

Cimetidine induces apoptosis of human salivary gland tumor cells

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Abstract. It has been reported that cimetidine, a histamine type-2 receptor (H2R) antagonist, inhibits the growth of glandular tumors such as colorectal cancer. However, its effects against salivary gland tumors are still unknown. We demonstrated previously that human salivary gland tumor (HSG) cells spontaneously express the neural cell adhesion molecule (NCAM) and also that HSG cell proliferation could be controlled via a homophilic (NCAM-NCAM) binding mechanism and that NCAM may be associated with perineural invasion by malignant salivary gland tumors. In the present study, we investigated the effects of cimetidine via the expression of NCAM on tumor growth and perineural/neural invasion in salivary gland tumor cells. Expression of both NCAM mRNA and protein was found to decrease in a dose-dependent manner upon treatment with cimetidine for 24 h. The MTT assay and confocal laser microscopy clearly showed that HSG cells underwent apoptosis after treatment with cimetidine. Activation of caspases 3, 7, 8 and 9 was observed in HSG cells after cimetidine treatment, thus confirming that the apoptosis was induced by the activated caspases. Apaf-1 activity was also detected in HSG cells in a dose-dependent manner after treatment with cimetidine. We also found that the cimetidine-mediated down-regulation of NCAM expression in HSG cells did not occur via blocking of the histamine receptor, even though H2R expression was observed on HSG cells, as two other H2R antagonists, famotidine and ranitidine, did not show similar effects. We demonstrated for the first time that cimetidine can induce significant apoptosis of salivary gland tumor cells, which express NCAM, at least in part by down-regulation of NCAM expression on the cells. These findings

suggest that the growth, development and perineural/neural invasion of salivary gland tumor cells can be blocked by cimetidine administration through down-regulation of NCAM expression, as well as induction of apoptosis.

Introduction

Cimetidine is a histamine type-2 receptor (H2R) antagonist, commonly prescribed to treat gastro-esophageal reflux disease as well as gastric and duodenal ulcers (1). It has been reported that cimetidine improves the survival of patients with malignant tumors (2,3), including gastric (4) and colorectal carcinomas (5). Since histamine has been shown to inhibit the growth of gastrointestinal cancers by several mechanisms including inhibition of cancer cell proliferation and enhancement of immune activity (3), cimetidine may act by enhancing the host immune response against tumor cells (6,7) or by blocking the cell growth-promoting activity of histamine in colon cancer and melanoma cell lines (5,8-10). However, it is still unclear whether cimetidine blocks the development of salivary gland tumors.

Adenoid cystic carcinoma (ACC) is a well-known and typical malignant salivary gland tumor. ACCs are biologically aggressive and can metastasize many years after excision of the primary tumor. Facial palsy due to perineural/neural invasion is a particularly frequent and generally accepted hallmark of ACCs (11-13), and therefore blocking of perineural/neural invasion could be a strategy for arresting the development of ACC. We have demonstrated previously (14) that the neural cell adhesion molecule (NCAM) is expressed spontaneously in the human salivary gland tumor cell line HSG, derived from submandibular salivary gland, and that NCAM induces the adhesion of cancer cells expressing NCAM peptide to neural cells *in vitro*. We further found that HSG cell proliferation could be controlled via a homophilic (NCAM-NCAM) binding mechanism and that NCAM may be associated with perineural/neural invasion by malignant salivary gland tumors (15).

In the present study, we examined the effects of cimetidine via the expression of NCAM on tumor growth and perineural/neural invasion in salivary gland tumor, *in vitro*. We demonstrated that cimetidine can induce apoptosis of HSG

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cells, which express NCAM, at least in part by down-regulation of NCAM expression on the cells.

