British Journal of Cancer (2000) **82**(1), 46–51

Article no. bjoc.1999.0875

Anti-HER2 antibody enhances the growth inhibitory effect of anti-oestrogen on breast cancer cells expressing both oestrogen receptors and HER2

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Summary Anti-oestrogen is effective for the treatment of oestrogen receptor (ER)-positive breast carcinomas, but most of these tumours become resistant to anti-oestrogen. It has been suggested that anti-oestrogen therapy may induce a HER2 signalling pathway in breast cancer cells and this may cause resistance to anti-oestrogen. Thus, it is conceivable that combined therapy with anti-oestrogen and anti-HER2 antibody might be more effective. In the present study, we investigated the effect of combined treatment with a humanized anti-HER2 monoclonal antibody, rhumAbHER2 (trastuzumab), and an anti-oestrogen, ICI 182,780, on the cell growth of three human breast cancer cell lines which respectively express different levels of ER and HER2. The combined treatment enhanced the growth inhibitory effect on ML-20 cells, which express a high level of ER and a moderate level of HER2, but showed no additive effect on either KPL-4 cells, which express no ER and a moderate level of HER2, or MDA-MB-231 cells, which express no ER and a low level of HER2. It is also suggested that both the antibody and anti-oestrogen induce a G1–S blockade and apoptosis. These findings indicate that combined treatment with anti-HER2 antibody and anti-oestrogen may be useful for the treatment of patients with breast cancer expressing both ER and HER2. © 2000 Cancer Research Campaign

Keywords: anti-HER2 antibody; anti-oestrogen; breast cancer; additive effect; apoptosis

HER2/c-erb B-2 is the homologue of the rat proto-oncogene *neu* (Schecter et al, 1984) and is located on chromosome 17q21 (Coussens et al, 1985; Fukushige et al, 1986). It encodes a 185-kDa transmembrane glycoprotein receptor (p185HER2) that has intrinsic tyrosine kinase activity (Maguire et al, 1989). An anti-HER2 monoclonal antibody, rhumAbHER2 (trastuzumab), is a humanized form of the murine 4D5 antibody which is directed to the external domain of HER2 and inhibits the growth of cells overexpressing HER2 (Carter et al, 1992; Tokuda et al, 1996; Baselga et al, 1998). Recently, clinical phase studies with rhumAbHER2 as a single agent were conducted in patients with metastatic breast cancer overexpressing HER2, and 12 and 15% response rates were reported (Baselga et al, 1996; Cobleigh et al, 1998). In addition, two clinical studies with chemotherapy combined with rhumAbHER2 were conducted and demonstrated that the addition of rhumAbHER2 to chemotherapy significantly increased not only the response rate but also the duration of response (Pegram et al, 1998; Slamon et al, 1998). Very recently, this antibody has been approved for clinical use with patients having HER2-overexpressing breast cancer in the United States.

It has been reported that either amplification of the *HER2* proto-oncogene or overexpression of HER2 has been observed in 25–30% of primary breast cancers and has correlated with a poor prognosis (Slamon et al, 1987; Wright et al, 1989). Approximately half of these cancers were oestrogen receptor (ER)-positive (McCann et al, 1991). It has been also suggested that, in the adjuvant setting, an anti-oestrogen, tamoxifen, may worsen disease-free survival of patients with breast cancer expressing both ER and HER2 (Bianco et al, 1998).

Anti-oestrogens are well known to be effective for the treatment of ER-positive breast carcinomas. Unfortunately, however, most of them eventually develop resistance to anti-oestrogens. It is also known that anti-oestrogens are less effective for the treatment of patients with breast cancer overexpressing HER1 or HER2 together with ER (Ross and Fletcher, 1998). Previous experiments have indicated that oestrogen down-regulates HER2 in ER-positive breast cancer cells and anti-oestrogen reverses the oestrogen-induced decrease in HER2 expression (Read et al, 1990). Thus, it is conceivable that anti-oestrogen may activate a HER2-signalling pathway and may cause resistance to anti-oestrogen. If so, it seems reasonable to use both anti-oestrogen and anti-HER2 antibody for the treatment of patients with breast cancer expressing both ER and HER2.

