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Radioimmunotherapy and Apoptotic Induction on Ck19-overexpressing Human Cervical Carcinoma Cells with Re-188-mAbCx-99

CHIH-HSIEN CHANG[1,2] , LAI-CHEN TSAI[3] , SHUI-TEIN CHEN[4], CHIOU-CHUNG YUAN[5], MEI-WHEY HUNG[3], BOR-TSUNG HSIEH[6], PEI-LING CHAO[3], TUNG-HU TSAI[7] and TE-WEI LEE[2]

[1]Institute of Pharmacology, National Yang-Ming University, Taipei; [2]Institute of Nuclear Energy Research, Taoyuan; Departments of [3]Medical Research and Education and [5]Obstetrics and Gynecology, Veterans General Hospital, Taipei; [4]Institute of Biological Chemistry, Academia Sinica, Taipei; [6]Chungtai Institute of Health Sciences and Technology, Taichung; [7]National Research Institute of Chinese Medicine, Taipei, Taiwan

Abstract. *Background: The overexpression of Ck19 antigen occurs frequently in human carcinomas. The strategy and mechanism of radioimmunotherapy using Re-188-mAbCx-99 to Ck19 on human cervical carcinoma cells was investigated in this study. Materials and Methods: Using mAbCx-99, the overexpression of Ck19 protein in lysates of cell lines and tissues from various patients' cervixes were verified by immunobinding and immunoblot analysis. The therapeutic effect of Re-188-mAbCx-99 on ME180 cells was examined in vitro by cell proliferation, apoptosis, DNA fragmentation and internucleosomal levels. Results: A relatively high expression of Ck19 was found in all human cervical carcinoma cell lines (4- to 44-fold) and in tissue lysates (26.8- to 79-fold) from patients (31 out of 34) with cervical, endometrial or ovarian carcinomas compared with that of benign or normal control samples. The growth inhibition of ME180, CC7T and Hela cells were significantly higher ($p<0.001$) in the Re-188-mAbCx-99-treated (60-80%) than in the Re-188-MOPCIgG1-treated lines (8-18%) after 72-h treatment. After 48 h of treatment with Re-188-mAbCx-99, ME180 cells significantly exhibited DNA fragmentation and morphological features of apoptosis. This effect markedly elevated the expression of p21, p53 and Bcl-xS protein, while the Mcl-1 and Caspase-8 proteins were down-regulated. Conclusion: We suggest that an elevated Ck19 level is associated with disease stage in most patients with cervical cancer. The therapeutic effect of Re-188-mAbCx-99 was verified through apoptosis on targeting the enriched Ck19 protein of carcinoma cells.*

Cervical carcinoma is a supravaginal disease that is prevalent in many countries. Screening for cervical carcinoma by cervicovaginal cytology indicates that approximately 80% of the current incidence and mortality of this disease occurs in a geographic area of under-served and under-screened women (1). Although several candidate biomarkers have been used to monitor cervical carcinoma (2, 3), the early diagnosis and treatment of cervical carcinoma remains difficult. Cytokeratin (Ck) fragments have been utilized in monitoring the diagnosis or treatment of cervical carcinoma (4, 5). The expression of cytokeratin modified and influenced the chemosensitivity of cancer cells to anti-cancer drugs (6, 7). Since Ck8, Ck18 and Ck19 are generally associated with simple epithelia and are grouped together by classification, Ck19 may also have an influence on the drug resistance of cancer cells. Elevated Ck19 levels were clinically found to be a useful marker for the progression of lung and oesophageal cancers (8, 9). Furthermore, the overexpression of Ck19 has not only been regarded as a tumor marker for prognostic evaluation of cervical carcinoma (10), but is also associated with apoptotic resistance to anti-cancer drugs (7, 10). Thus, the chemotherapeutic efficiency is restrained by the overexpression of Ck19 that will minimize the efficacy of chemotherapy in cervical cancers (7). Nevertheless, overexpression of Ck19 may provide an alternative approach to treat cervical cancers by radiolabelling mAb against Ck19.

Abbreviations: Ck, cytokeratin; RIT, radioimmunotherapy.

Correspondence to: Dr. Te-Wei Lee, Isotope Application Division, Institute of Nuclear Energy Research, 1000 Wenhua Road, Chiaan Village, Lungtan, Taoyuan 325, Taiwan. Fax: 886-3-4711416, e-mail: twlee@iner.gov.tw

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Beta-irradiation, used for systemic radioimmunotherapy (RIT), is an efficient treatment approach *in vitro* and *in vivo*. The strategy with radiolabelled mAbs has been shown to be beneficial in the treatment of hematopoietic neoplasm and solid tumors (11-13). Due to the fact that the indium-111 (In-111)-mAbCx-99 could be used to monitor the tumor in a cervical carcinoma-bearing nude mouse model (14, 15), we hypothesized that radiolabelling mAb against Ck19 may be used as a tool for the therapy of Ck19-overexpressing cervical carcinoma. Rhenium-188 (Re-188) ($T_{1/2}$ = 16.9 h) is produced from beta decay of the tungsten-188 (W-188) parent ($T_{1/2}$ = 69 d). In addition to the emission of high energy beta particles (maximal energy of 2.12 MeV, average energy of 769 keV), Re-188 also decays with emission of a gamma photon (energy of 155 keV, 15%) for imaging. Re-188 would be the radioisotope of choice in the study because it simultaneously provides an efficient model for both radioimmunotherapy and radioimmunodetection.

