We studied factors that control chemoresistance to 6 head and neck squamous cell carcinoma cell lines carrying p53 mutations. Cell lines were chosen, based on the presence of EGFR amplifications, the presence of H-ras mutations, and the absence of either. WST-1 viability assays showed that, in response to etoposide, Ca922 was most sensitive, HOC313 most resistant, and HSC6 and the others moderately sensitive. A similar tendency was shown by further analyses with cisplatin, 5-fluorouracil, LY294002, and combined treatment with LY294002 and TNF-related apoptosis-inducing ligand (TRAIL). Although both Ca922 and HOC313 had activating mutations upstream of Akt signaling, the constitutive phosphorylation of Akt at S473 was observed in chemosensitive Ca922, but not in chemoresistant HOC313, suggesting that constitutive Akt phosphorylation was not the primary determinant for chemoresistance in these cell lines. Further, by the combined treatment with LY294002 and TRAIL, apoptosis was induced in Ca922 and HSC6 but not in HOC313. Interestingly, caspase 8 was not detected in HOC313, while it was cleaved in the other 2 cell lines. Further, in Ca922 and HSC6 but not in HOC313, caspase 8 inhibitor restored loss of viability induced either with LY294002 and TRAIL or even with etoposide alone. These findings suggest that caspase 8 played an important role in chemoresistance against genotoxic drugs.

Key words: head and neck squamous cell carcinoma, caspase 8, Akt, etoposide, TRAIL

Introduction

Human head and neck squamous cell carcinoma, HNSCC, is the sixth most common solid tumor worldwide, accounting for about 5% of all new cancers diagnosed annually in the United States. Although recent advances in management with a multidisciplinary approach including chemotherapy in combination with radiotherapy or surgery have resulted in improved local and regional disease control, the overall survival rate has improved little during the last decade. Obviously, greater intervention will be required to significantly enhance HNSCC cancer therapy.

Chemotherapy and radiotherapy mostly depend on how effectively a drug induces apoptosis, which is regulated in part by growth regulatory factors and cell survival factors. Growth and survival signaling pathways are controlled by interaction between oncogenes and tumor suppressor genes, both of which also control apoptosis. The tumor suppressor p53 that is important
in preventing cancer is activated under the genotoxic stress imposed with many anti-cancer drugs. The activated p53 transactivates genes many products of which are involved in the induction of apoptosis either through an intrinsic mitochondria-initiated pathway or through an extrinsic death-receptor-initiated pathway. In the intrinsic pathway, p53-target gene products are involved in the release of cytochrome c resulting in activation of caspase 9, which in turn activates execution-type caspase 3. In the extrinsic pathway, p53 transactivates genes of death receptors (Fas and DRs) which sensitize cells to apoptosis induced by death receptor ligands. Although more than half of all human cancers have p53 mutations, p73, a p53 homologue, partly complements the defectiveness of mutant p53 while mutant p53 has been reported to modulate p73-dependent apoptosis.

Akt/PKB plays a key role in survival signaling through phosphatidylinositol 3 kinase (PI3K) and it was reported that activation of this pathway confers to cancer cells chemoresistance. Akt/PKB is activated in response to different growth factors, such as epidermal growth factor (EGF), and insulin through growth factor receptors and Ras which sensitize cells to apoptosis induced by death receptor ligands. Although more than half of all human cancers have p53 mutations, p73, a p53 homologue, partly complements the defectiveness of mutant p53 while mutant p53 has been reported to modulate p73-dependent apoptosis.

Recent studies have shown that genotoxic agents such as chemotherapy and ionizing radiation can sensitize cells to killing by TRAIL. TRAIL induces apoptosis in a variety of tumor cell lines more efficiently than in normal cells. The activated p53 transactivates genes many products of which are involved in the induction of apoptosis either through an intrinsic mitochondria-initiated pathway or through an extrinsic death-receptor-initiated pathway. In the intrinsic pathway, p53-target gene products are involved in the release of cytochrome c resulting in activation of caspase 9, which in turn activates execution-type caspase 3. In the extrinsic pathway, p53 transactivates genes of death receptors (Fas and DRs) which sensitize cells to apoptosis induced by death receptor ligands. Although more than half of all human cancers have p53 mutations, p73, a p53 homologue, partly complements the defectiveness of mutant p53 while mutant p53 has been reported to modulate p73-dependent apoptosis.

