Low-intensity ultrasound enhances the anticancer activity of cetuximab in human head and neck cancer cells

TAKASHI MASUI, ICHIRO OTA, MASATOSHI KANNO, KATSUNARI YANE and HIROSHI HOSOI

1Departments of Otolaryngology-Head and Neck Surgery and 2Oncology Center, Nara Medical University; 3Department of Otolaryngology, Kinki University School of Medicine, Nara Hospital, Nara, Japan

Received September 4, 2012; Accepted September 28, 2012

DOI: 10.3892/etm.2012.739

Abstract. The potential clinical use of ultrasound in inducing cell apoptosis and enhancing the effects of anticancer drugs in the treatment of cancers has previously been investigated. In this study, the combined effects of low-intensity ultrasound (LIU) and cetuximab, an anti-epidermal growth factor receptor (EGFR) antibody, on cell killing and induction of apoptosis in HSC-3 and HSC-4 head and neck cancer cells, and its mechanisms were investigated. Experiments were divided into 4 groups: non-treated (CNTRL), cetuximab-treated (CETU), ultrasound-treated (UST) and the combination of cetuximab and US-treated (COMB). Cell viability was assessed by trypan blue staining assay and induction of apoptosis was detected by fluorescein isothiocyanate (FITC)-Annexin V and propidium iodide (PI) staining assay at 24 h after cetuximab and/or US treatment. To elucidate the effect of cetuximab and US on EGFR signaling and apoptosis in head and neck cancer cells after the treatments, the expression of EGFR, phospho-EGFR, and the activation of caspase-3 were evaluated with western blotting. More cell killing features were evident in the COMB group in HSC-3 and HSC-4 cells compared with the other groups. No differences in EGFR expression among the CETU, UST and COMB groups was observed, while the expression of phospho-EGFR in the CETU group was downregulated compared with that in the CNTRL group. Phospho-EGFR expression was much more downregulated in the COMB group compared with that in the other groups. In addition, the activation of caspase-3 in the UST group was upregulated compared with that in the CNTRL group. Caspase-3 activation was much more upregulated in the COMB group than that in the other groups. These data indicated that LIU was able to enhance the anticancer effect of cetuximab in HSC-3 and HSC-4 head and neck cancer cells.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is known for its rapid clinical progression and poor prognosis. The survival rates for many types of HNSCC have improved little over the past 40 years. Anti-monoclonal antibody is often used as a first-line and primary treatment of malignancies, such as malignant lymphomas and breast cancer. Cetuximab, an anti-epidermal growth factor receptor (EGFR) antibody, is the only EGFR inhibitor approved in HNSCC. Several combined methods with cetuximab, such as irradiation or many types of anticancer drugs, have been applied to enhance the effect of treatment. However, the efficacy of such treatment is inadequate. Therefore, new therapeutic methods should be developed to improve the survival rate.

In non-surgical treatment, induction of apoptosis is a preferred mode of killing cancer cells with fewer side effects and immune reactions (1). Apoptosis is a physiologic process that contributes to the homeostasis of multi-cellular organisms and maintains the balance between cell proliferation and cell death (2), as a cell response to stress stimuli, such as exposure to chemotherapeutic drugs, oxidative stress, free radicals, X-rays, ultraviolet radiation, shear stress and ultrasound (US) irradiation.

Since the biological effect of US was first reported in 1927, numerous reports concerning the application of US have been published. The mechanisms of US-induced apoptosis on physical and biochemical levels have been discussed in several studies (3-7). In addition, US irradiation can be divided into low-intensity ultrasound (LIU) and high-intensity focused ultrasound (HIFU) depending on the intensity. LIU has a great potential in apoptosis therapy for cancer (8), while HIFU can thermally ablate tissues via hyperthermia in various types of cancer (9,10). LIU has been subjected to numerous studies to evaluate its biological effects and their possible application in therapeutic strategies in cancer treatment. These outcomes include the enhancement of cellular uptake of drugs (11) and the induction of apoptosis and cell killing (12,13). Besides, it is likely that the selectivity of LIU for cancer cells is higher than that for normal cells (14).