Apoptosis induced by Re-188 beta-irradiation has been studied in leukemia and lymphoma *in vitro* (16). It was valuable to determine whether Re-188-mAb directly induced apoptosis and its molecular mechanism in cervical carcinoma. The first purpose of the study was to evaluate the effect of Re-188-labelled mAbCx-99 (Re-188-mAbCx-99) in cervical carcinoma cells and the second was to investigate the possible therapeutic molecular mechanism related to apoptosis induced by Re-188-mAbCx-99 on Ck19-overexpressing cervical cancer cells. To study the effect of Re-188-mAbCx-99 in human cervical cancer ME180 cells, the alterations in protein levels of p21, c-Jun, p53, Mcl-1 and the Bcl-2 family in cells during apoptosis were examined.

Materials and Methods

Cell lines. Two epidermoid and one squamous human cervical carcinoma cell lines (Caski, ME180 and HeLa) and two control cell lines (a foreskin cell line, FS-4 and a mouse embryo cell line, 3T3) were obtained from the American Tissue Culture Collection (ATCC, Rockville, MA, USA). A cervical carcinoma cell line (CC7T) was kindly provided by Dr. T.M. Chang (Taipei-Veterans General Hospital, Taipei, Taiwan). All of these cell lines were maintained in DMEM medium containing 10% fetal calf serum (FCS), 3 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 40μg/ml gentamycin.

Patients. Samples from 26 patients in Taipei-Veterans General Hospital, Taiwan, were studied, including patients with 5 early-stage and 21 advanced cervical carcinoma, 8 endometrial or ovarian carcinoma, as well as 6 control benign neoplasia.

Preparation of Rhenium-188. Rhenium-188 was obtained from an alumina-based W-188/Re-188 generator, as described previously (17). Elution of the W-188/Re-188 generator with normal saline provided solutions of carrier-free Re-188 as sodium perrhenate ($NaReO_4$). The generator has demonstrated consistently high Re-188 yields and low parent breakthrough for a period of at least 3 months (18).

Labelling of antibody with Re-188. The mAbCx-99 is a murine IgG1 monoclonal antibody, specific for the epitope of an approximate 37-kDa glycoprotein of human cervical carcinoma cells (19). An irrelevant murine IgG1 (MOPCIgG1; Litton Bionetic Inc., Charleston, SC, USA) was used as the control immunoglobulin. Both mAbCx-99 and MOPCIgG1 were purified as previously described (13). MOPCIgG1 and mAbCx-99 were labelled with Re-188 through slight modification of the method of Griffiths *et al.* (20). The disulfide bridge of mAbCx-99 (100 μg in 200 μl PBS) was cleaved with 2-mercaptoethanol (3.6 μM) (E. Merck, Darmstadt, Germany) after stirring for 30 min at room temperature (RT). After purification using a Sephadex G-50 column, the reduced mAbCx-99 was stored frozen under inert gas in a microcentrifuge tube until required. Reduced mAbCx-99 was labelled with Re-188 immediately using the Osteolite kit containing medronate disodium 10 mg and stannous chloride 0.6 mg (Du Pont Merck Pharmaceutical, MA, USA) as an transchelator for 10 to 12 h at RT under inert gas, and the radiolabelled antibody was then purified with a Sephadex G-50 column. The specific activity was 8 to 12 μCi/μg of protein. Quality control was determined by spotting samples on instant thin-layer chromatography silica gel (ITLC-SG, Gelman), using normal saline (0.9% NaCl) as the mobile phase. The radioactivity was monitored with a Bioscan Imaging Scanner (Packard, USA).

Cell culture. In this study, ME180 cells ($5x10^5$) were seeded into 3.5-cm culture dishes for 24 h prior to treatment. The cells were then incubated with medium containing Re-188-mAbCx-99 or Re-188-MOPCIgG1 (80-120 μCi/ml) at 37˚C for 1 h. Control cells were fed with culture medium only. After incubation, the cells were washed and further cultured in fresh medium for 3 to 6 days. The growth of cells was determined by counting the viable cells with trypan blue staining. The morphological changes and cell survival of treated and untreated cells were examined under a microscope and recorded. These cells were also used for studying apoptotic characteristics.

Reactivity of mAbCx-99 to carcinoma cells. Approximately $2x10^4$ cervical carcinoma cells (ME180 and Hela) and the control cells (FS-4) were added separately into 96-well plates (Costar, Cambridge, MA, USA) and reacted with 50 μl culture medium containing Re-188-mAbCx-99 or Re-188-MOPCIgG1 ($3x10^5$ cpm/well) at RT for 1 h, respectively. The cells were washed with TBS (phosphate-buffered saline containing 0.05% Tween-20 and 1% bovine serum albumin, pH 7.2) and harvested using Cell Harvester (Cambridge Technology Inc., Watertown, MA, USA), and the radioactivity was measured using a gamma counter (LKB, Instrument Ltd., Wallac, Finland).

Immunofluorescent (IF) or flow cytometric analysis. The specific reactivity of mAbCx-99 towards the cervical cancer cell line ME180 was also determined by the IF method after fixing the adherent cells onto a chamber coverglass (Lab-Tek, Nagle Nunc, Naperville, IL, USA) with 70% ethanol for 30 min at 4˚C. Fixed cells were reacted sequentially with diluted 50 μl culture medium (1:200) containing mAbCx-99 or MOPCIgG1, and FITC-antimouse IgG (1:100 dilution) (Cappel, Turnhout, Belgium) for 30 min at RT. The cells were then microscopically examined with a Confocol Microscope (FV300, Olympus, Japan). For flow cytometric analysis, growing cells were trypsinized, and the processes for fixing with ethanol and reacting with antibodies were the same as