Materials and methods

Reagents. Mouse anti-human NCAM monoclonal antibodies for the 120- and 140-kDa isoforms, and for 180-kDa isoform (MAb NCAM antibody; CD56) were purchased from Santa Cruz Biotechnology and Becton-Dickinson (BD) Biosciences (CA, USA), respectively. Rabbit anti-human apoptotic protease-activating factor-1 (apaf-1) polyclonal antibody was also purchased from BD Biosciences. MAb β -actin and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] were obtained from Sigma (MI, USA). The biotinylated horse anti-mouse IgG (H+L) antibody and Streptavidin-peroxidase were obtained from Vector Laboratories (Burlingame, CA) and Gibco-BRL, Life Technologies (MD, USA), respectively. Mouse anti-human caspase-7 and -8 monoclonal antibodies and rhNCAM peptide were purchased from Santa Cruz Biotechnology, and caspase-3 and -9 were purchased from Medical and Biological Laboratories. For the detection of H2R by immunoblot analysis, rabbit anti-human H2R polyclonal antibody was purchased from Chemicon International (Temecula, CA). Cimetidine, famotidine, and ranitidine were purchased from Sigma Chemical Co. H2R blockers were dissolved in PBS.

Cell culture. The HSG cell line, derived from a human submandibular salivary gland, was established by Shirasuna *et al.* (16) and three human oral squamous cell carcinoma (HOSCC) cell lines, HSC-2 and -3 and Ca9-22 obtained from the American Type Culture Collection (ATCC, VA, USA) were respectively maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μ g/ml streptomycin, and grown to confluence in 25-cm² culture flasks at 37°C in a humidified 5% CO₂ incubator until use.

RNA extraction, Northern blot analysis and RT-PCR. Total RNA was extracted from HSG cells by the acid-guanidinium-phenol-chloroform (AGPC) method (17). Briefly, HSG monolayers were scraped off and dissolved in solution D (4 M guanidinium thiocyanate solution containing 25 mM sodium citrate, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol), then extracted with phenol-chloroform and chloroform. The RNA was alcohol-precipitated and the pellet was dissolved in sterile water. Total RNA (10 μ g) was separated by electrophoresis on a 1% formaldehyde-agarose gel. The RNA was transferred to Hybond-N nylon membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK), and the filter was cross-linked by using a UV-cross linker (Stratagene, La Jolla, CA, USA), then pre-hybridized with salmon-sperm DNA (0.15 mg/ml) for 4 h at 42°C. Blots were hybridized with a human NCAM cDNA probe according to the method reported previously (15).

Random hexamer-primed single-stranded cDNA was synthesized with an RNA LA PCRTM kit (Takara Shuzo) from 1.0 μ g of total RNA in a final volume of 20 μ l with 0.25 units of avian myeloblastosis virus reverse transcriptase at 42°C

Table I. The primer sequences used in this study.

Oligonucleotides	Sequence
H2R primer pairs	
Forward primer	5'-TTCATCGTGCCTTGGCTATC-3'
Reverse primer	5'-CTGAGAGGCGTTGGACCTC-3'
β -actin primer pairs	
Forward primer	5'-GTGGGGCGCCCCAGGCACCA-3'
Reverse primer	5'-CTCCTTAATGTCACGCACGATTTC-3'

for 60 min. An aliquot (1.0 μ l) of the reaction mixture was diluted with 10 μ l of PCR buffer containing 4 pmole each of 5' and 3' H2R primer sets was previously designated by Jutel *et al.* (18) and β -actin primer sets (Table I). The PCR reaction was then performed with 2.5 units of Takara LA TaqTM (Takara Shuzo). The samples were subjected to denaturation at 94°C for 2 min. This was followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 62°C for 30 sec, and extension at 68°C for 50 sec. The last cycle had an extension period of 7 min. The amplified DNA was electrophoresed on a 2.0% agarose gel, stained with ethidium bromide and visualized on a UV illuminator, then photographed.

Western blot analysis. For Western blot analysis, total cell extracts prepared from each cell line were analyzed with the appropriately diluted antibody according to the method reported previously (15).

