cells (NIH-3T3 cell lines that had been transfected with erbB-2 cDNA) and untransfected NIH-3T3 cells, human gastric carcinoma JRST cells, breast carcinoma SK-BR-3 cells, MRKnu-1 cells, MDA-MB-231 cells, and myeloid leukemia K562 cells were maintained in RPMI 1640 (GIBCO, Grand Island, NY) culture medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM l-glutamine and gentamycin sulfate (25 mg/ml). These cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo).

Expression of ErbB-2 receptor on cultured cells The 125I-labeled MoAb CH401, E401, E907 or chHA58 (1×10⁵ cpm/well) was incubated with the cultured cells (K562, NIH-3T3, MDA-MB-231, MRKnu-1, SK-BR-3, SV22 and JRST) (1×10⁴ cells/well) in multiwell plates for 2 h at
4°C. The plates were washed with phosphate-buffered saline (PBS), the wells were cut out, and the radioactivity was counted in a γ-counter.

**Cytolytic activity of anti-ErbB-2 MoAbs in vitro** K562, NIH-3T3, MDA-MB-231, MRKmu-1, SK-BR-3. SV22 and JRST cells were plated at a density of 4×10⁴ cells/ml. After the cells had been allowed to adhere for 2 h, medium containing MoAb CH401, E401, E907 or chHA58 (final concentration of 50 nM) was added to each well. After 48-h incubation, the cells were detached using trypsin, stained with trypan blue (0.5%) and counted.

**Internalization of anti-ErbB-2 MoAbs** Uptake of 125I-labeled MoAb CH401, E401 or E907 into SV22 cells was measured using the pH 2.8 desorption method. Live cells (SV22, 1×10⁴ cells/ml) were incubated with the 125I-labeled MoAb CH401, E401 or E907 (1×10⁶ cpm/50 µl) at either 0°C or 37°C. Duplicate aliquots (200 µl) were removed at intervals from 0 to 120 min after the addition of the labeled MoAb CH401, E401 or E907. The cell pellets were obtained, washed and resuspended in 1 ml of glycine buffer (0.05 M glycine-HCl, pH 2.8 containing 0.1 M NaCl), then kept for 20 min at room temperature. During this time, the samples were counted in a γ-counter to determine the total amount of bound radioactivity. The cells were washed again and the residual radioactivity was counted to determine the amount of MoAb CH401, E401 or E907, which was not dissociated by the acid buffer treatment. The percentage of internalization was determined by subtracting the percentage of residual radioactivity measured at 0°C from that measured at 37°C.

**Modulation of ErbB-2 expression by anti-ErbB-2 MoAbs** SV22 cells (2×10⁶/well) were plated in 96-well round-bottomed plates (Costar, Cambridge, MA) in medium containing 10% FCS and cultured for 24 h. Fifty nanomolarities MoAb CH401, E401 or E907 was then added to each well. The mixtures were cultured at 37°C. After 1, 2, 4 or 24 h, the cells were washed with PBS. After internalization of the MoAb, cells were incubated for 2 h at 4°C with 50 µl of 125I-labeled MoAb E919 (10⁶ cpm/50 µl), whose epitope is different from those of MoAbs CH401, E401 and E907. The cells were washed, and residual radioactivity was counted in a γ-counter to determine the amount of the ErbB-2 receptor that remained at the cell surface.

**Analysis of cell morphological changes** SV22 cells (0.4×10⁶ cells in 1 ml of medium/chamber) were incubated with 50 nM MoAb chHA58 or CH401 on polystyrene chamber slides for 48 h. After removal of the medium, the cells were stained with Giemsa’s solution. The cell morphology was observed with a light microscope and photographed.

**Fluorescence microscopical analysis for in situ staining of apoptotic cells** For in situ staining of apoptotic cells, the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (d-UTP)-biotin nick end labeling (TUNEL) method was applied. SV22 cells were incubated with MoAb chHA58 or CH401 (final concentration of 50 nM) on polystyrene chamber slides for 48 h. The medium and MoAb were removed, and then the cells were fixed in 4% paraformaldehyde and permeabilized in 0.1% Triton X-100. After being washed, the cells were stained using a commercially available kit (In Situ Cell Death Detection Kit; Boehringer Mannheim Corp., Indianapolis, IN). These slides were mounted with mounting medium under coverslips and viewed by fluorescence microscopy.

