Cytotoxic and Genotoxic Effects of Areca Nut-related Compounds in Cultured Human Buccal Epithelial Cells

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ABSTRACT

Because betel quid chewing has been linked to the development of oral cancer, pathobiological effects of an aqueous areca nut extract, four areca nut alkaloids (arecoline, guvacoline, guvacine, and arecaidine), and four nitrosated derivatives [N-nitrosoguvacoline, N-nitrosoguvacine, 3-(N-nitrosomethylamino)propionaldehyde and 3-(N-nitrosomethylamino)propionitrile] have been investigated using cultured human buccal epithelial cells. Areca nut extract in a dose-dependent manner decreases cell survival, vital dye accumulation, and membrane integrity, and it causes formation of both DNA single strand breaks and DNA protein cross-links. Depletion of cellular free low-molecular-weight thiols also occurs, albeit at quite toxic concentrations. Comparisons of the areca nut-related N-nitroso compounds and their precursor alkaloids, at concentrations up to 5 mM, indicate that 3-(N-nitrosomethylamino)propionaldehyde is the most potent on a molar basis to decrease both survival and thiol content and to cause significant formation of DNA single strand breaks. Arecoline, guvacoline, or N-nitrosoguvacoline decreases survival and cellular thiols, whereas arecaidine, guvacine, N-nitrosoguvacine, and 3-(N-nitrosomethylamino)propionitrile have only minor effects on these variables. Taken together, the present studies indicate that aqueous extract and, in particular, one N-nitroso compound related to areca nut, i.e., 3-(N-nitrosomethylamino)propionaldehyde, are highly cytotoxic and genotoxic to cultured human buccal epithelial cells, of potential importance in the induction of tumors in betel quid chewers.

INTRODUCTION

Epidemiological studies have demonstrated a clear causal association between chewing of betel quid containing tobacco and an increased risk for oral cancer. These studies have also indicated that most tumors arise in the buccal mucosa in contact with the quid (1–3). Besides tobacco, areca nut (also called “betel nut”) is often a major ingredient of the quid. More information is available on the adverse health effects of tobacco, whereas less is known about the corresponding effects of areca nut (1–3). There is limited evidence that aqueous extracts of the betel quid or areca nut alone are carcinogenic to experimental animals (1). Areca nut extracts are mutagenic in Salmonella typhimurium (4) and in Chinese hamster V79 cells (5). Increased frequencies of chromatid breaks and exchanges have been observed in Chinese hamster ovary cells (6). Although the nut constituents have been separated into a variety of extracts, subfractions, and components (6, 7), the agent(s) involved in the genotoxic and carcinogenic effects of areca nut has not yet been unequivocally identified.

Areca nut contains several alkaloids (Fig. 1), of which arecoline is the most abundant (8). This compound is mutagenic in both bacteria and mammalian cells (4, 5). It also increases the frequency of bone marrow cells with micronuclei and chromosomal aberrations in mice in vivo (5, 9) and causes chromosomal aberrations in Chinese hamster ovary cells in vitro (10). However, arecoline has not been clearly demonstrated to be carcinogenic in animals (1). Arecaidine, another areca nut alkaloid and a structural analogue of arecoline, is found as a urinary metabolite of arecoline in rats (11) and is formed in vitro after incubation of arecoline with liver homogenates (12). Arecaidine is also mutagenic in bacteria (4) and causes sister chromatid exchanges in mouse bone marrow cells in vivo (13). Very little is currently known about the effects of the other alkaloids in areca nut (guvacoline, guvacine, and arecolidine).

The alkaloids present in areca nut can undergo nitrosation reactions (Fig. 1). When arecoline is nitrosated in vitro, both NMPN3 and NMPA are formed (14). These compounds, together with N-nitrosoguvacoline and N-nitrosoguvacine, are also formed after in vitro nitrosation of betel quid (15). Similar nitrosations may occur in humans during chewing of betel quid, because NMPN (16), N-nitrosoguvacoline (15, 17), and N-nitrosoguvacine (15) have been detected in their saliva. In rats, NMPN is a potent and complete carcinogen (16, 18), which both methylates and cyanoethylates DNA in target tissues (19). N-Nitrosoguvacoline is not mutagenic in Drosophila (20), requires metabolic activation to exert mutagenicity in S. typhimurium (21), and was reported to be carcinogenic in rats in one study (22), but not in others (17, 20, 21, 23).

