Effects of Acetaldehyde on Cell Regeneration and Differentiation of the Upper Gastrointestinal Tract Mucosa

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Background: The tumor-promoting effect of ethanol on cancer of the upper respiratory–digestive tract is not well understood. Although ethanol itself is not carcinogenic, the first product of ethanol metabolism—acetaldehyde—is. Acetaldehyde can be produced from ethanol by oral bacteria, and high concentrations have been observed in human saliva after ethanol consumption. The purpose of this study was to investigate whether acetaldehyde administered orally to rats induces altered differentiation and proliferation in the animals’ upper gastrointestinal tracts.

Methods: Twenty Wistar rats were given either water containing acetaldehyde at a concentration of 120 mM or tap water to drink for 8 months. Tissue specimens were then taken from the tongue, epiglottis, and forestomach of each animal and immunohistochemically stained for markers of cellular proliferation (Ki67 nuclear antigen) or differentiation (cytokeratins 1, 4, 10, 11, 14, and 19). The mean epithelial thickness of each sample was measured via light microscopy, using an eyepiece containing grid lines. Differences between the control and acetaldehyde-treated groups were analyzed by use of the unpaired Student’s t test. All reported P values are two-sided.

Results: Although no tumors were observed, staining for cytokeratins 4 and 14 revealed an enlarged basal layer of squamous epithelia in the rats receiving acetaldehyde. In these animals, proliferation was significantly greater than that observed in the control animals for samples from the tongue (P < .0001), epiglottis (P < .0001), and forestomach (P < .0001). In addition, the epithelia from acetaldehyde-treated rats were significantly thicker than in epithelia from control animals (P < .05 for all three sites).

Conclusions: Acetaldehyde, administered orally to rats, can cause hyperplastic and hyperproliferative changes in epithelia of the upper gastrointestinal tract. This finding suggests that microbially produced acetaldehyde in saliva may explain the tumor-promoting effect of ethanol on these epithelia. [J Natl Cancer Inst 1997;89:1692–7]

Numerous epidemiologic studies (1–5) have provided evidence that chronic ethanol consumption and smoking are the main causes for cancer of the upper respiratory–digestive tract. Although ethanol acts as an independent risk factor for cancer, the mechanism by which ethanol acts remains unclear. Ethanol has no direct mutagenic effects and has failed to be carcinogenic in any animal model (1,2).

In contrast to ethanol, its first metabolite—acetaldehyde—has multiple mutagenic effects (6–10). Moreover, in an inhalation study, acetaldehyde caused laryngeal cancer in experimental animals (6).

Acetaldehyde derives from ethanol through aerobic oxidation by alcohol dehydrogenases, and, typically, is rapidly metabolized to acetate by aldehyde dehydrogenases. Consequently, acetaldehyde levels in the blood, observed after ethanol ingestion, are generally very low (11). However, microbes can oxidize ethanol to acetaldehyde in the upper gastrointestinal tract (12,13). The oral microflora has been shown to produce substantial amounts of acetaldehyde, which remains present in saliva for a long time (14). Moreover, acetaldehyde production capacity is increased in oral rinses from patients with cancer of the aerodigestive tract (15) than from control subjects.

Saliva is distributed over the whole upper gastrointestinal tract. Poor dental health, followed by bacterial overgrowth, is an independent risk factor of cancer of the mouth (16). Accordingly, acetaldehyde produced locally in saliva by bacteria after ethanol ingestion may play some role in the carcinogenic effect of ethanol in these regions.

Our purpose was to investigate whether acetaldehyde administered orally to rats induces the altered differentiation and hyperproliferation in the animals’ upper gastrointestinal tracts associated with ethanol consumption.