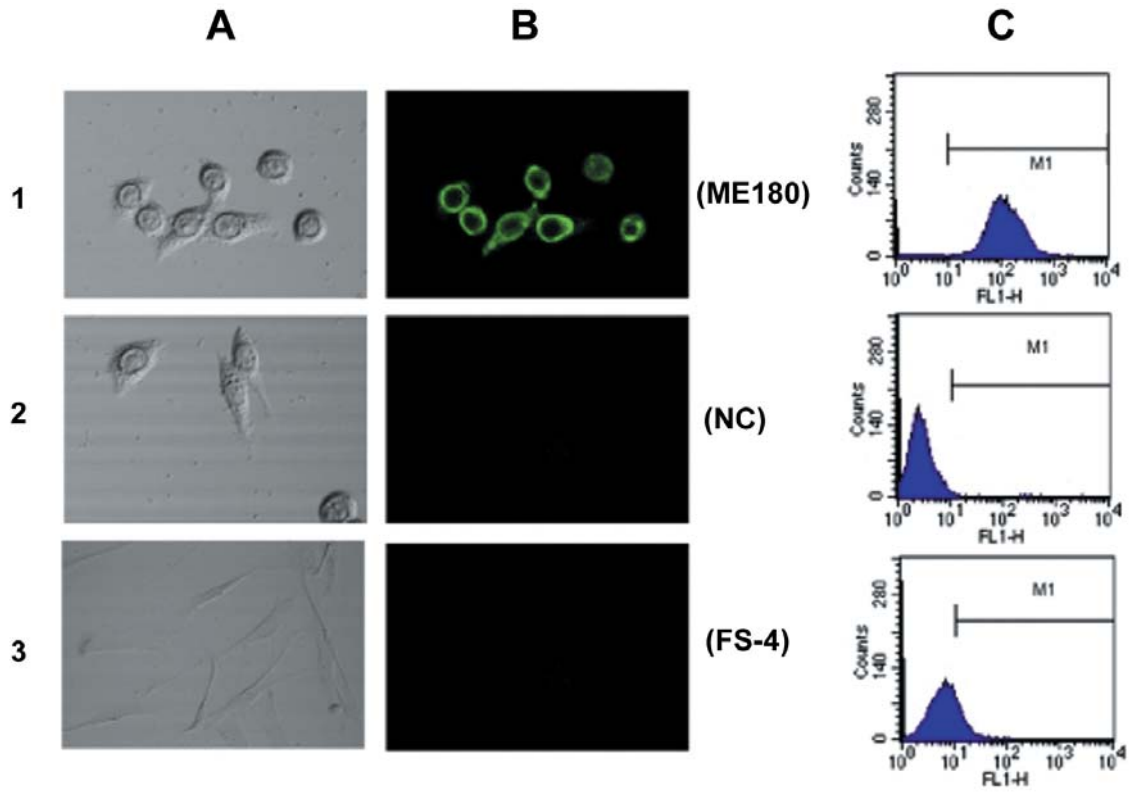

Figure 1. *Binding reactivity of mAbCx-99 to Ck19-expressing ME180 (1) and the control FS-4 cells (3) by immunofluorescent (B) and flow cytometric (C) analysis. Cell samples were fixed in 70% ethanol for immunofluorescent analysis. Some were trypsinized and fixed for flow cytometric analysis. The anti-Ck19 monoclonal antibody (mAbCx-99) and fluorescence isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulins were used to stain Ck19 proteins on ME180 (1B) and FS-4 cells (3B). MOPCIgG1 (2B) was also used to stain ME180 cells as a control sample. Phase microscopic view of ME180 (1A and 2A) and FS-4 (3A) cells were also examined. Cells containing Ck19 emitting fluorescence in channels were enumerated as a percentage of the total population (C).*

described above. The fluorescence staining of Ck19 was assayed by a FACStar flow cytometer with an argon laser tuned to the 488-nm line for excitation (Becton-Dickinson, Lincoln Park, NJ, USA).

Western blot analysis. Carcinoma cell or tissue extracts were prepared and Western blot was performed, as previously described (10). Aliquots of protein samples (5 μg/lane) were fractionated using 12% SDS-polyacrylamide gel electrophoresis and then electrotransferred onto a nitrocellulose membrane. After blocking, the blots were incubated subsequently with diluted mAbCx-99 (1:5000 in PBS), HRP-conjugated rabbit anti-mouse IgG (1:2000, DAKO Corp, Carpinteria, CA, USA) and an enhanced chemilluminescence reagent (ECL; Amersham, UK). The binding intensity of protein bands on the X-ray film was calculated using an ImageQuaNT™ Analyzer (Molecular Dynamics Inc., Sunnyvale, CA, USA). The relative intensity was calculated from the intensity of protein bands of the carcinoma cell extract over that of the control cell extract using ImageQuaNT™ software, according to the user's guide.

Western blot was also performed with rabbit anti-human antibodies for p21 or c-Jun (Transduction Lab., San Diego, CA, USA), Mcl-1, Bcl-xL, Bcl-xS or p53 (Santa Cruz, Biotec Inc., CA, USA) and Caspase-8 (PharMingen, San Diego, CA, USA) on whole cell extract preparation from treated and untreated ME180 cells. Signals were visualized with ECL analysis followed by exposure to X-ray films.

Cytotoxicity assay. The cytotoxic activity of Re-188-mAbCx-99 on ME180 cells was measured with an MTT (3-[4,5-dimenthylthiazol-2-yl]-diphenyl-tetrazolium bromide) assay kit (Chemicon Inc., Temecula, CA, USA). Adherent ME180 or control FS-4 cells ($2x10^4$/well) were treated with a medium containing Re-188-mAbCx-99 or Re-188-MOPCIgG1 (80-120 μCi/10 μg/ml) at 37˚C for 1 h. After washing, the treated cells were further incubated for 3 or 6 days and 50 μl of 0.5% MTT reagent was added for 4 h before assay. The formazan formation was extracted with 50 μl dimethyl sulfoxide (DMSO) and quantified in an ELISA Reader at 550 nm. The number of living cells was confirmed by trypan blue exclusion staining.

