Journal of Oral Science, Vol. 54, No. 1, 71-75, 2012

Original

Activation-induced cytidine deaminase mRNA expression in oral squamous cell carcinoma-derived cell lines is upregulated by inflammatory cytokines

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(Received 20 October 2011 and accepted 24 January 2012)

Abstract: Activation-induced cytidine deaminase (AID) induces cytosine deamination to generate somatic hypermutation and class switch recombination in immunoglobulin genes. AID expression is upregulated by inflammatory cytokines such as interferon-γ and tumor necrosis factor (TNF)-α, which in turn induce p53 mutations in inflammatory or cancer cells. In this study, the effects of growth factors, cytokines or sodium butyrate on AID mRNA expression were examined in human OSCC-derived cells using real-time RT-PCR. Expression of AID mRNA was detected in OSCC cells and the expression was increased by EGF, TNF-a, or sodium butyrate. These results suggest that aberrant AID expression may play an important role in the dysplasia-carcinoma sequence in the oral cavity. (J Oral Sci 54, 71-75, 2012)

Keywords: activation-induced cytidine deaminase (AID); cytokines; oral squamous cell carcinoma (OSCC).

Introduction

Activation-induced cytidine deaminase (AID), a member of the apolipoprotein B mRNA-editing catalytic polypeptide (APOBEC) family, has been identified in activated B cells (1,2). AID induces cytosine deamination to generate uracil, thereby changing a DNA C:G pair to a U:G mismatch (3). This mismatch induces DNA cleavage, thereafter generating somatic hypermutation and class switch recombination in immunoglobulin genes to produce high-affinity antibodies against specific antigens (4-6). Moreover, it has been reported that AID expression, in turn, induces p53 mutations in gastric epithelial cells (7) or cancer cells (8).

Although AID is normally expressed in activated B cells, its expression has also been demonstrated in some inflammatory tissues, such as *Helicobacter pylori*-infected gastric epithelium and human colonic epithelium, and in some malignancies such as non-Hodgkin lymphoma, hepatocellular carcinoma, lung cancer, and gastric cancer (7,9-15). In addition, AID expression is upregulated by NF-κB via stimulation with inflammatory cytokines, including interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-4 and IL-13 (7,14-16), suggesting that AID acts as a linking factor between inflammation and cancer.

Oral squamous cell carcinoma (OSCC) is one of the most frequently occurring cancers. It is an invasive epithelial neoplasm of the oral cavity showing various degrees of squamous differentiation, and a propensity for early and extensive lymph node metastasis (17). Kusama et al. have previously demonstrated p53 mutation in OSCC, suggesting that this mutation is involved in the early stages of the dysplasia-carcinoma sequence in the oral squamous epithelium (18). Moreover, recent

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studies have suggested an association between periodontal disease and the risk of various human malignant neoplasms, including poorly differentiated OSCC (19, 20). Chronic periodontitis, an inflammatory form of periodontal disease, is caused by microorganisms present in the plaque biofilm that forms around teeth (21), and our previous study has shown that butyric acid produced by periodontopathic bacteria enhances OSCC invasion (22).

Here we investigated the expression of AID mRNA in the human OSCC-derived cell lines Ca9-22, HSC-2, -3, and -4, and – using real-time RT-PCR – examined the effects of co-culture with sodium butyrate (NaB), IL-1β, IFN-γ, TNF-α or epidermal growth factor (EGF).

Materials and Methods

Cells

The human gingival squamous cell carcinoma-derived cell line Ca9-22, and the human tongue squamous cell carcinoma-derived cell lines HSC-2, -3, and -4, were provided by the RIKEN BRC through the National Bio-Resource Project of MEXT, Japan. Each cell line was routinely grown in RPMI1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 100 U/ml penicillin-streptomycin (GIBCO Invitrogen, Carlsbad, CA, USA), 10 U/ml fungizone (GIBCO Invitrogen), and 10% fetal bovine serum (GIBCO Invitrogen) in a humidified atmosphere of 5% CO_2 at 37˚C.

Total RNA extraction

Each cell line was incubated with 2 mM NaB, 50 ng/ml EGF, 50 ng/ml IL-1β, 50 ng/ml IFN-γ, or 50 ng/ml TNF-α. After 24 h of incubation, total RNAs were extracted in accordance with the instructions supplied with the PAREx kit (Takara) and adjusted to 0.1 μg/ml.

