Expression of Alcohol Dehydrogenase 3 in Tissue and Cultured Cells from Human Oral Mucosa

Jesper J. Hedberg,* Jan-Olov Höög,*
Jan A. Nilsson,[†] Zheng Xi,[†] Åsa Elfving,[†] and
Roland C. Grafström[†]

From the Department of Medical Biochemistry and Biophysics*
and the Institute of Environmental Medicine,[†] Karolinska
Institutet, Stockholm, Sweden

Because formaldehyde exposure has been shown to induce pathological changes in human oral mucosa, eg, micronuclei, the potential enzymatic defense by alcohol dehydrogenase 3 (ADH3)/glutathione-dependent formaldehyde dehydrogenase was characterized in oral tissue specimens and cell lines using RNA hybridization and immunological methods as well as enzyme activity measurements. ADH3 mRNA was expressed in basal and parabasal cell layers of oral epithelium, whereas the protein was detected throughout the cell layers. ADH3 mRNA and protein were further detected in homogenates of oral tissue and various oral cell cultures, including, normal, SV40T antigen-immortalized, and tumor keratinocyte lines. Inhibition of the growth of normal keratinocytes by maintenance at confluency significantly decreased the amount of ADH3 mRNA, a transcript with a determined half-life of 7 hours. In contrast, decay of ADH3 protein was not observed throughout a 4-day period in normal keratinocytes. In samples from both tissue and cells, the ADH3 protein content correlated to oxidizing activity for the ADH3-specific substrate S-hydroxymethylglutathione. The composite analyses associates ADH3 mRNA primarily to proliferative keratinocytes where it exhibits a comparatively short half-life. In contrast, the ADH3 protein is extremely stable, and consequently is retained during the keratinocyte life span in oral mucosa. Finally, substantial capacity for formaldehyde detoxification is shown from quantitative assessments of alcohol- and aldehyde-oxidizing activities including K_m determinations, indicating that ADH3 is the major enzyme involved in formaldehyde oxidation in oral mucosa. (*Am J Pathol* 2000, 157:1745–1755)

Humans are exposed to formaldehyde through various sources, eg, tissue fixatives, products containing formaldehyde preservatives, tobacco smoke, automotive emissions, and certain dental materials.^{1–3} Formaldehyde also occurs naturally in fruits and other foods. It is formed

during amino acid metabolism, oxidative demethylation, as well as in purine and pyrimidine metabolism. Estimates indicate that aldehydes, including formaldehyde, is present at 100 to 500 $\mu\text{mol/L}$ in biological fluids of mammals, including humans.^{2,4} Because of reactions with nucleophiles, including thiols and amines, the proportion of free, unbound formaldehyde is likely to be low.^{2,5}

Formaldehyde causes genotoxicity manifested as DNA damage, mutations, and tumors in experimental studies, and is therefore regarded as a probable human carcinogen.^{2,3} Because of its reactivity, inhaled formaldehyde will primarily react with the mucous membranes of the nasal and oral mucosa, respectively, dependent on route of inhalation.^{2,6,7} Human exposure to atmospheric formaldehyde, even considerably below the permissible exposure limit,^{8,9} causes many-fold increased levels of micronuclei and chromosome breakage in oral mucosa.^{8,9} The fact that oral mucosa is a target for formaldehyde genotoxicity from minute exposure levels implies a need for characterization of the enzymatic defense against formaldehyde in this tissue.

The alcohol and aldehyde dehydrogenase families (ADHs and ALDHs) have evolved into classes with broad substrate repertoires; the human isozymes are denoted ADH1–5 and ALDH1–10 according to current nomenclature.^{2,10–13} ADH3, identical to glutathione-dependent formaldehyde dehydrogenase, has conserved function/structure and exhibits high specificity for formaldehyde in complex with glutathione, S-hydroxymethylglutathione (HMGSH).^{14–16} Based on studies of *ADH3*–/– mice as well as tissue preparations and purified fractions from a variety of species, ADH3 is regarded as a formaldehyde scavenger.^{14,17–21} Notably, the distribution and activity of ADH3 vary among different tissues and cell types, including within epithelial structures of human and laboratory animals.^{22–27}

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Address reprint requests to Dr. Roland Grafström, Experimental Carcinogenesis, Institute of Environmental Medicine, Karolinska Institutet, Box 210, S-171 77 Stockholm, Sweden. E-mail: roland.grafstrom@imm.ki.se

Replicative cultures of normal keratinocytes and fibroblasts can be established from human oral mucosal tissue.²⁸ The serum-free methods developed for oral normal keratinocytes are also applicable to the various transformed oral keratinocyte lines, including SV40 T antigen-immortalized keratinocyte line SVpgC2a and the squamous carcinoma cell line SqCC/Y1.^{29,30} These transformed keratinocyte lines model the step-wise development of oral cancer on the basis that they reflect acquisition of immortality (the SVpgC2a cells), loss of p53 tumor suppressor functions, and eventually gain of the tumorigenic phenotype (the SqCC/Y1 cells).^{28,31,32} Studies of expression, activity, and regulation of human ADH and ALDH enzymes are not available in normal or transformed oral cell lines.

Based on overlapping substrate specificities,^{11,12} the various ADH and ALDH enzymes may participate in the defense against formaldehyde. For example, ADH3 and low- K_m ALDHs (ALDH1 and ALDH2) in rat liver equally contribute to formaldehyde metabolism.¹⁷ Low- K_m ALDHs oxidize free formaldehyde and exhibit activity for aliphatic aldehydes like propanal.¹² The human ADHs have affinity for different aliphatic alcohols, eg, octanol, and exhibit ethanol-oxidizing capacity to different degrees. In this regard, ADH1, ADH2, and ADH4 display high activity, whereas ADH3 almost lacks this activity.^{19,33–35} Assessment of the metabolic conversion of various aldehyde and alcohol substrates in human oral mucosa may clarify the existence of multiple activities for formaldehyde detoxification.