DNA fragmentation. DNA fragmentation, an apoptotic characteristic, was performed as previously described (21). ME180 cells ($5x10^5$) were seeded into culture dishes (3.5 cm) and allowed

Table I. *Reactivity of Re-188-mAbCx-99 to human cervical carcinoma cell lines.*

Cell line	Cell number ($2x10^4$/well)
Hela	47914±570
ME180	44197±1390
ME180*	2487±261
FS-4	2235±103

Human cervical carcinoma cell lines (ME180 and Hela) and control cell line (FS-4) were treated with Re-188-mAbCx-99 ($3x10^5$ cpm/well) or *Re-188-MOPCIgG1 for 1 h at 37˚C. After washing, the binding radioactivity of the treated cells was counted.

to attach 24 h prior to treatment. Adherent cells were treated with Re-188-mAbCx-99 or Re-188-MOPC (80-120 μCi/ml) at 37 ˚C for 1 h. The cells were then washed gently and further cultured for 12 to 120 h. After that, they were lysed periodically with 0.5 ml lysis buffer (5 mM Tris-HCl, pH 8.0, 1 mM EDTA and 0.25 NP-40), followed by the addition of RNase (20 μg/ml) at 37˚C for 1 h. The cells were then treated with proteinase K (300 μg/ml) for an additional 1 h, and DNA was then isolated. The DNA was resuspended in 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA and quantified. The same amount of DNA from each sample was loaded and electrophoresed on a 1.5% agarose gel in Tris-borate buffer (pH 8.0) containing 1 mM EDTA.

Quantitative internucleosomes. To study the role of Re-188-mAbCx-99 on cell apoptosis, another apoptotic parameter, internucleosomes, was defined according to the method described in the Cell Death Detection ELISA^{PLUS} kit (Roche Molecular Biochemicals, Mannheim, Germany). Cell lysates were placed onto a streptavidin-coated MTP (microtiter plate) and then mixed with anti-histone-biotin and anti-DNA-POD for 2 h of incubation. The nucleosome (cytoplasmic histone-associated-DNA-fragments) was captured by the MTP *via* its biotinylation. After washing with buffer solution, ABTS solution was then added for color development. The quantity of nucleosomes was measured at 405 nm against ABTS solution as a blank (reference wavelength approximately 490 nm).

Statistical analysis. The results were presented as means ± SD. The paired Student's *t*-test was used for group comparisons. Values of $p<0.05$ were considered significant.

Results

Expression of Ck19 in cervical carcinoma cells. The expression of Ck19 protein in cervical carcinoma cell lines (Caski, CC7T, ME180, HeLa) was tested by immunoblotting. From ImageQuaNT^{TM} analysis, the mean ratio of binding activity to Caski, CC7T, ME180 and HeLa cells was 38.5±5.8, 32.4±4.7, 30.5±4.1 and 5.1±1.2, respectively. The results showed that the Ck19 level in cervical carcinoma cell extracts was greatly elevated compared with that in control FS-4 (1.0±0.1) or 3T3 (0) cells. The expression of Ck19 density in those cervical carcinoma cell lines was approximately amplified from 4- to 44-fold mean ratio over that in the control cells. Our results indicated that the expression of Ck19 was significantly elevated in all cervical carcinoma cell lines compared with the control cell lines ($p<0.001$). Moreover, Ck19 was species-specific since it was undetectable in the mouse cell line 3T3.

Specific reactivity of Re-188-mAbCx-99. We used immunofluorescent or flow cytometric analysis to determine the binding reactivity between anti-Ck19 antibody and Ck19-expressing ME180 cells. From both assays, ME180 cells (98.6%) (Figure 1, 1B and 1C) in comparison with FS-4 control cells (8.4%) (Figure 1, 3B and 3C) reacted with mAbCx-99, or ME180 cells (0.5%) reacted with MOPC IgG 1 (Figure 1, 2B and 2C). Both results indicated the highly specific reactivity of mAbCx-99 to Ck19-expressing ME180 cells.

The specific reactivity of radiolabelled mAbCx-99 to the cervical carcinoma cell lines was also analyzed by the binding assay. The binding reactivity of Re-188-mAbCx-99 to ME180 (44,197±1390 cpm) or Hela (47,914±570 cpm) was much higher (>17-fold) than that of Re-188-MOPCIgG1 to ME180 (2,486±261 cpm) or Re-188-mAbCx-99 to the control FS-4 cells (2,235±103 cpm) (Table I). From Scatchard analysis of mAbCx-99-binding affinity, its association constant (K_a) was found to be $K_a = 4.44±0.81 \times 10^8$ mol^{-1} (Mean±S.E.M, for three experiments) (unpublished data).

Elevated expression of Ck19 in malignant cervical cancer tissues. To analyze the expression of Ck19 in human cervical cancer tissues, immunoblot analysis of tissue extracts was performed (Figure 2). The protein profile of tissue extracts from a portion of tissue samples was shown in immunoblot analysis (Figure 2A). After quantitative imaging analysis, the results indicated that all patients with advanced disease stage and two patients with early disease stage showed a significant elevation of Ck19 protein (Figure 2B). The relative intensity of Ck19 expression in endometrial (mean ratio±SD; 26.8±13), ovarian carcinomas (45.8±9.6) and cervical carcinoma tissues (34.8±24.3 to 79±18) was obviously higher than that in benign neoplasia (myoma and adenomyosis) (1.01±0.25) or normal cervical tissues (1±0.01). A correlation tendency was found between elevated Ck19 level and the clinical stage in most cases of patients with cervical cancer (88.5%; 23 out of 26 tissue samples).