The combined effects of US and several anticancer drugs, such as (DOX) and Cisplatin (DDP) have often been reported (15,16). Thus, sonication by US may also be considered a candidate for the enhancement of anticancer drugs.
However, few reports associated with anti-monomoclonal antibody are available. We hypothesized that US has the potential to become an enhancer of cetuximab owing to its effect of changing cell membrane structure and making cancer cells more sensitive to cetuximab as well as rituximab, as previously reported (17).

In the present study, we assessed the therapeutic potential of the combination of cetuximab and LIU in vitro and demonstrated that this combination induces the effect of tumor cell killing and increases the therapeutic efficacy in head and neck cancer HSC-3 and HSC-4 cells. In addition, we focused on EGFR signaling and apoptosis signaling through the mitochondria-caspase pathway to confirm cetuximab enhancement by US.

Materials and methods

Cell culture and drugs. Human HNSCC cells, HSC-3 and HSC-4, were employed in this study. The HSC-3 and HSC-4 cells, obtained from the Japanese Cancer Research Resource Bank (Tokyo, Japan), were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 100 U/ml penicillin, and 100 mg/ml streptomycin (Gibco, Grand Island, NY, USA) at 37°C in 5% CO₂. Cetuximab (Merck KgaA, Darmstadt, Germany) was dissolved in phosphate-buffered saline (PBS) at a concentration of 100 nM and then stored at 4°C until use.

US apparatus and intensity. A 1.0 MHz ultrasonic generator (KUS-2S; ITO Ultrasonic Co., Ltd., Tokyo, Japan) with a fixed duty factor of 50% and with 1 Hz pulse repetition frequency (PRF) was used. The sonication was conducted at an intensity of 0.5 W/cm² (= output intensity of 0.38 W/cm²) and exposure time of 1 min. The intensity and time were used in all of the sonication experiments.

To keep the transducer-facing directory upward for the sonication procedure, the transducer, with a diameter of 2.7 cm, was fixed with a clamp attached to a metal stand. A 3.5-cm culture dish was placed on the center of the transducer with intermediate gel (Fig. 1).

Experimental protocol. The experimental groups for this study were: i) non-treated (CNTRL), ii) cetuximab-treated (CETU), iii) US-treated (UST) and iv) the combination of cetuximab and US-treated (COMB). Cells (1x10⁶) in 1.5 ml medium were seeded in a 3.5-cm dish and incubated at 37°C for 12 h. At 30 min prior to sonication another 1.5 ml of fresh medium, with or without cetuximab at the final concentration of 100 nM, was added to each dish to avoid cavitation attenuation due to the high concentration of carbon dioxide accumulated in the dish, as previously described (18,19). Following the treatment, the cells were subjected to different analyses.

Measurement of cell viability. Cell viability was assessed by trypan blue staining assay at 24 h after cetuximab and US, as previously described (6). The number of cells was counted using a hemocytometer to estimate the viability. The cell viability was calculated as the number of viable cells in the treated group/the number of viable cells in the non-treated group. Each measurement was repeated three times independently.

Detection of apoptosis. Apoptosis was assessed by fluorescein isothiocyanate (FITC)-Annexin V and propidium iodide (PI) staining assay according to the manufacturer’s instructions (Roche Diagnostic GmbH, Penzberg, Germany). Following the treatment of cetuximab and US, the cells were incubated at 37.0°C for 24 h. The cells were then washed with cold PBS, stained with FITC-Annexin V/PI for 10 min and observed under a fluorescent microscope for typical morphological changes characteristic of apoptosis.