Materials and Methods

Animal Study

Twenty 8-week-old male Wistar rats were randomly divided into two groups. The rats were strain Hsd/wi, barrier bred locally in the Laboratory Animal Center of the University of Helsinki. Animals were kept in plastic cages under conventional conditions according to the institutional guidelines and the principles of the Animal Care Unit of the University of Helsinki. They were fed ad libitum with a standard diet (Altromin 1324 pellets, Altromin, Lage, Germany). The room’s temperature was maintained at 22 ± 2 °C and the rats lived in a 12-hour light/dark cycle. Ten animals received 120 mM acetaldehyde (Merck-Schuchardt, Hohenbrunn, Germany) in drinking water while the control rats received tap water. The solutions were changed every third day and the animals were weighed weekly. Their general condition, behavior, and fluid intake were recorded weekly. Time was used as the endpoint of the study and the animals were killed under ether anesthesia after 8 months.

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Immunohistochemistry

Parts of the tongue, the epiglottis, and the forestomach were removed and transferred to ice-cold isopropanol. Subsequently, the samples were snap-frozen in liquid nitrogen and stored at −80 °C. Serial cryostat sections (5 μm thick) were mounted on 3-aminopropyltriethoxysilane-coated slides (Sigma Chemical Co., St. Louis, MO). The frozen sections were fixed for 10 minutes in acetone at −20 °C and air-dried for 24 hours. Immunohistochemical analysis was performed by the Alkaline Phosphatase/Anti-Alkaline Phosphatase method (Dako, Glostrup, Denmark). The monoclonal antibodies used were MIB-5, specific for Ki67 antigen (Dianova, Hamburg, Germany); K8.60, specific for human cytokeratins 1, 10, and 11 (Progen, Heidelberg, Germany); M6B10, specific for human cytokeratin 4 (Progen); LL002, specific for human cytokeratin 14 (Medac, Hamburg, Germany); and Ki9.2, specific for human cytokeratin 19 (Progen). Primary monoclonal antibodies were applied to the sections in the recommended dilutions for 1 hour, at room temperature, in phosphate-buffered saline (pH 7.4). The secondary antibody (rabbit-anti-mouse immunoglobulin G; Dako) was administered for 30 minutes in a 1:20 dilution in phosphate-buffered saline (pH 7.4) containing one tenth of normal rat serum to abolish cross-reaction with rat species antigens. The slides were incubated for 30 minutes in a 1:50 dilution of monoclonal mouse Alkaline Phosphatase/Anti-Alkaline Phosphatase complex (Dako). Incubation with the secondary antibody, respectively the Alkaline Phosphatase/Anti-Alkaline Phosphatase complex was repeated twice each with a shortened incubation time of 10 minutes. Between all incubation steps, slides were carefully washed twice in phosphate-buffered saline (pH 7.4). Substrate for the alkaline phosphatase was freshly prepared for each experiment. In detail, 530 mg 2-amino-2-methyl-1,3-propandiole (Merck, Darmstadt, Germany); and 675 mg 2-morpholinoethanesulfonic acid were added to 100 mL Tris (0.05 M; pH 9.7). Naphtol as-bi-phosphate (50 mg) (Sigma Chemical Co.), dissolved in 600 μL N,N-dimethylformamide (Merck), was then added. Two hundred microliters of 5% New-Fuchsin solution in 2 M hydrochloric acid (Fluka Chemicals, Buchs, Switzerland) was incubated for 1 minute with 500 μL of 4% sodium nitrite (Merck) and mixed with the former solution. The substrate was filtered and the pH was adjusted to 8.7 with concentrated hydrochloric acid. The slides were incubated for 20 minutes in this substrate, rinsed twice in phosphate-buffered saline (pH 7.4), counterstained in Mayer's hemalum solution (Merck), and embedded in Elvanol (Mowiol, Calbiochem, La Jolla, CA). For each primary antibody and tissue, negative controls were run by replacing the primary antibody with nonimmune serum.

Histopathology

To assess proliferation, we counted at least 500 cells of the basal layer and estimated the proliferation index by counting the proportion of cells stained Ki67 positive. To avoid bias, analyses were done separately by two independent investigators (N. Homann and P. Kärkkäinen), who were blinded to both groups of rats.