**Electrophoretic analysis** After incubation with 50 nM MoAb chHA58, E907, CH401 or with serum withdrawal medium for 24 h, SV22 cells were centrifuged, washed with PBS and incubated in a lysis buffer (10 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1% SDS and 0.5 µg/ml of protease K) at 37°C for 24 h. DNA was extracted with phenol/chloroform/isoamyl alcohol as described previously with slight modifications, and treated with 100 µg/ml of RNase A for 1 h at 37°C. DNA precipitated with ethanol at −20°C overnight was pelleted by centrifugation and was dissolved in TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA). DNA concentrations were estimated by measuring the A260, and 2.5 µg of DNA was transferred onto 1.2% agarose gels (FMC BioProducts, Rockland, ME), after which electrophoresis was performed in TAE buffer (20 mM Tris-acetate and 1 mM EDTA) for 12 h at 25 V. The gels were stained with 1 µg/ml ethidium bromide and photographed under UV light.

**Flow cytometric analysis** (A) SV22 cells were incubated in RPMI1640 medium alone, or supplemented with 10% FCS and 50 nM MoAb CH401, E907 or chHA58 for 24, 48 and 72 h. Cells were stained using the TUNEL method as described above and analyzed with a FACscan flow cytometer (Becton-Dickinson, Mountain View, CA). (B) SV22 cells were incubated in RPMI 1640 medium alone, or supplemented with 10% FCS and 50 nM MoAb CH401, E907 or chHA58 for 12 and 24 h. After pretreatment, the cells were taken up and washed with PBS, and then cultured in RPMI 1640 medium supplemented with 10% FCS without a MoAb up to 48 h. The cells were assayed for apoptosis by the TUNEL method as described above.

**Analysis of cell cycle** SV22 cells were incubated in RPMI 1640 medium alone, or supplemented with 10% FCS and 50 nM MoAb CH401, E907 or chHA58 for 24, 48 and 72 h. After centrifugation and washing, 1 ml of nuclear stained solution (NSS, 5 mg of propidium iodide, 0.1 mg of sodium citrate, 0.03 ml of Triton-X 100, and 100 ml of distilled water) was mixed with the cell pellets. Following centrifugation, 250 µl of NSS was added to these cell pellets and mixed again. Two-hundred and fifty milliliters of RNase solution (500 U/ml) was then added to the resuspended cells for 15 min at room tem-
per temperature in the dark and 500 µl of PBS was added. The cells were analyzed by flow cytometry.

**Analysis of tyrosine phosphorylation** SV22 cells (1×10^6/100 µl reaction volume) were incubated with MoAb chHA58 (50 nM) or CH401 (50 nM) for 1 and 5 min. Reactions were terminated by the addition of 1 ml of ice-cold stop buffer (10 mM Tris, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 30 mM sodium pyrophosphate, 100 mM Na_3VO_4, 200 µl PMSF, 10 mg/ml leupeptin and 5 mg/ml aprotinin, pH 7.4). After centrifugation, the pellets were lysed in 40 µl of stop buffer supplemented with 1% Triton-X100. Following centrifugation, detergent-soluble proteins were resolved by 10% polyacrylamide/SDS gel electrophoresis (under reducing conditions) before electrophoretic transfer to a polyvinyl difluoride membrane (Millipore, Bedford, MA). The membrane was blocked with 4% bovine serum albumin, 0.5% Tween, and 0.1% azide. Proteins were then probed with anti-phosphotyrosine MoAb (0.1 mg/ml; Upstate Biotechnology, Lake Placid, NY) or anti-ErbB-2 MoAb (2.5 µg/ml; Oncogene Science, Cambridge, MA). The membrane was washed and incubated for 40 min with rabbit anti-mouse IgG (1 µg/ml; Pierce Chemicals, Rockford, IL), then washed again. Immunoreactive proteins were detected with protein A-horseradish peroxidase and the Enhanced Chemiluminescence (ECL) detection system from Amersham (Buckinghamshire, UK).