Effects of Re-188-mAbCx-99 on cell proliferation and morphological changes. To study the role of Re-188-mAbCx-99 on the cell apoptotic phenomenon, the effects of Re-188-

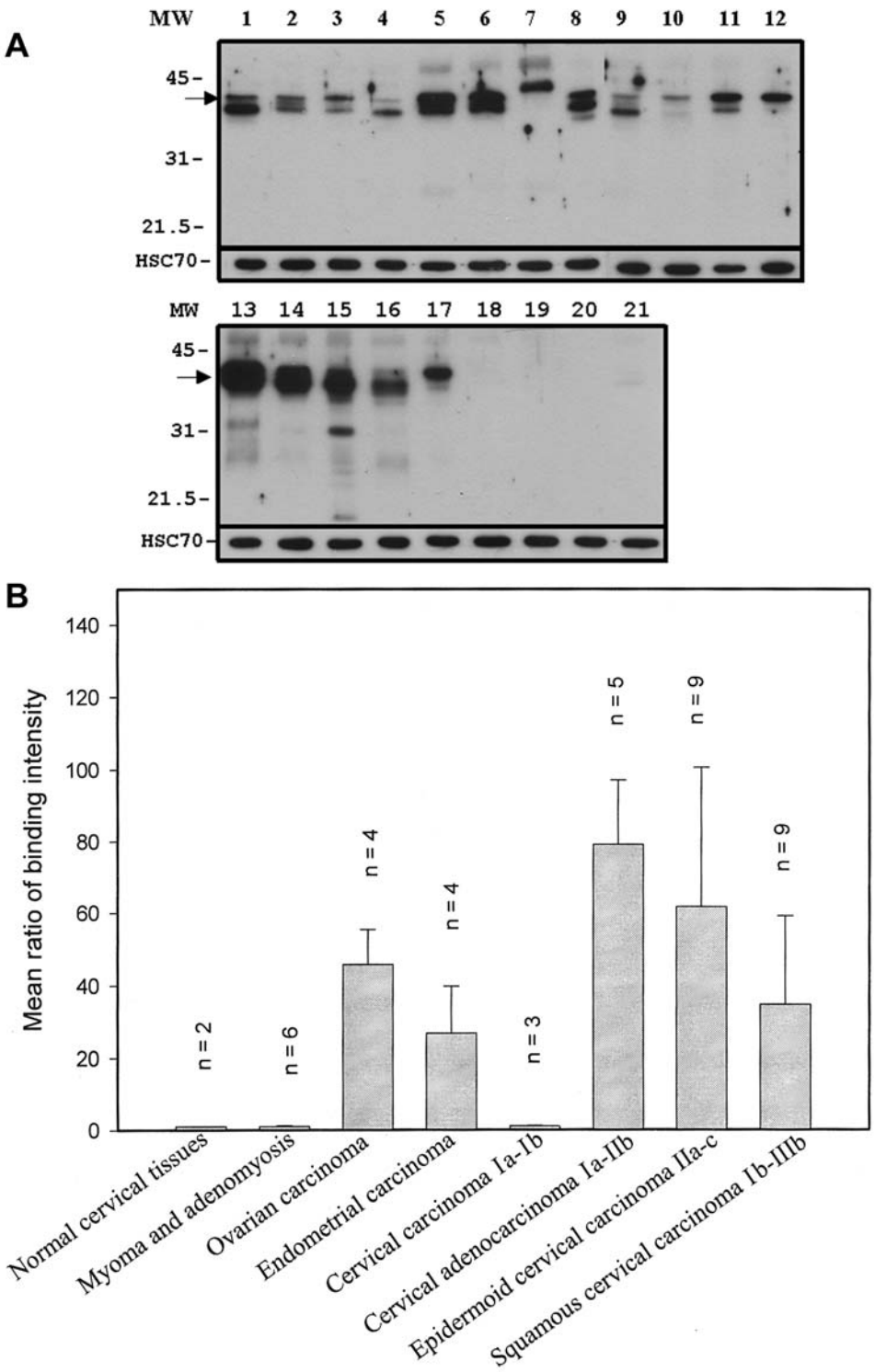

Figure 2. Western blot analysis of Ck19 levels in tissue lysates of some patients with cervical cancer, other carcinomas or benign tumors (A). Protein (5 μg/lane) of tissue lysates were fractionated on SDS-PAGE electrophoresis and transferred onto nitrocellulose paper. Blots were subsequently immunostained with mAbCx-99, HRP-conjugated rabbit anti-mouse IgG and an ECL reagent. The protein profiles of Ck19 in tissue extract from patients with squamous cervical carcinomas (lanes 9 to 12), cervical adenocarcinomas (lanes 13 to 17) and normal cervical tissues (lanes 20 and 21) are shown (A). Patients with endometrial (lanes 1 to 4), ovarian (lanes 5 to 8) and benign (lanes 18 and 19) tumors were used as a comparison. For imaging quantitative analysis, the mean ratio was calculated from the binding intensity of mAbCx-99 to Ck19 in cancer over that in normal cervical tissues (B). Number (n) of tissue samples was included in this experiment.