Induction of apoptosis by cimetidine (MTT assay). Near confluent cells (5x10⁴ cells/ml) were incubated for 24 h on a 96-microwell plate (Nalge Nunc). The cells were washed once with PBS and incubated with various concentrations of cimetidine (10⁻¹⁰-10⁻² M) for 24 h in RPMI-1640 medium with 10% FBS. The cells were washed once with PBS and incubated with 0.2 mg/ml MTT for 4 h. After removing the medium, cells were lysed with 100 μ l of DMSO, and the relative viable cell number was determined by measuring absorbance of the cell lysate at 540 nm.

Morphological observation of apoptotic cells. HSG cells (1x10⁴ cells/ml) were maintained with RPMI-1640 supplemented on a 10 cm² glass plate (Iwaki, Chiba, Japan). The cells were washed once with PBS and incubated 10⁻⁴ M of cimetidine for 24 h in RPMI-1640 medium with 10% FBS. The apoptotic cells were directly observed under a confocal laser microscope.

Results

Detection of NCAM mRNA in HSG cells after treatment with cimetidine. To examine how cimetidine affects to the expression of NCAM, HSG cells were treated with various doses of cimetidine (10⁻⁸-10⁻² M) for 24 h. Total RNA extracts from HSG cells were subjected to Northern blot analysis to determine the quantity of NCAM mRNA expressed in HSG cells. A single band of ~5.0 kb was found migrating above

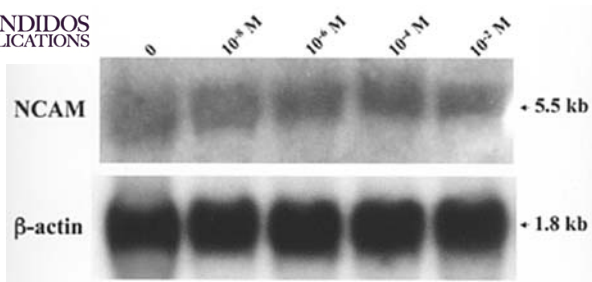


Figure 1. Northern blot analysis of NCAM mRNA extracted from HSG cells treated with various doses of cimetidine (10^{-8} - 10^{-2} M) for 24 h. The levels of NCAM mRNA expression at 5.0 kb gradually decreased in a dose-dependent manner. As an internal control, detection of β -actin mRNA was performed using a β -actin cDNA probe, revealing that the obtained total RNA was intact.

the 28S ribosomal band. Thereafter, the level of NCAM mRNA expression decreased gradually upon treatment with cimetidine for 24 h in a dose-dependent manner (Fig. 1). As an internal control, β -actin mRNA was monitored to assess the integrity of the total RNA extracted from the HSG cells, and this demonstrated that the total RNA obtained was intact.

Expression of NCAM protein in HSG cells after cimetidine treatment. SDS-solubilized cell extracts of HSG cells treated with various doses of cimetidine for 24 h, as described above, were subjected to Western blot analysis to determine the quantity of NCAM protein expressed in the cells. It was revealed that the concentration of NCAM peptide induced in HSG cells decreased gradually and dose-dependently after treatment with cimetidine for 24 h (Fig. 2).

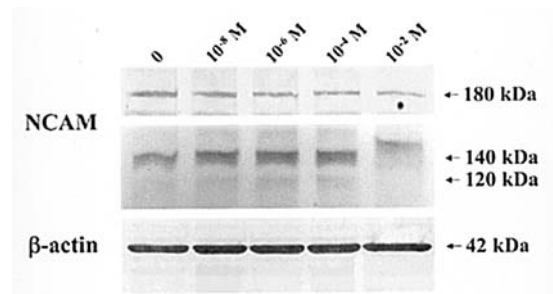


Figure 2. Western blot analysis of NCAM peptides extracted from HSG cells treated with various doses of cimetidine (10^{-8} - 10^{-2} M) for 24 h. The NCAM peptides induced in the HSG cells were the 120, 140 and 180 kDa isoforms, which gradually decreased in a dose-dependent manner after treatment with cimetidine for 24 h.