Expansion of the basal layer was analyzed by counting cells in epithelia of rats in which at least three cell rows near the basal membrane showed positive staining for cytokeratin 14.

We measured mean epithelial thickness on toluidine blue-stained slides of the investigated epithelium by light microscopy, using an eyepiece containing grid lines. The lines were brought parallel to the epithelium, and the epithelial thickness was calculated by multiplying by the corresponding magnification. Special attention was paid to assessing these measurements only in slides containing vertical cuts, and each epithelium was measured at three different sites.

Since hyperplasia and hyperproliferation are often encountered as a response to inflammation, we investigated changes in the number and the type of inflammatory cells in the lamina propria underlying the epithelia.

Statistical Analysis

All values are shown as the mean ± one standard deviation (1 SD), if not otherwise mentioned. Statistical calculations were performed by using unpaired Student’s t tests [GraphPad, InStat Software, version 2.02, San Diego, CA (17)]. The expansion of the basal layer and the proportion of epithelia harboring inflammatory cells were compared between both groups by using Fisher’s exact test [GraphPad, InStat Software, version 2.02 (18)]. P values of less than .05 were regarded as significant. All reported P values were generated from two-sided statistical tests.

Results

All animals developed and behaved normally during the experiment. In the acetaldehyde group, intake of acetaldehyde was 324 ± 24 mg acetaldehyde/kg body weight per day. The daily fluid intake was 58 ± 4 mL/kg body weight/day in the acetaldehyde-treated group versus 61 ± 5 mL/kg body weight/day in the control group (not statistically significant). The final body weights in the acetaldehyde and control groups were 590 ± 44 g and 618 ± 54 g, respectively (not statistically significant). There were neither macroscopically nor microscopically cancerous or dysplastic lesions in the upper gastrointestinal tract in either group.

The epithelia were significantly thicker in rats treated with acetaldehyde. This effect was seen in all three investigated anatomic sites (Table 1).

Only a few inflammatory cells, mainly mononuclear cells and mast cells, were seen (below the epithelium only) in three tongues, two epiglottic epithelia, and two forestomasches from the acetaldehyde-treated group and two tongues, two epiglottic tissues, and one forestomach from the control group (not statistically significant).

We discovered specific suprabasal expression of cytokeratin 19 in squamous epithelia of the upper gastrointestinal tract in three acetaldehyde-fed animals, but in only twof the control group. Strong, suprabasal expression of cytokeratins 1, 10, and 11 was seen in all studied epithelia.

All epithelia showed staining for cytokeratins 4 and 14, with largely suprabasal expression for cytokeratin 4, whereas cytokeratin 14 was present predominantly in cells of the basal layer. The staining pattern for cytokeratin 14 showed an expansion of positive cells in the basal layer in acetaldehyde-fed rats, and the region of staining varied from three cell rows beyond the basal membrane up to all cells outside of the keratinizing layer. This was observed in nine of 10 tongues, and in all epiglottic tissues and forestomasches. In control rats, a positive signal for cytokeratin 14 was largely restricted to one or two cell rows of the basal layer (eight tongues, seven epiglottic tissues, and six forestomasches, P<.05, P<.005, and P<.05 respectively; Fig. 1). Cytokeratin 4 exhibited an inverse pattern of staining, but these differences were less obvious because a very weak signal was sometimes present in cells of the basal layer.

There was a striking difference in the expression of the proliferation marker Ki67 between the two groups. In acetaldehydefed rats, positive staining occurred in two to three cell rows in the basal layer, whereas the expression of Ki67 in the control rats was limited to a single cell row in the mucosa (Fig. 2). The proliferation indexes were significantly higher in the acetaldehydefed rats (Table 1).
hyde group than in the control group for all investigated anatomic sites (43% ± 6.6% versus 26.9% ± 6% for the tongue, 36% ± 7.2% versus 21% ± 9.5% for the epiglottis, and 31% ± 3.8% versus 18% ± 3.4% for the forestomach; for all sites, \( P < .001 \); Fig. 3).

