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NOTE

Toxicology

Cow's milk neutralizes the cytotoxicity of acrolein, a putative carcinogen in cigarette smoke

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ABSTRACT. Cigarette smoke is a strong and independent risk factor for esophageal cancer, while the consumption of cow's milk has been proposed as a protective factor. The mechanistic role of milk in preventing cancer, however, has not been clarified. We focused our study on acrolein, an abundant unsaturated aldehyde present in cigarette smoke. Acrolein is a highly toxic compound and a putative carcinogen. Using a cell culture system, we found that (1) acrolein caused necrosis in Ramos Burkitt's lymphoma cells, (2) the necrosis was inhibited by preincubation of acrolein with milk, and (3) acrolein formed adducts with milk proteins. These results indicated the protective effects of cow's milk against acrolein-induced cytotoxicity via protein-acrolein adduct formation.

KEY WORDS: acrolein, cow's milk, cytotoxicity

The consumption of cow's milk has been reported to improve health and to decrease risks of diabetes, metabolic syndrome and colon cancer [4]. However, there is some controversy over the benefits of milk in cancer prevention and some studies showed instead a causative effect of milk on some types of cancer, such as prostate [11] and ovarian cancers [10]. In spite of this controversy, the health benefit of milk consumption may outweigh its harm. One study on esophageal cancer also reported the beneficial effect of cow's milk [5]. The intake of cigarette and alcohol increases esophageal cancer risk while the consumption of milk, vegetables and seafood decreases the risk. Although acetaldehyde, the main metabolite of alcohol, has gained increasing attention as a causative factor of esophageal cancer [1], we have focused on another aldehyde in cigarette smoke, acrolein. Acrolein (2-propenal, CH_2=CHCHO) is one of the most abundant chemicals in cigarette smoke and has strong cytotoxicity, causing oxidative damage and mitochondrial dysfunction [13]. Acrolein is also a strong inhibitor of aldehyde dehydrogenase, which is an acetaldehyde detoxification enzyme [7], suggesting that acrolein may augment the toxicity of acetaldehyde. Acrolein is also generated endogenously from unsaturated fatty acids during oxidative stress [3]. Furthermore, it is well known that acrolein is metabolized from cyclophosphamide, a chemotherapeutic drug, and causes bladder inflammation and cancer [14]. According to Tang *et al*., acrolein is a major lung and bladder carcinogen and its carcinogenicity arises via induction of DNA damage and inhibition of DNA repair [13].

We have been interested in the beneficial effects of cow's milk, especially its role in cancer prevention. Accordingly, we focused on esophageal cancer and expected that acrolein might be a causative chemical in cigarette smoke. We hypothesized that milk might scavenge acrolein and prevent its toxicity. In this study, using a cell culture system, we investigated whether commercial cow's milk could directly neutralize the toxicity of acrolein. Ideally, esophageal epithelial cells should be used in this study, but they are difficult to obtain. As acrolein has strong cytotoxicity [13], we thought that any cell can be killed by acrolein. Thus, we used the human Burkitt's lymphoma cell line Ramos (JCRB, Osaka, Japan) that we had used in our previous cell death experiment [9]. First, the morphological and biochemical changes of acrolein-treated cells was determined, since the sensitivity of Ramos cells to acrolein was unknown. Ramos cells (0.5×10^6 cells), cultured in 1 m*l* of RPMI1640 medium supplemented with 10% fetal bovine serum and 100 U/m*l* of penicillin-streptomycin, were incubated with 100 μM of acrolein (Tokyo Chemical Industry, Tokyo, Japan) at 37°C for the indicated time. To determine the cell death pattern, apoptosis vs necrosis, we also induced cell death with 1 μM of staurosporine (Merk Japan, Tokyo, Japan), a protein kinase C inhibitor and a well-known apoptosis inducer [9]. Cell viability was determined by trypan blue dye exclusion test and the stained cells were counted as dead cells [12]. Chromatin condensation, DNA fragmentation and Poly (ADP-ribose) polymerase (PARP) cleavage, hallmarks of apoptosis [8], were examined using Hoechst staining, agarose gel electrophoresis and Western blot analysis, respectively. For chromatin condensation, cells