The existence of species specificity, and a current lack of correlative analysis of ADH3 mRNA and protein in epithelial tissues, accentuates the need for an analysis of ADH3 in human oral mucosa. The current study investigated the presence and distribution of ADH3 mRNA and protein in tissue using *in situ* hybridization and immunohistochemistry, respectively. Further, ADH3 mRNA, protein, and activity was determined by Northern blot, Western blot, and enzymatic analyses, respectively, in preparations from oral tissue and cell cultures. To study an association of ADH3 expression with proliferation, subconfluent dividing oral keratinocytes were compared with cells grown to and maintained at confluency, a protocol known to efficiently inhibit cell proliferation.^{28,36} The markedly different half-lives indicated for ADH3 mRNA and protein in oral epithelium *in vivo* were substantiated by measurements in normal keratinocyte cultures. Finally, the oxidation of formaldehyde and other aldehyde and alcohol substrates were studied in lysates from tissue and cell lines. The results provide novel aspects of the regulation of ADH3 in human epithelia, and further show that primarily this enzyme is responsible for formaldehyde detoxification in oral mucosa.

Materials and Methods

Cell Cultures

Human buccal tissue was obtained from noncancerous patients undergoing maxillofacial surgery with approval

from the Karolinska Institutet ethical committee. Primary keratinocyte lines were derived after incubation of tissue with 0.17% trypsin in phosphate-buffered saline (PBS) at 4°C for 18 to 24 hours, and the subsequent seeding of keratinocyte aggregates and single cells at 5×10^3 cells/cm² onto fibronectin/collagen-coated dishes in serum-free epithelial medium with elevated amino acid supplements (EMA).²⁹ EMA was reconstituted from MCDB 153 medium and supplemented with 1 μ mol/L hydrocortisone, 0.77 μ mol/L insulin, 1.64 nmol/L epidermal growth factor, 100 μ mol/L each of ethanolamine and phosphoethanolamine, and 50 μ g/ml Gentamicin (Life Technologies Ltd., Paisly, Scotland).⁵ The immortal cell line SVpgC2a, derived by transfection and stable integration of the SV40T antigen into buccal keratinocytes,³⁰ and the buccal carcinoma cell line SqCC/Y1²⁹ were cultured in EMA. Primary outgrowths of fibroblasts were obtained from tissue explants maintained in CRML 1066 medium supplemented with 10% fetal bovine serum, 440 nmol/L hydrocortisone, 1.83 nmol/L epidermal growth factor, 0.25 μ mol/L ethanolamine, 0.25 nmol/L phosphoethanolamine, and 50 μ g/ml Gentamicin (Life Technologies Ltd.), and the resulting cell lines grown and transferred in a 1:1 mixture of MCDB 153 and M199 media and was supplemented with 1.25% fetal bovine serum, 440 nmol/L hydrocortisone, 0.83 nmol/L epidermal growth factor, 0.25 μ mol/L ethanolamine, 0.25 nmol/L phosphoethanolamine, 63 nmol/L transferrin, and 50 μ g/ml Gentamicin.³⁷ The normal cell types were used in passages 1 to 5, the SVpgC2a line in passages 59 to 64, and the SqCC/Y1 line in passages 115 to 120. The optimal seeding density and the length of time required to reach the preferred state of confluence were different for each cell line. Normal keratinocytes were seeded at 5×10^3 cells/cm² to reach 75% confluence, SVpgC2a at 4.1×10^3 cells/cm² (100% confluence), SqCC/Y1 at 1×10^4 cells/cm² (90% confluence), and normal fibroblasts at 7×10^3 cells/cm² (100% confluence) at 4 to 7 days.²⁸ The term confluency (100%) was regarded as the stage/moment when the cultures were (first) grown to fully occupy the dish surface area as determined from visual inspection under a phase contrast microscope. In the experiments in which normal keratinocytes were cultured beyond confluency, the cells were seeded as above, and the cultures were allowed to grow for 6 to 8 days to reach the state of confluency. Thereafter, the assessments of the cultures were based on time; cultures were analyzed at 5, 10, and 15 days after their growth to the confluent stage.

In Situ Hybridization

Tissue specimens were frozen on dry-ice. Frozen sections (14 μ m) were prepared and mounted on Probe On⁺ slides (Fisher Scientific, Pittsburgh, PA). Specific oligonucleotide probes complementary to the human ADH3 gene, (nucleotides 1170 to 1215),³⁸ sense probe (nucleotides 1215 to 1170), and β -actin gene (nucleotides 1244 to 1288) were used for *in situ* hybridization. Probes were labeled at the 3' end with [α -³⁵S]dATP using terminal deoxynucleotidyl-transferase (Amersham Pharmacia Bio-

tech, Buckinghamshire, UK). Sections were covered with hybridization buffer containing 50% formamide, 4× standard saline citrate, 1× Denhardt's solution, 1% sarcosyl, 0.02 mol/L phosphate buffer, pH 7.0, 10% dextran sulfate, 500 μ g/ml heat-denatured salmon sperm DNA, 200 mmol/L dithiothreitol, and 10^7 cpm/ml of the labeled probe. Slides were incubated for 16 to 18 hours at 42°C placed in a box humidified with 50% formamide and 4× standard saline citrate. Sections were sequentially rinsed in four changes of 1× standard saline citrate at 55°C for 60 minutes, dehydrated in 60% and 95% ethanol, air-dried, and exposed for 2 to 4 weeks to NTB nuclear track emulsion (Kodak, Rochester, NY) diluted 1:1 with distilled water.

Immunohistochemistry

Human ADH1, ADH2, and ADH3 were recombinantly expressed in *Escherichia coli* and purified to homogeneity as described earlier.^{35,39} Homogenous ADH3 protein was subsequently used to raise antiserum against ADH3 in a White New Zealand rabbit. The antiserum was used without further purification and tested for reactivity against human ADH1, ADH2, and ADH3. Human oral mucosa was fixed in 4% formaldehyde and embedded in paraffin wax. Paraffin sections (4 μ m) were prepared for immunostaining. A three-step immunoperoxidase staining method was performed to detect the expression of ADH3 in human oral mucosa using standard procedures.⁴⁰ The sections were weakly counterstained with hematoxylin. In negative controls the anti-ADH3 serum was replaced by null serum.