We have recently developed serum-free methods for replicative cultures of normal human buccal epithelial cells (4). The present study was undertaken to investigate in vitro whether areca nut extract causes pathobiological effects associated with carcinogenesis directly in these human target cells. Therefore, to investigate cytotoxicity, thiol reactivity, and genotoxicity, several biological end points, including colony-forming efficiency, vital dye uptake ability, cell membrane integrity, intracellular free low-molecular-weight thiol status, and two types of DNA damage, i.e., single strand breaks and DNA protein cross-links, were studied. Effects of several areca nut alkaloids and their N-nitroso derivatives (see Fig. 1 for chemical structures) were also investigated in buccal epithelial cells in an attempt to determine whether these compounds are responsible for some of the cytopathic actions related to areca nut.

MATERIALS AND METHODS

Chemicals. Arecoline and arecaidine were purchased from Sigma Chemical Co. (St. Louis, MO). The following compounds were synthesized as described previously: guvacoline (24), guvacine (24), N-nitrosoguvacoline (15), N-nitrosoguvacine (15), NMPN (14), and NMPA (14). Arecoline, arecaidine, guvacoline, and guvacine were dissolved in the exposure medium immediately before addition to the cells. Stock

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3 The abbreviations used are: NMPN, 3-(N-nitrosomethylamino)propionitrile; NMPA, 3-(N-nitrosomethylamino)propionaldehyde.

4 K. Sundqvist, Y. Liu, K. Arvidsson, K. Ormstad, L. Nilsson, and R. C. Grafström. Growth regulation of serum-free cultures of epithelial cells from human buccal epithelial cells in vitro whether NMPN or NMPA is added to the exposure medium immediately before addition to the cells. Arecoline, arecaidine, guvacoline, and guvacine were dissolved in the exposure medium immediately before addition to the cells. All exposure media immediately before addition to the cells. All exposure media immediately before addition to the cells.
solutions of N-nitrosoguvacoline (3.6 mM) and N-nitrosoguvacine (3.1 mM) in dimethyl sulfoxide and of NMPN (3.0 mM) and NMPA (0.5 mM) in sterile, distilled water were used and added to the cells as required, using appropriate solvent controls. All other reagents were of analytical grade.

Preparation of Aqueous Areca Nut Extract. Areca nut extract was prepared by extraction of 10 g dry, powdered areca nut with 250 ml distilled water for 1 h (7). The extract was filtered through a sintered glass funnel, freeze dried, and frozen at −70°C (7). For each experiment, aliquots of the extract were dissolved in serum-free BEG-1 medium 4 without thiols, i.e., no cysteine, immediately before exposure of the cells.

Media Solutions and Growth Factors. Porcine Zn-free insulin was a gift of the Eli Lilly Co. (Stockholm, Sweden). Fibronectin was obtained from Collaborative Research, Labassco (Stockholm, Sweden); hydrocortisone, polyvinylpyrrolidone, and transferrin were from Sigma Chemical Co.; 2.5% trypsin solution was from GIBCO (Stockholm, Sweden); Vitrogen collagen was Flow Laboratories (Stockholm, Sweden). Basic tissue culture medium MCDB 153 was obtained from NordVacc AB (Huddinge, Sweden). Human epidermal growth factor was kindly provided by Dr. G. Nascimento from the Chiron Corp. (Emeryville, CA).