**RESULTS**

**ErbB-2 expression on cultured cells and cytolitic activity of anti-ErbB-2 MoAbs** The relationship between ErbB-2 expression and killing activity of an anti-ErbB-2 MoAb *in vitro* was investigated. We developed a mouse-human chimeric form (CH401) of mouse MoAb E401 as described in “Materials and Methods.” As shown in Fig. 1, all the ErbB-2-positive human cultured cell lines, MRKnu-1, SK-BR-3, SV22 and JRST, were susceptible to the cytolitic activity of MoAb CH401 and E401, while ErbB-2-negative cell lines, K562, NIH3T3, and MDA-MB-231 were not affected at all. The cytolitic activity was found to occur in a concentration- and time-dependent manner (data not shown). MoAb E907, which recognizes an epitope distinct from that of MoAb E401, and anti-ICAM-1 mouse-human chimeric MoAb chHA58 used as a control, did not have cytolitic activity at all. Although E401 and E907 had similar binding activities, their cytolitic activities were markedly different. It is noteworthy that, in response to CH401 or E401 treatment, erbB-2 gene-transfected SV22 cells were killed but paren-

![Fig. 1. ErbB-2 expression on cultured cells (A) and cytolitic activities of MoAbs CH401, E401, E907, and chHA58 (B).](image-url)

- A. Cultured cells were incubated for 2 h at 4°C with ^125^I-labeled MoAbs. Following washing, bound radioactivity of cells was counted in a γ counter.  
- B. Cells were incubated with MoAbs. Aliquots of cells were removed at 48 h and viable cells were determined by trypan blue staining. The percentage cell survival was calculated as (the viable cell number/non-treated cell number per milliliter)×100. Data are shown as the mean value±SD of three independent experiments. All the ErbB-2-positive cells were susceptible to cytolitic activity of MoAb CH401 or E401, while the ErbB-2-negative cells were not. □ MoAb CH401, □ MoAb E401, □ MoAb E907, □ chHA58.
Anti-ErbB-2 Monoclonal Antibody Induces Apoptosis

Fig. 2. Internalization of anti-ErbB-2 MoAb (A) and down-modulation of ErbB-2 receptors by anti-ErbB-2 MoAbs (B). A, Uptake of 
$^{125}$I-labeled MoAb CH401 (○), E401 (●) or E907 (□) into SV22 cells was measured at 37°C. The percentage internalization of the
MoAbs was evaluated after pH 2.8 desorption by subtracting the background uptake at 0°C. B, Down-regulation of cell surface ErbB-
2 protein by MoAb CH401 (○), E401 (●) or E907 (□) was investigated. The percentage modulation was calculated by dividing the
cpm of the binding of MoAb E919 at the indicated time by the total cpm initially bound to the cell surface. (Anti-ErbB-2 MoAb E919
recognizes an epitope distinct from those of MoAbs CH401, E401 and E907.) Data are shown as the mean value±SD of three indepen-
dent experiments. For MoAbs CH401, E401 and E907, the levels of internalization and down-modulation were almost the same.

Fig. 3. Morphological analysis (A) and DNA fragmentation (B) of SV22 cells treated with MoAb CH401. A, SV22 cells were incu-
bated with 50 nM MoAb CH401 or chHA58 (as a control) on chamber slides for 48 h. The cells were stained with Giemsa’s solution.
Condensation of nuclei corresponding to apoptosis in MoAb CH401-treated cells was observed. B, Cells were stained by the TUNEL
method, and nuclear fragmentation in cells was observed. (×40)
tal NIH-3T3 cells were not. These results indicated that the anti-tumor effects of CH401 and E401 were specific to ErbB-2-positive cells and that they were cytolytic.

**Internalization of anti-ErbB-2 MoAbs** At 60 min after the MoAb treatment, uptakes of 125I-labeled MoAb CH401, E401 and E907 into SV22 cells were 23.7%, 24.6% and 21.9%, respectively, as shown in Fig. 2A. These MoAbs were similar in terms of internalization into ErbB-2-positive cells.

**Modulation of ErbB-2 expression by anti-ErbB-2 MoAbs** To assess whether MoAb CH401, E401 or E907 could remove ErbB-2 protein from the cell surface, we examined ErbB-2 expression on SV22 cells after the addition of MoAb CH401, E401 or E907 to cell cultures at 37°C during the indicated incubation periods. As shown in Fig. 2B, addition of CH401, E401 or E907 to cultured SV22 cells caused rapid down-modulation of cell surface ErbB-2 expression up to 24 h. There was no significant difference in the down-modulation percentages induced by these MoAbs.

