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SHORT COMMUNICATION

Induction of activation-induced cytidine deaminase by a not-directly mutagenic carcinogen: a novel potential molecular mechanism

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Abstract
Objective The molecular mechanisms underlying the carcinogenic activity of not-directly mutagenic (Ames mutagenicity test-negative) carcinogens are not fully understood. Given recent findings that ectopic expression of activation-induced cytidine deaminase (AID) in somatic cells plays a critical role in carcinogenesis, we investigated whether several of the established not-directly mutagenic carcinogens induce AID expression.
Methods We prepared cells with stable expression of luciferase reporter gene containing the promoter of AID. We then used this system to examine the AID promoter activity of the non-genotoxic carcinogen: butyl benzyl phthalate, bisphenol A, di (2-ethylhexyl) phthalate, cadmium chloride (Cd), and butylated hydroxyanisole.
Results Results showed that Cd increased the promoter activity of AID and actually induced AID gene expression.
Conclusion A not-directly mutagenic carcinogen, cadmium, has the potential to induce the AID gene, suggesting that this might represent a novel molecular mechanism of carcinogenesis of cadmium.

Keywords Not-directly mutagenic carcinogens · AID · Screening

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Introduction

Most chemical carcinogens are capable of inducing DNA damage and are 'genotoxic' in their carcinogenic mode of action. There is, however, a group of carcinogens that induce cancer in a non-genotoxic manner [1]. These not-directly mutagenic carcinogens are generally negative on the Ames mutagenicity test and positive on in vivo testing in rodents. A wide variety of molecular mechanisms to explain this cancer induction have been proposed, including receptor-mediated endocrine modulation, non-receptor-mediated endocrine modulation, tumor-promoting induction of tissue-specific toxicity and inflammatory responses, immunosuppression, and inhibition of gap junction intercellular communication [2, 3]. Despite these proposals, however, the molecular mechanisms of non-genotoxic carcinogens remain largely unknown.

Recent studies have shown that ectopic expression of activation-induced cytidine deaminase (AID) in somatic cells play a critical role in carcinogenesis [4–7], and transgenic mice with overexpression of AID are prone to tumorigenicity [8]. Physiologically, AID initiates somatic hypermutation, class-switch recombination, and gene conversion in immunoglobulin genes [9], and its expression in activated B cells in the germinal center of lymphoid tissue is strictly regulated [10]. However, AID is induced by TNF-α, IL-4, and estrogen in some types of cancer cells and epithelial cells [5, 6].

Accordingly, we hypothesized that several non-genotoxic carcinogens might cause carcinogenesis via the induction of ectopic AID expression. Here, we prepared stable expression cells with a reporter gene system containing the AID promoter and then used this system to screen established non-genotoxic carcinogens for their potential to induce AID expression.

Materials and methods

Cell culture

Human cancer cell lines, PANC-1, A549, normal human embryo fibroblast cell (JCRB1006.0), and Romas were cultured at 37 °C and in 5 % CO_2 in Dulbecco's modified Eagle's medium (DMEM), and RPMI, respectively (Life Technologies, Carlsbad, CA, USA), containing 10 % fetal bovine serum and an antibiotic–antimycotic mix (Life Technologies).

Reagents

Butyl benzyl phthalate (BBP), bisphenol A (BPA), di (2-ethylhexyl) phthalate (DEHP), and TNF-α were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Cadmium chloride (Cd), butylated hydroxyanisole (BHA), and 17β-estradiol were purchased from Sigma-Aldrich (St. Louis, MO, USA). IL-4, MG-132, and ICI 182,780 were purchased from PeproTech (Rocky Hill, NJ, USA), Calbiochem (Gibbstown, NJ, USA), and Tocris Bioscience (Ellsville, MO, USA), respectively.

