Cepharanthin-enhanced radiosensitivity through the inhibition of radiation-induced nuclear factor-κB activity in human oral squamous cell carcinoma cells

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Received February 2, 2007; Accepted March 22, 2007

Abstract. We have already demonstrated that human head and neck cancer cells have significantly enhanced levels of transcription factor nuclear factor (NF)-κB activity compared to their normal counterparts, suggesting that NF-κB plays an important role in the development of head and neck cancer. However, it has been reported that chemotherapeutic agents and radiation activate NF-κB activity in cancer cells, thus making the cells radioresistant and chemoresistant. In addition, we have shown that the suppression of NF-κB activity enhanced apoptosis in oral squamous cell carcinoma cells. In this study, we examined whether cepharanthin-induced inhibition of NF-κB activity enhances radiosensitivity in human oral carcinoma cells. Cepharanthin is a biscoclaurine alkaloid extracted from the roots of *Stephania cepharantha hayata*, and is widely used in Japan for the treatment of patients with leucopenia, nasal allergy, and venomous snakebites. γ-irradiation (IR) induces NF-κB activity in oral carcinoma cells through the activation of upstream molecules, including Akt and IκB kinase. However, a luciferase assay revealed that cepharanthin suppresses IR-induced NF-κB activity in oral squamous cell carcinoma cells, thereby enhancing the radio-sensitivity. Western blot analysis showed an enhanced cleavage of poly-(ADP-ribose) polymerase protein in carcinoma cells by both cepharanthin treatment and IR exposure compared to IR or cepharanthin alone. In an *in vivo* study, B88 cells were s.c. inoculated into the backs of nude mice. Tumor-bearing nude mice received either cepharanthin, IR alone, or a combination of cepharanthin and IR. The combined treatment suppressed tumor growth significantly more than either cepharanthin or IR alone. Cepharanthin inhibited the production of IR-induced IL-6 and IL-8, which are downstream targets of NF-κB. In quantitative real-time RT-PCR, IR also induced the expression of anti-apoptotic proteins [cellular inhibitor of apoptosis protein (cIAP)-1 and -2] in carcinoma cells. Treatment of cancer cells with cepharanthin combined with exposure to IR decreased cIAP-1 and -2 mRNA expression. These findings suggested that the combination of radiotherapy and cepharanthin could enhance radiosensitivity in the treatment of human oral cancer.

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Key words: oral cancer cells, nuclear factor-κB, cepharanthin

Introduction

NF-κB represents a family of dimeric transcriptional factors characterized by a 300-amino-acid region called the Rel homology domain (1). In unstimulated cells, inhibitor of NF-κB (IκB) proteins localize with NF-κB dimers in the cytoplasmic compartment by masking the nuclear localization sequences of NF-κB subunits. NF-κB is activated by various stimuli, including inflammatory cytokines and the receptors that initiate the signal transduction cascade, leading to activation of the IκB kinase (IKK) complex (IKKα, ß, and γ). Following activation of IKK, IKK complex phosphorylates IκB at serine residues, which target IκB for ubiquitination and degradation via a proteasome-dependent pathway. After IκB degradation, NF-κB allows nuclear accumulation, which promotes sequence-specific DNA binding and transcriptional activation of target genes, including anti-apoptotic proteins [TNF receptor-associated factor (TRAF)-1, TRAF-2, cellular inhibitors of apoptosis proteins (cIAP)-1, -2], proinflammatory cytokines [interleukin (IL)-1α, -6, -8], growth factor vascular endothelial growth factor (VEGF), and protein degradative enzyme [matrix metalloproteinase (MMP)-9] (2-7). Akt, which functions downstream of Ras and phosphatidylinositol 3'-kinase (PI3K), can control transcriptional activation through a mechanism dependent on IKK function and p65 phosphorylation.

Surgical resection, radiation therapy, and chemotherapy have proved to be highly effective in eradicating early stages (I and II) of oral squamous cell carcinoma. However, 5-year survival rates of advanced stages (III and IV) have not improved. It is apparent that a different approach to the treatment of oral cancer is needed. Treatment options are limited, and patients with oral cancer frequently fail to respond to standard therapies. Recent advances in the understanding of the molecular events underlying initiation, progression, and metastatic spread may reveal novel therapeutic targets in oral

cancer. Despite the fact that concurrent chemo-radiotherapy markedly improves disease control and survival, the resistance of tumor cells to radiation remains a major therapeutic problem. Based on these considerations, we have investigated the molecular targets for improving treatment of oral cancer by radiotherapy.

Many human solid tumor cell lines display increased nuclear NF-κB levels or increased NF-κB transcriptional activity. NF-κB is activated in head and neck squamous cell carcinoma, multiple myeloma, prostate cancer, leukemia, thyroid cancer, breast cancer, and so on (8-11). We have previously shown that human head and neck cancer cells have significantly enhanced levels of NF-κB activity compared to their normal counterparts, suggesting that NF-κB plays an important role in the development of head and neck cancer (12). We have also demonstrated that the production of angiogenic factors and growth factors in response to radiotherapy and chemotherapy is one of the principal mechanisms of inducible radioresistance and chemoresistance in human oral cancers, and that the introduction of srIκBα cDNA into human oral squamous cell carcinoma (SCC) cells constitutively inhibits the nuclear translocations of NF-κB, thereby drastically decreasing tumorigenicity, in part by down-regulating the expression of angiogenic factors (2). We also suggested that NF-κB may be an important therapeutic target for improving conventional radiotherapy in oral SCC (2,12).