MTT assay. To confirm the viability of HSG cells after treatment with various doses of cimetidine for 24 h, MTT assay was performed. The MTT assay showed a significant and dose-dependent reduction in the number of HSG cells upon treatment with cimetidine for 24 h (Fig. 3A). Moreover, to confirm whether cimetidine affected HSG cells specifically, we also treated three HOSCC cell lines, HSC-2, -3 and Ca9-22, for 24 h with the same doses of cimetidine, and MTT assays were carried out. It was found that the HOSCC cell lines were almost completely resistant to cimetidine-induced apoptosis (Fig. 3B). These results suggested that HSG cells, which express NCAM, might be induced to undergo apoptosis by treatment with cimetidine.

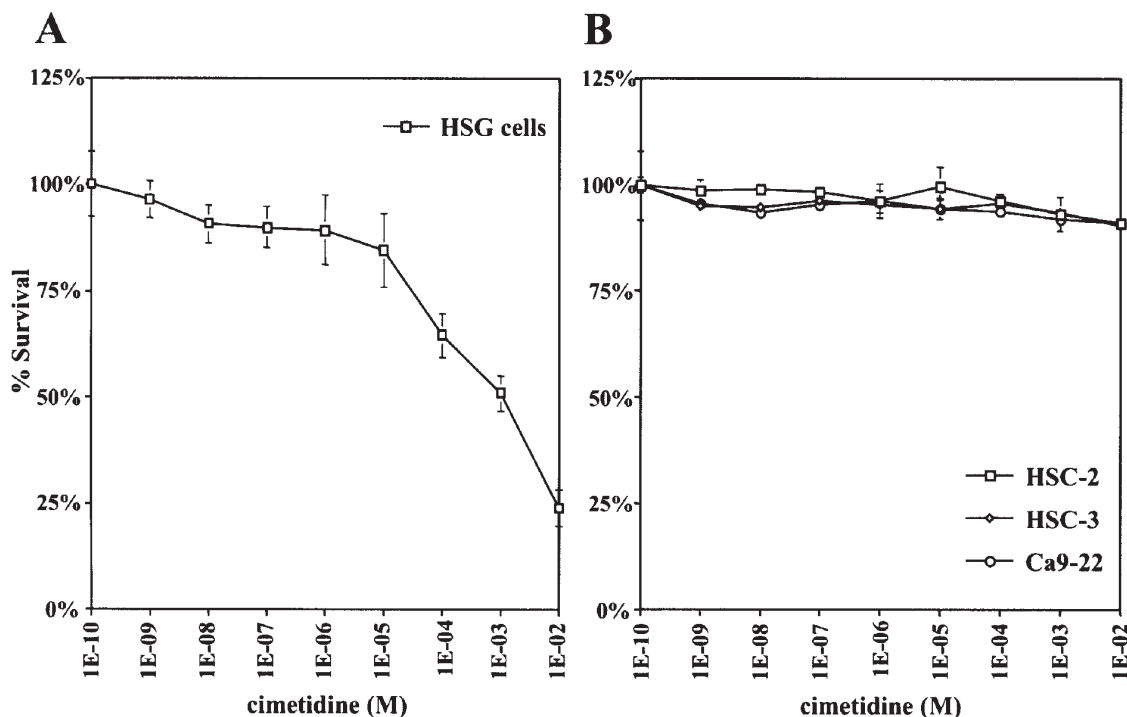


Figure 3. MTT assay. Dose-response survival curves based on the MTT assay were generated for HSG cells treated with different concentrations of cimetidine (10^{-10} - 10^{-2} M) for 24 h. There was a significant and dose-dependent reduction in the number of HSG cells (A). However, HSC-2, HSC-3, and Ca9-22 cells (B) were almost completely resistant to cimetidine-induced apoptosis (n=3 experiments; means \pm SD).