Stable expression of cells with luciferase reporter gene including the AID promoter

Our previous report demonstrated that AID mRNA was induced by TNF-α in PANC-1 cells [11]. We therefore used PANC-1 cells to develop a reporter gene system to detect AID promoter activity. The promoter sequence of AID was amplified by PCR from PANC-1 genomic DNA and cloned into a pGL4.14 [luc2/Hygro] luciferase plasmid vector (Promega, Madison, WI, USA). PCR fragments approximately 500, 1,000, and 1,600 bp upstream of the AID transcription initiation site were amplified using the following primers:

hAID promoter 500 F-5′-CAGTCTCGAGGCCTGTCT GTACTGAGGTTC-3′
hAID promoter 1000 F-5′-CAGTCTCGAGCCCTAGCT GCATTGCTTAGC-3′
hAID promoter 1600 F-5′-CAGTCTCGAGCTGCCCA GGTTTTGCCAAGC-3′
hAID promoter R-5′-CAGTAAGCTTCAGAGTGTCTT CTTGCCTCC-3′

The fidelity of AID reporter constructs pGL4.14 AID 500, pGL4.14 AID1000, and pGL4.14 AID1600 was confirmed by sequencing analysis.

To establish a stable transfectant, PANC-1 cells were seeded at a concentration of 1×10^5 cells/well in 6-well plates and transfected with linearized AID reporter constructs and pGL4.14 using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol. 24 h after transfection, the cells were split and cultured in DMEM containing 800 μg/ml hygromycin B (Roche Diagnostics GmbH, Mannheim, Germany). After selection, clones were maintained in DMEM containing 500 μg/ml hygromycin B. Genomic DNAs were isolated from clones using a QIAamp DNA mini kit (Qiagen GmbH, Hilden, Germany) and insertion of AID promoter-luciferase fragments into the genome was confirmed by PCR.

Promoter assays

PANC-1 cells with stable expression of the reporter gene including the AID promoter were seeded at a density of 1×10^4 cells per well on 96-well plates and incubated for 24 h. To normalize luciferase activity, the Renilla luciferase (RLuc) expression plasmid pGL4.74 (Promega) was transfected as an internal control using Lipofectamine 2000 (Life Technologies). Treatment with TNF-α, IL-4, or the agent to be tested was for 24 h on the basis of our preliminary finding that AID mRNA was induced between 6 and 24 h in PANC-1 cells. Luciferase and RLuc activities were then measured using the Dual-Glo Luciferase Reporter Assay System and a Glomax 96 Microplate Luminometer (Promega). Given that a previous study reported that TNF-α and/or IL-4 enhanced AID expression [6], we tested the effect of IL-4 on the promoter activity of AID to confirm whether the system was functional.

Reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR

mRNA levels of AID were determined by RT-PCR. Cells were harvested at 24 h after treatment with the agent tested and total RNA was isolated using a FastPure RNA kit (Takara Bio Inc., Shiga, Japan) or RNeasy Minikit (Qiagen) according to the manufacturer's protocol. First-strand cDNA was synthesized using a ReverTra Ace qPCR RT kit (Toyobo Co., Ltd., Osaka, Japan). To detect the appropriate genes, first-strand cDNA was amplified by PCR using sets of oligonucleotide primers. β-actin was used as the internal control for RT-PCR. Quantitative real-time RT-PCR for human AID amplification was also performed, as described elsewhere [12].The primer sequences used in the present study for RT-PCR and quantitative real-time PCR were as follows: AID-forward 5′-AAATGTCCGCTGGGCTAAG G-3′, AID-reverse 5′-GGAGGAAGAGCAATTCCACGT-3′; FAM-labeled Taqman probe: TCGGCGTGAGACCTA CCTGTGCTAC, β-actin-forward 5′-ATG ATA TCG CCG CGC TCG TCG TC-3′, β-actin-reverse 5′-CCA GAG GCG TAC AGG GAT AGC AC-3′; 18S rRNA -forward 5′-AAA CGG CTA CCA CAT CCA AG-3′ and rRNA-reverse 5′-CAA TTA CAG GGC CTC GAA AG-3′. Real-time PCR

was conducted using the ABI-PRISM 7900 Detection System and TaqMan Gene Expression Master Mix for AID or Power SYBR Green Master Mix for rRNA (Life Technologies). mRNA from Romas cells was used as positive control.