FITC-Annexin V/PI staining assay was performed to detect the phosphatidylserine expression using FITC-Annexin V labeling as an endpoint indicator of early apoptosis, and the PI uptake as an indicator of necrosis. Annexin V-positive and PI-negative cells were considered to be early apoptosis, while Annexin V-positive and PI-positive cells were considered to be secondary necrosis (6,15). Apoptosis was calculated as the number of apoptotic cells/the number of total cells in each group. Each measurement was repeated three times independently.

Antibodies and immunoblot analysis. EGFR, phospho-EGFR, and activation of caspase-3 signaling on HSC-3 cells after the treatment were also evaluated with western blotting. Cells were cultured in 3.5-cm dishes for 6 h after the treatment, respectively. The cells were collected and frozen in 100 µl RIPA buffer, and stored in a -30°C ultra low temperature freezer. Briefly, total protein extracts were prepared according to the freeze-thaw lysis method (20) and protein concentrations were measured with the bovine serum albumin (BSA) protein assay. Extract samples containing 20 µg of protein were then separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. The membranes were incubated with anti-EGFR, anti-phospho-EGFR, and anti-caspase-3 antibody [Cell Signaling Technology, Danvers, MA, USA; diluted 1:2000, 1:1000 and 1:1000 in PBS with Tween-20 (PBST) respectively] at 4°C overnight, and then with peroxidase-conjugated secondary anti-rabbit or goat immunoglobulin G (IgG) (Cell Signaling Technology; diluted 1:1000 in PBST) for 1 h.
After rinsing in PBST (4 times, 5 min each), immuno detection was performed using an enhanced chemiluminescence (ECL) western blot analysis detection reagents and analysis system. The membranes were subsequently exposed to X-ray film, as previously described (21).

**Statistical analysis.** Data were presented as the mean ± standard deviation (SD). Significant differences between groups were assessed using two-way factorial analysis of variance (ANOVA). $P<0.01$ was considered to be a statistically significant difference.

**Results**

Enhancement of cetuximab-induced cell killing by US. Cell viability was assessed by trypan blue staining assay at 24 h after cetuximab and US. The cell viabilities of HSC-3 cells (Fig. 2A) and HSC-4 cells (Fig. 2B) in the CETU group were 71.2 and 87.1% compared to those of the CNTRL group, respectively. The cell viabilities in the UST group were 73.0 and 79.8%, while those in the COMB group decreased to 39.3 and 60.6%, respectively, thus showing a synergistic enhancement of cetuximab-induced cell killing by US.
Enhancement of cetuximab-induced apoptosis by US. In order to estimate the enhancement of cell killing, the induction of apoptosis was assessed by FITC-Annexin V/PI staining assay (Figs. 3 and 4). FITC-Annexin V/PI staining assay was performed to detect the phosphatidylserine expression on the cell membrane as an endpoint of early apoptosis, showing the exact percentage of cells in viable cells (Annexin V−/PI−), early apoptotic cells (Annexin V+/PI−) and secondary necrosis (Annexin V+/PI+) under a fluorescent microscope. Fig. 3 shows (A) DAPI, (B) Annexin V, (C) PI and (D) merged images of the combined treatment on HSC-3 cells, respectively. The rates of apoptotic features of HSC-3 cells were 2.7 and 1.5%, respectively, while in the CETU group, they were 3.7 and 1.8%, respectively. No statistically significant difference was observed between these two groups. In the UST group, the apoptotic features were 8.6 and 5.2%, respectively, while those in the COMB group were 15.2 and 9.5%, respectively. These data suggest that US induced apoptosis in these cells and that the combination of US and cetuximab enhanced apoptosis synergistically.