In the present study, we investigated the effects of combined treatment with a steroidal, pure anti-oestrogen, ICI 182,780, and a humanized anti-HER2 monoclonal antibody, rhumAbHER2, in three breast cancer cell lines which respectively express different levels of ER and HER2 to explore the possible additive effect of the combined treatment.

Received 12 April 1999
Revised 21 June 1999
Accepted 22 June 1999

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MATERIALS AND METHODS

Human breast cancer cell lines

The ML-20 cell line is a transfectant of the MCF-7 cell line with the pCHC β-Gal expression vector encoding the bacterial *lacZ* gene (Kurebayashi et al, 1993). The MDA-MB-231 cell line was kindly provided by Dr Robert B Dickson (Lombardi Cancer Center, Georgetown University Medical Center, Washington, DC, USA). The KPL-4 cell line was established in our laboratory and its characterization has been published elsewhere (Kurebayashi et al, 1999). This cell line was derived from the malignant pleural effusion of a Japanese patient with recurrent breast cancer. All of the cell lines were routinely cultured in Dulbecco's modified Eagle's medium (DMEM; ICN Biochemicals, Costa Mesa, CA, USA) supplemented with 5% fetal bovine serum (FBS; ICN Biochemicals Japan, Osaka, Japan).

Reagents

The steroidal, pure anti-oestrogen, ICI 182,780, was kindly provided by Zeneca Pharmaceuticals (Macclesfield, UK). ICI 182,780 was dissolved with 100% ethanol and added to the medium at a final ethanol concentration of 0.1%. The humanized anti-ErbB-2 monoclonal antibody, rhumAbHER2, was kindly provided by Mitsubishi Chemical Co. (Tokyo, Japan).

ER analysis

Oestrogen receptor (ER) levels in the cell pellet of the three cell lines were measured by an enzyme immunoassay using ER-EIA kits (Dinabot, Tokyo, Japan) following the manufacturer's recommendation.

Flow cytometric analysis of HER family member expression

Approximately 1×10^6 cells per sample were harvested with trypsin, stained with first antibodies for 1 h and washed with phosphate-buffered saline (PBS) twice. Then they were stained with secondary fluoroscein isothiocyanate (FITC)-antimouse antibody (Becton Dickinson, San Jose, CA, USA) for 30 min and washed with PBS twice. The level of each HER family member was analysed by a flow cytometer (Becton Dickinson). The first antibodies were: anti-HER1 monoclonal antibody (Oncogene Science, Uniondale, NY, USA), anti-HER2 monoclonal antibody (Ab-2, NeoMarker, Freemont, CA, USA), anti-HER3 monoclonal antibody (NeoMarker) and anti-HER4 monoclonal antibody (NeoMarker). The level of each HER family member was expressed as the product of the specific peak fluorescence intensity divided by the background peak intensity (Kurebayashi et al, 1999).

Cell growth in vitro

Three cell lines were respectively plated at a density of 5×10^4 cells per well in 12-well plates (SB Medical, Tokyo, Japan) and grown in DMEM supplemented with 5% fetal bovine serum (FBS) at 37°C in a 5% carbon dioxide atmosphere for 2 days. The cells were then washed with PBS and incubated with phenol red-free RPMI-1640 medium (Gibco-BRL, Bethesda, MD, USA) supplemented with 2% dextran-coated charcoal-stripped FBS (Kurebayashi et al, 1998) plus 10^{-9}–10^{-7} M ICI 182,780, 0.1–10 μg ml^{-1} rhumAbHER2 or 10^{-7} M ICI 182,780 and/or 10 μg ml^{-1} of rhumAbHER2. The culture medium was changed every other day. Triplicate wells were trypsinized 4 days after switching the culture medium and the cell number was counted with a Coulter counter (Coulter Electronics, Harpenden, UK).