Cepharanthin (6', 12'-dimethoxy-2, 2'-dimethyl-6, 7-[methylenebis(oxy)] oxyacanthan) (CE) is a biscoclaurine alkaloid extracted from the roots of *Stephania cepharantha hayata*. Although the exact mechanism by which CE kills tumor cells has not been elucidated, CE is known as a membrane-interacting agent that has membrane-stabilizing activity, it is also an immunomodulatory effector that enhances the cytotoxic effects of natural killer cells and macrophages, suggesting that it may be involved in the regulation of cytokine signaling pathways (13,14). CE has been used in Japan for the treatment of leukopenia induced by anticancer drugs and radiation therapy, snake venom-induced hemolysis, and nasal allergy (13,14).

Thus, we report here that CE-induced inhibition of NF-κB activity would enhance radiosensitivity in human oral carcinoma cells. In both *in vitro* and *in vivo* studies, cell and tumor growth were suppressed significantly more by the combined CE and IR treatment than by either CE or IR alone. CE inhibited the production of IR (7.5 and 15 Gy)-induced IL-6 and IL-8 proteins and the mRNA expression of anti-apoptotic proteins, cIAP-1 and -2, in carcinoma cells.

Materials and methods

Cells and media. Three human oral SCC cell lines (B88, BHY, and HNt) were previously established in our laboratory (2,12). These cell clones were cultured in DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA) and 100 μg/ml penicillin-streptomycin (Invitrogen) in the presence of 5% CO_2 in an incubator at 37˚C.

In vitro cell growth assay. Cells ($5x10^3$ cells per well) were seeded on 96-well plates (Becton Dickinson Labware, Franklin lakes, NJ) in DMEM supplemented with 10% serum. Twenty-four hours later, the cells were either treated with cepharanthin (CE) (Kaken Syouyaku, Tokyo, Japan), exposed to IR in a Hitachi Medical MBR-1505R2 X-ray generator (Tokyo, Japan), or both. After 72 h, 10 ml of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well and incubated for 4 h. The blue dye taken up by cells was dissolved in dimethyl sulfoxide (100 μl/well), and the absorbance was measured with a spectrophotometer (BioRad Laboratories, Hercules, CA) at 540 nm. All assays were run in triplicate.

Labeling of oligonucleotides and electrophoretic mobility shift assay (EMSA). The probe consisted of NF-κB-specific double-stranded oligonucleotides with the sequence 5'-AG TTGAGGGGACTTTCCCAGGC-3' containing the κB site. Oligonucleotides were end-labeled with [γ-^{32}P]ATP using polynucleotide kinase. EMSA was carried out as described previously (2). In brief, 5 μg of nuclear extract was mixed with the labeled probes in a 20-μl volume in buffer (10 mM HEPES, pH 7.9, 50 mM KCl, 0.2 mM EDTA, 2.5 mM DTT, 10% glycerol, and 50 ng/ml poly dI-dC). The specificity of the complex was analyzed by incubation with an excess of unlabeled competitor oligonucleotides (100-fold molar excess of the labeled probe). Samples were run on 5% polyacrylamide gels. Gels were dried at 80˚C for 2 h, then exposed to X-Omat AR-5 film (Eastman Kodak, Rochester, NY) at -70˚C.

Luciferase assay. The luciferase assay was performed using a transient transfection system. A total of $1x10^5$ cells were plated in six-well tissue culture plates (Becton Dickinson Labware). The reporter plasmid (0.4 μg), consisting of NF-κB-responsive elements (BD Biosciences Clontech, Palo Alto, CA) and the luciferase gene, was transfected into cell clones using an Effectene transfection reagent (Qiagen, Hilden, Germany). Forty-eight hours after transfection, the cells were treated with CE or exposed to IR. The cell lysate was prepared, and luciferase activity was determined by a luciferase assay system (PicaGene Toyo-Inki, Tokyo, Japan) using a luminometer (LUMAT LB9507; Perkin Elmer Life Sciences, Boston, MA).

Western blot analysis of IκBα and poly-(ADP-ribose) polymerase (PARP). After the cells were treated with CE or exposed to IR, they were collected and lysed. Whole cell lysates were subjected to electrophoresis on 10% SDS-polyacrylamide gels, then transferred to a nitrocellulose membrane. The membranes were incubated with the anti-IκBα antibody (Cell Signaling Technology, Beverly, MA) and anti-PARP antibody (BD Biosciences Clontech). The antibody was detected using a chemiluminescence Western blotting kit (Amersham, Tokyo, Japan) according to the manufacturer's instructions.