mRNA Preparation and Northern Blot Analyses

Total RNA was prepared according to the acid guanidinium thiocyanate phenol-chloroform extraction method.⁴¹ Snap-frozen tissue specimens were homogenized with a Polytron instrument in denaturing solution,⁴¹ and cells in culture were recovered by addition of denaturing solution directly to the dish. Poly A⁺ RNA was enriched by using oligo dT coupled to a solid phase matrix, Oligotex (Qiagen, Hilden, Germany), and eluted according to the manufacturer's recommendations. mRNA (0.7 μ g) or total RNA (25 μ g) was subjected to electrophoresis under denaturing conditions in 1% agarose containing 6.5% formaldehyde. After electrophoresis, the RNA was blotted to Hybond-N+ nylon membranes (Amersham Pharmacia Biotech) and cross-linked by oven-baking or UV exposure according to the manufacturer's recommendations. ADH3 mRNA was probed with a 390-bp *EcoRI/KpnI* fragment from the ADH3 cDNA clone³⁸ and a 2-kb human β -actin fragment was used as control. The probes were labeled with [α -³²P]dCTP (megaprime DNA labeling system; Amersham Pharmacia Biotech) and hybridizations were performed as described.²⁵ Quantification of signals was performed by phosphorImager analysis using ImageQuant soft ware (Molecular Dynamics, Sunnyvale, CA) and obtained values were correlated to the

amount of RNA, determined spectrophotometrically (OD_{260}), loaded on the gel.

Determination of mRNA Half-Life

Normal keratinocytes at 70 to 80% confluency were exposed to the adenosine analog 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (100 μ mol/L; Sigma, St. Louis, MO), an agent known to inhibit RNA polymerase II and effectively reduce transcription by 90%.⁴² 5,6-Dichloro-1- β -D-ribofuranosylbenzimidazole was dissolved in dimethyl sulfoxide to a final working concentration of 0.1%. Cells were harvested at indicated time points and total RNA extraction, Northern blot analyses, and quantification of signals were performed as described above. Data points were plotted on a semilogarithmic scale and half-lives were calculated from the best-fit line by linear regression analyses.⁴²

Determination of Protein Half-Life

Normal keratinocytes were seeded at 5×10^3 cells/cm² in 35-mm dishes and grown for 1 day in EMA. Metabolic labeling was performed in EMA free of unlabeled methionine but supplemented with 50 μ Ci/ml [³⁵S]methionine for 2 hours at 37°C. Subsequently, the cells were washed once with PBS and then incubated in complete EMA. At indicated time points, cells were lysed by addition of 1 ml immunoprecipitation buffer (50 mmol/L, Tris/HCl, pH 8, 150 mmol/L NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate), incubated for 15 minutes at 4°C followed by collection of cell lysates. The lysates were precleared overnight at 4°C with null serum (final dilution, 1:50) and 200 μ l 30% suspension of Protein A Sepharose CL-4B (Amersham Pharmacia Biotech, Uppsala, Sweden). Specific immunoprecipitation of ADH3 was performed by addition of ADH3 antiserum to the precleared lysate (final dilution, 1:100). The mixture was incubated for 2 hours at 4°C followed by addition of 50 μ l 30% Protein A Sepharose CL-4B and incubation for additionally 2 hours at 4°C. To ascertain the specificity of the immunoprecipitation reaction, a separate sample was immunoprecipitated with antiserum preabsorbed with 100 μ g of purified ADH3 for 3 hours at 4°C before the analysis. Immune complexes were washed four times in immunoprecipitation buffer and twice in double-distilled H₂O before the sample was boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer for 10 minutes. Precipitated proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted to polyvinylidene difluoride transfer membranes (Bio-Rad, Hercules, CA). Membranes were dried and labeled proteins were visualized by exposure to X-OMAT films (Kodak) using intensifying screens. Quantification of signals was performed by phosphorImager analysis using ImageQuant software.

Figure 1. Distribution of ADH3 mRNA in human oral epithelium. Sections from frozen tissue specimens were analyzed by *in situ* hybridization under high stringency conditions with probes specific for ADH3 (**A**) and β -actin (**B**). ADH3 mRNA is preferentially located in the basal and lower prickle-cell layers. As cells migrate toward the superficial layers, ADH3 transcription is markedly reduced. β -actin mRNA is found in the basal and prickle-cell layers and shows less prominent reduction toward the epithelial surface. Original magnification, $\times 100$.

Western Blot Analyses

Cell lysates from tissue specimens and cultured cells were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted to polyvinylidene difluoride transfer membranes. The membrane was blocked with 5% fat-free dry milk (Semper, Stockholm, Sweden), 0.05% Tween 20 in Tris-buffered saline, subsequently incubated with a 1:5,000 dilution of antiserum against ADH3, washed, and finally incubated in a 1:3,000 dilution of Protein A-HRP Conjugate (Bio-Rad, Hercules, CA). Immunoreactive bands were detected by the ECL detection reagents according to the manufacturer's recommendations (Amersham Pharmacia Biotech). Band intensities were quantified using a Personal Densitometer (Molecular Dynamics, Sunnyvale, CA) and normalized to controls with recombinantly expressed and purified ADH3, of which a minimum of three samples ranging from 5 to 100 ng of enzyme had been loaded on the gel.

Lysate Preparations and Enzyme Assays

Tissue specimens were collected at surgery, immediately snap-frozen and stored in liquid nitrogen. Before homog-

enization with a Polytron instrument, the tissue specimen was thawed in PBS or 10 mmol/L Tris/HCl, pH 8, 1 mmol/L dithiothreitol. Cultures of normal cells were harvested at the preferred confluency states optimized for passage, as specified under Cell Cultures. All cells were washed once with PBS and were then scraped and collected in a small volume of PBS or 10 mmol/L Tris/HCl, pH 8, 1 mmol/L dithiothreitol. After harvesting, the cells were completely disrupted by sonication and centrifuged at $48,000 \times g$ for 1 hour. The resulting lysates were applied to a small gel filtration column, (PD-10; Amersham Pharmacia Biotech) in efforts to eliminate low molecular weight compounds and possible background activity as previously described.⁴³ ADH and ALDH activities were measured using ethanol, octanol, formaldehyde, propanal, and HMGSH. Ethanol- and octanol-oxidizing activities were measured in 0.1 mol/L glycine/NaOH, pH 10, with 33 mmol/L ethanol or 2 mmol/L octanol. Propanal oxidizing activity was measured in 0.1 mol/L of phosphate buffer, pH 7.5, with 1 mmol/L propanal. HMGSH- and formaldehyde-oxidizing activities were measured in 0.1 mmol/L phosphate buffer, pH 8, and 1 mmol/L formaldehyde with or without 1 mmol/L GSH, respectively. Apparent K_m values for the latter two substrates were