Cell cycle analysis

Three cell lines were plated at a density of 5×10^4 cells per well in 12-well plates and grown in DMEM supplemented with 5% FBS for 3 days. Then the cells were washed with PBS and treated with phenol red-free RPMI-1640 medium supplemented with 2% dextran-coated charcoal-stripped FBS plus 10^{-7} M ICI 182,780, 10 μg ml^{-1} rhumAbHER2 or both. Triplicate wells were trypsinized, harvested a day after and stained with propidium iodide using the CycleTest Plus DNA Reagent Kit (Becton Dickinson). Flow cytometry was performed with a FACSort flow cytometer (Becton Dickinson), and the DNA histogram was analysed by a CELLQuest Version 1.2.2 (Becton Dickinson).

Detection of apoptosis by flow cytometry

The ML-20 and KPL-4 cell lines were plated at a density of 4×10^5 cells per well in 6-well plates (SB Medical, Tokyo, Japan) and grown in DMEM supplemented with 5% FBS for 2 days. Then the cells were washed with PBS and treated with phenol red-free RPMI-1640 medium supplemented with 2% dextran-coated charcoal-stripped FBS plus 10^{-7} M ICI 182,780, 10 μg ml^{-1} rhumAbHER2 or both. Duplicated wells were trypsinized and harvested 3 days after. The percentages of apoptotic cells were measured with a FACSCaliber flow cytometer (Becton Dickinson Immunocytometry Systems, Mansfield, MA, USA) using an in situ cell death detection kit (Boehringer Mannheim, Germany) according to the manufacturer's recommendations as described elsewhere (Otsuki et al, 1998).

Detection of apoptosis by immunocytochemistry

The ML-20 and KPL-4 cell lines were plated at a density of 1×10^6 cells in T-75 flasks (Corning Japan, Tokyo, Japan) and grown in DMEM supplemented with 5% FBS for 3 days. Then the cells were washed with PBS and treated with phenol red-free RPMI-1640 medium supplemented with 2% dextran-coated charcoal-stripped FBS plus 10^{-7} M ICI 182,780, 10 μg ml^{-1} rhumAbHER2 or both. Duplicated flasks were trypsinized and harvested 2 days after. The cell pellets were fixed with 5% buffered-formalin and embedded in paraffin. DNA breaks in the sections were detected by nick end labelling using an ApopTag in situ apoptosis detection kit (Oncor Inc., Gaithersburg, MD, USA) as described elsewhere (Kuwashima et al, 1996). The areas of most intense staining were identified in each sample, and approximately 500 cells per sample were examined. The percentage of cells showing positive staining was calculated in each sample.

Statistical analysis

ER content, percentages of control cell numbers, percentages of the S phase or G0/G1 phase of the cell cycle and percentages of apoptotic cells were expressed as means ± s.e.m. These values for

Table 1 Comparison of expression levels of HER family members in three human breast cancer cell lines

	ML-20	KPL-4	MDA-MB-231
HER1	2	2	55
HER2	14	13	5
HER3	21	19	2
HER4	4	1	1

The expression of each member was measured by flow cytometric analysis as described in the Materials and Methods. Each value represents the specific peak fluorescence intensity divided by the background peak intensity.

the control and treated groups were compared using analysis of variance (ANOVA) with StatView computer software (ATMS Co., Tokyo, Japan). All the experiments in this study were repeated at least twice and the reproducibility of the results was confirmed.

RESULTS

ER levels and HER family member expression

The ML-20 cells expressed a high level of ER (112 ± 9.9 fmol mg^{-1} protein) but neither the KPL-4 cells nor the MDA-MB-231 cells expressed a detectable level of ER (the limit of detection of the ER assay was 5.0 fmol mg^{-1} protein).

Flow cytometric analysis showed that both the ML-20 cells and the KPL-4 cells expressed a moderate level of HER2 and HER3 and a low level of HER1 and HER4. The MDA-MB-231 cells expressed a low level of HER2, HER3 and HER4 and a high level of HER1 (Table 1).

