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Epidermal growth factor receptor inhibitors enhance susceptibility to Fas-mediated apoptosis in oral squamous cell carcinoma cells

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Summary Molecular inhibition of epidermal growth factor receptor (EGFR) signaling is a promising cancer treatment strategy. We examined whether inhibition of EGFR signaling would affect the susceptibility of oral squamous cell carcinoma (OSCC) cells to Fas-mediated apoptosis. Treatment of OSCC cells with an anti-EGFR monoclonal antibody, C225, and an EGFR tyrosine kinase inhibitor, AG1478, which target the extracellular and intracellular domains of the receptor, respectively, inhibited phosphorylation of EGFR and its downstream effector molecule Akt and amplified the induction of Fas-mediated apoptosis. In OSCC cells treated with EGFR inhibitors, Fas-mediated apoptosis was accompanied by caspase-8 activation but not Bid cleavage. Caspase-3 and -8 inhibitors reduced the effect of EGFR inhibitors on Fas-mediated apoptosis in OSCC cells, but a caspase-9 inhibitor did not. These results indicate that the pro-apoptotic activity of EGFR inhibitors in OSCC cells depends on the extrinsic pathway of the caspase cascade. Although EGFR inhibitors did not affect the expression of Fas, the Fas-associated death domain protein, or procaspase-8 in OSCC cells, the inhibition downregulated cellular FLICE-inhibitory protein (c-FLIP). Moreover, knockdown of c-FLIP in HSC-2 cells with a small interfering RNA strongly enhanced Fas-mediated apoptosis. These results suggest that the EGFR signaling pathway may, in part, regulate Fas-mediated apoptosis in OSCC cells through c-FLIP expression.

Introduction

The survival rate for patients with squamous cell carcinoma (SCC) of the oral cavity (OSCC) remains poor despite advances in diagnosis and treatment.[1] OSCC usually develops

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in areas of the epithelium exposed to carcinogens and likely results from the accumulation of cellular and genetic alterations, which lead to aberrant expression of many proteins involved in cell growth regulation.[2] Blockade or modification of the function of one or several of these proteins may impede or delay the development of the cancer.

The epidermal growth factor receptor (EGFR) plays an important role in the regulation of cell proliferation, differentiation, development, and oncogenesis.[3,4] The activation of downstream effectors of the EGFR signaling pathway can also lead to cell proliferation and tumor growth, as well as to the progression of invasion and metastasis.[5] EGFR is expressed at high levels in a number of tumor types and in most SCC,[6] and is associated with lower rates of survival.[7,8] As such, EGFR has been identified as an important target for cancer therapy. Several targeted strategies have been developed to specifically inhibit aberrant EGFR signaling. The clinically efficacy of these agents appears to rely on multiple anticancer mechanisms, including inhibition of cell cycle progression, inhibition of metastasis, and an increase in the susceptibility of cells to apoptosis. Inhibition of the EGFR, most commonly using monoclonal antibodies (mAbs) directed against the external ligand-binding domain[9,10] or small-molecule tyrosine kinase (TK) inhibitors,[11,12] has been the focus of much attention in recent years. However, despite promising anticancer activity in clinical trials, including trails for SCC,[9–12] neither class of EGFR-signaling antagonists appears to be curative. Thus, new treatment modalities for the management of SCC patients are urgently required, and additional strategies to target EGFR or to combine EGFR inhibition with other therapeutic approaches are under investigation. Various additive or cooperative effects have been reported for SCC, both in vitro and in vivo, when an anti-EGFR mAb or EGFR-TK inhibitor is combined with other chemotherapeutic agents or radiotherapy.[13–16]