Figure 2. Distribution of ADH3 protein in human oral epithelium. Immunohistochemical analyses were performed on sections from formalin-fixed tissue. A three-step peroxidase-staining protocol was used with primary antiserum against ADH3 (A) or null serum as negative control (B). The sections were counterstained with hematoxylin to visualize the structures. ADH3 is expressed throughout the entire epithelium except for in the outmost nonviable keratinized layers. Generally diffused cytoplasmic staining is seen. The cells of the connective tissue also stain for ADH3. Original magnification, $\times 100$.

determined using varying concentrations of formaldehyde (1 $\mu\text{mol/L}$ to 1.5 mmol/L) with or without GSH as above. All experiments were performed at 37°C with a NAD^+ concentration of 2.4 mmol/L. NADH production was monitored using a Hitachi U-3000 spectrophotometer. One unit (U) of activity corresponded to 1 μmol NADH produced per minute, based on an absorption coefficient of 6,220 $\text{mol/L}^{-1} \text{cm}^{-1}$ for NADH at 340 nm. Background activities without substrate were subtracted from raw data. In the case of HMGSH oxidation, the formaldehyde oxidation rate was used as background. Protein concentrations were determined colorimetrically with bovine serum albumin as standard.⁴⁴ To fit lines to the obtained data points, and to calculate the apparent K_m constant for HMGSH and formaldehyde, respectively, a weighted nonlinear-regression analysis program was used (Fig. P for Windows; Biosoft, Ferguson, MO).

Results

Expression of ADH3 mRNA in Oral Mucosa

Expression of ADH3 mRNA in human oral tissue was determined by application of *in situ* hybridization (Figure 1A). An β -actin probe was used as a positive control (Figure 1B) and ADH3 sense probe as negative control (not shown). Pronounced amounts of ADH3 transcripts were uniformly distributed along the epithelium in both basal and parabasal cells. The transcript levels markedly decreased more superficially, implying that ADH3 transcripts were not present in the upper half of the prickle layer. Buccal and gingival specimens showed similar

basal and parabasal distributions of transcripts in their respective epithelium (data not shown), the latter lacking detectable transcripts also in the keratinized layer. The β -actin transcripts showed a more gradual basal-suprabasal reduction compared to ADH3. Sections from five individuals showed similar expression patterns for ADH3 and β -actin in oral tissue.

Generation of ADH3-Antiserum

ADH3 was recombinantly expressed, purified to homogeneity,³⁵ and antibodies were subsequently raised in a rabbit. Antiserum was tested for reactivity toward purified ADH1, ADH2, and ADH3. The antibody showed more than 20-fold higher specificity toward ADH3 than ADH1, ie, 5 ng of ADH3 were easily detected in a Western blot analysis with the antiserum whereas 100 ng of ADH1 were barely detected. Immunoreactivity toward ADH2 was not detected.

Expression of ADH3 Protein in Oral Mucosa

Expression of ADH3 protein throughout the epithelium except for the keratinized layer (Figure 2A) was demonstrated by application of immunohistochemistry with the raised ADH3 antiserum. The staining pattern was generally diffuse, although occasional cells showed dot-like expression. A tendency for higher expression was noted in the upper prickle-cell layer (but below the keratohya-

Figure 3. Northern blot analysis of ADH3 expression in human oral tissue and cell cultures. **A:** Hybridization with a 390-bp cDNA fragment coding for ADH3 revealed two ADH3 transcripts with approximate sizes of 1.6 and 2.8 kb (arrows) in all samples. **B:** The relative abundance of ADH3 transcripts among the samples are shown. The densitometric values were normalized to tissue (set to 1). The cell lines, as indicated, exhibited higher levels of ADH3 transcripts than tissue.

line granule layer). The cells of the connective tissue also showed immunopositivity. No immunoreactivity was detected in the negative control (Figure 2B). Sections from five individuals showed similar expression pattern.

Figure 4. ADH3 and β -actin mRNA in confluent cultures of normal oral keratinocytes. The cells were grown to confluency (day 0) and kept at confluency for the indicated number of days. Northern blot analyses were performed with probes specific for ADH3 and β -actin. Signals were quantified by densitometry and standardized to signals obtained at day 0. The levels of ADH3 mRNA (filled columns) are significantly reduced after 10 and 15 days of cultivation at confluency. The data indicate that the level of β -actin (open columns) may also become reduced at these time points (the results did not statistically differ from the earlier time points). The data were obtained from three separate experiments and expressed as mean ($\pm$ SEM). *, Significantly different from day 0 and 5 ($P < 0.05$, ANOVA with Kramer multiple comparisons test).

ADH3 mRNA Levels in Normal and Transformed Oral Keratinocytes

Two ADH3 transcripts were detected in preparations from human oral tissue specimens and various oral cell lines (Figure 3A). Quantification of signals revealed that tissue exhibited lower levels of ADH3 transcripts than the cell lines (Figure 3B). The fibroblasts and transformed keratinocyte lines clearly exhibited higher mRNA levels than normal keratinocytes. Two separate experiments generated similar ratios among the cell lines.

ADH3 mRNA expression was then analyzed in normal keratinocytes grown to confluency and cultures maintained at confluency for up to 15 days. ADH3 transcripts were detected in RNA preparations from all time points (Figure 4). The densitometric analysis showed that the cultures retained similar ADH3 mRNA abundance for the 5 days after confluency. However, the level of ADH3 decreased to $\sim$ 10% after 10 and 15 days. The levels of β -actin transcripts were also lowered by maintenance of cells at confluency, although the decreases noted were statistically insignificant.

Determination of mRNA Half-Lives in Normal Keratinocytes

The half-lives of ADH3 and β -actin transcripts were determined in normal keratinocytes after 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole-induced inhibition of transcription. Total RNA yields were similar in all preparations from the respective time points, indicating that overall RNA metabolism was unaffected.⁴² Northern blot analyses, followed by a densitometric assessment, revealed a transcript half-life of 7 and 30 hours for ADH3 and β -actin mRNA, respectively (Figure 5, A and B). Keratinocytes

Figure 5. ADH3 and β -actin mRNA stability in normal oral keratinocytes. Cells were treated with 100 μ mol/L of 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole and total RNA was extracted at the indicated times after treatment. **A:** Northern blot analyses were performed with probes specific for ADH3 and β -actin. **B:** Signals were quantified by densitometry and obtained data are expressed as percentage of the maximum level (time = 0 hours). mRNA half-life was calculated to 7 hours and 30 hours for ADH3 (■) and β -actin (●), respectively.

from two individuals were analyzed in separate experiments with similar results.