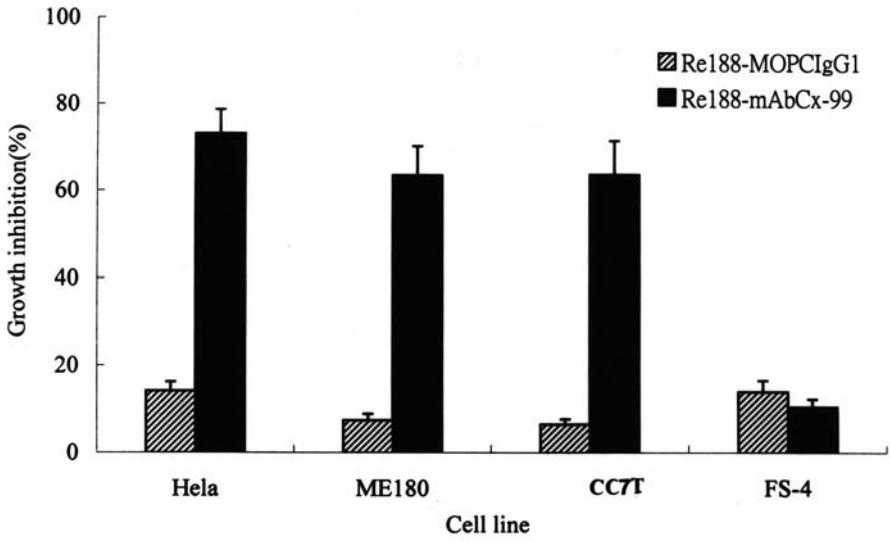

Figure 3. *Effect of Re-188-mAbCx-99 on growth inhibition of carcinoma cells. Human adherent cervical carcinoma cells (ME180, Hela and CC7T) and the control cells (FS-4) ($2x10^4$/well) were incubated with a culture medium containing Re-188-mAbCx-99 or Re-188-MOPC (80-120 μCi/ml). After 1 h of incubation, the cells were washed and further cultured with fresh medium for 3 days. The cells were trypsinized, stained with trypan blue solution and counted. The inhibition of cell proliferation was measured by the percentage of the number of living cells in the treated group over that in the untreated group.*

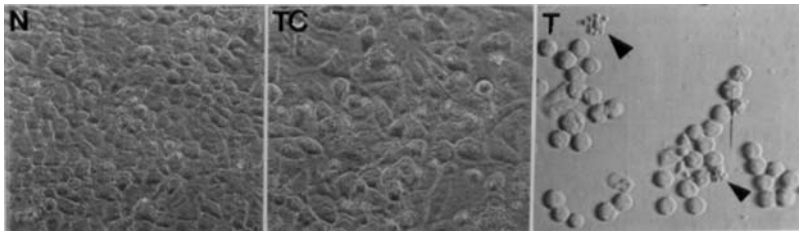

Figure 4. *Effects of Re-188-mAbCx-99 on cell morphological changes. Adherent ME180 cells ($5x10^5$) were co-cultured with the medium containing Re-188-mAbCx-99 (80-120 μCi/ml) (T), Re-188-MOPC (TC) or fresh medium (N) for 1 h at 37°C. After washing, the cells were further cultured in fresh medium for 3 days. Morphological changes of the experimental cells were analyzed by phase-contrast microscope (x 400). An example of the condensed and fragmented cell nuclei is indicated with an arrow. These changes are apoptotic characteristics.*

mAbCx-99 on cell proliferation and morphological changes were assessed. In this study, the MTT assay was also used to evaluate the effects of Re-188-mAbCx-99 (80-120 μCi/10μg/ml) on the proliferation of Ck19-overexpressing ME180 cells. It was found that the proliferation of ME180 cells was significantly inhibited by treatment with Re-188-mAbCx-99 ($p<0.001$; Table II), but not with free Re-188 or Re-188-MOPCIgG1 for 3 to 6 days; however, this effect was not found in the same reaction on the control FS-4 cells ($p>0.05$). In the trypan blue exclusion test, the growth inhibition of the Re-188-mAbCx-99-treated ME180, CC7T and Hela cells (60-80%) was significantly higher than that of the Re-188-MOPCIgG1-treated ones or Re-188-mAbCx-99-treated FS-4 cells (8-18%)(Figure 3). In terms of morphological changes, cells began to round up, developed plasma membrane projections and were eventually fragmented into membrane-bound vesicles after 48 h post-treatment with Re-188-mAbCx-99 (Figure 4, T). However, these apoptotic phenomena were not detected in untreated cells (Figure 4, N) or cells treated with Re-188-MOPCIgG1 (Figure 4, TC).

Re-188-mAbCx-99 induced DNA fragmentation. Cell apoptosis was also assayed by DNA fragmentation after post-treatment with Re-188-mAbCx-99 for 12 h, 24 h, 48 h, 72 h and 120 h. The results indicated that the DNA fragmentation of ME180 cells was much enhanced after 48 h post-treatment with Re-188-mAbCx-99. This tendency was not found in the control groups of cells treated with Re-188-MOPCIgG1 or culture medium (Figure 5).

Table II. *Cytotoxic effect of Re-188-mAbCx-99 on human cervical carcinoma cell line (ME180) and control normal cell line (FS-4).*

Experimental groups	Days after treatment	Absorbance (570 nm)	
		ME180	FS-4
Untreated control	3	1.573±0.21	1.356±0.14
	6	1.626±0.166	1.536±0.24
Free Re-188-treated	3	1.521±0.268	1.248± 0.295
	6	1.723±0.258	1.629±0.295
Re-188-MOPCIgG1-treated	3	1.324±0.139	1.247±0.142
	6	1.585±0.139	1.587±0.142
Re-188-mAbCx-99-treated	3	0.705±0.115*	1.288±0.237
	6	0.513±0.069*	1.388±0.139

Cells ($2x10^4$/ well) were cultured in the 96-well plate 24 h prior to treatment with free Re-188, Re-188-MOPC or Re-188-mAbCx-99 (80-120 μCi/10μg/ml). After 1 h of incubation, the cells were washed and further cultured in fresh DMEM medium for 3 and 6 days. The cytotoxic effect of the radiolabelled conjugates on cell proliferation was assayed by MTT method at the end of the culture period. *Statistically significant difference between Re-188-mAbCx-99-treated group and other treated or untreated control groups ($p<0.001$).