Growth inhibitory effect of ICI 182,780, rhumAbHER2 or their combination

Both ICI 182,780 (10^{-9}–10^{-7} M) alone and rhumAbHER2 (0.1–10 μg ml^{-1}) alone dose-dependently inhibited the growth of ML-20 cells (Figure 1 A, B). The inhibition rates were 0.40 for 10^{-7} M ICI 182,780 (P = 0.0008 in comparison with the control) and 0.20 for 10 μg ml^{-1} rhumAbHER2 (P = 0.0094). rhumAbHER2 (0.1–10 μg ml^{-1}) also dose-dependently inhibited the growth of KPL-4 cells (Figure 1C). The inhibition rate was 0.14 for 10 μg ml^{-1} rhumAbHER2 (P = 0.0021). No growth inhibitory effect of ICI 182,780 was observed in either KPL-4 or MDA-MB-231 cells. rhumAbHER2 showed no effect on the MDA-MB-231 cell growth (data not shown).

To investigate a possible additive effect of rhumAbHER2 and ICI 182,780, 10 μg ml^{-1} rhumAbHER2 and 10^{-7} M ICI 182,780 were concomitantly administered. The combined treatment inhibited the growth of ML-20 cells more than the respective agent alone (Figure 2A). The inhibition rates were 0.36 for 10^{-7} M ICI 182,780, 0.21 for 10 μg ml^{-1} rhumAbHER2 and 0.55 for their combination. In contrast, no additive effect was observed in KPL-4 and MDA-MB-231 cells (Figure 2 B, C).

Effects of ICI 182,780 and rhumAbHER2 on cell cycle progression and apoptosis

Both ICI 182,780 (10^{-7} M) alone and rhumAbHER2 (10 μg ml^{-1}) alone significantly increased the percentage of the G0/G1 phase

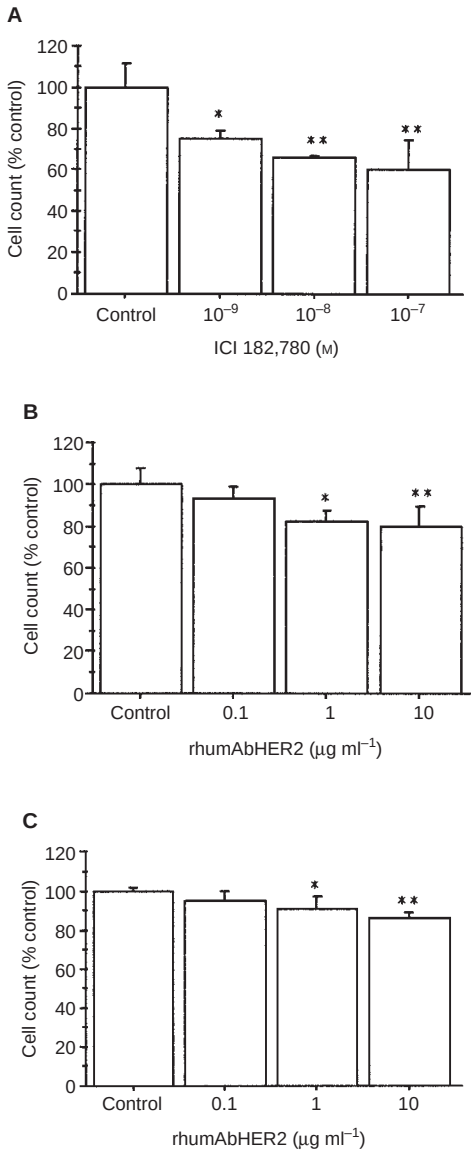

Figure 1 Growth inhibitory effects of ICI 182,780 (10^{-9}–10^{-7} M) in ML-20 cells (**A**), and those of rhumAbHER2 (0.1–10 μg ml^{-1}) in ML-20 cells (**B**) and in KPL-4 cells (**C**). The cells were incubated with medium containing the reagents for 4 days and counted with a Coulter counter. The values represent the percentages of the control and means of triplicate samples. Bars, SE. *P < 0.05 in comparison with the control. **P < 0.01 in comparison with the control