Recently, strong synergistic anticancer effects have been reported for strategies in which EGFR-signaling antagonists are combined with activation of death receptors or their ligands, including Fas and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL).[17–19] The initiation of apoptosis is controlled by the integration of pro-apoptotic and anti-apoptotic signal transduction pathways that mediate stimuli such as the deprivation of survival factors, cell-damaging stress, the action of growth factors and cytokines, or signaling through death receptors.[20] Fas, a death receptor, is recognized as part of a major pathway for the induction of apoptosis in cells and tissues and for the removal of unwanted cells in the human body.[21,22] Fas is widely expressed in many cell types, either constitutively or following activation of the cells. The activation of the Fas/Fas ligand (FasL) system may be one of the mechanisms responsible for the induction of apoptosis in cancer cells of varying histotypes.[23] However, although cancer cells, including OSCC cells, express Fas on their cell surfaces, cancer cells are relatively resistant to Fas-mediated apoptosis.[24,25]

Signal transduction through the Fas/FasL system has been well studied, and many signaling molecules have been found to modulate Fas-mediated apoptosis.[21,22] Stimulation of Fas results in clustering of the receptors, which in turn leads to the recruitment of the adaptor molecule Fas-associated death domain protein (FADD) and the receptor-proximal caspase-8 to the death-inducing signaling complex (DISC).[21,22] Caspase-8 becomes activated upon recruitment to the DISC and initiates apoptosis by subsequent cleavage of downstream effector caspases.[26] In some cell types (type I), activation of caspase-8 is sufficient for subsequent activation of the effector caspase-3 to execute cellular apoptosis (extrinsic pathway).[27] In other cell types (type II), amplification through the mitochondrial pathway (intrinsic pathway), which is initiated when caspase-8 cleaves Bid, is required for cellular apoptosis. In the intrinsic pathway, the truncated Bid induces the release of cytochrome-*c* from mitochondria, and cytochrome-*c* binds to Apaf-1 to activate caspase-9, which in turn activates the effector caspase-3.[27]

To avoid inappropriate cell death, death receptor signals must be tightly controlled.[21,22] Therefore, the enhancement of Fas-mediated apoptosis can be modulated by anti-apoptotic factors.[28] One proximal regulators of the Fas-induced death program is cellular FLICE-inhibitory protein (c-FLIP).[28–30] In most Fas-resistant cells, c-FLIP forms stable complexes with Fas, FADD, and caspase-8.[29,30] The association of caspase-8 and c-FLIP within the DISC blocks the autoproteolytic cleavage of caspase-8.[31] c-FLIP is expressed at higher levels in cancer cells than in normal tissue cells, enabling cancer cells to overcome Fas-mediated apoptosis.[32,33] The intracellular mediators of apoptosis in OSCC cells are still largely unknown, and it is unclear how Fas-mediated apoptosis is attenuated by the EGFR signaling pathway but c-FLIP may also control the susceptibility of OSCC cells to Fas-mediated apoptosis.

Here, we analyzed whether EGFR inhibitors can affect the susceptibility of OSCC cells to Fas-mediated apoptosis. We demonstrate that an EGFR inhibitor enhances Fas-mediated apoptosis through the downregulation of c-FLIP in OSCC cells.

Materials and methods

Cell lines and culture conditions

Cells of the human OSCC cell line, HSC-2, were grown as adherent monolayers. Cells were maintained in DMEM (GIBCO BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated FBS (GIBCO BRL), 2 mM L-glutamine, and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37 °C in a humidified atmosphere of 5% CO_2.

Detection of Fas-mediated apoptosis

The binding of annexin V-FITC was used as a sensitive method for measuring apoptosis and was assessed using a modification of a previously described method.[34,35] HSC-2 cells (1×10^5/well) were seeded into 12-well plates and cultured for 24 h to allow adherence. OSCC cells were preincubated with or without the anti-EGFR mAb C225 (4 nM; Calbiochem, San Diego, CA, USA) or the EGFR TK inhibitor AG1478 (8 μM; Calbiochem) for 2 h. Treated OSCC cells were exposed to anti-Fas (CH-11, MBL, Nagoya, Japan; 200 ng/ml) for 24 h and then harvested. Annexin V-FITC (MEBCYTO apoptosis kit, MBL) was allowed to specifically bind to the cells, according to the manufacturer's instructions. After incuba-