Effect of an inhibitor of NF-κB or of estrogen receptor on AID promoter activity

PANC-1 cells with stable expression of the reporter gene and AID promoter were seeded at a density of 1×10^4 cells per well on 96-well plates and incubated for 24 h. To normalize for luciferase promoter activity, the RLuc expression plasmid pGL4.74 (Promega) was transfected as an internal control using Lipofectamine 2000 (Life Technologies). After pre-incubation for 1 h with MG-132 (0.3 μM), a selective inhibitor for NF-κB, which is widely used in the research fields, or ICI 182,780 (0.5, 1 μM), an estrogen receptor blocker, treatment with TNF-α (100 ng/ml) or Cd (25 μM) was given for 6 h in the case of MG-132 or 24 h in the case of ICI 182,780, after which luciferase activity was measured using the Dual-Glo Luciferase Reporter Assay System and a Glomax 96 Microplate Luminometer (Promega).

Statistical analysis

Data are expressed as the mean ± SD. Significance was determined by the Mann–Whitney's *U* test or Kruskal–Wallis with Bonferroni's multiple comparison test using IBM SPSS Statistics ver.19.0 (IBM Japan Ltd. Tokyo, Japan). *P* values <0.05 were considered to be significant.

Results

Cells with stable expression of the reporter plasmids pGL4.14 AID 500, pGL4.14 AID1000, and pGL4.14 AID1600 were treated with TNF-α (100 ng/ml) for 24 h. Among these three constructs, the ratio of luciferase to RLuc was highest in the cells with pGL4.14 AID1600 (data not shown). We therefore used cells with pGL4.14 AID1600 in the following experiment.