Effects of EGFR signaling and apoptosis following the treatments. To elucidate the effects of EGFR signaling and apoptosis on HSC-3 cells after the treatments were administered, the expression of EGFR, phospho-EGFR, and the activation of caspase-3 were evaluated using western blotting (Fig. 5). No statistically significant differences in EGFR expression among the CETU, UST and COMB groups was observed, while the expression of phospho-EGFR was much more downregulated in the COMB group compared with the remaining groups, although phospho-EGFR was downregulated in the CETU group. These data suggest that US induced the inhibition of EGFR-signaling pathway by cetuximab. To elucidate the induction of apoptosis, the activation of caspase-3 was estimated. The activation, the cleavage of caspase-3, in the UST group was upregulated compared with that in the CNTRL group. Additionally, caspase-3 activation was much more upregulated in the COMB group compared with that in the remaining groups, suggesting that US induced apoptosis through the activation of caspase-3 by cetuximab.

Discussion

In recent years, there has been a focus on the potential of LIU as well as HIFU in clinical applications. These outcomes include the enhancement of cellular uptake of drugs and the induction of cell killing or apoptosis (15,16,22). LIU primarily inhibits cell proliferation through its heating, cavitation and mechanical effect. Such investigations indicate that the biological effectiveness of US is linked to the acoustic conditions (23). In general, the impulsive pressures generated by the collapse of cavitation bubbles associated with US may...
Ultrasonic enhancement of anticancer drug by LIU are available. However, response rates as a single agent have been less than satisfactory (13%) and of limited duration (2-3 months) (41).

However, response rates as a single agent have been less than satisfactory (13%) and of limited duration (2-3 months) (41). Thus, we suggest that US has the potential to become an enhancer of cetuximab owing to its effect of changing cell membrane structure and making cancer cells more sensitive to cetuximab as well as rituximab, as previously reported (17).

In the present study, we demonstrated that there were more tumor cell killing features in the COMB group than in the other groups and that the phosphorylation of EGFR was down-regulated, while the activation of caspase-3 was upregulated in the COMB group compared with that in the remaining groups. These data suggest that LIU was able to increase the induction rate of cetuximab in head and neck cancer cells, leading to enhancement of the effect of cetuximab in the COMB group. In addition, as previously reported, the activation of caspase-3 also increased when apoptosis was induced by US alone in cancer cells (22). These results suggest that an increase of the induction rate of cetuximab by US could subsequently activate caspase-cascade reaction more than US alone.

In summary, the present study has shown the synergistic enhancement of apoptosis induction by the combination of LIU and cetuximab in HNSCC cells. The mitochondria-caspase pathway is considered to be crucial in this process. These data suggest that LIU enhanced the anticancer effect of cetuximab in HSC-3 and HSC-4 cells of HNSCC. Although the precise involvement of the EGFR and caspase signaling modifications by cetuximab in the enhancement remains to be elucidated, they may be involved in the latent effect. The critical mechanisms remain to be further investigated. The majority of studies on US effects have been performed in vitro to evaluate clinical cancer therapies. However, it should be noted that the results of in vitro studies on the biological effects of therapeutic US cannot be translated to the same cells in vivo, even if the same US exposure is used. Although it is still too early to discuss its clinical efficiency, LIU may prove to be a valid treatment option for HNSCC.