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Materials and Methods

Chemicals

Human recombinant EGF and TRAIL were purchased from Sigma-Aldrich Co. (St. Louis, MO) and R&D Systems (Minneapolis, MN), respectively. A PI3K inhibitor, LY294002 (Alexis Biochemicals, San Diego, CA), and anti-cancer drugs including etoposide, cisplatin, and 5-FU (Sigma-Aldrich Co.), were used. The caspase 8/6 inhibitor, Ac-IETD-CHO, was from Peptide Institute Inc. (Osaka, Japan).

Antibodies

Anti-phospho S473 (recognizes Akt1, -2, and -3) and T308 (recognizes Akt1) of Akt, anti-total-Akt, anti-PTEN, anti-PARP, anti-caspase 8, anti-caspase 9, and anti-cleaved-caspase 3 were purchased from Cell Signaling (Beverly, MA). Anti-β-tubulin was purchased from Sigma-Aldrich Co.

Cell lines and culture

HNSCC cell lines, (Ca922, HSC6, HOC313, ZA, HSC3, and OM-1) were used. All cell lines were cultured in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% (v/v) FBS, and 10 μg/ml gentamycin (Sigma-Aldrich Co.). Cells were incubated at 37 °C with 5% CO2/95% air in a humidified CO2 incubator.

EGF treatment

HNSCC cells were plated at 4 x 10^5 cells/well in 60 mm dishes with DMEM containing 10% FBS, and incubated for 24 h. The medium was changed to
DMEM without FBS (serum-free), and the cells were further incubated for 48 h. EGF (50 ng/ml) was added for 10 min before cell lysates were prepared.

**Preparation of cell lysates and Western blotting**

Cell extracts were prepared and Western blotting was conducted essentially as described previously\(^27\). Briefly, cells in 60 mm culture dishes were washed once with ice-cold PBS, and lysed with 100 \(\mu\)l of ice-cold NP-40 lysis buffer [20 mM TrisHCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% NP-40 2.5 mM sodium PPI, 1 mM \(\beta\)-glycerophosphate, 1 mM Na\(_4\)VO\(_4\), and 1 mM phenylmethylsulfonly fluoride, containing protease inhibitor cocktail (Roche Diagnostics, Boehringer-Mannhein, Germany)] for 10 min, collected by scraping, sonicated and centrifuged at 10,000 \(\times g\) for 10 min. Protein concentrations of the supernatants (extracts) were quantified using a DC protein assay kit (Bio-Rad Co., Hercules, CA). Equal amounts of protein were loaded, and the lysates were separated by SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were blocked for 1h in blocking buffer (1xTBS, 5% skim milk, and 0.2% Tween 20) and incubated with appropriately diluted primary antibody in the blocking buffer overnight at 4°C. Nitrocellulose membranes were washed three times in wash buffer (0.1% NP-40, 0.1% Tween 20, and 1xTBS). Protein bands that reacted with the primary antibody were detected using horseradish peroxidase-linked goat anti-mouse or goat anti-rabbit IgG antibodies and visualized with the enhanced chemiluminescent detection reagents (New England Biolabs, MA).

**Drug sensitivity assessed by cell proliferation (WST-1) assay**

HNSCC cells were seeded in 96-well plates at a density of 5,000 cells/well in a final volume of 100 \(\mu\)l. Twenty-four hours after seeding, the medium was replaced with fresh medium containing a concentration of anti-cancer drug in a final volume of 100 \(\mu\)l. The culture was maintained in a CO\(_2\) incubator for an additional period of 24 h. At the end of the incubation, 10 \(\mu\)l of WST-1 labeling solution (WST-1 cell proliferation assay kit; Dojin Chemicals, Tokyo) was added, and the cells were returned to the incubator for 2 h. The absorbance of the formazan product formed by metabolically active cells was detected at 450 nm with a reference wavelength at 650 nm in a 96-well plate reader. Cell viability was determined as a percentage of the control.

**Treatment with TRAIL and/or LY294002**

Cells were replaced with fresh medium containing TRAIL and/or LY294002. For combination experiments, TRAIL and/or LY294002 were added at the same time and both were incubated for 24 h, before the preparation of cell extract or quantification of apoptosis (see below).