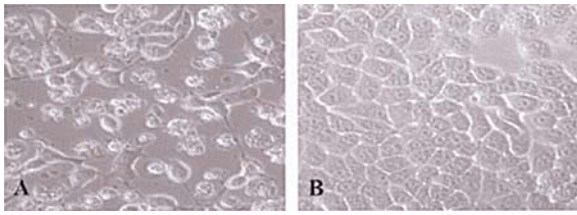


Figure 4. Confocal laser microscopy. Apoptosis induced with 10^{-4} M cimetidine for 24 h was directly observed as blebbing of the cellular membrane, shrinkage of the cytoplasm and release of apoptotic vesicles on HSG cells under a confocal laser microscope (original magnification, $\times 40$) (A). No apoptosis was observed on the surfaces of control HSG cells without cimetidine treatment (original magnification, $\times 40$) (B).

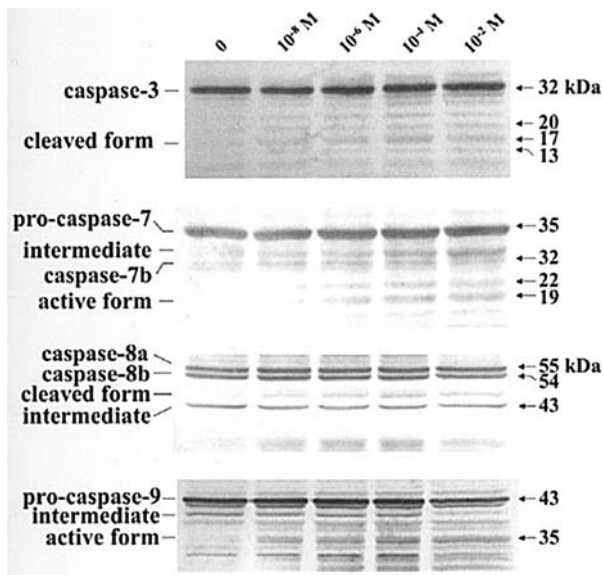


Figure 5. Western blot analysis of activated caspase extracted from HSG cells treated with various doses of cimetidine (10^{-8} - 10^{-2} M) for 24 h. Cleavage product formation mediated by activated caspases 3, 7, 8 and 9 was observed, and this increased in a dose-dependent manner after treatment with cimetidine.

Induction of apoptosis in HSG cells. Since the process of apoptosis rapidly induces dramatic effects on cellular structure, the effects of cimetidine on HSG cells were visualized by confocal laser microscopy. HSG cells were treated with or without 10^{-4} M cimetidine for 24 h, then imaged by confocal laser microscopy (Fig. 4A and B). The results indicated that cimetidine induced HSG cell death characterized by various hallmarks of apoptosis, including cellular membrane blebbing, cytoplasm shrinkage and the release of apoptotic vesicles.

Detection of activated caspases in HSG cells after cimetidine treatment. Regardless of circumstance, the induction and execution of apoptotic events require the activation of caspases, a family of aspartate-specific cysteine proteinases. To investigate how the apoptotic machinery is regulated by cimetidine in HSG cells, Western blot analysis was carried out using antibodies for individual caspases. Activated caspases 3, 7, 8 and 9 were observed in HSG cells after treatment with cimetidine (Fig. 5), thus confirming that activated caspase



Figure 6. Western blot analysis of apaf-1 protein extracted from HSG cells treated with various doses of cimetidine (10^{-8} - 10^{-2} M) for 24 h. Apaf-1 was constitutively expressed in HSG cells, and the concentrations were clearly increased by cimetidine in a dose-dependent manner. As a positive control, apaf-1 protein detection in HeLa cells was performed.