**Induction of apoptosis by anti-ErbB-2 MoAb** To study the anti-tumor mechanism of MoAb CH401, morphological changes of the dead cells after MoAb CH401 treatment were investigated. SV22 cells treated with CH401 were greatly shrunken and chromatin condensation was observed as shown in Fig. 3A. To determine whether SV22 cell death involved apoptosis, we assessed internucleosomal DNA fragmentation after CH401 treatment (Fig. 4). Analysis of DNA extracts by agarose gel electrophoresis showed an internucleosomal DNA ladder, which is characteristic of apoptosis, while no ladder formation was seen in the assay of cells treated with MoAb chHA58 or E907 or with serum withdrawal medium (serum-free) up to 24 h, as shown in Fig. 4. This DNA ladder formation showed a concentration- and time-dependent pattern.
Anti-ErbB-2 Monoclonal Antibody Induces Apoptosis

To confirm these results, SV22 cells treated with CH401 were stained by the TUNEL method. Apoptotic cells were detected after the treatment with CH401 (Fig. 3B). Additionally, marked DNA fragmentation was recognized in CH401-treated cells in a time-dependent manner, as shown in Fig. 5A. In contrast, no fragmentation was noted in chHA58-treated cells. Furthermore, cultured cells with MoAb E907 or without serum showed little fragmentation even at 72 h.

Next, to determine whether the effect of CH401 treatment was cytostatic or cytotoxic, reversible or irreversible, cells were shifted to a 10% serum-containing medium without CH401 after treatment with CH401 for 12 or 24 h, and the DNA fragmentation of cells was analyzed (Fig. 5B). Twelve-hour preincubation with CH401 was sufficient to induce DNA fragmentation. These data indicate that 12-h incubation with CH401 was sufficient to trigger apoptosis. In contrast, no detectable DNA fragmentation of cells was observed in the absence of serum or after preincubation with MoAb E907 or chHA58 for 12 h and 24 h.

Cell cycle analysis To explore whether MoAb CH401 may act by interfering with cell cycle traversal in SV22 cells, flow cytometric analysis was performed (Fig. 6). The results indicate that SV22 cells accumulated in the G1 phase after exposure to CH401 for 24 h, as shown in Fig. 6. On the other hand, when the cells were incubated in the absence of serum, with E907 or with chHA58, the percentage of G0/G1 phase cells was essentially unchanged for 24 h. Furthermore, the percentage of apoptotic cells was more than 5 times greater after MoAb CH401 treatment (81.5%) than after incubation without serum (15.1%). The G1 phase growth arrest of SV22 cells treated with serum-withdrawal medium was first detected at 48 h, but the G1 population of cells increased slowly compared with that of MoAb CH401-treated cells.

Analysis of tyrosine phosphorylation To assess whether tyrosine kinase was activated after MoAb CH401 treatment, SV22 cells were incubated with the MoAb for 1 or 5 min and then subjected to anti-phosphotyrosine immunoblot analysis. The treatment of SV22 cells induced rapid (within 1 min) tyrosine phosphorylation of ErbB-2 protein (Fig. 7).

DISCUSSION

Many MoAbs to the ErbB-2 receptor have been generated, and reported as tumor-development inhibitory
reagents.2-15 Some of them have an inhibitory effect on tumor growth per se, independent of the host’s immunity, such as complement-mediated cytotoxicity or antibody-dependent cellular cytotoxicity.16-20 The mechanism of tumor growth inhibition by anti-ErbB-2 MoAbs previously reported was considered not to be a killing effect on cells, but to be a purely cytostatic one.20 Consequently, its functional activity was insufficient to affect optimal tumor destruction. Two kinds of anti-tumor effects of anti-ErbB-2 MoAbs have been identified in the literature.20-23 One is a continuous down-modulation of ErbB-2 receptors, which results in the inhibition of ligand binding to ErbB-2 receptors.29 The other is an internalization of the antibody into cells without a significant effect on receptor down-regulation, where tumor-inhibitory potential correlates with the kinetics of the cellular uptake of antibodies by endocytosis.21, 22 In contrast, another study showed that growth inhibition did not always correlate with internalization of ErbB-2 receptors, based on a comparison of the internalization rate and clonogenic growth of tumor cells in the presence of effective and ineffective antibodies.23 The induction of cellular differentiation by MoAbs was also proposed as one mechanism of their tumor-inhibitory effects.16 It is conceivable that several independent mechanisms may lead to tumor-growth inhibition, but at the present time, there is still little knowledge of the precise mechanisms that govern the induction of tumor growth inhibition by anti-ErbB-2 MoAbs.