Assessment of ADH3 Protein Stability in Normal Oral Keratinocytes

The cells were metabolically labeled with 35 S-methionine followed by a chase period with unlabeled amino acids for up to 4 days. One band of ~40 kd corresponding to the ADH3 protein was detected by autoradiography (Figure 6). Band intensities, confirmed by densitometry (data not shown), were unchanged throughout the experiment showing that the ADH3 protein was stable throughout the time of the assay. Notably, the specificity of the immunoprecipitation was controlled by pre-absorption of the an-

Figure 6. Determination of ADH3 protein stability in normal oral keratinocytes. ADH3 was immunoprecipitated at the indicated time points after metabolic labeling of the cells. Precipitated proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electroblotted to polyvinylidene difluoride transfer membranes, and visualized by autoradiography. As a control, one sample was immunoprecipitated with antiserum that had been preabsorbed with homogeneous ADH3 protein. One band corresponding to ADH3 was detected in all samples (arrow). The band was absent in the control. Degradation of the ADH3 protein could not be detected throughout the 4 days demonstrating high stability of ADH3. Background bands corresponding to larger proteins were detected at various intensities in all samples, including the control.

tiserum with pure ADH3 protein, a procedure used to visualize background after neutralization of binding sites in the antiserum.

Protein Content Analyses and Correlation to Activity in Normal and Transformed Oral Keratinocytes

Total protein from lysates of the various cell cultures and tissue lysates were subjected to Western blot analyses (Figure 7, A and B). Generally one band at the approximate size of 40 kd was detected. After quantification of the respective bands by densitometry and normalization to band intensities from purified recombinant ADH3, the amounts of ADH3 protein were determined in each sample (Figure 7C). The fractions thus calculated showed that the cell lines contained similar amounts of ADH3. The tissue containing extracellular protein, ie, connective tissue, showed lower levels than the cell lines. For the purpose of allowing a comparison of the amounts of protein with enzymatic activity, HMGSH oxidizing activities were then determined in lysates. The respective activities were related to the activity exerted by purified recombinant ADH3, 4 U/mg.¹⁹ Further, the amounts of ADH3 protein found by Western blot analyses in cell lysates were related to the amount of protein loaded on the gel (Figure 7C). Notably, the two methods of determination showed extensive correlation for all samples. In agreement, the lysate from tissue showed several-fold lower activity than those of the cell lines.

Figure 7. Western blot analyses of ADH3 expression in human oral tissue and cell cultures. Total protein from lysate of the cell lines (15 μ g) and tissue (45 μ g), respectively, was analyzed. ADH3 protein (recombinantly expressed and purified to homogeneity) was used as control. **A:** Analysis of cultured cells. **B:** Analysis of tissue. **C:** Quantification of ADH3 protein in lysates by Western blot analyses (open columns) and activity measurements (filled columns). The fraction of ADH3 protein and activity in the sample is represented as per thousands of the total protein amount and activity of pure protein, respectively. To obtain an estimate of the amount of ADH3 protein, the intensities from Western blot analyses were quantified by densitometry and the obtained values were normalized to band intensities monitored for known amounts of pure ADH3. ADH3 activity in lysates was determined by spectrophotometric measurements of NADH production using HMGSH as substrate and correlated to the specific activity for recombinantly isolated ADH3. Oral tissue showed significantly lower protein and activity levels than the cell lines. The data were obtained from three separate experiments and expressed as mean ($\pm$ SEM).

Alcohol and Aldehyde Oxidizing Activities in Cell Lysates

Metabolic activities of different ADH and ALDH activities were determined in lysates from oral tissue and the various cell lines (Table 1). In addition to HMGSH, octanol and ethanol were used to determine ADH activities. Propanal and formaldehyde were used to determine low- K_m

ALDH activities. Metabolism of all substrates were detected in both tissue and cell lines. Oral tissue exhibited significant activity for both octanol and ethanol. The cell lines showed more than 10-fold higher octanol than ethanol activity. The values for octanol and HMGSH oxidation correlated in the cell lines, although in tissue, a higher ratio for octanol/HMGSH oxidation was detected. The ethanol metabolizing capacity of each cell line was significantly lower than for tissue. The activity for free formaldehyde oxidation was similar in tissue and the cell lines. The activity for propanal showed some variation, ie, the SVpgC2a cells showed lower activity than fibroblasts.

To further determine the respective role of ADH3 versus low- K_m ALDH in oral mucosa, the apparent K_m for HMGSH and free formaldehyde, respectively, was determined in tissue lysates. The analysis of separate preparations of tissue from three individuals showed that the apparent K_m (mean $\pm$ SEM) for HMGSH (substrate for ADH3) was $11 \pm 2 \mu\text{mol/L}$, whereas the apparent K_m for free formaldehyde (substrate for low- K_m ALDHs) was $360 \pm 90 \mu\text{mol/L}$.

Finally, efforts were made to analyze ADH3 protein content and HMGSH oxidation in confluent keratinocyte cultures (to complement the mRNA analysis depicted in Figure 4). After 5 days at confluency, the protein content and enzymatic activity were similar as in cells that had just reached confluency (data not shown). However, at 10 and 15 days, the cultures could not be analyzed for ADH3 protein or activity because of extensive protein cross-linking (resulting in protein insolubility and decreased antibody access to antigen).^{36,45}