In vivo tumor growth assay. The effect of combined CE treatment and IR exposure was assessed by inoculation of cells into 5- to 6-week-old female athymic BALB/c nude mice (Japan Clea, Osaka, Japan). B88 ($5x10^6$ cells) cells were injected s.c. into the backs of 5 mice. Tumors at the inoculation site were monitored and measured. When the tumors reached 50-100 mm^3 in volume, they were either

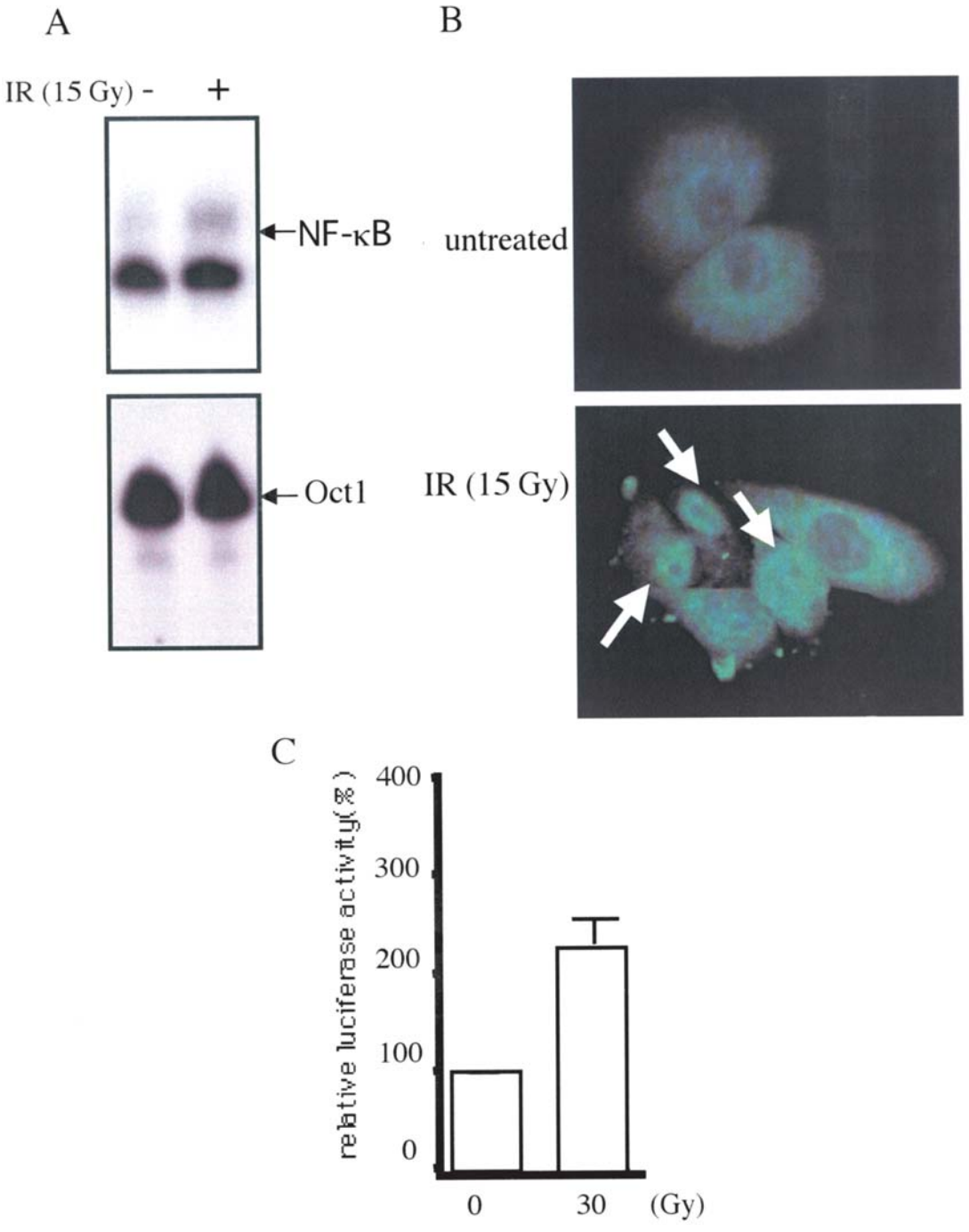


Figure 1. (A) EMSA for NF-κB and Oct1 activation in B88 cells after treatment without or with IR (15 Gy). The specificity of the complex was confirmed by incubation with a 100-fold excess of unlabeled κB oligonucleotide. (B) Immunofluorescence staining of p65 protein in oral carcinoma cells. B88 cells were grown on coverslips in 35-mm dishes. After the cells were exposed to IR (15 Gy), they were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. The expression of p65 protein was located in IR-stimulated nuclei. The arrow shows the localization of p65 protein in the nuclei. (C) NF-κB luciferase reporter assay of NF-κB activity in B88 cells. The reporter plasmid was transfected into B88 cells exposed to IR (30 Gy). The results are representative of three experiments.

exposed to IR (1.5 Gy/day, 5 times/week) or treated with CE (10 mg/kg, ip, 3 times/week) for 2 weeks. Tumor volume was recorded three times a week. The mice were maintained under pathogen-free conditions and were handled in accordance with the Guidelines for Animal Experimentation of Tokushima University.