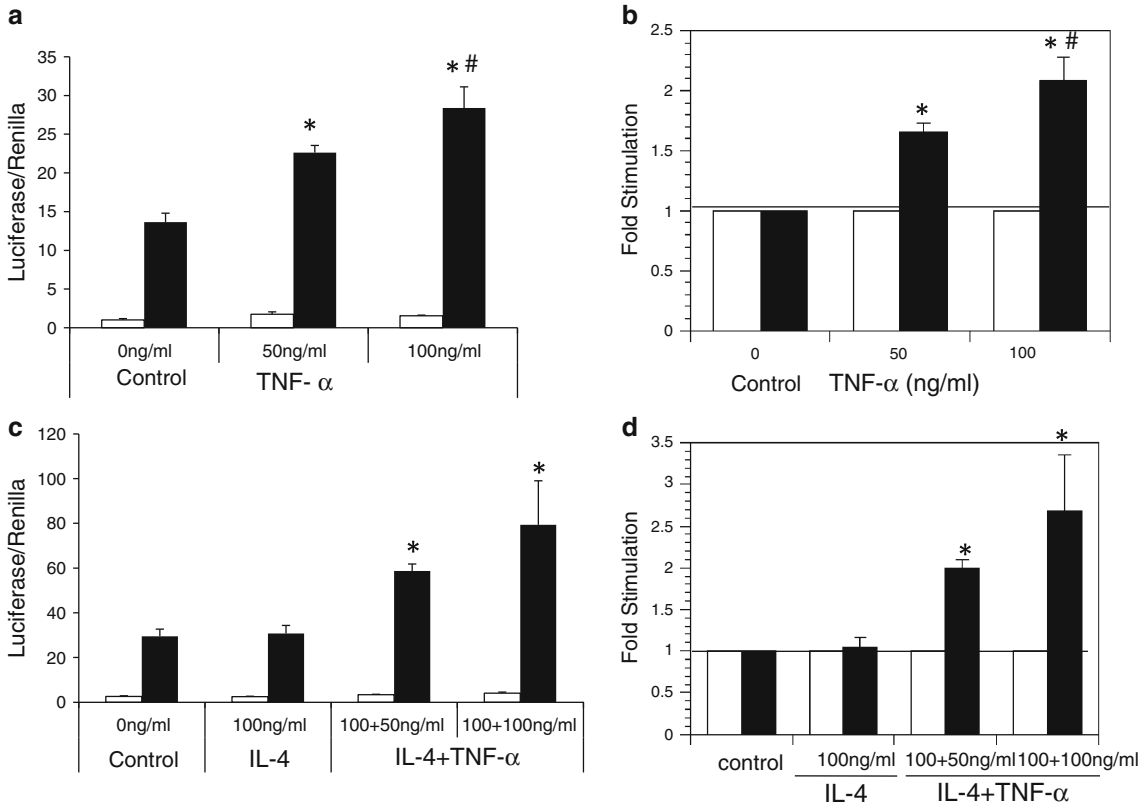

Fig. 1 AID promoter activity by TNF-α treatment. **a** Effect of TNF-α on AID promoter activity. Data represent the mean ± SD of the ratio of luciferase to Renilla luciferase. *Closed bar* indicates pGL4.14 -AID1600. *Open bar* indicates pGL4.14 (null vector). **b** Relative ratio of luciferase to Renilla compared to that of the control. **c** Effect of IL-4 on AID promoter activation by TNF-α. *Closed bar* indicates pGL4.14 -AID1600. *Open bar* indicates pGL4.14 (null vector). **d** Relative ratio of luciferase to Renilla luciferase to that of the control. ($n = 4$ in each experiment) $^*p < 0.05$, compared with control and $\#p < 0.05$, compared with cells treated with 50 ng/ml

Treatment with TNF-α enhanced AID promoter activity in a dose-dependent manner (Fig. 1a, b). The results showed that co-treatment with IL-4 further enhanced the AID promoter activity of TNF-α (Fig. 1c, d). These results indicated that the system was functional.

Using the reporter gene system, we then tested the following agents, namely BPA, DEHP, BBP, BHA, and Cd. Optimal dose was determined by examining whether RLuc activity as an internal control and cell viability were not decreased. Results showed that Cd tended to increase the ratio of luciferase to RLuc in a dose-dependent manner, whereas the others showed no enhancement of the promoter activity of AID (Fig. 2).

Furthermore, mRNA induction was confirmed in a dose-dependent manner at 24 h after treatment with Cd in the PANC-1 cells (Fig. 3a). In addition, real-time PCR showed that Cd treatment 4-times induced mRNA of AID in A549 cells derived from human lung cancer (Fig. 3b). Treatment with TNF-α (100 ng/ml) also induced AID mRNA in A549 cells; in addition co-administration of Cd plus TNF-α further induced AID mRNA (Fig. 3b), suggesting that an inflammatory condition of the lung could be a further risk for Cd-induced carcinogenesis. Furthermore, we examined AID gene induction using normal embryo fibroblast cells, which are not cancer cell lines. The results showed that the Cd administration induced dose-dependent induction of AID mRNA (Fig. 3c).

To investigate the mechanism of the enhancement of AID promoter activity by Cd, we examined the effects of MG-132 or ICI182, 780 on the promoter activity of Cd. Administration of MG-132 significantly reduced the enhancement of promoter activity by TNF-α or Cd (Fig. 4a). In contrast, treatment with 17β-estradiol (10 nM or 100 nM) did not elevate AID promoter activity (Fig. 4b). Furthermore, treatment with ICI182, 780, a potent antagonist for estrogen receptor, showed no effect on the increase in the ratio of luciferase to RLuc activity by Cd treatment (Fig. 4c).

