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Short communication

Avoiding the ingestion of cytotoxic concentrations of ethanol may reduce the risk of cancer associated with alcohol consumption

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ABSTRACT

Alcohol consumption is a known risk factor for cancer. Almost 6% of all cancers worldwide are attributable to alcohol use. Approximately half of them occur in tissues highly exposed to ethanol, such as the oral cavity, pharynx, upper larynx and esophagus. However, since ethanol is not mutagenic and the mutagenic metabolite of ethanol (acetaldehyde) is mainly produced in the liver, it is unclear why alcohol consumption preferentially exerts a local carcinogenic effect. Recent findings indicate that the risk of cancer in a tissue is strongly correlated with the number of stem cell divisions accumulated by the tissue; the accumulation of stem cell divisions leads to the accumulation of cancer-promoting errors such as mutations occurring during DNA replication. Since cell death activates the division of stem cells, we recently proposed that the possible cytotoxicity of ethanol on the cells lining the tissues in direct contact with alcoholic beverages could explain the local carcinogenic effect of alcohol. Here we report that short-term exposures (2–3 s) to ethanol concentrations between 10% and 15% start to cause a marked cytotoxic effect on human epithelial keratinocytes in a concentration-dependent manner. We propose that choosing alcoholic beverages containing non-cytotoxic concentrations of ethanol, or diluting ethanol to non-cytotoxic concentrations, may be a simple and effective way to reduce the risk of cancers of the oral cavity, pharynx, larynx and esophagus in alcohol users. This preventive strategy may also reduce the known synergistic effect of alcohol drinking and tobacco smoking on the risk of these cancers.

1. Introduction

Alcohol consumption is carcinogenic to humans. According to the International Agency for Research on Cancer (IARC), alcohol consumption causes cancer of the oral cavity, pharynx, larynx, esophagus, colorectum, liver and female breast (IARC, 2012). The risk is particularly high for tissues directly exposed to ethanol. For example, compared with non-drinkers, the relative risk for heavy alcohol users is 5.13 for oropharyngeal cancer, 4.95 for esophageal cancer, 2.65 for laryngeal cancer, 2.07 for liver cancer, 1.44 for colorectal cancer and 1.61 for breast cancer (Bagnardi et al., 2015). It has been estimated that almost 6% of the total number of cancer cases and deaths world-wide are attributable to alcohol consumption, and that approximately half of them occur in tissues highly exposed to ethanol, such as the oral cavity, pharynx, larynx and esophagus (Praud et al., 2016).

The mechanism by which alcohol consumption preferentially exerts a local carcinogenic effect remains unclear (IARC, 2012; Connor, 2017). Since ethanol is not mutagenic, it is widely accepted that the carcinogenic activity of alcohol consumption is mediated by acetaldehyde, a mutagenic metabolite of ethanol. Upon ingestion of alcoholic beverages, ethanol is converted into acetaldehyde, which is then oxidized to the non-toxic compound acetate. However, this mechanism does not explain why alcohol consumption preferentially exerts a local carcinogenic activity, because most of the ingested ethanol is not converted into acetaldehyde until it reaches the liver. The low amounts of acetaldehyde produced before ethanol reaches the liver might induce mutations in the cells lining the oral cavity, pharynx, upper larynx and esophagus. However, these possible mutations would be eliminated when the cells lining these tissues are replaced by new cells during physiological tissue renewal. We cannot forget that carcinogenesis requires the multistep accumulation of DNA changes over years or decades, and that the cells lining these tissues are typically replaced every few weeks (Squier and Kremer, 2001; Lopez-Lazaro, 2016). We recently hypothesized that the cytotoxic activity of ethanol could explain why alcohol consumption increases the risk of these cancers (Lopez-Lazaro, 2016). In short, the cytotoxicity of ethanol on the cells lining the oral cavity, pharynx, larynx and esophagus forces the stem cells located in deeper layers to divide more often than usual to replace the damaged epithelia. The accumulation of cell divisions in stem cells promoted by regular alcohol consumption leads to a variety of cancer-related errors