Discussion

Epidemiologic studies (1–5) in the past decades have revealed striking evidence that ethanol ingestion is an independent risk factor for upper gastrointestinal cancer. The relative risks associated with ethanol consumption are significantly different in distinct anatomic sites of the upper gastrointestinal tract, with the lowest values for the larynx (4,5). It is believed that these differences are due to local effects of ethanol and that the higher risk for anatomic sites, such as tongue and hypopharynx, may be caused by longer contact with the ingested alcohol at these sites (19).

Ethanol could exhibit systemic co-carcinogenic effects by displacing potential cancer-protective nutrients in the diet, influencing the toxification of procarcinogens by inducing metabolic enzymes, and increasing the oxidative exposure (2,19–22). Despite these hypothetical mechanisms, the tumor-promoting effect on the mucosa of the upper gastrointestinal tract may, as pointed out above, largely be local, caused by a direct contact of ethanol with the mucosa (4,5,19).

Accelerated cell division in response to ethanol intake has been observed in the oral cavity and the esophagus (23,24). Accordingly, this is assumed to be one of the major effects of local ethanol-associated carcinogenesis (25). Direct mucosal damage may be an explanation for hyperproliferation following ethanol consumption. This remains speculative, however, since ethanol up to a concentration of 20% seems to be rather harmless (26). Interestingly, the two major anatomic sites—the upper gastrointestinal tract and the rectum—where ethanol-associated hyperproliferation has been demonstrated, are regions with very high concentrations of microbially produced acetaldehyde (14,27). Moreover, inactive aldehyde dehydrogenase-2 (and, accordingly, an intraepithelial accumulation of acetaldehyde) has been shown to be a risk factor for multiple cancers of the upper respiratory–digestive tract (28).

Consequently, there is some experimental evidence for acetaldehyde as the underlying factor behind alcohol-associated carcinogenesis.

Moreover, it has been shown that normal salivary function is essential for this increased cell proliferation in the esophagus. In animals that did not produce saliva due to the removal of salivary glands, a normal proliferation pattern was observed after ethanol ingestion (23). This observation supports a link to acetaldehyde, since very high acetaldehyde levels are detectable in saliva in the presence of ethanol (14). These acetaldehyde levels are reduced significantly after the reduction of the oral microflora, suggesting that acetaldehyde is mainly formed in...
by microbes. In animals whose salivary gland had been removed, redistribution of ethanol via saliva does not occur. Without this redistribution, a considerable decrease in local acetaldehyde production probably occurs. Thus, there is experimental evidence that the ethanol-associated hyperproliferation may mainly be induced by its metabolite acetaldehyde, which is produced by the oral microflora following the salivary redistribution of ethanol.

Abnormal proliferation is a characteristic sign of cancer, and increased cell division can be observed at early stages of carcinogenesis in the upper gastrointestinal tract (29–32). Hyperproliferation is consistently noted in the esophaguses of residents of regions with a high incidence of esophageal cancer and in patients with Barrett’s esophagus, a sometimes precancerous ulcer of the lower esophagus (29,30). Hyperplastic lesions in the oral cavity show increased cell division, and sequentially enhanced cell proliferation is observed in various stages of preneoplastic lesions during head and neck carcinogenesis (33,34). Increased cell proliferation can itself trigger the development of cancer through the accumulation of replication errors during DNA synthesis and by higher susceptibility of proliferating cells to ingested carcinogens (31).

We were able to demonstrate epithelial hyperplasia in rats receiving acetaldehyde. Hyperplasia, as pointed out above, is one early event in multistep carcinogenesis (29,30,33,34). It can be observed after mechanical irritation and inflammatory processes, and it can be reversible. Since there was no increase in the number or the type of inflammatory cells in the animals of our study, it is unlikely that the hyperplasia is generated by a chronic inflammatory reaction to acetaldehyde.