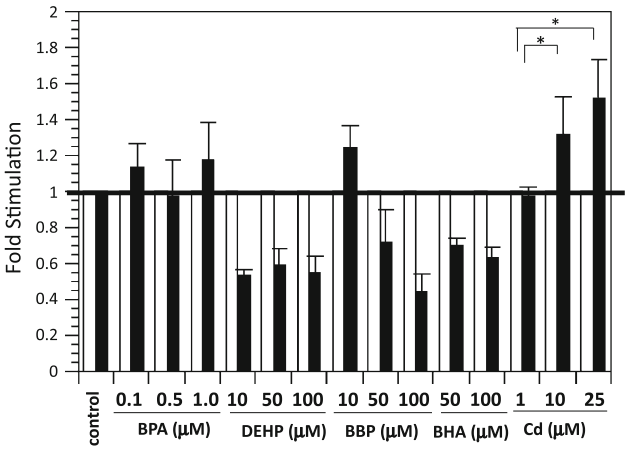

Fig. 2 Effect of BPA, DEHP, BBP, BHA, and Cd on AID promoter activity. Data are presented as the relative ratio of luciferase to Renilla luciferase. *Open bar* indicates the value of the control, and *closed bars* indicate the value of each concentration of chemical agent ($n = 4$ in each experiment). *BPA* bisophenol A, *DEHP* di (2-ethylhexyl) phthalate, *BBP* butyl benzyl phthalate, *BHA* butylated hydroxyanisole, *Cd* cadmium chloride. $*p < 0.05$

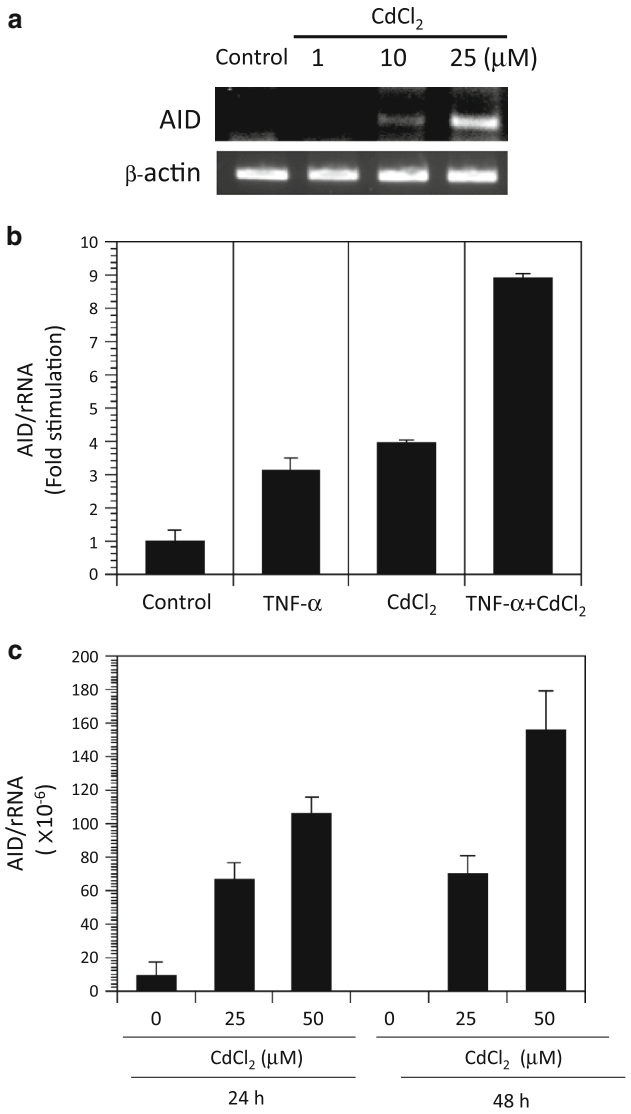

Fig. 3 AID mRNA expression by treatment with Cadmium (Cd). **a** AID mRNA was determined by RT-PCR in PANC-1 cells at 24 h after treatment with Cd. **b** AID mRNA was quantitatively determined by real-time RT-PCR 24 h after treatment with Cd (25μM) or TNF-α (100 ng/ml), or Cd (25μM) plus TNF-α (100 ng/ml) in A549 cells (duplicate examination). **c** AID mRNA was determined by real-time RT-PCR using normal embryo fibroblast cells. The results showed that the Cd administration induced dose-dependent induction of AID mRNA (triplicate examination)