Real-time reverse-transcription polymerase chain reaction (real-time RT-PCR)

Real-time RT-PCR was performed using a Thermal Cycler Dice Real Time System (Takara) following the standard protocol. A One-Step SYBR PrimeScript RT-PCR Kit II (Takara) was used for the RT-PCR reaction. The primers employed, which were based on sequences for AID and GAPDH, are shown in Table 1. Each PCR mixture (final reaction volume, 20 μl) contained 10 μl One Step SYBR RT-PCR buffer 4, 0.8 μl PrimeScript 1step Enzyme Mix 2, 0.8 μl forward primer (0.4 μM), 0.8 μl reverse primer (0.4 μM), and 100 ng/ml total RNA. PCR conditions were 95˚C for 10 s, followed by 45 cycles of 95˚C for 5 s, 60˚C for 30 s. Dissociation was performed according to a melting program.

Table 1 The primers employed

Product name	Sequences (5' → 3')		Accession number
AID	Forward:	tcttgatgaaccggaggaag	NM_020661
	Reverse:	agccgttcttattgcgaaga	
GAPDH	Forward:	gcaccgtcaaggctgagaac	NM_002046
	Reverse:	tggtgaagacgccagtgga	

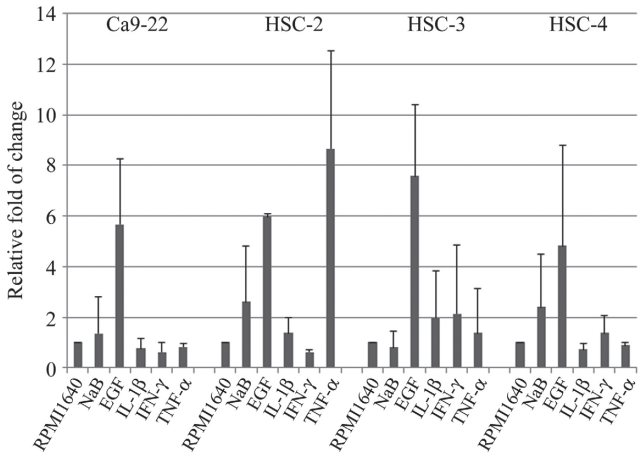

Fig. 1 Effects of NaB, EGF and inflammatory cytokines on expression of AID mRNA. Expression levels were normalized to that of the control RNA (RPMI1640). Expression of AID mRNA was increased by EGF in all of the cell lines examined. TNF-α enhanced the expression of AID markedly in HSC-2, and NaB enhanced AID expression slightly in the Ca9-22, HSC-2, and -4 cell lines.

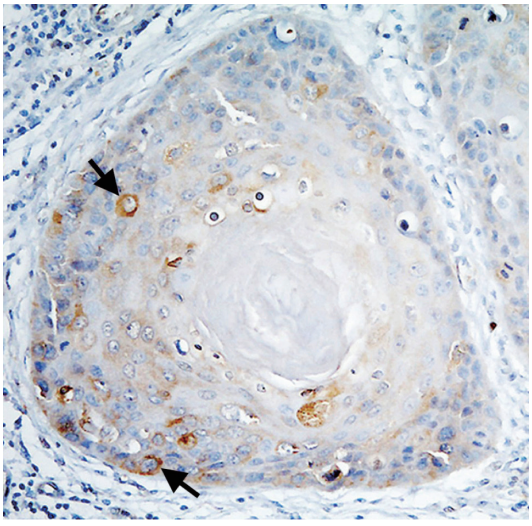

Fig. 2 Localization of AID in human OSCC tissues. Positive reaction products for AID were observed in the cytoplasm of the tumor cells (arrow). Positive cells were scattered in OSCC cancer nests (original magnification: ×200).