(P = 0.0001 and P = 0.01 respectively, in comparison with the control) and decreased the percentage of S phase (P = 0.0001 and P = 0.0015 respectively) in ML-20 cells. In addition, the combined treatment promoted the G1–S blockade more than the respective agent alone (Table 2). In KPL-4 cells, on the other hand, rhumAbHER2 tended to increase the percentage of G0/G1 phase (P = 0.39) and decrease the percentage of S phase (P = 0.12), but no additive effect of the combined treatment was observed (Table 2).

Both ICI 182,780 (10^{-7} M) alone and rhumAbHER2 (10 μg ml^{-1}) alone also induced apoptosis in the ML-20 cell line. In the flow cytometric analysis, ICI 182,780 alone or rhumAbHER2 alone tended to increase the percentage of TUNEL-positive cells but a

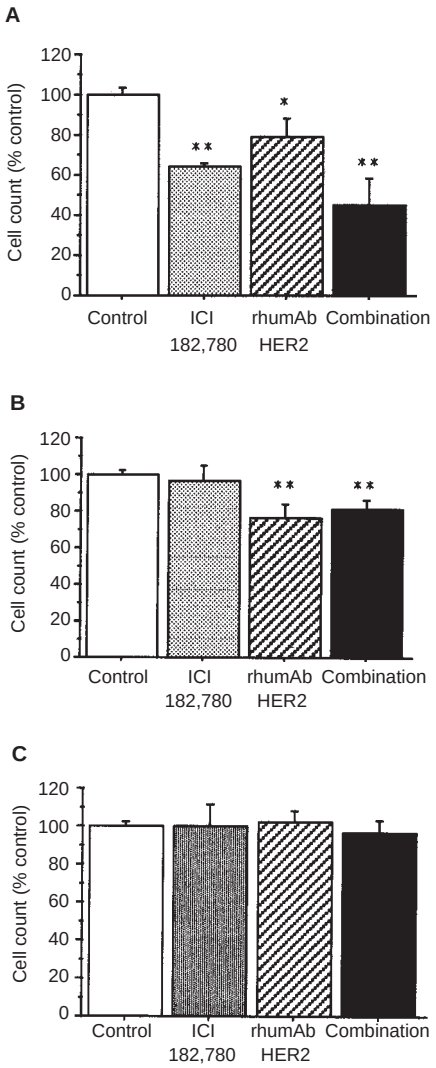

Figure 2 Growth inhibitory effects of ICI 182,780 alone, 10 μg ml^{-1} rhumAbHER2 alone or their combination in ML-20 cells (**A**), KPL-4 cells (**B**) or MDA-MB-231 cells (**C**). The cells were incubated with medium containing the reagents for 4 days and counted with a Coulter counter. The values represent the percentages of the control and means of triplicate samples. Bars, s.e.m. *P < 0.05 in comparison with the control. **P < 0.01 in comparison with the control

Table 2 Effects of ICI 182,780 alone, rhumAbHER2 alone or their combination on cell cycle progression in ML-20 and KPL-4 human breast cancer cell lines

Cell line	Treatment	% S	% G0/G1
ML-20	Control	16.7 ± 0.3	75.6 ± 0.2
	ICI 182,780	7.9 ± 1.1[b]	85.3 ± 1.4[b]
	rhumAbHER2	13.7 ± 1.3[b]	78.4 ± 0.7[a]
	Combination	6.3 ± 0.4[b]	86.6 ± 0.2[b]
KPL-4	Control	25.8 ± 0.5	60.9 ± 0.6
	ICI 182,780	27.8 ± 1.2	59.2 ± 1.0
	rhumAbHER2	23.8 ± 0.8	62.4 ± 1.4
	Combination	24.8 ± 1.3	62.1 ± 2.1

10^{-7} M ICI 182,780 alone, 10 μg ml^{-1} rhumAbHER2 alone or their combination was added to the culture medium and the cells were incubated for 3 days. Percentages of the S phase and G0/G1 of the cell cycle were measured by flow cytometric analysis as described in the Materials and Methods. The values represent means ± s.e.m. of triplicate samples. [a]P < 0.05 in comparison with the control. [b]P < 0.01 in comparison with the control.