Enzyme-linked immunosorbent assay (ELISA) for quantitative determination of IL-6 and IL-8. IL-6 and IL-8 contained in conditioned medium (CM) from untreated control, CE-treated, or IR-exposed cells were measured by a microtiter-based sandwich enzyme immunoassay system, which is commercially available and specifically estimates the total amounts of IL-6 and IL-8. After cultivation for 72 h, CM were subjected to ELISA using immunoassay kits for IL-6 and IL-8 (BioSource International, Camarillo, CA).

Immunofluorescence staining. B88 cells were grown on coverslips in 35-mm dishes. After the cells were exposed to IR, they were fixed in 4% paraformaldehyde and permeabilized

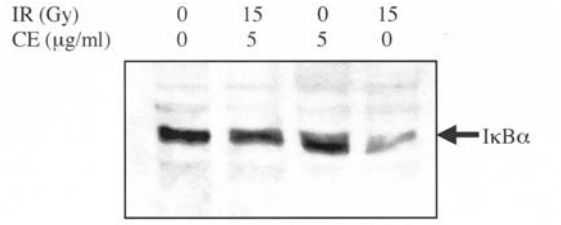


Figure 2. Western blot analysis of IκBα protein levels in B88 cells. B88 cells were pretreated and exposed with or without 5 μg/ml cepharanthin and IR (15 Gy). Whole cell fractions extracted from untreated cells and treated cells were subjected to SDS-PAGE. IκBα protein was visualized with IκBα-specific antibody as described under Materials and methods.

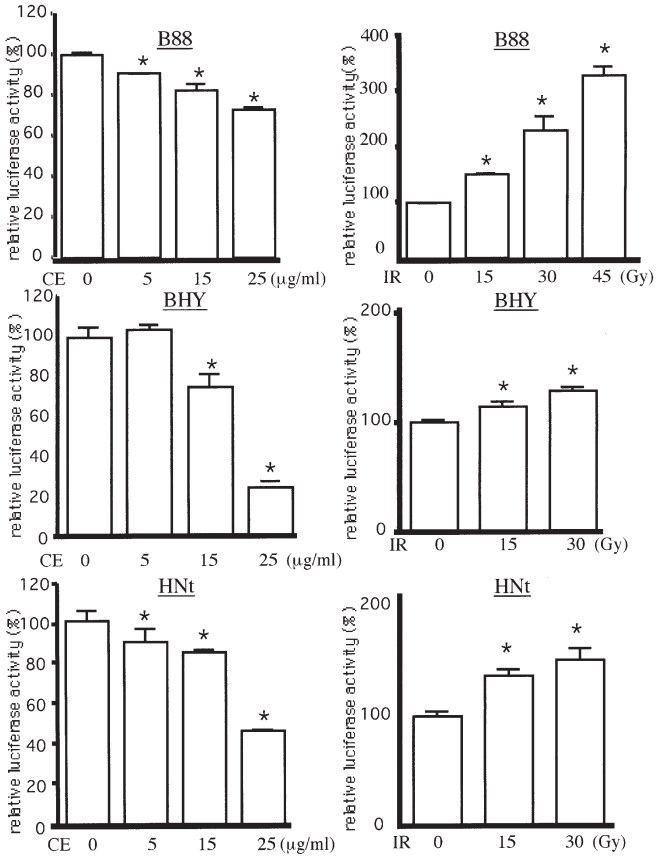


Figure 3. NF-κB luciferase reporter assay of NF-κB activity in B88, BHY, and HNt cells. The reporter plasmid was transfected into B88, BHY, and HNt cells exposed to IR (0, 15, 30, and 45 Gy) or pretreated with CE (0, 5, 15, and 25 μg/ml). The results are representative of three experiments.

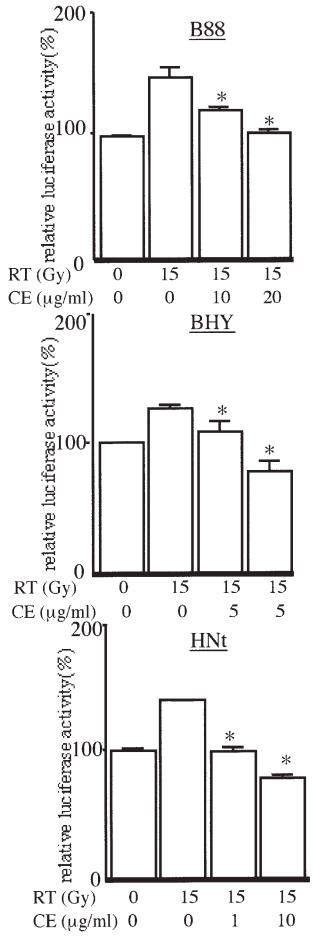


Figure 4. NF-κB luciferase reporter assay of NF-κB activity in B88, BHY, and HNt cells combined with exposure to IR (15 Gy) or pretreatment with CE (0, 1, 5, 10, 15, and 20 μg/ml). The results are representative of three experiments. Pretreatment with CE combined with exposure to IR significantly decreased as compared to IR alone at $P<0.05$ (Mann-Whitney U test).

with 0.1% Triton X-100. Cells were incubated with polyclonal p65 antibody (LAB Vision, Fremont, CA) and Alexa Fluor 488 goat anti-rabbit IgG (H+L). The coverslips were rinsed and mounted.