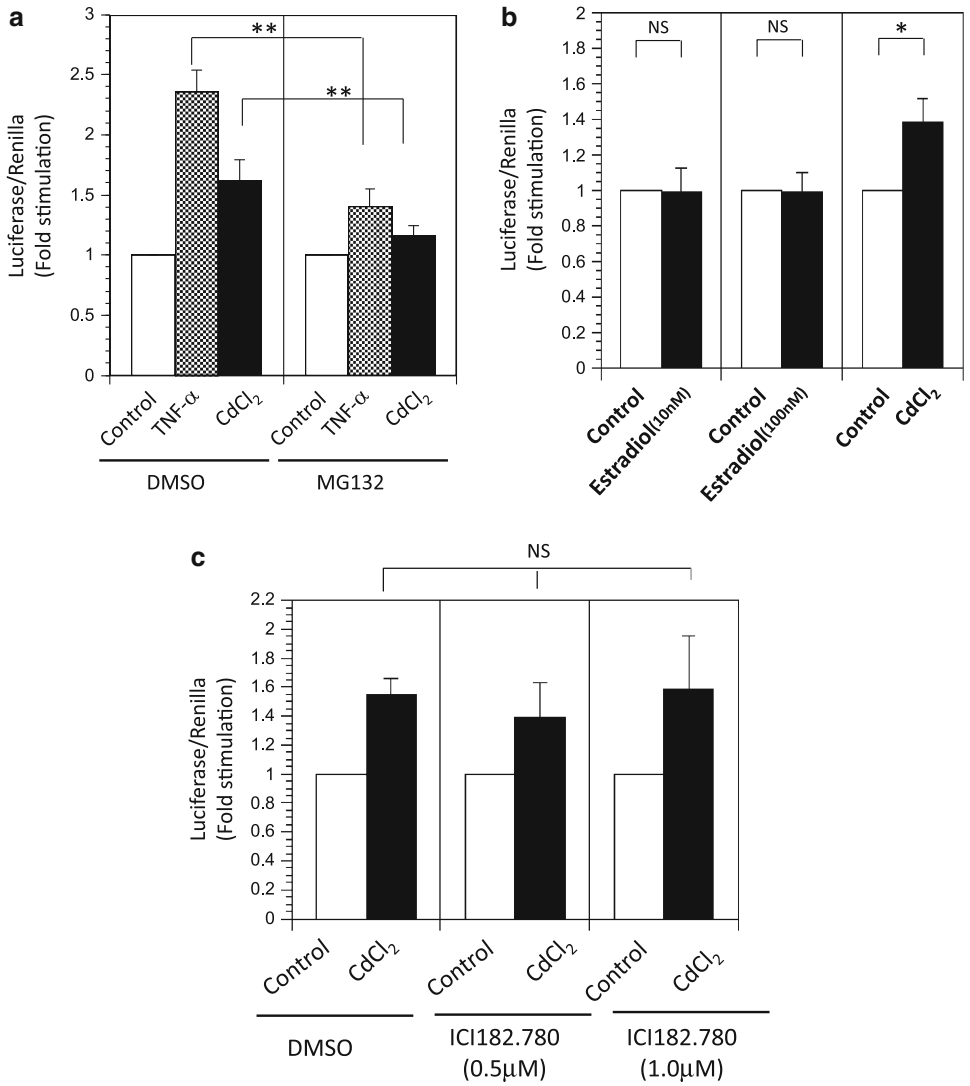

Fig. 4 Effect of an NF-κB inhibitor or estrogen receptor blocker on promoter activation by Cadmium (Cd). **a** Effect of pre-incubation with MG-132 (0.3 μM), a selective inhibitor of NF-κB, on the increase in the promoter activity of AID by treatment with TNF-α (100 ng/ml) or Cd (25 μM). Promoter activity was presented as the ratio of luciferase to Renilla of pGL4.14-AID1600. **b** Effect of 17β-estradiol (10 or 100 nM) on the promoter activity of AID, compared to that by treatment with Cd (25 μM). **c** Effect of pre-incubation with ICI182,780 (0.5 or 1 μM), an estrogen receptor blocker, on the promoter activity of AID by treatment with Cd (25 μM). ($n = 5$ in each experiment). *$p < 0.05$, **$p < 0.01$, NS = not significant

Discussion

In this study, we found that the Ames-negative carcinogen, Cd, induced AID gene expression. Importantly, this finding identifies a novel molecular mechanism of carcinogenesis for genotoxic test-negative carcinogens.