Tissue and Cell Culture Conditions. Buccal mucosa was obtained from noncancerous surgery or autopsy donors and maintained in culture according to methods developed by Sundqvist et al. 4 The tissue was cut into 5-× 5-mm explants and placed in culture dishes with the epithelial side up. The explants were incubated in serum-free BEG-2 growth medium under air plus 5% CO2 at 36.5°C. BEG-2 medium is a buccal epithelial growth medium based on serum-free MCDB 153 (diluted with 10% double distilled, sterile H2O to lower the osmolality to physiological levels, i.e., from 340 mOsm/liter to 305 mOsm/liter) and supplemented with insulin (5.2–7.8 μg/ml), transferrin (8.70 μg/ml), phosphoethanolamine and ethanolamine (each at 435 mM), hydrocortisone (63.5 ng/ml), epidermal growth factor (4.35 ng/ml), and bovine pituitary extract (20 μg/ml). BEG-2 medium on culture dishes surface treated with fibronectin (10 μg/ml) and collagen (30 μg/ml). 5 For all experiments, secondary cultures were used and exposed to the indicated agent for 3 h in thiol-free BEG-1 medium.

Colony-forming Efficiency Assay. Cells were seeded at 250 cells/cm2 in 60-mm dishes and incubated for 24 h prior to treatment. Subsequently, the medium was exchanged for fresh BEG-2 medium without the agent and the cells were incubated for 8 days before fixation in 10% formalin and staining with 1% aqueous crystal violet. The percentage of viability was calculated as 100 times the ratio of number of viable cells to the sum of viable and nonviable cells. More than 90% of unexposed cells were scored positive for vital dye accumulation, i.e., neutral red uptake.

Trypan Blue Exclusion Assay. Cells were inoculated onto 35-mm dishes at 6000 cells/cm2 and incubated for 24 h prior to treatment. Subsequently, they were washed twice with BEG-1 medium, and then 0.16% trypan blue (Sigma) dissolved in BEG-1 medium was added for 5 min. The dye was removed, and the number of viable cells (which exclude trypan blue) and nonviable cells (which appear entirely blue or have blue nuclei) were counted in four randomized fields per dish in a phase contrast microscope. The percentage of viability was calculated from duplicate dishes at 100 times the ratio of number of viable cells to the sum of viable and nonviable cells. More than 90% of unexposed control cells excluded trypan blue.

Assays for Free Low-Molecular-Weight Thiols. Cells were inoculated onto 35-mm dishes at 6000 cells/cm2 and incubated for 24 h prior to treatment. Subsequently, the cells were washed twice with Dulbecco’s phosphate-buffered saline without Ca2+ or Mg2+ (27), and the cellular free low-molecular-weight thiol status was determined by two separate methods. To measure the amounts of total free thiols, cells were lysed and precipitated at 1500 × g for 8 min, and 0.25 ml of the supernatant was analyzed for thiol content according to Saville (28). To measure the content of glutathione, cellular thiols were trapped using monobromobimane, and the thiolute conjugates were separated by high-pressure liquid chromatography (29). An estimate of the cellular content of glutathione disulfide was then determined as previously described (29).

Alkaline Elution Assay of DNA Damage. Cells were inoculated onto 60-mm dishes at 3-5 × 104 cells/cm2 in BEG-2 medium. Experimental cells, i.e., buccal epithelial cells, were labeled with [3H]thymidine, and internal standard, i.e., L-1210 cells, was labeled with [3H]thyminidine as previously described (29). Following exposure to the indicated agent, the assays for DNA single strand breaks and DNA protein cross-links were performed, and the numbers of DNA lesions were calculated as previously described (29, 31).

RESULTS

The toxicity of an aqueous areca nut extract in cultured human buccal epithelial cells was determined using three different assays, i.e., colony-forming efficiency, neutral red uptake, and trypan blue exclusion (Fig. 2). The dose required to decrease colony-forming efficiency to 50% of control was about 3 μg/ml following a 3-h exposure. In the neutral red and trypan blue assays, 50% of the cells were stained after exposure to about 150 and 540 μg/ml, respectively. Increased planar cell surface area, an indicator of differential proliferation in epithelial cells, was not apparent in the exposed cells (data not shown).