Statistical analysis. Statistical analysis was performed using the Mann-Whitney U test; P-values <0.05 were considered to indicate statistical significance.

Results

Effects of IR on NF-κB binding ability and activity in B88 cells. To assess IR's effects on NF-κB binding ability and activity, as well as on the translocation of p65 from cytosol to the nuclei, we performed EMSA, luciferase assay, and immunofluorescence staining for p65. As shown in Fig. 1A, exposure of B88 cells to IR (15 Gy) induced a significant increase in NF-κB binding ability compared with untreated cells. p65 protein also translocated to the nuclei by stimulation with IR (Fig. 1B). Luciferase assay revealed the enhancement of NF-κB activity in B88 cells by exposure to IR (Fig. 1C). Therefore, it was confirmed that IR enhances NF-κB activity in human oral cancer cells.

Inhibition of IκBα protein degradation by exposure to IR in carcinoma cells. To examine whether IR or CE would activate NF-κB activity, Western blot analysis was used to investigate IκBα protein expression after either IR exposure, CE treatment, or both (Fig. 2). Whereas IκBα protein was degraded by IR exposure (15 Gy), treatment of carcinoma cells with 5 μg/ml CE did not affect IκBα protein expression. Pretreatment with CE followed by IR exposure partially inhibited the degradation of IκBα protein in B88 cells.

Luciferase assay to determine the effects of IR and CE on NF-κB activity in carcinoma cells. We next investigated whether CE, IR, or both would increase NF-κB activity in

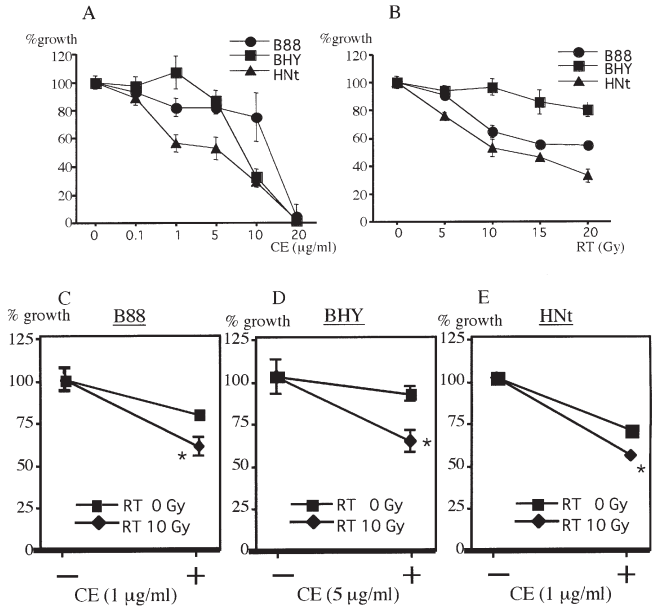


Figure 5. (A and B) Cells ($5x10^3$/well) were seeded in 96-well plates. After 24 h, cells were either exposed to IR (0-20 Gy) or treated with CE (0.1-20 μg/ml) alone. After 72 h, *in vitro* cell growth was evaluated by MTT assay. (B) Following a 1-h pretreatment with CE, B88, BHY, and HNt cells were exposed to IR and *in vitro* cell growth was evaluated by MTT assay as % growth.

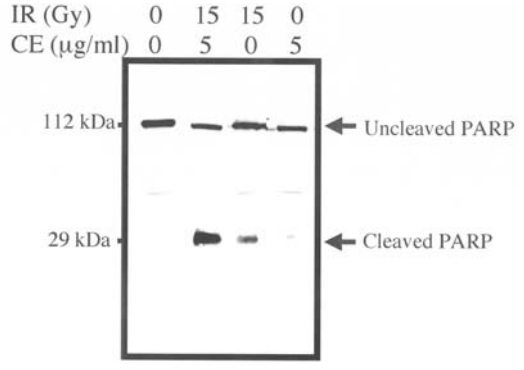


Figure 6. Western blot analysis of poly-(ADP-ribose) polymerase (PARP) cleavage in carcinoma cells treated with either CE (5 μg/ml) or IR (15 Gy) alone, or with the combination of CE and IR for 72 h. Whole cell fractions extracted from the cells were subjected to SDS-PAGE. The molecular weights of uncleaved and cleaved PARP were 112 and 29 kDa.

carcinoma cells. The luciferase assay revealed that CE treatment suppressed the transcriptional activity of NF-κB in a dose-dependent manner in B88, BHY, and HNt cells compared with untreated controls (Fig. 3). However, IR enhanced NF-κB activity in all of the cells, especially in B88 cells, in a dose-dependent manner. Pretreatment with CE followed by exposure to IR significantly suppressed the IR-induced NF-κB activity in B88, BHY, and HNt cells (Fig. 4). Accordingly, it is evident that CE inhibited IR-induced NF-κB activity in oral carcinoma cells.