**Flow cytometric analysis**

Adherent cells were harvested by trypsinization, and then centrifuged at 1000 \(\times g\) for 5 min. Cells were fixed in ice-cold 70% methanol by adding dropwise, and then incubated at -20 °C for 30 min. Cells were centrifuged and incubated with propidium iodide (25 \(\mu\)g/ml) supplemented with RNase A (30 \(\mu\)g/ml) for 30 min at room temperature. Quantification of sub G1 population was determined by flow cytometric analysis using a FACS scan (Becton Dickinson, NJ) and by manual gating using CellQuest software (Becton Dickinson, NJ). Gating was performed on blinded samples.

**Statistics**

All assays were performed in triplicate each time. The data were analyzed for statistical significance using Student’s *t*-test.

**Results**

**Sensitivity of HNSCC cell lines to anti-cancer drugs**

Six HNSCC cell lines were examined for sensitivity to etoposide by incubating for 24 h at different concentrations (0-300 \(\mu\)g/ml), and the cytotoxicity was assessed with the WST-1 assay. All 6 cell lines chosen were established in our faculty and varied in the status of genes commonly mutated in HNSCC, although all had mutations of p53 (Table). HOC313 and ZA have ras activation mutations, while Ca922, HSC3, and ZA have an amplification of EGFR. As shown in Figure 1, HOC313 which carried a H-ras codon 12 activation mutation exhibited the most resistance to etoposide, while Ca922 which had an amplification of EGFR was most sensitive to the drug. The other cell lines showed intermediate sensitivity. It is apparent that the sensitivity to etoposide was neither directly correlated to the ras mutation as ZA had the mutation in H-ras, nor to EGFR amplification as HSC3 and ZA had EGFR amplification.

We then used two additional anti-cancer drugs, cis-
platin and 5-FU also among the first choice for HNSCC treatment. The relative sensitivity was essentially the same for each of the cell lines (Figure 2) although the figure showed results with 3 cell lines, HOC313 (resistant), HSC6 (intermediate) and Ca922 (sensitive). It is thus suggested that the sensitivity to etoposide, cisplatin, and 5-FU may be controlled at least in part by a common mechanism.


Constitutive phosphorylation of Akt did not correlate to drug sensitivity

To find a common mechanism to explain the sensitivity, we examined whether the level of phosphorylated Akt is related to the chemosensitivity, as Akt is a survival factor and these drugs are reported to induce apoptosis. Cells were serum-starved for 48 h, and then stimulated with EGF for 10 min. Levels of phosphorylated Akt were compared before and after EGF treatment using antibodies against phosphorylated serine at codon 473 (S473). As shown in Figure 3, the phosphorylation of S473 was maintained at high levels in 4 of 6 HNSCC cell lines, Ca922, HSC6, ZA, and HSC3, even after the serum starvation. The total amount of Akt protein changed little in each of the six cell lines. There was no apparent correlation between the phosphorylation of Akt at S473 and the presence or absence of p53, H-ras, or EGFR mutations. However, 2 cell lines, HOC313 and OM-1, exhibited down regulation of S473 phosphorylation after serum starvation.
although the level was slightly higher in OM-1 than HOC313. In these two cell lines, S473 was phospho-
ylated upon stimulation of EGF, although levels were lower than those in the other 4 cell lines.

In contrast, most HNSCC cell lines showed phos-
phorylation of threonine at 308 (T308) upon EGF

Fig. 3. AKT/PKB Ser473 phosphorylation is constitutive in a subset of HNSCC cell lines.

whether the loss of cell viability was due to apoptot-
"CASPAR 8 AS A DETERMINANT IN CHEMOSENSITIVITY"

Synergistic induction of cytotoxicity by the combi-
nation of TRAIL and LY294002 in HNSCC cell lines

Loss of cell viability was accompanied by an increase in the subG1 population as determined by flow cytometric analysis

Whether the loss of cell viability was due to apoptot-
ic cell death or not was studied by flow cytometric analysis. HNSCC cell lines (Ca922, HSC6, and HOC313) were treated with TRAIL (0.1 or 1 μg/ml), LY294002 (50 or 100 μM), or a combination of the two for 24 h and stained with propidium iodide and subjected to flow cytometric analysis. As shown in Figure 5, fractions of the subG1 population and relative viability obtained in the WST-1 assay coincided. When the values for single LY294002 treatments were compared with those for co-treatment (TRAIL and LY294002), subG1 fractions in co-treated Ca922 and HSC6 cells increased 2-3 fold while in HOC313, the increase was marginal (from 15.2% to 18.8%), suggesting that a reduction of viability represents mostly, if not completely, apoptosis.