The extract decreased the cellular content of total free low-molecular-weight thiols at the highest concentration tested, 1 mg/ml (Fig. 2). When the cellular content of glutathione was specifically measured, a similar decrease was observed without any concomitant oxidative formation of glutathione disulfide (data not shown).

The extract was also assayed for its ability to cause DNA damage as measured by the sensitive alkaline elution method (29, 31). A concentration of 300 μg/ml caused significant amounts of DNA single strand breaks and DNA protein cross-links (Fig. 3), i.e., 1.2 DNA single strand breaks/106 Da and...
EFFECTS OF ARECA NUT CONSTITUENTS IN HUMAN BUCCAL CELLS

Fig. 2. Effects of aqueous areca nut extract on colony-forming efficiency, neutral red uptake, trypan blue exclusion, and content of total free low-molecular-weight thiols in human buccal epithelial cells. Means of three experiments ± SE (bars). ◼, colony-forming efficiency; ◼, neutral red uptake; ◼, trypan blue exclusion; ◼, free thiol contents.

Fig. 3. DNA single strand breaks (A) and DNA protein cross-links (B) caused by aqueous areca nut extract. Abscissa, corrected time scale; [3H]DNA from L1210 cells which had been irradiated with 300 rad; about 30% of this DNA was retained on the filter after 12 h of alkaline elution (29, 31). Three separate experiments were performed, and the results are shown as one representative elution profile. ◼, control; ◼, extract 300 µg/ml; ◼, 600 µg/ml; ◼, 1000 µg/ml.

Fig. 4. Effects of areca nut alkaloids (A) and N-nitroso compounds (B) on colony-forming efficiency of human buccal epithelial cells. Means ± SE (bars) from three separate experiments. ◼, arecoline; ◼, arecaidine; ◼, guvacoline; ◼, guvacine; ◼, N-nitrosoguvacoline; ◼, N-nitrosoguvacine; ◼, NMPN; ◼, NMPA.

Fig. 5. Effects of areca nut alkaloids (A) and N-nitroso compounds (B) on cellular thiol levels of human buccal epithelial cells. Means ± SE (bars) from three separate experiments. ◼, arecoline; ◼, arecaidine; ◼, guvacoline; ◼, guvacine; ◼, N-nitrosoguvacoline; ◼, N-nitrosoguvacine; ◼, NMPN; ◼, NMPA.

Table 1 Frequencies of DNA single strand breaks following exposure to areca nut-related alkaloids and N-nitroso compounds in cultured human buccal epithelial cells

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration (mM)</th>
<th>No. of breaks/10¹⁰ Da</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arecoline</td>
<td>5</td>
<td>0.5</td>
</tr>
<tr>
<td>Arecaidine</td>
<td>5</td>
<td>0.2</td>
</tr>
<tr>
<td>Guvacoline</td>
<td>5</td>
<td>0.5</td>
</tr>
<tr>
<td>Guvacine</td>
<td>5</td>
<td>0.3</td>
</tr>
<tr>
<td>N-Nitrosoguvacoline</td>
<td>5</td>
<td>0.5</td>
</tr>
<tr>
<td>N-Nitrosoguvacine</td>
<td>5</td>
<td>0.4</td>
</tr>
<tr>
<td>NMPN</td>
<td>5</td>
<td>0.3</td>
</tr>
<tr>
<td>NMPA</td>
<td>0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>NMPA</td>
<td>0.3</td>
<td>1.0</td>
</tr>
<tr>
<td>NMPA</td>
<td>1.0</td>
<td>2.6</td>
</tr>
</tbody>
</table>

* The cells were exposed to the various agents and assayed for DNA single strand breaks as described in "Materials and Methods."