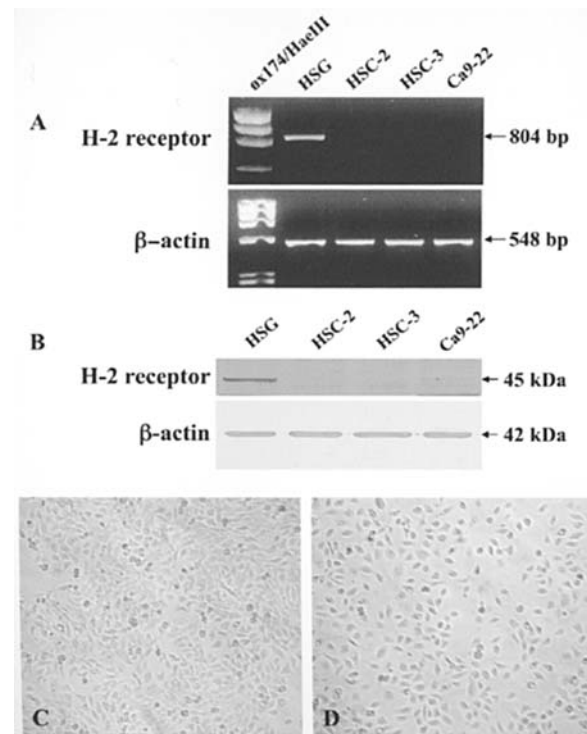



Figure 7. RT-PCR and Western blot analysis of H2R mRNA and protein extracted from HSG and HOSCC cells. Both H2R mRNA (A) and protein (B) were detected only in HSG cells. Then, HSG cells were treated with two other H2R antagonists, famotidine and ranitidine, at 10^{-4} M for 24 h. Neither famotidine (C) nor ranitidine (D) induced apoptosis of HSG cells.

mediates apoptosis in the HSG cells upon stimulation with cimetidine.

Apaf-1 activity. To examine whether this pathway of apoptotic signals involves the DNA-damage or death receptor pathway, we evaluated the expression of apaf-1 in HSG cells after treatment with cimetidine. Apaf-1 was constitutively expressed in HSG cells, and the concentrations were clearly increased by cimetidine in a dose-dependent manner (Fig. 6). This result indicated that cimetidine induces apoptosis of HSG cells via the DNA-damage signal through the mitochondria.

Involvement of H2R in apoptosis. To confirm whether H2R is expressed in HSG and HOSCC cells, RT-PCR and Western blot analysis were carried out. Both H2R mRNA and protein were detected only in HSG cells (Fig. 7A and B). These results suggest that cimetidine induces apoptosis of HSG cells via

 SPANDIDOS PUBLICATIONS Then, HSG cells were treated with two other H2R antagonists, famotidine and ranitidine, at 10^{-4} M for 24 h.

Neither famotidine nor ranitidine induced apoptosis of HSG cells (Fig. 7C and D), indicating that this action of cimetidine is probably not mediated via the H2R.

Discussion

Cimetidine, the most studied H2R antagonist, has been demonstrated to possess anti-tumor activity against colon, gastric and kidney cancers, and melanomas (5,8-10). According to a recent study by Lefranc *et al* (19), this activity involves a number of different mechanisms of action, characterized by three overall characteristics: a) a direct inhibitory effect on tumor growth by blocking the cell growth-promoting activity of histamine via activation of H2 receptors, and an indirect effect involving inhibition of tumor-associated angiogenesis; b) an immunomodulatory effect through enhancement of the host's immune response to tumor cells; c) an inhibitory effect on cancer cell migration and adhesion to endothelial cells, thus inhibiting tumor neo-angiogenesis and metastasis development. In this study, we investigated the effect of cimetidine via the expression of NCAM on tumor growth and perineural/neural invasion in salivary gland tumor using an *in vitro* cell culture system. These experiments clearly demonstrated that cimetidine effectively induced apoptosis of HSG cells by preventing the induction of NCAM.