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(e.g., mutations arising during DNA replication) that increase their risk of malignant transformation. The local carcinogenic effect of ethanol decreases or ends when alcoholic beverages reach a non-empty stomach, because the stomach content dilutes ethanol to non-cytotoxic concentrations (Lopez-Lazaro, 2016). However, although ethanol is a known cytotoxic agent (Tapani et al., 1996; Slomiany et al., 1997), direct evidence of the cytotoxicity of short-term exposures to ethanol on human epithelial cells was lacking. Here, we provide such evidence, analyze recent data that strongly support the mechanism of carcinogenesis we propose, and discuss a simple preventive strategy to reduce the risk of cancer of the oral cavity, pharynx, larynx and esophagus in alcohol users.

2. Materials and methods

The human keratinocyte cell line HaCaT (Cell Line Service; L#300493-4212) was maintained in Dulbecco's Modified Eagle's Medium (DMEM) at 37 °C in a humidified atmosphere containing 5% CO_2. DMEM was supplemented with 50 µg/mL penicillin, 50 µg/mL streptomycin and 10% fetal bovine serum. Cell culture reagents were obtained from Biowest and from Thermo Fisher Scientific. Ethanol absolute (≥99.8%, AnalaR NORMAPUR, ACS, Reag. Ph. Eur.) was obtained from VWR Chemicals.

Exponentially growing cells were seeded into 96-well plates until they formed a monolayer. Then, solutions of PBS or culture medium (DMEM) containing several concentrations of ethanol were gently added and immediately removed from the cells (exposure times were between 2 and 3 s). After a 20-h recovery period in ethanol-free medium, cell viability was estimated with the MTT assay. This colorimetric technique is based on the capacity of viable cells to transform the MTT salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into a formazan dye. After treatments and recovery periods, the medium was removed, and 125 µL MTT (1 mg/mL in medium) was added to each well for 4 h. Then, 80 µL 20% SDS in 0.02 M HCl were added to the plates, which were incubated overnight at 37 °C. Finally, optical densities were measured at 540 nm on a multi-well plate spectrophotometer reader (Calderon-Montaño et al., 2013). Cell viability was expressed as percentage in relation to controls. Data were averaged from at least three independent experiments; in each experiment, 3 wells were used for each ethanol concentration. Data were expressed as means ± standard error of the mean (SEM). Paired, two-tailed, *t*-test was used for statistical analysis. A P-value < 0.05 is considered to correspond with statistical significance and is indicated with an asterisk (*), a P-value < 0.01 is indicated with a double asterisk (**), and a P-value < 0.001 is indicated with a triple asterisk (***).

3. Results and discussion

The lining of the oral cavity, pharynx, larynx and esophagus is mainly formed by squamous epithelium made of keratinocytes. The soft tissues of the oral cavity and esophagus are covered by a non-keratinized stratified squamous epithelium, whereas regions associated with mastication (i.e., the gingiva and hard palate) are covered by a keratinizing epithelium resembling that of the skin epidermis (Squier and Kremer, 2001). We therefore selected a human keratinocyte cell line for our experiments. After choosing several concentrations of ethanol typically present in popular alcoholic beverages (beer, wine and some distilled alcoholic beverages), we exposed the cells for only 2–3 s to PBS or culture medium containing 5%, 10%, 15% or 40% of ethanol. A drastic reduction in cell viability was observed for the highest ethanol concentration (Fig. 1A and B). To more closely simulate alcohol consumption, we exposed the cells to the ethanol solutions for 2–3 s every minute during five consecutive minutes. A marked cytotoxic effect was observed for ethanol 15%, but not for ethanol 10% (Fig. 1C and D). Further experiments revealed that ethanol started to cause a concentration-dependent cytotoxic effect between 10% and 15% (Fig. 1E and F). Similar results were obtained in another non-malignant cell line (immortalized human fibroblastic hTERT-BJ cell line) commonly used in our laboratory (results not shown). Our data clearly show that short-term exposures to concentrations of ethanol present in alcoholic beverages are cytotoxic to human epithelial keratinocytes (Fig. 1).