Acknowledgements

We thank Dr Takashi Kondo (University of Toyama, Japan) for his critical input. This study was supported in part by Grants-in Aids for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1. Fulda S and Debatin KM: Apoptosis pathways in neuroblastoma therapy, Cancer Lett 197: 131-135, 2003.
2. Mandal D, Mazumder A, Das P, Kundu M and Basu J: Fas-, caspase-8-, and caspase 3-dependent signaling regulates the activity of the aminophospholipid translocase and phosphatidylserine externalization in human erythrocytes. J Biol Chem 280: 39460-39467, 2005.
3. Ashsh H, Rozenzajin LA, Blass M, et al: Apoptosis induction of human myeloid leukemic cells by ultrasound exposure. Cancer Res 60: 1014-1020, 2000.
4. Lagneaux L, de Meulenenaer EC, Delforge A, et al: Ultrasonic low-energy treatment: a novel approach to induce apoptosis in human leukemic cells. Exp Hematol 30: 1293-1301, 2002.
5. Honda H, Zhao QL and Kondo T: Effects of dissolved gases and an echo contrast agent on apoptosis induced by ultrasound and its mechanism via the mitochondria-caspase pathway. Ultrasound Med Biol 28: 673-682, 2002.
6. Feril LB Jr, Kondo T, Zhao QL and Ogawa R: Enhancement of hyperthermia-induced apoptosis by non-thermal effects of ultrasound. Cancer Lett 178: 63-70, 2002.
7. Feril LB Jr, Kondo T, Zhao QL, et al: Enhancement of ultrasound-induced apoptosis and cell lysis by echo-contrast agents. Ultrasound Med Biol 29: 331-337, 2003.
8. Abdollahi A, Domhan S, Jenne JW, et al: Apoptosis signals in lymphoblasts induced by focused ultrasound. Faseb J 18: 1413-1414, 2004.
9. Huber PE, Jenne JW, Rastert R, et al: A new non-invasive approach in breast cancer therapy using magnetic resonance imaging-guided focused ultrasound surgery. Cancer Res 61: 8441-8447, 2001.
10. Blana A, Walter B, Rogenhofer S and Wieland WF: High-intensity focused ultrasound for the treatment of localized prostate cancer: 5-year experience. Urology 63: 297-300, 2004.
11. Larkin JO, Casey GD, Tangney M., et al: Effective tumor treatment using optimized ultrasound-mediated delivery of bleomycin. Ultrasound Med Biol 34: 406-413, 2008.
12. Feril LB Jr, Kondo T, Cui ZG, et al: Apoptosis induced by the sonomechanical effects of low intensity pulsed ultrasound in a human leukemia cell line. Cancer Lett 221: 145-152, 2005.
13. Feng Y, Tian ZM, Wan MX and Zheng ZB: Low intensity ultrasound-induced apoptosis in human gastric carcinoma cells. World J Gastroenterol 14: 4873-4879, 2008.
14. Lepkowicz F and Salzberg S: Distinct sensitivity of normal and malignant cells to ultrasound in vitro. Environ Health Perspect 105 (Suppl 6): 1575-1578, 1997.
15. Yoshida T, Kondo T, Ogawa R, et al: Combination of doxorubicin and low-intensity ultrasound causes a synergistic enhancement in cell killing and an additive enhancement in apoptosis induction in human lymphoma U937 cells. Cancer Chemother Pharmacol 61: 559-567, 2008.
16. Watanabe Y, Aoi S, Horie S, et al: Low-intensity ultrasound and microbubbles enhance the antitumor effect of cisplatin. Jpn J Cancer Res 99: 2525-2531, 2008.
17. Danno D, Kanno M, Fujimoto S, Feril LB Jr, Kondo T and Nakamura S: Effects of ultrasound on apoptosis induced by anti-CD20 antibody in CD20-positive B lymphoma cells. Ultrasound Sonochem 15: 463-471, 2008.
18. Mendelsohn J: Blockade of receptors for growth factors: an anticancer therapy - the fourth annual Joseph H Burchenal Award Lecture. Clin Cancer Res 6: 747-753, 2000.
19. Feril LB Jr and Kondo T: Major factors involved in the inhibition of ultrasound-induced free radical production and cell killing by pre-sonication incubation or by high cell density. Ultrasound Sonochem 12: 353-357, 2005.