Treatment with TRAIL plus LY294002 promoted the cleavage of Poly (Adenosine Diphosphate-Ribose) Polymerase (PARP) and the activation of caspases.

Apoptosis is characterized by the cleavage of PARP and activation of caspases and inactivation of Akt by LY294002. In Western blotting, the phosphorylated Akt disappeared after LY294002 treatment, confirming that Akt as a survival factor was inactivated by LY294002 (Figure 6, the top panels). Then, we examined the effect of the combination of TRAIL and LY294002 on the cleavage of PARP and the activation of caspases, in particular, caspase 8, caspase 9 and caspase 3. HNSCC cell lines (Ca922, HSC6, and HOC313) were treated with TRAIL (0.1 μg/ml), LY294002 (50 μM), or a combination of the two for 24 h and then analyzed. Figure 6 shows that, in all cell lines, TRAIL (0.1 μg/ml) alone failed to induce PARP cleavage. In contrast, PARP (116-kDa) was cleaved, yielding a characteristic 89-kDa fragment in the presence of LY294002 (50 μM) alone. In Ca922 and HSC6, the amount of characteristic 24-kDa fragment was markedly enhanced, but not in HOC313, when TRAIL and LY294002 were combined. Here, in HOC313 treated with even only LY294002, the cleavage product of PARP (around 89-kDa) was detected in the absence of active forms of cleavage caspase 3 and caspase 9. Though the mechanism was unclear, this caspase-independent PARP cleavage may be caused by different protease (s) such as cathepsin-B30,31.

These results show that the combination of TRAIL and LY294002 synergistically induces apoptotic death in Ca922 and HSC6 cells. Similar results were observed for the activation of caspases. TRAIL (0.1 μg/ml) alone or LY294002 (50 μM) alone slightly reduced procaspase 8 levels, but the combination of the two markedly reduced the amounts in Ca922 and HSC6. In HOC313, surprisingly, we could not detect caspase 8, suggesting that the expression was...
silenced or the gene was deleted. Active forms of cleaved caspase 3 and caspase 9 were detected in samples of Ca922 and HSC6 cells which were treated with both TRAIL (0.1 μg/ml) and LY294002 (50 μM). Although both procaspase 3 and -9 were detected (data not shown), no active forms were detected at a significant level in HOC313 cells treated with both TRAIL and LY294002. Taken together, these results indicate that the synergistic induction of apoptosis in Ca922 and HSC6 by the combination of TRAIL and LY294002 is due to the promotion of caspase activation in the HNSCC cell lines.

Caspase 8 inhibitor restored the viability lost upon TRAIL/LY294002 or etoposide treatment in Ca922 and HSC6 but not in HOC313.

To confirm that caspase 8 was responsible for the chemosensitivity in Ca922, caspase 8 inhibitor was added to TRAIL/LY294002-treated cells. Loss of viability at 12 h was restored by caspase 8 inhibitor to half the level in HOC313, although caspase 8 exerted no effect on HOC313 (Figure 7A), suggesting that the loss of caspase 8 expression in HOC313 rendered the cell tolerant to TRAIL/LY294002 treatment.

We then studied whether caspase 8 played an important role also in the loss of viability induced by the treatment with etoposide, a genotoxic drug. As shown in Figure 7B, the inhibitor restored the viability lost on treatment with etoposide (100 μg/ml) in Ca922 and HSC6 but not in HOC313.

**Discussion**

We studied factors which control chemoresistance to HNSCC. Among 6 HNSCC cell lines, Ca922 was the most sensitive, HOC313 the most resistant and HSC6 intermediate in its response to etoposide, cisplatin, 5-FU, LY294002 and a combination of TRAIL and LY294002 in this study.
Many factors influence chemoresistance. Enhanced expression of ATP-binding cassette (ABC) transporters is one of them by which a multidrug-resistant cancer cell egests an anti-cancer drug to outside the cell\textsuperscript{32}. Gene expression was examined in microarrays by comparing Ca922 and HOC313 and the results showed that the observed drug resistance was not due to the multidrug-resistant mechanism (Matsumura, et al., unpublished data). The genes examined included those for ten members of the ABC family and the known cisplatin resistance associated protein and expression levels were 0.64-1.47 of that in normal keratinocytes.