Discussion

Human oral mucosa is a tissue with documented exposure and sensitivity to formaldehyde genotoxicity, although assessment of the capacity for removal of formaldehyde through metabolism has so far received minimal attention. Thus, this effort included analyses at the mRNA, protein, and activity levels for ADH3, a key enzyme in cellular formaldehyde metabolism.^{14,17-21} ADH3 was detected in oral tissue and cell lines through several methodological approaches, clearly demonstrating its expression. Interestingly, *in situ* hybridization and immunohistochemical analyses showed that the mRNA transcript and protein levels were differentially distributed in the oral epithelium. Presence of mRNA was confined to the basal and parabasal cells, whereas the protein was present throughout the viable cell layers. These results show that the basal, proliferative keratinocytes actively transcribe and translate ADH3. The ADH3 protein thus synthesized is then retained during the entire keratinocyte life span. This finding was substantiated by protein stability assessment *in vitro*. Thus, the apparent permanence of the protein in keratinocytes demonstrates a half-life for ADH3 protein well above their expected life span during transit from the basal to the outmost layer *in vivo* (10 to 14 days). The noted ADH3 mRNA and protein distribution may be a rare solution to gene expression in squamous epithelia, reflecting a uniquely high protein

Table 1. Alcohol and Aldehyde Oxidizing Activities in Human Oral Tissue Specimens, Cultures of Fibroblasts, and Cultures of Normal as well as Transformed Human Buccal Epithelial Cells

	S-hydroxy- methylglutathione	Formaldehyde	Propanal	Octanol	Ethanol
Tissue	2.9 ± 0.6	1.3 ± 0.2	2.0 ± 0.3	6.7 ± 0.3 [†]	8.3 ± 1.0
Normal keratinocytes	14.5 ± 1.8*	1.2 ± 0.7	3.0 ± 0.5	12.3 ± 2.0	0.4 ± 0.4*
SVpgC2a	10.7 ± 1.1* [†]	1.1 ± 0.4	0.5 ± 0.4 [†]	8.7 ± 1.3 [†]	0.5 ± 0.3*
SqCC/Y1	15.4 ± 1.8*	1.5 ± 0.4	1.8 ± 0.4	12.2 ± 1.2*	1.0 ± 0.4*
Normal fibroblasts	17.9 ± 1.4*	1.5 ± 0.4	4.4 ± 1.6	16.8 ± 1.4	1.0 ± 0.3*

For each indicated substrate, data are expressed as units (U) per g total protein in cell lysate, where one U is 1 μ mol NADH formed per minute under conditions described in Materials and Methods. Data are presented as mean values ($\pm$ SEM).

*Significantly different from tissue ($P < 0.05$, ANOVA with Kramer multiple comparisons test).

[†]Significantly different from fibroblasts ($P < 0.05$, as above).

stability of ADH3. Gene expression commonly involves co-localized mRNA and protein. Less commonly, mRNA is present throughout the epithelium despite lack of, or mosaic, protein expression, as for certain keratins in buccal mucosa.⁴⁶

Northern blot analysis of tissue and different oral cell lines revealed two transcripts for ADH3, in agreement with the existence of two different polyA signals (Figure 3).^{25,38} The cell types analyzed included normal keratinocytes and fibroblasts, and the transformed keratinocyte lines SVpgC2a and SqCC/Y1. Higher transcript levels were found in the cell lines than in tissue. Further, the fibroblasts and transformed keratinocyte lines showed higher transcript levels than normal keratinocytes. Culturing *in vitro* will clearly enrich the fraction of proliferative cells and normal fibroblasts, SVpgC2a and SqCC/Y1 have previously been shown to have significantly higher cloning efficiency than normal keratinocytes.^{28,47} Notably, the ADH3 transcript level in proliferative cultures of normal keratinocytes was significantly reduced by prolonged maintenance at confluency, a protocol known to efficiently inhibit cell growth of keratinocytes and other cell types.^{28,36} Thus, the *in vitro* and *in vivo* analyses both support that ADH3 mRNA abundance may be a manifestation of proliferative potential. The time required to lower ADH3 mRNA expression in confluent cultures (Figure 4) was longer than the determined mRNA half-life (Figure 5). Thus, the regulation of ADH3 mRNA levels is likely exerted at the transcriptional level. Concurrent studies on mRNA and protein stabilities are rare.⁴⁸ The noted ADH3 and β -actin mRNA half-lives in oral keratinocytes are in ranges reported as normal for various tissues and cell types.⁴⁹

Western blot and activity analyses of tissue and the respective cell lines showed marked correlation between the amount of protein and the ability for oxidation of HMGSH (Figures 2 and 7 and Table 1). In each case, the ADH3 protein had comparable activity to isolated enzyme, demonstrating that ADH3 is metabolically active in the oral mucosa. Further, metabolism of various human ADH and ALDH substrates was determined in both tissue and cultured cells in efforts to assess if additional activities besides that of ADH3 play a role in formaldehyde detoxification (Table 1). The estimates consistently indicate that ADH3 is the major activity responsible for formaldehyde metabolism in oral mucosa. Although presence of ALDH activity was indicated by the oxidation of free

formaldehyde and propanal, the metabolic rates were lower than for HMGSH oxidation through ADH3 with a 40-fold higher apparent K_m for free formaldehyde oxidation than for HMGSH oxidation. Moreover, ADH3 represents the dominating ADH activity in cultured oral cells, as shown by the comparison of octanol and ethanol oxidation. The noted lack of ethanol oxidation in cell lines compared to tissue agrees well with other studies.^{50,51} Overall, the *in vitro* analysis of different cell types, the normal *versus* the transformed state, as well as the principle of short-term *versus* extended culture, underscore an essential metabolic role of ADH3.

Using a combination of different methodological approaches, the present study demonstrates capacity for metabolism of formaldehyde in the micromolar range through ADH3 in human oral epithelium. Thus, the documented genotoxicity in this tissue^{8,9} is unlikely explained by absence of ability for metabolic detoxification. Future studies should consider if formaldehyde damage occurs at concentrations below those that undergo metabolism, including multiple exposures. Formaldehyde may exert cytotoxic and genotoxic effects through interaction with endogenous cellular constituents, eg, glutathione, resulting in altered redox state and gene transcription, or by inhibition of DNA repair.²⁸ Nongenotoxic concentrations of formaldehyde lowers the significant dose-effect levels of other mutation-inducing agents implying that formaldehyde can increase the genotoxicity of chemical and physical agents in a synergistic manner.⁵²⁻⁵⁴ These studies suggest mechanisms for low-dose formaldehyde genotoxicity and that other exposures/factors may easily influence the outcome of formaldehyde inhalation studies.