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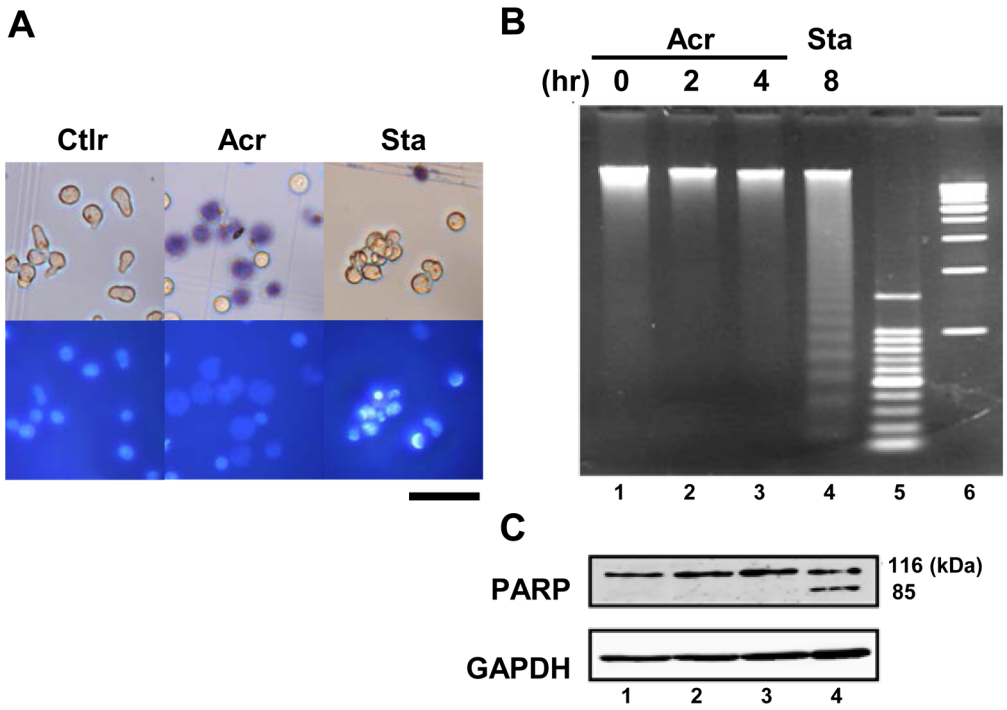

Fig. 1. Necrosis is induced by acrolein treatment. (A) Ramos cells were treated with acrolein (Acr, 100 μM for 4 hr) or staurosporine (Sta, 1 μM for 8 hr) and examined under microscope after mixing with Hoechst 33342 (1 μg/m*l*) and trypan blue (0.2%). Upper panels: phase contrast microscopic images, lower panels: fluorescent microscopic images. Ctlr: control Ramos cells. Scale bar: 100 μm. (B) DNA fragmentation was not detected in Ramos cells treated with acrolein (Acr, 100 μM) for 0, 2, and 4 hr (lanes 1–3, respectively) but was with the apoptosis-inducing reagent staurosporine (Sta, 1 μM) for 8 hr (lane 4). Cell viabilities determined by trypan blue dye exclusion test were 100, 70, and 40% (lanes 1–3, respectively). Lanes 5 and 6 are 100 bp- and 1 kb-DNA ladder makers, respectively. (C) Western blot analysis of PARP (top) and GAPDH (bottom). Samples correspond to those in Fig. 1B. Data are representative of two to three independent experiments with similar results.

(1 m*l*) incubated with acrolein for 4 hr or staurosporin for 8 hr as shown above were mixed with 1 μl of Hoechst 33342 (1 mg/m*l*, Dojindo, Kumamoto, Japan) and an equal amount of trypan blue (0.4% solution, Wako, Osaka, Japan) and examined under microscope. For the DNA fragmentation assay, cells were harvested by centrifugation after incubation with acrolein (0, 2, and 4 hr) or with staurosporine (8 hr), suspended in 60 μl of Phosphate buffered saline (PBS) and lysed by addition of 300 μl of 7 M guanidine. DNA was isolated from the lysate with the Wizard DNA purification resin (Promega, Madison, WI, U.S.A.) as described [8]. The isolated DNA was analyzed by electrophoresis in a 1.5% agarose gel in the presence of ethidium bromide (0.5 μg/m*l*). For Western blot analysis, cells were harvested as shown above, suspended in 50 μl of PBS and lysed in 50 μl of sample buffer (4% sodium dodecyl sulfate, 100 mM Tris-HCl pH 6.8, 2 mg/m*l* Bromophenol Blue, 20% glycerol, 200 mM dithiothreitol). The samples were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% gel and transferred to a nylon membrane. The membrane was incubated with anti-PARP antibody (Wako) or anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (MBL, Nagoya, Japan) and secondary antibody, horseradish peroxidase (HRP)-conjugated anti-mouse IgG (ZYMED, San Francisco, CA, U.S.A). Signals were visualized with the Enhanced chemiluminescence (ECL) kit (Amersham, Piscataway, NJ, U.S.A.). GAPDH is expressed at high levels in most cells and was therefore used as a protein loading control. Representative data are shown in Fig. 1. Acrolein-treated cells underwent necrosis and showed trypan blue staining (Fig. 1A, top middle), but did not chromatin condensation (Fig. 1A, bottom middle), DNA fragmentation (Fig. 1B, lane 3) nor PARP cleavage (Fig. 1C, lane 3, top). By contrast, PARP cleavage from a 116 kDa protein to an 85 kDa fragment (Fig. 1C, lane 4, top), chromatin condensation (Fig. 1A, bottom right), DNA fragmentation (Fig. 1B, lane 4) were readily detected in staurosporine-treated Ramos cells, although only few cells were stained with trypan blue (Fig. 1A, top right). Thus, the Ramos cells treated with acrolein suffer necrotic death and 100 μM acrolein is sufficient to induce necrosis.