DISCUSSION

Aqueous extract of whole areca nut was found to be toxic over a wide range of concentrations in cultured human buccal epithelial cells as judged by three different assays. Colony-forming efficiency was significantly decreased at about 1–3 µg/ml, whereas about 100-fold higher concentrations were required to affect vital dye accumulation and membrane integrity as indicated by the results of the neutral red uptake and trypan blue exclusion assays, respectively. In these experiments, a marked adherence of the extract to the cells, even after extensive rinsing, was observed by both light and scanning electron microscopy (data not shown). Thus, continuous exposure to remaining extract during the course of the 8-day colony-forming efficiency assay may be the reason why low concentrations of the extract decrease colony survival but have little or no effect

2.2 DNA protein cross-links/10¹⁰ Da. Formation of both types of DNA lesions was dose dependent; at 1000 µg/ml, 3.9 DNA single strand breaks/10¹⁰ Da and 6.6 DNA protein cross-links/10¹⁰ Da were formed.

The effects of four areca nut alkaloids, i.e., arecoline, arecaidine, guvacoline, and guvacine, and of four nitrosation products of these alkaloids, i.e., N-nitrosoguvacoline, N-nitrosoguvacine, NMPN, and NMPA, on survival, thiol status, and frequency of DNA single strand breaks were also measured in buccal epithelial cells. In order to decrease colony-forming efficiency to 50% of the control value, concentrations of 0.15 mM NMPA, 1.6 mM arecoline, 1.7 mM N-nitrosoguvacoline, or 2.1 mM guvacoline were required (Fig. 4). Arecaidine, guvacine, N-nitrosoguvacine, and NMPN at concentrations up to 5 mM had not significant effect on survival.

When effects on the cellular amounts of total free low-molecular-weight thiols were assayed, a 25% decrease was induced by 80 µM NMPA, 0.57 mM arecoline, 0.88 mM guvacoline, 2.9 mM N-nitrosoguvacoline, and 3.8 mM N-nitrosoguvacine (Fig. 5). Arecaidine, guvacine, and NMPN at concentrations up to 5 mM had no significant effect.

Both the alkaloids and the N-nitroso compounds were also assayed for their ability to cause DNA single strand breaks. Only NMPA caused a significant increase in the amounts of single strand breaks, i.e., more than 0.5 single strand break per 10¹⁰ Da, and this increase was dose dependent (Table 1).
on vital dye accumulation or membrane integrity. The extract
did not appear to increase planar cell surface area, indicating
that accelerated terminal differentiation was not involved in
cell killing. Although the colony-forming efficiency may be the
most sensitive of these toxicity assays, the other assays have
the advantage of providing information on cell viability that
can be directly related to parallel measurements of intracellular
thiol status and DNA damage (32).

Thiols are involved in a variety of intracellular functions,
including regulation of proliferation, and are also important in
the cellular defense against many reactive foreign compounds
(33, 34). Because only the highest and quite cytotoxic concen-
tration (1 mg/ml) of extract caused thiol depletion, aqueous
extract probably contains relatively few sulphydryl-reactive com-
ponents of minor importance to explain its cytotoxicity. Because
thiol depletion was not coupled with oxidation of glutathione
to glutathione disulfide, these data also indicate that exposure
of intact cells to extract was not coupled with oxidative stress,
an effect which has recently been associated with areca nut
extracts in vitro (7).

Assay of genotoxicity using the sensitive alkaline elution
technique indicated that the extract caused both DNA single
strand breaks and DNA protein cross-links in cultured human
buccal epithelial cells. These measurements of DNA damage
indicate that the extract contains agents which are reactive
toward cellular DNA but do not provide information on the
chemical nature of these types of genetic lesions. Thus, these
results imply the need for studies using additional genotoxicity
end points as well as comparisons of effects by areca nut-related
subfractions and defined compounds.