It is likely that the observed effects are caused by interference of acetaldehyde with the DNA. This remains, however, speculative, since we did not investigate the underlying genetic events, e.g., the formation of certain DNA-base adducts or the inactivation or mutation of tumor suppressor genes or oncogenes. It should be pointed out that no irreversible events, such as severe dysplasia or benign or malignant tumors, occurred in our experiment. The acetaldehyde concentration used in this study was rather high, but there are several methodologic reasons for this approach. Acetaldehyde evaporates very easily due to its low boiling point and high vapor pressure, and it could be metabolized by aldehyde dehydrogenases present in the mucosa and saliva. Furthermore, acetaldehyde administered via drinking water will be diluted by saliva, simultaneous food intake, and gastric juice in the forestomach. Consequently, the effective concentration of acetaldehyde will obviously be substantially reduced over that administered, but it remains unclear to what extent.

It can be calculated from our recent study (14) that in some individuals with a permanent alcohol intoxication level of 0.7%, a total of approximately 100 mg acetaldehyde is produced daily in the saliva. Consequently, the amount—but not the concentration—of acetaldehyde administered in this study is comparable to that of heavy drinkers and alcoholics. In contrast to severe alcoholics, who have a high risk for upper gastrointestinal tract cancer (1,2,19,21), the rats in our study were rather young, their
nutritional status was normal, and they did not receive other additional ingested or inhaled carcinogens. This, together with some uncertainty in the amount of acetaldehyde administered, could be an explanation for the lack of observed cancers in our animals. It should be stated, that the observed intermediate end points, hyperplasia and hyperproliferation, need to be validated in regard to their future progress to cancer.

To our knowledge, this is the first in vivo study showing a hyperproliferating effect of acetaldehyde in the target tissues of ethanol-associated carcinogenesis. Acetaldehyde has been shown to decrease cell replication (6,8,35,36), both in cell culture and in gastric epithelia in vivo. The initial reaction to a carcinogen is a delay in cell division and DNA synthesis, followed by hyperproliferation, that can be observed after chronic treatment with low doses of carcinogens. These phenomena may relate to cell division following DNA damage and/or mutation of proliferation-associated oncogenes or tumor suppressor genes (37,38). The DNA-modifying effects of acetaldehyde have been investigated intensively, and acetaldehyde should be considered a potent carcinogen (6–10). Whether the antagonistic effects of acetaldehyde on cell proliferation reflect these subsequent stages of proliferation via carcinogen treatment, or whether acetaldehyde affects different cell types differently, remains unclear. Future studies could elucidate this crucial point by using different concentrations, administration routes, and end points of acetaldehyde treatment.

Dedifferentiation is another important characteristic of cancer. In this context, the deregulation of cytoskeleton components such as cytokeratins have been suggested as markers (39). The combined expression of the pairs 4/13, 5/14, and 6/16 has been described for squamous nonstratifying epithelia (40–42). The observed differences in the staining pattern of cytokeratin 4 and 14 may indicate that these cytokeratins act as reliable markers for the determination of single-cell species during the differentiation process. Our results clearly indicate that acetaldehyde treatment leads to an enlargement of the basal proliferative compartment of the epithelia. Cytokeratins 1, 10 and 11 are only expressed in cells with the capability to terminally differentiate and in our study acetaldehyde did not appear to interfere in this process (40). Suprabasal expression of cytokeratin 19 has been reported to be associated with different stages of hyperplastic and dysplastic lesions in squamous epithelia (43), but this earlier result has not been confirmed in later studies (44,45). In our study, all epithelia showing suprabasal cytokeratin 19 staining were histopathologically normal and we conclude that its expression is neither a reliable tool for dedifferentiation nor specific for dysplasia.

In this study, we have demonstrated that acetaldehyde administered in drinking water to rats causes hyperplastic and hyperproliferative changes in the tongue, epiglottis, and the forestomach, tissues that are generally regarded as targets of ethanol-associated carcinogenesis. Consequently, microbially produced acetaldehyde in saliva may contribute to the tumorigenic effect of ethanol.

References


Notes

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