Cd is an environmental pollutant. Exposure occurs at certain workplaces and in the general population from foods. Cd is used during various industrial operations and is constantly introduced into the atmosphere through smelting, electroplating, and welding in the manufacture of Cd alloys and production of nickel–cadmium batteries [13]. The International Agency for Research on Cancer (IARC) has classified Cd as a Group 1 carcinogen [13]. A recent review of substances classified as human carcinogens by the IARC confirmed that there was sufficient evidence for Cd-induced lung cancer in humans [14]. Despite the strong evidence supporting the carcinogenic potential of Cd, the mechanisms underlying Cd-induced carcinogenesis are not clearly understood, and its lower ability causes direct interactions with DNA in cell extracts or with isolated DNA [15]. In a recent review by Hartwig, the combination of multiple mechanisms was suggested to give rise to genomic instability in Cd-treated cells, which is relevant not only for tumor initiation but also in tumor development [16]. He mentioned that the molecular mechanisms included the interference with the cellular response to DNA damage, resistance to apoptosis, and increased susceptibility to other DNA damaging agents and endogenous mutagens by disturbance of the nucleotide repair system [16]. In addition, Joseph [17] also reviewed the molecular mechanisms of Cd carcinogenesis

and emphasized the importance of aberrant gene expression including immediate early response genes and stress-response gene, inhibition of DNA damage repair, induction of oxidative stress, and inhibition of apoptosis. Particularly, generation of reactive oxygen species by Cd resulting in oxidative stress plays a central role in Cd carcinogenesis [17].

Our data showed Cd-induced AID expression in some cancer cells and non-cancer somatic cells. It is known that Cd exposure shows a risk for lung cancer [14]. Thus, we tested the potential of AID induction by Cd using A549 cells derived from human lung alveolar epithelial cells. In addition, to investigate whether Cd can induce AID in non-cancer somatic cells, we tested AID induction using embryo fibroblast cells. In both cells, AID gene was clearly induced by Cd treatment. Furthermore, Cd is a highly toxic metal. Thus, it is possible that the exposure of Cd by respiratory root induces inflammation in the lung. Consequently, TNF-α could be induced under the inflammatory condition and moderate the AID expression by Cd exposure. Thus, we tested the effects of TNF-α on the modification of AID induction by Cd. Our results showed that the co-treatment with TNF-α and Cd had synergistic effects on the induction of AID. These results suggest that AID induction might be involved in lung carcinogenesis by Cd exposure.

TNF-α acts as a potent inducer of the AID gene via the activation of NF-κB [5]. Pauklin et al. demonstrated that estrogen activates AID transcription directly via binding to two estrogen-responsive elements (EREs) in the AID promoter [18]. It is reported that Cd activates NF-κB [19] and also estrogen receptor alpha [20]. Our study showed that pre-incubation with an NF-κB inhibitor but not an estrogen receptor antagonist suppressed the enhancement of AID promoter activity by Cd. This result suggests that Cd might have the potential to induce AID in somatic cells via the activation of NF-κB. AID expression in somatic cells has been shown to lead to cytogenetic alteration, which is consistent with the results of cytogenetic studies among subjects with occupational exposure to Cd [21, 22].

Our study shows that among Ames-negative carcinogens, a metal compound has the potential to induce ectopic expression of the AID gene. Aberrant AID expression results in mutation and chromosomal instability and might be involved in carcinogenesis and progression. The reporter gene system established here might therefore be useful in screening Ames test-negative compounds for their carcinogenic potential.

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Conflict of interest The authors declare no conflict of interest.

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