Effects of IR and CE on in vitro growth of oral carcinoma cells. We examined the effects of IR and CE on the viability of carcinoma cells (Fig. 5A and B). Cell viability was evaluated by MTT assay after 72-h treatment with CE or IR at various

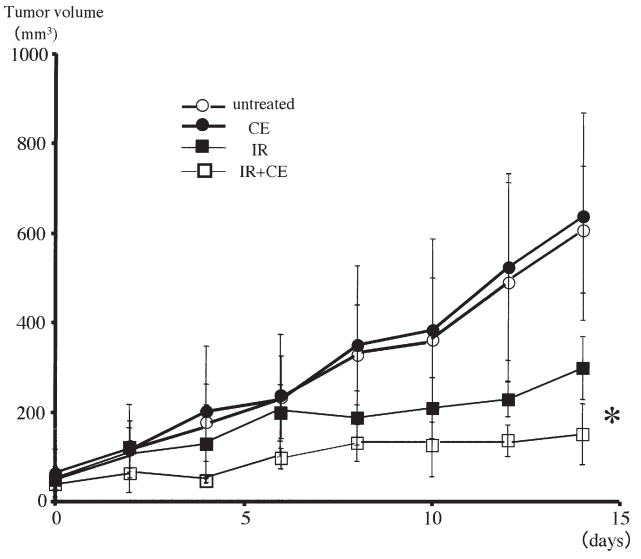


Figure 7. The role of cepharanthin and IR in *in vivo* tumorigenicity of B88 cells. Cells ($5x10^6$) were inoculated into the backs of nude mice (n=5). When the tumors reached 50-100 mm^3 in volume, they were either exposed to IR (1.5 Gy/day, 5 times/week) or treated with CE (10 mg/kg, i.p., 3 times/week) for 2 weeks. Tumor diameters along two orthogonal axes were recorded, and tumor volume was calculated by assuming a spherical shape of the tumor three times a week. Tumor volume was recorded as the mean volume for each treatment group (n=5); bars, SD. *The combination of cepharanthin treatment and exposure to IR significantly decreased tumor growth as compared to the untreated control, CE, and IR alone groups at $P<0.05$ (Mann-Whitney U test).

CE concentrations or IR doses. Cell growth decreased at concentrations of up to 1 μg/ml of CE, and the decreases were marked at 15-20 μg/ml of CE. IR also inhibited cell growth in a dose-dependent manner, and HNt cells were much more sensitive to IR than were the other carcinoma cells. To determine whether CE treatment of carcinoma cells enhances IR's anticancer effect, B88, BHY, and HNt cells were exposed to IR (10 Gy) after pretreatment with CE (1 or 5 μg/ml). Exposure of carcinoma cells to IR after pretreatment with CE resulted in a synergistic effect on growth suppression as compared to no CE pretreatment (Fig. 5C, D and E).

Augmentation of apoptosis by combination of IR exposure and CE pretreatment in carcinoma cells. To investigate whether the enhanced cytotoxicity was due to apoptosis, the cleavage of PARP in carcinoma cells was examined by Western blot analysis (Fig. 6). The expression of cleaved PARP with a molecular weight of 29 kDa was significantly enhanced by the combined IR and CE treatment compared with untreated controls, IR, or CE alone.

The effect of in vivo therapy combining CE and IR on tumor growth in nude mice. On the basis of the above *in vitro* findings, we evaluated the effects of CE and IR on *in vivo* tumor growth. B88 cells were injected s.c. into the backs of nude mice (n=5), and the treatment with IR or CE was started when the tumor volume reached 50-100 mm^3. As shown in Fig. 7, tumors exposed to IR with CE pretreatment had remarkably inhibited growth of B88 cells compared with untreated controls, IR exposure alone, or CE treatment alone.

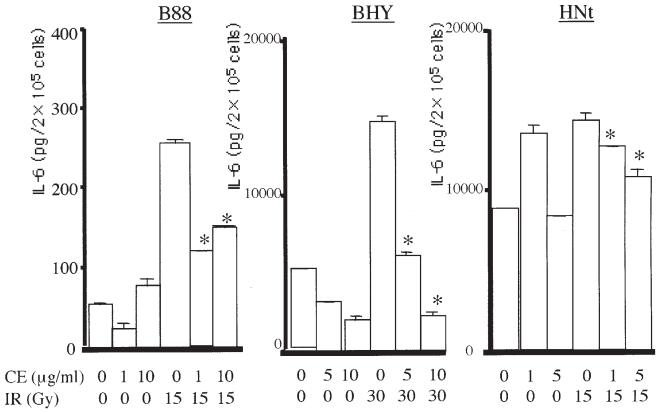


Figure 8. Expression of IL-6 proteins in the culture supernatants determined by ELISA in B88, BHY, and HNt cells. After treatment with cepharanthin (1, 5, 10 μg/ml) and/or exposure to IR (15, 30 Gy), the supernatants were collected. IL-6 levels were normalized to cell number, and they were estimated as pg/2x10^5 cells. *The combination of cepharanthin treatment and IR exposure significantly decreased IL-6 production as compared to the untreated control, CE, and IR alone groups at P<0.05. Bars, SD.