In conclusion, the overall analysis of ADH3 demonstrates differential distribution of mRNA and protein within an epithelial structure, protein stability during the expected keratinocyte life span, as well as an association between proliferation and mRNA abundance. Finally, comparison of activities of the currently known human ADH and ALDH activities, including enzyme kinetics, indicate that ADH3 acts as the primer guardian against formaldehyde toxicity in human oral mucosa. Additional experimental approaches, including altering gene expression, may further address the respective roles of ADH3 and ALDH activities in formaldehyde detoxification. Finally, future similar studies of ADH3 in other tissues

will serve to reference the apparent capacity of the oral mucosa for formaldehyde metabolism.

References

- Grafström RC: In vitro studies of aldehyde effects related to human respiratory carcinogenesis. *Mutat Res* 1990, 238:175–184
- IARC: Monograph on the evaluation of carcinogenic risks to humans, vol 62. Wood Dust and Formaldehyde. Lyon, International Agency for Research on Cancer, 1995, pp 217–362
- Grafström RC, Jernlov MI, Dypbukt JM, Sundqvist K, Atzori L, Zheng X: Aldehyde toxicity and thiol redox state in cell cultures from human aerodigestive tract. Correlations between in Vitro and in Vivo Investigations in Inhalation Toxicology. Edited by U Mohr, KB Adler, DI Dungworth, CC Harris, CG Plopper, R Saracci. Washington DC, ILSI Press, 1996, pp 319–336
- Heck HD, White EL, Casanova-Schmitz M: Determination of formaldehyde in biological tissues by gas chromatography/mass spectrometry. *Biomed Mass Spectrom* 1982, 9:347–353
- Nilsson JA, Zheng X, Sundqvist K, Liu Y, Atzori L, Elfving Å, Arvidson K, Grafström RC: Toxicity of formaldehyde to human oral fibroblasts and epithelial cells: influences of culture conditions and role of thiol status. *J Dent Res* 1998, 77:1896–1903
- IARC: Monograph on the evaluation of carcinogenic risk of chemicals to humans, Vol 38. Tobacco Smoking. Lyon, International Agency for Research on Cancer, 1986
- Cassee FR, Groten JP, Feron VJ: Changes in the nasal epithelium of rats exposed by inhalation to mixtures of formaldehyde, acetaldehyde, and acrolein. *Fundam Appl Toxicol* 1996, 29:208–218
- Suruda A, Schulte P, Boeniger M, Hayes RB, Livingston GK, Steenland K, Stewart P, Herrick R, Douthit D, Fingerhut MA: Cytogenetic effects of formaldehyde exposure in students of mortuary science. *Cancer Epidemiol Biomarkers Prev* 1993, 2:453–460
- Titenko-Holland N, Levine AJ, Smith MT, Quintana PJ, Boeniger M, Hayes R, Suruda A, Schulte P: Quantification of epithelial cell micronuclei by fluorescence in situ hybridization (FISH) in mortuary science students exposed to formaldehyde. *Mutat Res* 1996, 371:237–248
- Jörnvall H, Höög J-O: Nomenclature of alcohol dehydrogenases. *Alcohol Alcohol* 1995, 30:153–161
- Edenberg HJ, Bosron WF: Alcohol dehydrogenases. *Comprehensive Toxicology*, vol 3. Edited by FP Guengerich. New York, Pergamon Press Inc., 1997, pp 119–131
- Yoshida A, Rzhetsky A, Hsu LC, Chang C: Human aldehyde dehydrogenase gene family. *Eur J Biochem* 1998, 251:549–557
- Duester G, Farrés J, Felder MR, Holmes RS, Höög J-O, Parés X, Plapp BV, Yin SJ, Jörnvall H: Recommended nomenclature for the vertebrate alcohol dehydrogenase gene family. *Biochem Pharmacol* 1999, 58:389–395
- Uotila L, Koivusalo M: Formaldehyde dehydrogenase from human liver. Purification, properties, and evidence for the formation of glutathione thiol esters by the enzyme. *J Biol Chem* 1974, 249:7653–7663
- Koivusalo M, Baumann M, Uotila L: Evidence for the identity of glutathione-dependent formaldehyde dehydrogenase and class III alcohol dehydrogenase. *FEBS Lett* 1989, 257:105–109
- Danielsson O, Jörnvall H: "Enzymogenesis": classical liver alcohol dehydrogenase origin from the glutathione-dependent formaldehyde dehydrogenase line. *Proc Natl Acad Sci USA* 1992, 89:9247–9251
- Dicker E, Cederbaum AI: Effect of acetaldehyde and cyanamide on the metabolism of formaldehyde by hepatocytes, mitochondria, and soluble supernatant from rat liver. *Arch Biochem Biophys* 1984, 232:179–188
- Danielsson O, Shafqat J, Estonius M, Jörnvall H: Alcohol dehydrogenase class III contrasted to class I. Characterization of the cyclo-oxygenase, the existence of multiple forms as for the human enzyme, and distant cross-species hybridization. *Eur J Biochem* 1994, 225:1081–1088
- Estonius M, Höög J-O, Danielsson O, Jörnvall H: Residues specific for class III alcohol dehydrogenase. Site-directed mutagenesis of the human enzyme. *Biochemistry* 1994, 33:15080–15085
- Uotila L, Koivusalo M: Expression of formaldehyde dehydrogenase and S-formylglutathione hydrolase activities in different rat tissues. *Adv Exp Med Biol* 1997, 414:365–371
- Deltour L, Foglio MH, Duester G: Metabolic deficiencies in alcohol dehydrogenase Adh1, Adh3, and Adh4 null mutant mice—overlapping roles of Adh1 and Adh4 in ethanol clearance and metabolism of retinol to retinoic acid. *J Biol Chem* 1999, 274:16796–16801
- Keller DA, Heck HD, Randall HW, Morgan KT: Histochemical localization of formaldehyde dehydrogenase in the rat. *Toxicol Appl Pharmacol* 1990, 106:311–326
- Maier KL, Wippermann U, Leuschel L, Josten M, Pflugmacher S, Schröder P, Sandermann Jr H, Takenake S, Ziesenis A, Heyder J: Xenobiotic-metabolizing enzymes in the canine respiratory tract. *Inhal Tox* 1999, 11:19–35
- Hur MW, Edenberg HJ: Cell-specific function of cis-acting elements in the regulation of human alcohol dehydrogenase 5 gene expression and effect of the 5'-nontranslated region [published erratum appears in *J Biol Chem* Dec 29;270(52):31414]. *J Biol Chem* 1995, 270:9002–9009
- Estonius M, Svensson S, Höög J-O: Alcohol dehydrogenase in human tissues: localisation of transcripts coding for five classes of the enzyme. *FEBS Lett* 1996, 397:338–342
- Haselbeck RJ, Duester G: Regional restriction of alcohol/retinol dehydrogenases along the mouse gastrointestinal epithelium. *Alcohol Clin Exp Res* 1997, 21:1484–1490
- Cheung C, Smith CK, Höög J-O, Hotchkiss SA: Expression and localization of human alcohol and aldehyde dehydrogenase enzymes in skin. *Biochem Biophys Res Commun* 1999, 261:100–107
- Grafström RC, Noren UG, Zheng X, Elfving A, Sundqvist K: Growth and transformation of human oral epithelium in vitro. *Recent Results Cancer Res* 1997, 143:275–306
- Sundqvist K, Kulkarni P, Hybbinette SS, Bertolero F, Liu Y, Grafström RC: Serum-free growth and karyotype analyses of cultured normal and tumorous (SqCC/Y1) human buccal epithelial cells. *Cancer Commun* 1991, 3:331–340
- Kulkarni PS, Sundqvist K, Betsholtz C, Höglund P, Wiman KG, Zhivotovsky B, Bertolero F, Liu Y, Grafström RC: Characterization of human buccal epithelial cells transfected with the simian virus 40 T-antigen gene. *Carcinogenesis* 1995, 16:2515–2521
- Edington KG, Loughran OP, Berry IJ, Parkinson EK: Cellular immortality: a late event in the progression of human squamous cell carcinoma of the head and neck associated with p53 alteration and a high frequency of allele loss. *Mol Carcinog* 1995, 13:254–265
- Raybaud-Diogene H, Tetu B, Morency R, Fortin A, Montell RA: p53 overexpression in head and neck squamous cell carcinoma: review of the literature. *Eur J Cancer B Oral Oncol* 1996, 32:143–149
- Ditlow CC, Holmquist B, Morelock MM, Vallee BL: Physical and enzymatic properties of a class II alcohol dehydrogenase isozyme of human liver: pi-ADH. *Biochemistry* 1984, 23:6363–6368
- Moreno A, Pares X: Purification and characterization of a new alcohol dehydrogenase from human stomach. *J Biol Chem* 1991, 266:1128–1133
- Hedberg JJ, Strömberg P, Höög J-O: An attempt to transform class characteristics within the alcohol dehydrogenase family. *FEBS Lett* 1998, 436:67–70
- Poumay Y, Pittelkow MR: Cell density and culture factors regulate keratinocyte commitment to differentiation and expression of suprabasal K1/K10 keratins. *J Invest Dermatol* 1995, 104:271–276
- Liu Y, Arvidson K, Atzori L, Sundqvist K, Silva B, Cotgreave I, Grafström RC: Development of low- and high-serum culture conditions for use of human oral fibroblasts in toxicity testing of dental materials. *J Dent Res* 1991, 70:1068–1073
- Sharma CP, Fox EA, Holmquist B, Jörnvall H, Vallee BL: cDNA sequence of human class III alcohol dehydrogenase. *Biochem Biophys Res Commun* 1989, 164:631–637
- Svensson S, Lundsjö A, Cronholm T, Höög J-O: Aldehyde dismutase activity of human liver alcohol dehydrogenase. *FEBS Lett* 1996, 394:217–220
- Takahashi H, Fujita S, Okabe H, Tsuda N, Tezuka F: Immunophenotypic analysis of extranodal non-Hodgkin's lymphomas in the oral cavity. *Pathol Res Pract* 1993, 189:300–311
- Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987, 162:156–159
- Harrold S, Genovese C, Kobrin B, Morrison SL, Milcarek C: A com-