Table 3 Effects of ICI 182,780 alone, rhumAbHER2 alone or their combination on apoptosis measured by flow cytometric or immunocytochemical analysis in ML-20 and KPL-4 human breast cancer cell lines

Assay method	Treatment	Cell line	
		ML-20	KPL-4
Flow cytometry	Control	4.9 ± 1.0	4.2 ± 0.7
	ICI 182,780	8.7 ± 2.6	4.2 ± 0.2
	rhumAbHER2	6.4 ± 0.0	4.9 ± 1.1
	Combination	10.5 ± 0.7[a]	4.4 ± 0.9
Immunocytochemistry			
	Control	1.0 ± 0.5	2.7 ± 0.2
	ICI 182,780	4.3 ± 0.8[b]	2.9 ± 0.9
	rhumAbHER2	2.9 ± 1.0[b]	5.0 ± 0.4[b]
	Combination	7.5 ± 1.5[b]	4.7 ± 0.6[b]

10^{-7} M ICI 182,780 alone, 10 μg ml^{-1} rhumAbHER2 alone or their combination was added to the culture medium and the cells were incubated for 2 days. Percentages of apoptotic cells were measured by flow cytometric or immunocytochemical analysis as described in the Materials and Methods. The values represent means ± s.e.m. of triplicate samples. [a]P < 0.05 in comparison with the control. [b]P < 0.01 in comparison with the control.

significant increase was noted with their combination (P = 0.018 in comparison with the control, Table 3). In KPL-4 cells, rhumAbHER2 alone slightly increased the percentage of TUNEL-positive cells (Table 3). In the immunocytochemical analysis, both ICI 182,780 and rhumAbHER2 significantly induced apoptosis (P = 0.0001 and P = 0.0048 respectively) in ML-20 cells. The combined treatment also induced apoptosis more than the respective agent alone (Table 3). In KPL-4 cells, rhumAbHER2 alone significantly induced apoptosis (P = 0.0001, Table 3), but no additive effect of the two agents on apoptosis was observed in KPL-4 cells.

A small difference was observed between the two apoptosis analyses in the percentages of apoptotic cells. The percentages in the flow cytometric analysis tended to be higher than those in the immunocytochemical analysis. These findings suggest the possibility that the immunocytochemical analysis is more specific in detecting apoptotic cells than the flow cytometric analysis.

DISCUSSION

Antioestrogen resistance frequently occurs in hormone-dependent breast cancer following successful treatment with anti-oestrogen. It has been demonstrated that HER2-overexpressing MCF-7 cells which were transfected with a full-length of *HER2* cDNA were no longer sensitive to the anti-oestrogen tamoxifen (Benz el al, 1992). Thus, it has been speculated that HER2 overexpression may be one of the causes of anti-oestrogen resistance in human breast cancer.

Previous reports have suggested a direct interaction between ER and HER2-signalling pathways. It has been demonstrated that oestrogen down-regulates HER2 expression and anti-oestrogen partly reverses this oestrogen-induced HER2 down-regulation (Read et al, 1990). Overexpression of a ligand for HER3 or HER4, heregulin, which also stimulates the HER2-signalling pathway, down-regulates ER-mediated transcription (Tang et al, 1996). Our preliminary data on the regulation of HER2 expression by various

hormones in breast cancer cells also suggest that oestrogen down-regulates HER2 expression and anti-oestrogen up-regulates it (unpublished data). In addition, a recent clinical report suggested that an anti-oestrogen, tamoxifen, improves the outcome of patients with breast cancer without HER2-overexpression, while showing a paradoxical detrimental effect in patients with HER2-positive breast cancer (Bianco et al, 1998). These findings suggest that anti-oestrogen may promote breast cancer cell growth through the HER2-signalling pathway in ER- and HER2-positive breast cancer and prompted us to investigate a possible additive effect of anti-oestrogen and a blocker of HER2-signalling pathway, anti-HER2 antibody, in breast cancer cells.