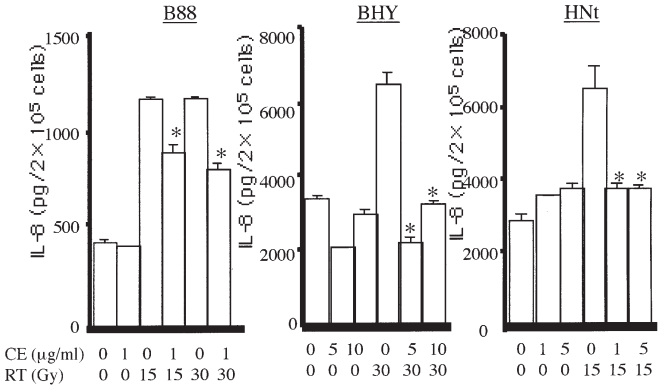
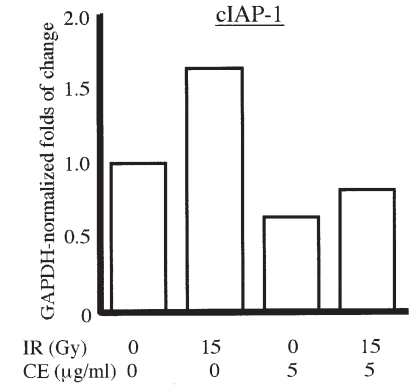


Figure 9. Expression of IL-8 proteins in the culture supernatants determined by ELISA in B88, BHY, and HNt cells. After treatment with cepharanthin (1, 5, 10 μg/ml) and/or exposure to IR (15, 30 Gy), the supernatants were collected. IL-8 levels were normalized to cell number, and they were estimated as pg/2x10^5 cells. *The combination of cepharanthin treatment and IR exposure significantly decreased IL-8 production as compared to the untreated control, CE, or IR alone groups at P<0.05. Bars, SD.

Expression of IL-6 and IL-8 proteins in conditioned medium after treatment with IR or CE. We next investigated the effects of IR and CE on the production of IL-6 and IL-8 by cancer cells. Because IL-6 and IL-8 genes contain NF-κB binding sites in their 5' promoter regions, their expression is regulated by NF-κB. As shown in Figs. 8 and 9, ELISA was used to quantify IL-6 and IL-8 proteins in conditioned medium. Although IR augmented the production of IL-6 and IL-8 proteins in B88, BHY, and HNt cells, CE showed no significant effect on the production of IL-6 and IL-8 proteins in any of the cells. CE pretreatment followed by exposure to IR significantly suppressed the production of IL-6 and IL-8 from cancer cells.

Effects of IR and CE on the expression of cIAP-1 and cIAP-2 mRNAs in B88 cells. Quantitative RT-PCR was performed to further investigate whether or not CE treatment or IR exposure affects the expression levels of two anti-apoptosis proteins,



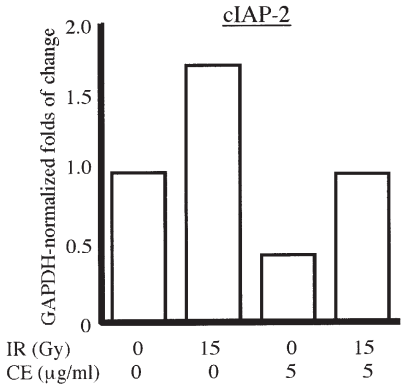


Figure 10. Quantitative analysis of cIAP-1 and cIAP-2 mRNA transcription in B88 cells. RT-PCR products were quantitatively analyzed using the ABI 7000 sequence detection system. The data shown are the ratio of GAPDH-normalized fold change in PCR product in IR-exposed or CE-treated cells to the GAPDH-normalized amount of PCR product in untreated cells.

cIAP-1 and cIAP-2, in oral cancer cells. As shown in Fig. 10, real-time RT-PCR revealed that cIAP-1 and cIAP-2 mRNA expression was statistically increased by IR exposure compared with untreated controls, whereas CE treatment significantly decreased cIAP-1 and cIAP-2 expression. The combination of IR (15 Gy) and CE (5 μg/ml) reduced cIAP-1 and cIAP-2 mRNA expression levels more than did IR alone.

Discussion

Multiple modalities for the treatment of oral cancer, such as surgery, radiation, and chemotherapy, have contributed to the upward trend in the 5-year survival rates. In addition, remarkable progress has been made in the elucidation of genetic and epigenetic events in the development of oral cancer, including the sequential accumulation of alterations in oncogenes, tumor suppressor genes, and their related protein products (15). Important therapeutic molecular targets for the treatment of oral cancer have been reported as clinical studies (16,17). Patients who are diagnosed with an advanced stage of oral cancer are subjected to lengthy, high-risk operations, often resulting in functional loss, disfigurement, and diminished quality of life. Since the 5-year survival rates of patients at advanced stages (stages III and IV) have not improved, it is apparent that a different approach to the treatment of oral cancer is needed. The combination of chemotherapy and radiotherapy has been implemented for oral cancer patients at

stage III or IV as a primary treatment method. Chemotherapy acts as a radiation sensitizer, enhancing the effect of radiation treatment. We have focused on NF-κB as a molecular target for improving the effectiveness of both radiotherapy and chemotherapy, and as a radiation sensitizer.