- parison of apparent mRNA half-life using kinetic labeling techniques vs decay following administration of transcriptional inhibitors. *Anal Biochem* 1991, 198:19–29
43. Iborra FJ, Renau-Piqueras J, Portoles M, Boleda MD, Guerri C, Pares X: Immunocytochemical and biochemical demonstration of formaldehyde dehydrogenase (class III alcohol dehydrogenase) in the nucleus. *J Histochem Cytochem* 1992, 40:1865–1878
44. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976, 72:248–254
45. Hohl D: Cornified cell envelope. *Dermatologica* 1990, 180:201–211
46. Bloor BK, Su L, Shirlaw PJ, Morgan PR: Gene expression of differentiation-specific keratins (4/13 and 1/10) in normal human buccal mucosa. *Lab Invest* 1998, 78:787–795
47. Grafström RC: Carcinogenesis studies in human epithelial tissues and cells in vitro: emphasis on serum-free culture conditions and transformation studies. *Acta Physiol Scand Suppl* 1990, 592:93–133
48. Ross J: mRNA stability in mammalian cells. *Microbiol Rev* 1995, 59:423–450
49. Hargrove JL, Schmidt FH: The role of mRNA and protein stability in gene expression. *FASEB J* 1989, 3:2360–2370
50. Dong YJ, Peng TK, Yin SJ: Expression and activities of class IV alcohol dehydrogenase and class III aldehyde dehydrogenase in human mouth. *Alcohol* 1996, 13:257–262
51. Garle MJ, Fry JR: A comparison of hepatic enzyme activities and their modulation by dexamethazone in freshly isolated and cultured hepatocytes and in the differentiated hepatoma cell line, 2sFou. *ATLA* 1996, 24:31–37
52. Grafström RC, Fornace Jr AJ, Autrup H, Lechner JF, Harris CC: Formaldehyde damage to DNA and inhibition of DNA repair in human bronchial cells. *Science* 1983, 220:216–218
53. Grafström RC, Curren RD, Yang LL, Harris CC: Genotoxicity of formaldehyde in cultured human bronchial fibroblasts. *Science* 1985, 228:89–91
54. Grafström RC, Hsu I-C, Harris CC: Mutagenicity of formaldehyde in Chinese hamster lung fibroblasts: synergy with ionizing radiation and N-nitroso-N-methylurea. *Chem Biol Interact* 1993, 86:41–49