Next, we examined whether cow's milk could prevent necrosis of acrolein-treated Ramos cells. Non-adjusted milk (Meiji Oishii Gyunyu, Meiji Holdings Co., Ltd., Tokyo, Japan) was processed by centrifugation at 1,700 × g for 30 min to remove fat and the supernatant was used for these experiments. Various amounts of milk (0, 10, 20, and 50 μl) were mixed with PBS (50−0 μl) to get 50 μl of sample mixtures. One microliter of acrolein (100 mM stock solution) was added to each mixture. The samples and a control sample (50 μl PBS) were incubated at 37°C for 15 min. Then the preincubated samples were added to 1 m*l* of Ramos

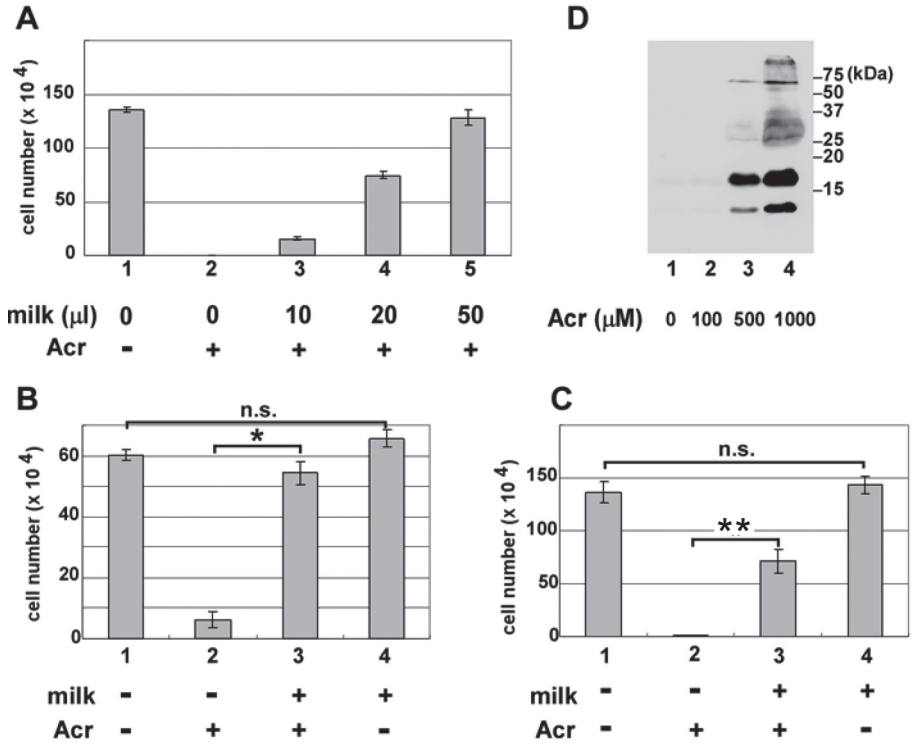

Fig. 2. Milk neutralizes acrolein toxicity. (A) Acrolein (Acr) was preincubated with various amounts of milk at 37°C for 15 min and the mixed samples were cultured with Ramos cells (0.5×10^6 cells) at 37°C. The final concentration of acrolein was 100 μM. After 24 hr, viable cell number was determined by trypan blue dye exclusion test. The amounts of milk were 0, 0, 10, 20, 50 μl (lanes 1–5, respectively). The cell numbers in the acrolein-contained samples were shown in lanes 2–5 (+). (B) (C) Acrolein was preincubated with PBS or milk at 37°C for 15 min and the mixed samples were cultured with Ramos cells (0.5×10^6 cells) at 37°C. The final concentration of acrolein was 100 μM. After 4 hr (B) or 24 hr (C), viable cell number was determined by trypan blue dye exclusion test. The amounts of milk were 0, 0, 20, 20 μl (lanes 1–4, respectively). The cell numbers in the acrolein-contained samples were shown in lanes 2 and 3 (+). Asterisks (*) indicate a statistically significant. *: $P<0.01$ (n=4) in (B), **: $P<0.05$ (n=4) in (C). n.s.: not significant. Tukey's test was used for the analysis. (D) Western blot analysis of acrolein-adduct formation. Increasing amounts of acrolein were incubated with milk at 37°C for 30 min (lanes 2–4). The acrolein concentrations were 0, 100, 500 and 1,000 μM (lanes 1, 2, 3, and 4, respectively).

cells (0.5×10^6 cells) and incubated at 37°C for 24 hr. The final concentration of acrolein was 100 μM. The neutralizing effect of milk on acrolein toxicity was determined by comparing the number of viable cells recovered in the treated and untreated cultures. As shown in Fig. 2A, the cell growth after 24 hr incubation was correlated with the amount of milk, suggesting that milk could indeed neutralize acrolein toxicity. To confirm this result and to estimate the contribution of milk to cell growth, we repeated the experiment using 20 μl of milk and examined cell growth at 4 hr and 24 hr (Fig. 2B and 2C, respectively). As shown in Fig. 2B and 2C, milk itself did not contribute to cell growth (compare lanes 1 and 4 of Fig. 2B and 2C) but prevented the acrolein-induced cell death (compare lanes 2 and 3 of Fig. 2B [$P<0.01$, n=4] and 2C [$P<0.05$, n=4]), indicating that milk neutralizes acrolein-toxicity.