NF-κB is one of the main transcription activators. Many studies have shown that the inhibition of this pathway could suppress tumor growth (18-20). One major mechanism by which NF-κB works is the inhibition of IκB phosphorylation, resulting in the retention of NF-κB in the cytoplasm. NF-κB is an extensively anti-apoptotic transcription factor whose DNA binding is potently and rapidly induced by TNF-α, IR, and other stimuli in almost all cells. Constitutive NF-κB activation has been observed in a wide variety of cancers including those of the prostate, colon, and lung. It is associated with a resistance to apoptosis because many of its target genes code for anti-apoptotic molecules (21-23). We have shown that several human head and neck carcinoma cell lines, including B88, BHY, and HNt cells used in this study, express high levels of NF-κB activity (12). Thus, the introduction of a super-repressor form of IκBα cDNA and constitutive inhibition of the nuclear translocation of NF-κB have caused a drastic decrease in the tumorigenicity of oral cancer cells via the downregulation of the expression of angiogenic factors, growth factors, and MMP-92. In addition, the suppression of NF-κB activity in B88 cells led to the enhancement of radiosensitivity and chemosensitivity to IR and 5-FU. The results of many reports together suggest that NF-κB would be an important therapeutic molecular target for the treatment of patients with oral cancer. Many findings indicate that the blockade of NF-κB signaling pathways has produced a new and effective strategy for anticancer therapy (16,17). The NF-κB pathway, by enhancing the expression of genes that increase cellular proliferation and survival in addition to promoting angiogenesis and metastasis, plays an oncogenic role. Furthermore, following treatment with radiotherapy or chemotherapy, activation of the NF-κB pathway in cancer led to the induction of apoptosis inhibitors, resulting in reduced cell death as well as reduced effects of treatment by chemotherapeutic agents and radiation. In this study, we investigated the effect of IR on NF-κB activity and binding ability in three oral cancer cell lines. In all three lines, IR enhanced NF-κB activity in a dose-dependent manner, resulting in the increase of downstream signaling. We also demonstrated that IR induced the expression of IL-6 and IL-8, both of which contribute to tumor growth and angiogenesis, as well as that of cIAP-1 and cIAP-2, which work anti-apoptotically.

Cepharanthin has been widely used for the treatment of patients with leukopenia, nasal allergy, and venomous snakebites (14). Although the exact mechanisms underlying its actions have not been elucidated, cepharanthin may play a role in the regulation of signaling pathways of cytokines. It has been shown that cepharanthin is an effective agent for the reversal of resistance in P-glycoprotein (P-gp)-overexpressing cells, which are involved in multidrug resistance (24-27). It has been reported that cepharanthin not only reversed the multidrug resistance mediated by P-gp, but also enhanced the cytotoxic effects of two anticancer agents, doxorubicin and vincristine (28,29). We had already reported that cepharanthin suppressed TNF-α-induced MMP-9 production through the inhibition of NF-κB activity in human salivary gland acinar cells (13,14). We have not yet identified in detail the mechanisms involved in the cepharanthin-induced inhibition of NF-κB activity. Although several possibilities can be suggested, in this study we have focused on the inhibition of NF-κB activity. Following pretreatment with cepharanthin, IR exposure induced a synergistic growth-inhibitory effect *in vitro* and *in vivo* compared with cepharanthin alone or IR alone. Cepharanthin inhibited IR-induced NF-κB activity in a dose-dependent manner. Therefore, it also suppressed the IR-induced production of IL-6 and IL-8 protein, which contained NF-κB binding sites in their 5' promoter regions and which NF-κB regulated. In this study, we investigated whether cepharanthin can work as an NF-κB inhibitor and augment the radiosensitivity of carcinoma cells. Although IR has been used as an effective anticancer modality for pre- and post-operative treatment in oral cancer, it has been demonstrated that IR stimulates the production of angiogenic factors and growth factors as well as the expression of anti-apoptosis genes.

Ikeda *et al* reported that cepharanthin enhanced sensitivity to doxorubicin (ADM) and vincristine (VCR) and enhanced apoptosis induced by ADM and VCR via inhibition of p-glycoprotein expression (21). In addition, Okamoto *et al* demonstrated that the combination of transcriptional inhibitor K-12 and cepharanthin through suppression of NF-κB synergistically inhibited HIV production in tumor necrosis factor α-stimulated U1 cells, a promonocytic cell line chronically infected with the virus (30). On the basis of these findings, in this study we investigated whether cepharanthin increases the antitumor effects of IR. Although IR would induce cell death via apoptotic pathway induction, NF-κB could protect cancer cells from IR-induced apoptosis and regulate the anti-apoptotic proteins (TRAF-1, -2, cIAP-1, -2, and bcl-2). Cepharanthin markedly inhibited tumor growth by suppressing IR-induced expression of cIAP-1 and -2 genes.

In conclusion, this study showed that cepharanthin can work as an NF-κB inhibitor. Although IR significantly enhanced IL-6 and IL-8 production as well as cIAP-1 and -2 mRNA expression, cepharanthin reduced those increases. These findings suggest that cepharanthin not only suppresses NF-κB in oral cancer cells but also sensitizes tumor cells to radiotherapy. Therefore, cepharanthin treatment may be a potential approach to controlling the growth of human oral cancer. Accordingly, the combination of radiotherapy and cepharanthin could lead to enhanced radiosensitivity in the treatment of oral cancer.

Acknowledgements

This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan.

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