If we regularly drink alcoholic beverages containing cytotoxic concentrations of ethanol, the stem cells located in deeper layers of the affected tissues will probably divide more often than usual to renew the damaged cells and thus maintain tissue function. It has been estimated that the stem cells of the oral cavity, pharynx, larynx and esophagus divide approximately every 2–3 weeks (Squier and Kremer, 2001; Tomasetti and Vogelstein, 2015). These division rates will probably increase if the ingestion of cytotoxic concentrations of ethanol shortens the life of the cells lining these tissues. The accumulation of cell divisions in stem cells can increase their risk of malignant transformation, because cell division is associated with a variety of cancer-promoting errors. For example, it is well known that cell division can lead to spontaneous mutations arising during DNA replication and to chromosome aberrations occurring during mitosis. These DNA alterations can affect cancer-related genes and disorder genetic programs controlling stem cell behavior and fate. In addition, cell division exposes the DNA of the cell to the genotoxic activity of DNA-damaging agents, such as acetaldehyde and tobacco carcinogens. During cell division, the DNA unwinds to be copied during DNA replication and the nuclear membrane disintegrates during mitosis; these cellular events facilitate the interaction between DNA-damaging agents and the DNA of the cell. Therefore, the accumulation of cell divisions in stem cells promoted by alcohol consumption can lead to the accumulation of DNA alterations in stem cells and increase their risk of malignant transformation (Lopez-Lazaro, 2016).

Two lines of evidence strongly support the mechanism of carcinogenesis discussed in this manuscript. First, ethanol is a known cytotoxic agent (Tapani et al., 1996; Slomiany et al., 1997), which can induce a marked cytotoxic effect under conditions that simulate alcohol consumption (Fig. 1). The ingestion of cytotoxic concentrations of ethanol will therefore reduce the lifespan of the cells directly exposed to ethanol. Since cell death inevitably leads to cell division in self-renewing tissues, the regular consumption of cytotoxic concentrations of ethanol will promote the accumulation of stem cell divisions in tissues in direct contact with alcoholic beverages; otherwise, the functionality of these tissues would be compromised. Second, increasing evidence indicates that the accumulation of cell divisions in stem cells plays a major role in carcinogenesis (Tomasetti and Vogelstein, 2015; Tomasetti et al., 2017; Lopez-Lazaro, 2015; Lopez-Lazaro, 2017). Cancer incidence increases dramatically with age in tissues that accumulate stem cell divisions with age. For example, cancers of the oral cavity, pharynx, larynx and esophagus are diagnosed almost one hundred times more frequently in people over 60 years old than in people under 30 (Lopez-Lazaro, 2016; Howlader et al., 2017). In addition, most cancers originate in self-renewing tissues, that is, in tissues that accumulate numerous stem cell divisions throughout life. Cancer almost never arises from organs and tissues composed of cells that rarely divide, even though these cells are also exposed to naturally-occurring DNA damage and to environmental carcinogens. The variation in cancer incidence among tissues with different renewal capacities are striking; some cancers occur even millions of times more often in some tissues than in others (Tomasetti and Vogelstein, 2015). Importantly, these large differences in cancer risk can be explained by the number of stem cell divisions accumulated by the tissues (Tomasetti and Vogelstein, 2015). The correlation between the number of stem cell divisions occurring in a tissue during a person's life and the risk of being diagnosed with cancer in that tissue is highly positive and statistically significant (Spearman's rho = 0.81; P < 0.001) (Tomasetti and Vogelstein, 2015). This striking correlation has been confirmed in 69 countries representing over half of the world's population (Tomasetti et al., 2017). These recent findings strongly suggest that the