The cytotoxic mechanism of CH401 was independent of down-regulation of ErbB-2 receptors or of internalization of the MoAb, since our study of the therapeutic effects of CH401 indicated that the percentages of these reactions were rather low as compared with those in the cases of other anti-ErbB-2 MoAbs previously reported.23, 24 Furthermore, the levels of these reactions to CH401 were almost the same as those to E907, which did not have any cytolytic activity, as shown in Figs. 1 and 2.

In this study, the analysis of tumor growth inhibition by CH401 made it clear that the cytotoxicity was induced by programmed cell death, as judged from morphological observation (Fig. 3A), DNA fragmentation study (Figs. 3B, 4 and 5) and the cell cycle arrest of the MoAb-treated cells (Fig. 6). Since 12-h preincubation with CH401 was sufficient to induce apoptosis of cells, the anti-tumor effect of CH401 was considered to be cytocidal rather than cytostatic. These results strongly suggest that the binding of CH401 to the ErbB-2 receptor not only caused a ligand-binding block, but also induced rapid tyrosine phosphorylation of ErbB-2 protein, resulting in signal activity to induce apoptosis, as shown in Fig. 7.

The apoptosis induced by CH401 was different from that caused by the anti-Fas antibody, since the apoptosis in the Fas antigen pathway is rapidly (within several hours) induced by a low dose (several ng/ml) of antibody and is independent of the cell cycle.25 Based on the facts described above, CH401 was estimated to induce the apoptotic effect through a signaling pathway different from that of the anti-Fas antibody. It is known that the binding of anti-epidermal growth factor (EGF) receptor antibody to cells results in G1 phase growth arrest and apoptosis because of the inaccessibility of EGF to the receptor,26 which is equivalent to growth factor withdrawal. To discover the difference between the anti-EGF receptor antibody and CH401, DNA fragmentation was compared after cells had been pre-treated with CH401 or pre-incubated in serum-free medium. Twelve-hour pretreatment with CH401 was sufficient to induce apoptosis and this reaction was irreversible, whereas 24-h preincubation in serum-free medium was not sufficient and was reversible (Fig. 5B). Furthermore, the level of phosphotyrosylated proteins of the ErbB-2 receptor increased just after a 1-min treatment with CH401, as shown in Fig. 7. These data suggested that CH401, bound to an epitope distinct from the ligand binding site, activated the ErbB-2 receptor and was able to induce apoptosis of cells via a signaling pathway. Therefore, the ErbB-2 receptor appears to have two opposing functions: stimulating cell proliferation by ligand binding to the receptor and triggering the apoptotic program by binding to a distinct epitope of the receptor. These results suggest that there might exist an unknown ligand for the ErbB-2 receptor which can bind and induce apoptosis by signaling activity.

Our current study focused on defining the intracellular signal transduction pathway that induced apoptosis after the binding of CH401 to the ErbB-2 receptor and determining the distinct epitope recognized by CH401, which is the binding site of an unknown apoptotic factor. In the present study, two anti-ErbB-2 MoAbs, CH401 and E907, were selected and compared to each other. Although there was no appreciable difference of internalization rate or down-regulation of ErbB-2 receptors between these MoAbs, CH401 showed a strong anti-tumor effect and induction of apoptosis and E907 did neither. MoAbs CH401 and E907 recognized different but similar spatially close epitopes on the ErbB-2 receptor. Consequently, CH401 is unique, and should be distinguished from the other anti-ErbB-2 MoAbs reported before, including E907. We speculate that the difference of epitopes may explain the duality of the ErbB-2 receptor; one for a growth factor and the other for an apoptotic factor. Mouse-human chimeric MoAb CH401 has therapeutic potential for ErbB-2-overexpressing cancers, since it binds to a tumor-associated target molecule (oncogene product ErbB-2 receptor) and, moreover, induces ErbB-2-specific programmed cell death. This approach may be particularly valuable as a new type of cancer therapy.
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