NCAM is a membrane glycoprotein receptor of the immunoglobulin supergene family that mediates cell-to-cell adhesion via homophilic binding (NCAM to NCAM) and cell-to-substrate adhesion via heterophilic binding (NCAM binding to another ligand or counter-receptor) (20). NCAM is recognized to play an important role in perineural invasion in various neoplasms, such as bile duct cancer, gallbladder carcinoma, melanoma and adenoid cystic carcinoma of the head and neck (11-13,21-23). Previously, we also demonstrated by immunohistochemical methods that adenoid cystic carcinoma of the parotid gland is distinctly positive for NCAM (14). Moreover, we found that HSG cell proliferation could be controlled via a homophilic (NCAM-NCAM) binding mechanism and that NCAM may be associated with perineural/neural invasion by malignant salivary gland tumors (15).

However, it is unknown how NCAM-expressing cells such as HSG behave when exposed to cimetidine. In the present study, therefore, we treated HSG cells with cimetidine *in vitro*, and found that the levels of NCAM mRNA and protein decreased in a dose-dependent manner after treatment with cimetidine for 24 h. Data from Western blot analysis revealed that lysates from HSG cells underwent a change similar to degradation at a cimetidine concentration of 10^{-2} M. MTT assay was then performed to examine the viability of HSG cells treated with cimetidine, and the data suggested that the HSG cells underwent apoptosis.

Apoptosis is an active process of cell death characterized by cytoplasmic shrinkage, membrane blebbing, nuclear fragmentation and internucleosomal DNA cleavage. Disruption of apoptosis and cell proliferation are key events in tumorigenesis. To investigate the appearance of HSG cells undergoing apoptosis, confocal laser microscopy and detection of activated caspases were respectively carried out, and the findings

confirmed that HSG cells obviously underwent apoptosis upon treatment with cimetidine. From analysis of apaf-1 activity, we further found that cimetidine induced apoptosis of HSG cells via the DNA-damage signal through the mitochondria, inducing release of cytochrome *c* to the cytoplasm. However, cimetidine had no effect on HOSCC cell lines, which do not express NCAM. These results imply that the effect of cimetidine is specific to NCAM-expressing tumor cells, such as HSG cells; furthermore, blocking the activity of NCAM through cimetidine, as well as the homophilic (NCAM-NCAM) binding mechanism, rather than regulating a signaling pathway of cell proliferation, may in fact induce a negative signal such as apoptosis in HSG cells. In addition, homophilic (NCAM-NCAM) binding may activate multiple signaling pathways that differ among cell types. Simultaneously, it was confirmed whether H2R is expressed in HSG and HOSCC cells, as cimetidine is an H2R antagonist. Both H2R mRNA and protein were detected only in HSG cells, thus suggesting that cimetidine induces apoptosis of HSG cells via the H2R. However, neither famotidine nor ranitidine, two other H2R antagonists, induced apoptosis of HSG cells. This observation indicated that the cimetidine-mediated down-regulation of NCAM expression, as well as induction of apoptosis in HSG cells, did not occur via blocking of the histamine receptor. It has been reported that NCAM expression is regulated by NF- κ B (24), and that NF- κ B activity is induced by NCAM (25); in other words, homophilic NCAM binding can increase NF- κ B activity, then NF- κ B regulates NCAM expression. Some studies have also indicated that cimetidine may have antioxidant activity (26-28), and antioxidants have been shown to block the NF- κ B activation cascade (29). If cimetidine inhibits NF- κ B activation, the expression of NCAM would be down-regulated, and consequently HSG cell proliferation, which requires homophilic NCAM binding, would be blocked. Therefore, the exact mechanisms by which cimetidine suppresses development of cancers, including the regulation of NF- κ B activation, remain to be elucidated.

The present findings suggest that the growth, development and perineural/neural invasion of salivary gland tumor cells can be blocked by cimetidine administration through down-regulation of NCAM expression, as well as induction of apoptosis. Although malignant glandular tumors are known to be generally resistant to radiation therapy and chemotherapy, the clinical application of cimetidine as an anti-cancer drug might be a useful approach for devising future therapeutic strategies against NCAM-expressing tumors such as adenoid cystic carcinoma. Further studies will be required to identify the signal transduction pathway utilized by glandular tumors after treatment with cimetidine and to establish a strategy for cimetidine-based salivary gland tumor therapy.

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