Milk compounds, especially milk protein, were expected to neutralize acrolein and therefore we examined acrolein-protein adduct formation by Western blot analysis using an antibody that detects acrolein-lysine adducts [15]. One microliter of various concentrations of acrolein (5, 25, or 50 mM) was added to 50 μl of milk. The samples and a control sample (50 μl milk) were incubated at 37°C for 30 min. Final concentrations of acrolein in the samples were 0, 100, 500 and 1,000 μM. After incubation, the samples were mixed with the same volume of sample buffer. Ten microliter of the resultant mixtures were separated by SDS-PAGE using a 15% gel and transferred to a nylon membrane. The membrane was incubated with anti-acrolein antibody (NIKKEN SEIL, Tokyo, Japan) and secondary antibody, HRP-conjugated anti-mouse IgG (ZYMED). Signals were visualized with an ECL kit (Amersham). As shown in Fig. 2D, strong adduct formation was detected after the incubation of milk with acrolein (especially, lanes 3 and 4). Thus, milk proteins scavenge acrolein and neutralize its toxicity.

In this paper, we showed that commercial cow's milk could prevent acrolein toxicity. We also showed that milk scavenges

acrolein by adduct formation, which quite likely accounts for its neutralization capacity. Acrolein is one of the abundant chemicals in cigarette smoke and is also generated *in vivo* under oxidative stress. Several reports have suggested links between acrolein and the risk of cancer, including lung and bladder cancers [2, 13]. As for the correlation between acrolein and esophageal cancer, we expect two scenarios: (1) Direct induction of DNA damage and inhibition of DNA repair. (2) Inhibition of aldehyde dehydrogenase, resulting in augmented acetaldehyde toxicity. Given that acetaldehyde is a known carcinogen of esophageal cancer [1], acrolein may cause esophageal cancer in a direct or indirect manner.

As mentioned above, milk and dairy products may have both beneficial and adverse effects with regard to the risk of different cancers. Several reports have suggested that milk or milk product consumption may promote some types of cancer, such as prostate cancer [6, 11]. A recent report, however, suggests that the probable harmful effect of milk and dairy product consumption related to cancer is dose-dependent and that for normal people this could only occur with absolutely excessive and indiscriminate consumption rather than regular moderate daily intake [4]. The evidence indicating healthful effects of milk and milk product consumption on prevention of cancer is considerably greater than that showing a harmful impact.

Here, we showed that commercial cow's milk can neutralize acrolein toxicity and may help prevent esophageal cancer. Although this conclusion should be confirmed with future animal studies, our results may have a substantial impact on the dairy industry.

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