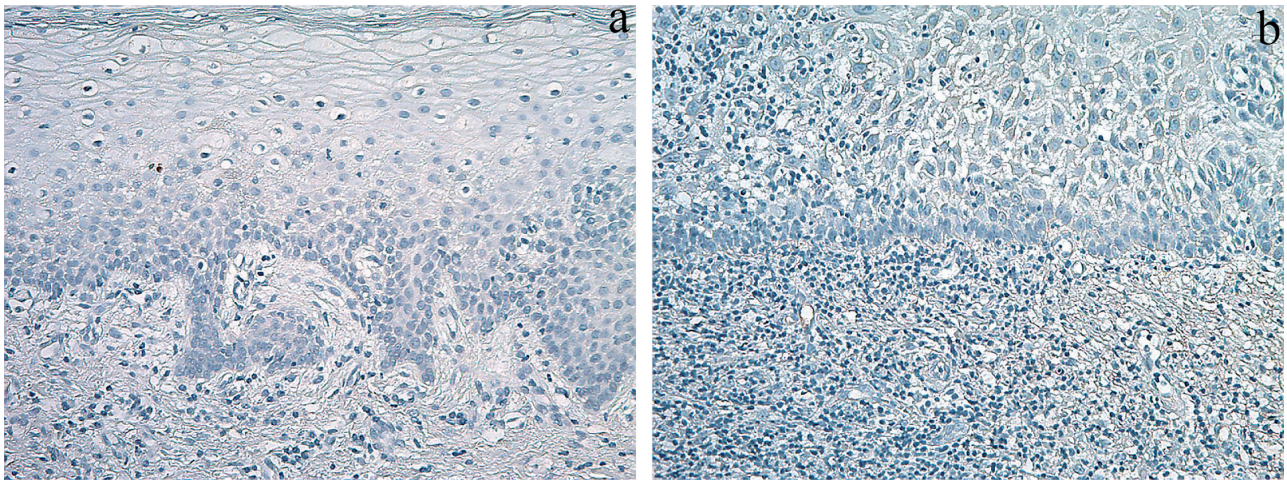

Fig. 3 Localization of AID in human oral epithelium. No positive cells were observed in the tissues either with (a) or without (b) inflammation (original magnification: ×100).

Results and Discussion

AID mRNA expression was demonstrated by real-time RT-PCR, and the effects of incubating the human OSCC-derived cell lines with NaB, EGF or inflammatory cytokines were examined. Exposure to EGF enhanced the expression of AID mRNA in all of the cell lines studied. Similarly, AID mRNA expression was also enhanced markedly by TNF-α in the HSC-2 cell line, and slightly by NaB in the Ca9-22, HSC-2 and HSC-4 cell lines, as well as being upregulated by IL-1β and IFN-γ in the HSC-3 cell line (Fig. 1).

AID induces DNA cleavage, thereby generating somatic hypermutation and class switch recombination in immunoglobulin genes (3-6). This DNA cleavage also induces p53 mutation in gastric epithelial cells and cancer cells (7,8). Among the cell lines used in this study, point mutation of p53 has been reported in HSC-4 and Ca9-22 (18,23). Also, p53 mutation is frequently detected in oral epithelial dysplasia as well as OSCCs (18). These observations suggest a role of aberrant AID expression in human OSCC tumorigenesis.

In a preliminary experiment, we investigated the localization of AID. Immunoreactivity for AID was also demonstrated in the cytoplasm of tumor cells using paraffin-embedded tissues from 16 cases of OSCC (8 lingual and 8 gingival squamous cell carcinomas) (Fig. 2). In contrast, no AID expression was found in any of the non-tumorous squamous epithelia associated with inflammation in the same specimens used in this study (Fig. 3). Although AID induces somatic hypermutation in DNA sequences, AID protein was detected in the cytoplasm by immunohistochemistry (Fig. 2). Similar results have been reported for the Ramos cell line and primary B cells (24,25). Patenaud et al. suggested that the C-terminal region of AID may be retained in the cytoplasm through anchor sequences (26), and in this study, we used an antibody recognizing the C-terminal region of AID.

AID mRNA expression was enhanced by EGF in all of the cell lines examined. It has been reported that EGF stimulates cell invasion through ERK, AP-1 and NF-κB signaling in human gastric carcinoma cells (27). In addition, it has been shown that AID expression is upregulated by NF-κB activation after treatment with IFN-γ, TNF-α, IL-4 and IL-13 in gastric epithelium (7), hepatocytes (14), and colorectal cancers (15,16). These observations suggest that NF-κB activated by EGF upregulates the expression of AID, being consistent with a previous study in which NF-κB was shown to activate IL-1β in murine astrocytes (28). In the present study, however, AID expression was not effected by IL-1β. Furthermore, TNF-α enhanced the expression of AID only in HSC-2 cells. A further study involving stimulation with other kinds of inflammatory cytokines, such as IL-4 or IL-13, will be necessary to clarify the molecular mechanism responsible for AID expression.

As shown in Fig. 1, AID expression was increased slightly by NaB. Butyric acid/NaB is a short-chain fatty acid that acts as a histone deacetylase inhibitor. Pulukuri et al. suggested that histone deacetylase inhibitor induces the expression of urokinase plasminogen activator and its activity in human cancer cells, resulting in enhanced invasion (29). In addition, our previous study suggested that NaB stimulates human oral cancer cell invasion via the transmembrane mucoprotein podoplanin (22). Thus, butyric acid may act as not only a cancer progression factor, but also a cancer development factor through

induction of AID expression. Further studies including immunohistochemistry using epithelial dysplasic tissues with or without inflammation and co-culture of OSCC-derived cell lines with siRNA for AID will be necessary to clarify the role of AID in the dysplasia-carcinoma sequence in the oral cavity.

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