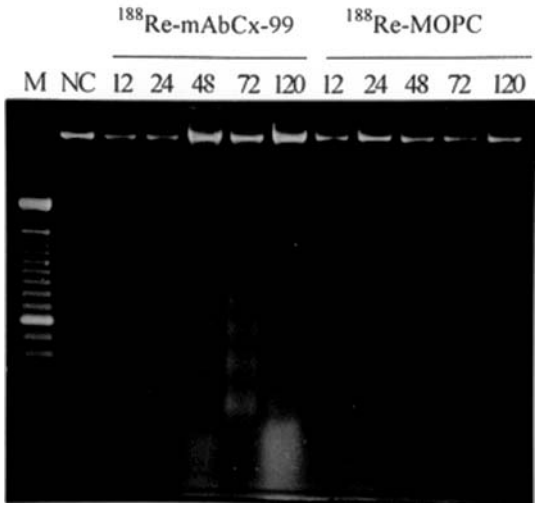

Figure 5. *Effects of Re-188-mAbCx-99 on DNA fragmentation in apoptotic ME180 cells. Adherent cells were cultured in a 35-mm culture dish and treated with Re-188-mAbCx-99 or Re-188-MOPCIgG1 (80-120 μCi/ml) for 1 h. After incubation, the treated cells were washed and cultured with fresh medium. The cells were harvested at the time indicated and their DNA was isolated. DNA was analyzed by 1.5% agarose gel electrophoresis and visualized by UV illumination. NC: untreated control, M: protein markers.*

Internucleosomal level in apoptotic cells. Cell apoptosis was further assessed by quantitative internucleosomal level. Figure 6 shows that the internucleosomal level of ME180 cells was elevated in 2 days, and greatly enhanced 3 days after treatment with Re-188-mAbCx-99 (T), but the internucleosomal level in cells treated with Re-188-MOPCIgG1 (TC) or in control cells (NC) remained unchanged.

Modulation of p53, p21, c-Jun and Bcl-2 family expression by Re-188-mAbCx-99. To study the effect of Re-188-mAbCx-99 on the protein levels of p21, c-Jun, p53, Mcl-1 and the Bcl-2 family, we examined the change of expression of these proteins in cells during apoptosis. The apoptosis triggered by Re-188-mAbCx-99 was associated with overexpression of oncoproteins, Bcl-xS, p21 and p53 in ME180 cells. The basal level of these proteins (Bcl-xS, p21 and p53) increased after treatment with Re-188-mAbCx-99 for 24 h. Conversely, the expression of anti-apoptotic protein, Mcl-1 and Caspase-8 declined after treatment with Re-188-mAbCx-99 (Figure 7). The caspase-8 level decreased indicating the proteolytic activation of caspase-8 during apoptosis.

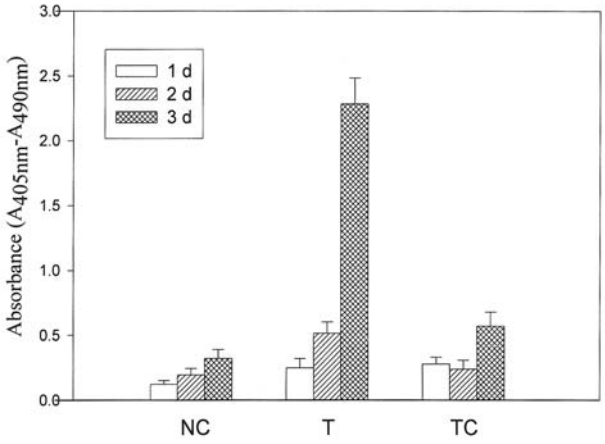

Figure 6. *Internucleosomal level of apoptotic cells was measured with a Cell Death Detection ELISA^{PLUS} kit. Adherent cells were treated either with Re-188-mAbCx-99 (80-120 μCi/ml) (T), Re-188-MOPCIgG1 (TC) or a control medium (NC) for 1 h. After incubation, the cultured supernatants were removed and replaced with fresh medium. The cells were harvested at the indicated time (1, 2 and 3 days) and nucleosomes were quantified according to the instruction guide of the kit.*

Discussion

The experiments described here showed that Re-188-radiolabelled mAbCx-99 could be used to detect the expression of Ck19 *in vitro* in most cervical carcinoma cell lines and cervical carcinoma tissues. In order to characterize the mechanism of action of beta-irradiation in more detail, we used only human ME180 cells for most studies. Although apoptosis induced by beta-irradiation has been studied, we demonstrated that the Re-188-mAbCx-99 specific for Ck19 could be successfully applied in

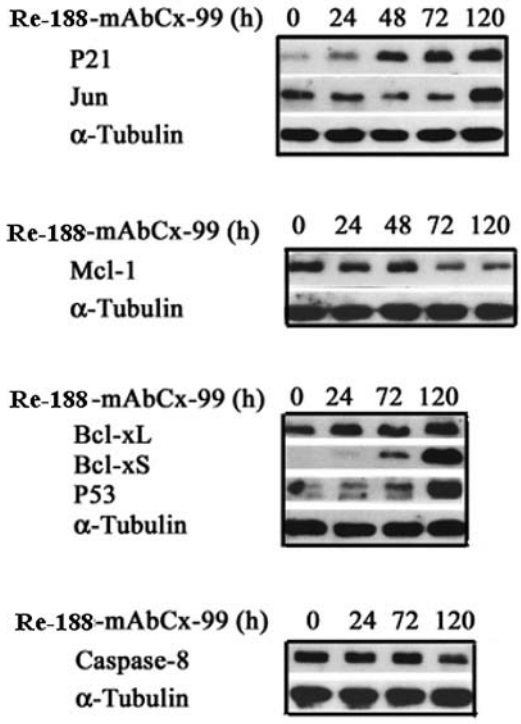

Figure 7. *Effects of Re-188-mAbCx-99 on protein expression of c-Jun, p21, Mcl-1, Bcl-xS, Bcl-xL, p53 and caspase-8. Whole cell extract (10μl) of untreated and Re-188-mAbCx-99-treated ME180 cells from indicated time points (0, 24, 48, 72 and 120 h) were analyzed by immunoblotting for c-Jun, p21, Mcl-1, Bcl-xS, Bcl-xL, p53 and caspase-8 protein expression as described in "Materials and Methods". The a-Tubulin level demonstrates approximately equal loading in each lane. The immunoblots shown here represent the typical result from several independent times.*

radioimmunotherapy of human ME180 cells. This study showed that apoptosis is induced in human cervical carcinoma cells by Re-188 beta-irradiation.