It is widely accepted that chemotherapeutic drugs kill cancer cells through the induction of apoptosis\textsuperscript{2}. Factors determining chemoresistance are a loss of proapoptotic factors and gain of anti-apoptotic factors\textsuperscript{2}. Among the proapoptotic factors, p53 plays a key role in inducing apoptosis when cancer cells sustain genotoxic damage by stabilizing and activating the wild-type p53. As a result of the activation, genes for proteins involved in both the intrinsic and extrinsic apoptotic pathways are transactivated\textsuperscript{3}. The cell lines used in this study all carry mutant p53 genes. Recently, cisplatin-induced sensitivity to apoptosis was reported in Saos-2 cells expressing exogenously different point mutants of p53. Ca922 had the mutation 248W with either the arginine or proline allele at codon 72, one of the mutants which confers the most resistance to Saos-2\textsuperscript{9}. HSC6 produced a C-terminal truncated form of p53, and thus is anticipated to have the least anti-apoptotic effect. Taken together, it is unlikely that the

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![Fig. 6. Combined treatment with TRAIL and LYZ294002 results in cleavage of Caspase 3, -8 and -9 and PARP with a greater propensity in Ca922 and HSC6.](image)

Cells were grown and treated with drugs as mentioned in Figure 5, and then harvested and lysed. Total cell lysates were Western blotted with specific antibodies described in the figure. Stripping and reprobing of the membranes with anti-β-tubulin showed equal loading of protein in each of the lanes. Data are representative of three independent experiments.
mutations in the present HNSCC cell lines had a significant effect on drug resistance. It has been reported that Akt is often constitutively activated in cancer cells via alterations of upstream signaling proteins, which confer chemoresistance. The amplification of EGFR in Ca922, in addition to the activation of H-ras in HOC313, was anticipated to contribute to enhanced Akt activity, while in HSC6, we could not detect any genetic abnormalities upstream of Akt signals. In fact, in the Ca922 and HSC6 cell lines, serine 473 of Akt was constitutively phosphorylated even after serum deprivation, but the phosphorylation was down-regulated in HOC313. In addition, we could not detect any abnormality in the size or amount of PTEN which is known to down-regulate Akt phosphorylation. From these results, it is unlikely that the level of Akt activity by itself determined the chemosensitivity in the present cell lines, since HOC313 was most resistant to these drugs but not constitutively active. This was the case also when we examined sensitivity to LY294002, a PI3K inhibitor which inhibits the phosphorylation of Akt. Our data thus suggested that constitutive signaling to Akt might not be an important factor when evaluating chemotherapeutic effectiveness in HNSCC.

We could not detect caspase 8 protein expression in HOC313. This finding is consistent with the result that co-treatment with TRAIL and LY294002 was of no effect. It has been reported that caspase 8 is not only activated in the extrinsic pathway, i.e. death receptor pathway, but can be also activated independently of death receptors during genotoxic stress-induced apoptosis. It is thus suggested that loss of caspase 8 in HOC313 may block this pathway, leading to cell death by anti-cancer drugs which cause genotoxic damage. In fact, we showed that caspase 8 inhibitor inhibited loss of viability in Ca922 not only with TRAIL and LY294002, but with etoposide. Hence, caspase 8 played an important role in the cell death induced with genotoxic anti-cancer drugs as well as with death receptor ligand. The mechanism of caspase 8’s silencing in HOC313 cells is not known.

Two mechanisms have been proposed, promoter methylation for small cell lung carcinoma cell lines and neuroblastoma, and point mutations. We looked at caspase 8 expression in three other cell lines, but HOC313 was the only case, suggesting that silencing of caspase 8 is not a frequent event in HNSCC.

Recently, loss of the caspase 8 activation pathway in HEp-2 cells was proposed as a mechanism of resistance to cisplatin. Our data is consistent with this finding and further suggested that the role of caspase 8 in chemoresistance may extend to most, if not all, genotoxic agents. It was recently reported that the genotoxic drug etoposide could activate caspase 8 without receptor-triggered dimerization which also supports a key role for caspase 8 in the induction of apoptosis.

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References

17. del Peso L, Gonzalez-Garcia M, Page C, et al. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science (Wash DC) 1997;278:88-89.