In areca nut, alkaloids are a pharmacologically active group
of compounds (1) which together constitute about 1% by weight
of the nut and also are the precursors for formation of several
specific N-nitroso compounds (cf. Fig. 1). Areca nut alkaloids
in the millimolar concentration range may be of importance for
the long-term effects of betel quid chewing on buccal mucosa,
because arecoline has been detected in saliva obtained during
chewing in concentrations up to about 140 µg/ml (15), corre-
sponding to 0.9 mm. In cultured human buccal epithelial cells,
only certain alkaloids up to 5 mm exerted measurable patho-
biological effects, supporting previous studies that structure-
activity relationships exist for this group of compounds (5, 11).
At similar concentration ranges, guvacine and arecoline de-
creased both colony survival and thiol content, whereas guva-
cine and arecaidine had no appreciable effects on these cellular
variables. The cytotoxic and thiol-depleting properties of gu-
vacine and arecoline appear to be chemically related to the
presence of a methylester group (cf. Fig. 1). The lower polarity
and lower degree of ionization of these compounds in aqueous
solution may facilitate their passage over biological membranes.
In contrast, arecaidine becomes ionized (11), and guvacine
may form a carboxylate anion in aqueous solutions, properties
which may explain the lack of biological effects of these agents.
Because arecoline is known to react readily with free thiols such
as glutathione or N-acetyl cysteine both in vitro and in vivo (11),
conjugation is a likely mechanism for thiol depletion by meth-
ylester-containing alkaloids in buccal epithelial cells. The cel-
lar toxicity by arecoline and by guvacine may be mechanistic-
ally linked to thiol depletion, and this effect may also render
the cells more vulnerable to other reactive agents.

Several reports have shown that arecoline is genotoxic (4, 5,
9), whereas arecaidine was found to be less genotoxic than
arecoline (4, 5). However, neither compound was shown to
react with any of the four nucleic acid bases in vitro (11). In
cultured human buccal epithelial cells, none of four areca nut
alkaloids caused significant amounts of DNA single strand
breaks at the highest concentration that was tested, 5 mm.

Of the areca nut-related N-nitroso compounds investigated
in cultured human buccal epithelial cells, NMPA was by far the
most potent on a molar basis; it caused toxicity, thiol depletion,
and DNA single strand breaks. The aldehyde moiety of NMPA
may be causative in this regard because aldehydes are highly
reactive compounds which have been shown to decrease sur-
vival, to deplete thiols, and to cause various types of DNA
damage in cultured human epithelial cells (32, 35). Theoret-
cally, both alkylating species and several reactive aldehydes
may be generated during NMPA breakdown, and these metabolites
may also have contributed to the observed effects. The fact that
NMPA caused marked pathobiological effects in cultured hu-
man buccal epithelial cells, whereas the other N-nitroso com-
pounds did not, may indicate specificity in terms of metabolism
of these compounds. Cultured human buccal epithelial cells
have previously been shown to metabolize benz(a)pyrene ac-
tively at rates substantially higher than their fibroblastic coun-
terparts (36). Moreover, several types of tobacco-specific N-
nitroso compounds have been shown to be actively metabolized
by cultured rat buccal epithelial cells (37). In rats, NMPN and
NMPA were shown to be strongly and weakly carcinogenic,
respectively (16–18). Thus, the present study also suggests the
possibility of species variations in susceptibility to these N-
nitroso compounds. Because methods to grow human buccal
epithelial cells and to maintain buccal mucosal tissue in explant
culture at defined conditions have only recently become avail-
able, these in vitro approaches may aid in the further charac-
terization of the capability of human oral tissues to metabolize
N-nitroso compounds.

In summary, some pathobiological effects of areca nut extract
and several areca nut compounds have now been identified in
human target cells by using recently developed serum-free cul-
ture conditions. Areca nut extract is clearly both cytotoxic
and genotoxic. When the alkaloids are compared on a weight basis
to the extract, no single agent has detectable effects on the cells
at concentrations of the extract that cause decreased colony
survival and DNA single strand breaks. Therefore, additive or
synergistic effects could be considered among the various al-
kaldoids. Moreover, other classes of components including pol-
yphenols (1) are likely to contribute to the marked toxicity of
the extract. NMPA was the most potent in causing cytotoxicity,
thiol depletion, and DNA damage, effects that indicate both
carcinogenic and cocarcinogenic properties of this agent. Fur-
ther studies will be of importance to address the contribution
of various betel quid constituents, including tobacco, as well as
the role of areca nut compounds and salivary N-nitrosation
reaction products in betel quid carcinogenesis in human oral
epithelium.

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