20. Bollag DM and Edelstein SJ: Protein methods. Wiley-Liss, New York, 1991.
21. Hayashi K, Motoyama S, Koyota S, et al: REG I enhances chemoresistance in squamous cell esophageal cancer cells. Cancer Sci 99: 2491-2495, 2008.
22. Feng Y, Tian Z and Wan M: Bioeffects of low-intensity ultrasound in vitro: apoptosis, protein profile alteration, and potential molecular mechanism. J Ultrasound Med 29: 963-974, 2010.
23. Tezal A, Sens A, Tuchscherer J and Mitragoti S: Frequency dependence of sonophoresis. Pharm Res 18: 1694-1700, 2001.
24. Chen WS, Matula TJ and Crum LA: The disappearance of ultrasound contrast bubbles: observations of bubble dissolution and cavitation nucleation. Ultrasound Med Biol 28: 793-803, 2002.
25. Miller DL and Dou C: Membrane damage thresholds for 1- to 10-MHz pulsed ultrasound exposure of phagocytic cells loaded with contrast agent gas bodies in vitro. Ultrasound Med Biol 30: 973-977, 2004.
26. Hallow DM, Mahajan AD, McCutchen TE and Prausnitz MR: Measurement and correlation of acoustic cavitation with cellular bioeffects. Ultrasound Med Biol 32: 1111-1122, 2006.
27. Kodama T, Tomita Y, Koshiyama K and Blomley MJ: Transfection effect of microbubbles on cells in superposed ultrasound waves and behavior of cavitation bubble. Ultrasound Med Biol 32: 905-914, 2006.
28. Larina IV, Evers BM and Esenaliev RO: Optimal drug and gene delivery in cancer cells by ultrasound-induced cavitation. Anticancer Res 25: 149-156, 2005.
29. Honda H, Kondo T, Zhao QL, Feril LB Jr and Kitagawa H: Role of intracellular calcium ions and reactive oxygen species in apoptosis induced by ultrasound. Ultrasound Med Biol 30: 683-692, 2004.
30. Firestein F, Rozenszajn LA, Shemes-Darvish L, Elimelech R, Radny J and Rosenschein U: Induction of apoptosis by ultrasound application in human malignant lymphoid cells: role of mitochondria-caspase pathway activation. Ann N Y Acad Sci 1010: 163-166, 2003.
31. Feril LB Jr, Tsuda Y, Kondo T, et al: Ultrasound-induced killing of monocyte U937 cells enhanced by 2,2’-azobis(2-amidino-propane) dihydrochloride. Cancer Sci 95: 181-185, 2004.
32. Koshiyama K, Kodama T, Yano T and Fujikawa S: Structural change in lipid bilayers and water penetration induced by shock waves: molecular dynamics simulations. Biophys J 91: 2198-2205, 2006.
33. Duvshani-Éshet M, Adam D and Machluf M: The effects of albumin-coated microbubbles in DNA delivery mediated by therapeutic ultrasound. J Control Release 112: 156-166, 2006.
34. Taniyama Y, Tachibana K, Hiraoka K, et al: Local delivery of plasmid DNA into rat carotid artery using ultrasound. Circulation 105: 1233-1239, 2002.
35. Kagiya G, Ogawa R, Tabuchi Y, et al: Expression of heme oxygenase-1 due to intracellular reactive oxygen species induced by ultrasound. Ultrasound Sonochem 13: 388-396, 2006.
36. Yuan J: Molecular control of life and death. Curr Opin Cell Biol 7: 211-214, 1995.
37. Majno G and Joris I: Apoptosis, oncosis, and necrosis. An overview of cell death. Am J Pathol 146: 3-15, 1995.
38. Wyllie AH, Kerr JF and Currie AR: Cell death: the significance of apoptosis. Int Rev Cytol 68: 251-306, 1980.
39. Kroener G, Galluzzi L and Brenner C: Mitochondrial membrane permeabilization in cell death. Physiol Rev 87: 99-163, 2007.
40. Ciardiello F and Tortora G: A novel approach in the treatment of cancer: targeting the epidermal growth factor receptor. Clin Cancer Res 7: 973-977, 2001.
41. Verhoren JB, Trigo J, Hitt R, et al: Open-label, uncontrolled, multicenter phase II study to evaluate the efficacy and toxicity of cetuximab as a single agent in patients with recurrent and/or metastatic squamous cell carcinoma of the head and neck who failed to respond to platinum-based therapy. J Clin Oncol 25: 2171-2177, 2007.