It has been suggested that concomitant treatment with an anti-oestrogen, tamoxifen, and a murine monoclonal anti-HER2 antibody, 4D5, enhanced the anti-proliferative activity in ER- and HER2-positive BT474 breast cancer cells (Witters et al, 1997). In contrast, results of another experiment indicated that rhumAbHER2, which was used in the present study and a humanized form of 4D5, has no growth-inhibitory effect on MCF-7 cells (Lewis et al, 1993). The ML-20 cell line used in the present study is a transfectant of the MCF-7 cell line with a bacterial *lacZ* gene and its growth characteristics in vitro and in vivo are indistinguishable from the wild-type MCF-7 cell line (McLeskey et al, 1998). We confirmed that ML-20 cells express a high level of ER and a moderate level of HER2 (Table 1). rhumAbHER2 alone (0.1–10 μg ml^{-1}) dose-dependently inhibited the growth of ML-20 cells (Figure 1). The discrepancy in the effects of this antibody may be explained as follows. The phenol red-free medium supplemented with dextran-coated charcoal FBS used in the present study contains less endogenous oestrogen than the medium which was used in the other study. The lower level of oestrogen in our medium may induce HER2 expression in ML-20 cells and may enhance the growth inhibitory effect of rhumAbHER2.

To explore the mechanisms responsible for the growth inhibitory effect of ICI 182,780 and rhumAbHER2, their influences on cell cycle progression and apoptosis were investigated. It has been reported that anti-oestrogen causes a G1–S blockade and induces apoptosis in ER-positive breast cancer cells (Perry et al, 1995; Watts et al, 1995). It is also suggested that a murine anti-HER2 antibody induces apoptosis in HER2-overexpressing cancer cells (Kita et al, 1996). In the present study, ICI 182,780 significantly induced a G1–S blockade and apoptosis in ER-positive ML-20 cells but not in ER-negative KPL-4 cells. rhumAbHER2 also significantly induced a G1–S blockade and apoptosis in ML-20 cells, which express a moderate level of HER2, but not in MDA-MB-231 cells, which express a low level of HER2. In KPL-4 cells, rhumAbHER2 caused a significant induction of apoptosis and a slight G1–S blockade. Interestingly, the combined treatment with ICI 182,780 and rhumAbHER2 enhanced the induction of either a G1–S blockade or apoptosis in ML-20 cells which express both ER and HER2 (Tables 2 and 3). These findings in the present study support the findings in previous reports indicating that either ICI 182,780 alone or rhumAbHER2 alone causes a G1–S blockade and apoptosis in breast cancer cells and this results in the retardation of cell growth.

Combined treatment with rhumAbHER2 and a certain cytotoxic agent, such as cisplatin, paclitaxel or doxorubicin/cyclophosphamide, has been reported to be a promising approach for the treatment of advanced breast cancer (Pegram et al, 1998; Slamon et al, 1998). However, the action mechanisms responsible for their additive effect remain to be elucidated. In addition, an unexpected adverse effect of their combination; that is, cardiotoxicity, has been reported (Slamon et al, 1998). rhumAbHER2 may also enhance the anti-tumour effect of hormonal agents, such as anti-oestrogen, as indicated in this study. Thus, it is conceivable that combined treatment with a HER2-signalling blocker and a hormonal agent might be a new promising therapeutic approach for ER- and HER2-positive breast cancer.

ACKNOWLEDGEMENTS

This work is partly supported by grants from the Ministry of Education, Science, Sports and Culture of Japan (to J Kurebayashi), from the Ministry of Health of Japan (to H Sonoo) and by Research Grants (no. 10–113 to J Kurebayashi and no. 10–307 to H Sonoo) from Kawasaki Medical School.

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