Immunohistochemical staining of cancer tissues with polyclonal or monoclonal antibodies has been widely used to detect residual cancer cells and to evaluate the prognosis of cancer patients (1). Ck19 has been recognized as a tumor marker of human cervical carcinoma (5, 22). The overexpression of Ck19 in cervical carcinoma cell lines (ME180, CC7T, Hela and Caski) in the current study was verified by Western blotting and a binding assay with specific antibody, mAbCx-99. Indeed, its expression was very low in the normal foreskin cell line FS-4. Our findings demonstrated that the tumor tissues from all patients with advanced stages of cervical, endometrial and ovarian carcinomas had significantly elevated Ck19 levels in comparison with normal or benign neoplasm tissues (Figure 2). Therefore, the estimation of the Ck19 level seems to be helpful for monitoring the disease status and may provide information about the prognosis for cervical and ovarian cancer. However, it was not applicable for the early diagnosis since elevated Ck19 levels were insignificant in several patients (3 out of 5) with early cervical carcinoma stage (Figure 2). By immunohistochemical staining, it has been reported that an elevated Ck19 level was clinically found in 48% of esophageal SCC patients (9), 45% of squamous cell lung cancer patients, 39% of lung adenocarcinoma patients and 35% of small cell lung cancer patients (8). In the present study, the Ck19 level was found to be greatly elevated in approximately 88.5% (23 out of 26) of all cervical carcinoma, especially in advanced disease stages (100%; all of the 22 patients), suggesting that it may be a useful marker for cervical cancer prognosis. Furthermore, overexpression of Ck19 in cervical cancers could be a candidate antigen for radioimmunotherapy.

Although cytokeratins are mainly expressed in the cytosol of tumor cells, the results from the specific reactivity of Re-188-mAbCx-99 suggested that the CK19 was expressed on the surface of the ME180 cell line. These results agreed with previous studies that the presence of cytokeratin molecules could be expressed on the surface of ME180 (23, 24) and some malignant epithelial cells (25-27). A slight expression of Ck19 on the cell surface was presented on the MCF7 breast cancer cell line (26). Ditzel *et al.* (28) showed that cell surface cytokeratin could be recycled into an intracellular compartment. According to our results, we suggested that CK19 could be slightly expressed on the surface of ME180 and that the expression could be detected by the highly sensitive radioactivity of Re-188-mAbCx-99 binding with CK19 on the surface of ME180.

The p53, p21 and Bcl-2 family proteins are involved in apoptosis triggered by a variety of apoptotic agents including irradiation. It is suggested that cells are susceptible to apoptosis when the expression of these genes are deregulated (29). In previous reports, apoptosis induced by irradiation was associated with an increased expression of mutated p53 protein and up/down-regulated expression of the Bcl-2 family proteins in some cancers (16, 30, 31). Although induction of the tumor cell towards apoptosis after radioimmunotherapy has been reported (16, 32), the molecular mechanisms by which beta-irradiation induces cell death are still not understood in cervical cancers. In this study, we showed that immunotherapy with Re-188-mAbCx-99 increased the expression of several apoptotic-related genes including p53, p21 and c-Jun. p53 can transactivate numerous genes coding for proteins acting in the apoptotic process, such as inducing cell cycle arrest *via* expression of p21. The Bcl-2 family proteins, including Bcl-2, Bcl-xS, Bcl-xL and Mcl-1, have been described as mediators of either cell survival or cell death. We found that an apoptotic-promoting Bcl-xS protein was up-regulated markedly, while an anti-apoptotic protein, Mcl-1, was down-regulated by Re-188 irradiation in ME180 cells (Figure 7). This indicated that the changes of these oncoproteins could be attributed to Re-188-mAbCx-99-mediated radioimmunotherapy.

Re-188 irradiation-induced apoptosis was accompanied with a mitochondrial- dependent mechanism and declining mitochondrial transmembrane potential (16). In our study, we examined the activation of caspase-8 during Re-188-mAbCx-99 treatment. The results indicated that the mitochondria play a role in beta-irradiation-mediated apoptosis. Since the expression of Ck19 in cervical carcinoma cells is associated with apoptotic resistance to chemotherapy (7, 10), it may also restrain the efficacy of radiotherapy with radiolabelling mAb. Our data conversely indicated that radioimmunotherapy on the Ck19-enriched cancer cells by using Re-188-mAbCx-99 is effective. Re-188 demonstrated several characteristics that make it a potential therapeutic radiopharmaceutic for targeting therapy. Radioimmunotherapy with Re-188-mAbCx-99 may have a potential clinical implication in human cervical carcinoma, or other Ck19-overexpressing cancers. It may be a good therapeutic choice for those cancers with apoptotic resistance to chemotherapy.

In conclusion, Re-188-mAbCx-99 was successfully employed to treat Ck19-enriched cervical carcinoma. Direct evidence of the therapeutic effects of Re-188-mAbCx-99 on cervical cancer cells was the obvious inhibition of cell proliferation and viability. In addition, the apoptotic activity of Re-188-mAbCx-99 was associated with the activation of p53, p21, c-Jun and caspase-8 and down-regulation of Mcl-1 protein. Although an elevated expression of Ck19 (6, 7, 10) has been associated with apoptotic resistance to chemotherapy, our data clearly show that Re-188-mAbCx-99 has a therapeutic effect on Ck19-overexpressing cervical cancer cells. However, this would be further determined by animal xenograft studies, to demonstrate the therapeutic efficacy of Re-188-mAbCx-99 *in vivo*.

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