Short Communication

BENZO[A]PYRENE-INDUCED ORAL CARCINOGENESIS AND CHEMOPREVENTION: STUDIES IN BIOENGINEERED HUMAN TISSUE

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ABSTRACT:

Oral cancer, originating from smoking-induced lesions of the basal cells in the complex stratified oral epithelium, is difficult to treat. Early detection of premalignant lesions, e.g., leukoplakia, has suggested the possibility of chemopreventive measures, such as topical application of antimitogenic/antiproliferative dietary or pharmaceutical agents. As an extension of a study in human oral epithelial cell monolayers, we determined the carcinogen, i.e., benzo[a]pyrene (BaP), transport, bioactivation, and DNA binding in a bioengineered human gingival epithelial tissue construct and the chemopreventive effects of dietary polyphenols. Short-term experiments showed that both types of compounds can traverse this tissue as well as be effectively taken up by the tissue. The model cigarette smoke carcinogen BaP very slowly, but to a great extent, accumulated in the tissue with maximal uptake at 24 h. Such exposure clearly resulted in DNA binding of BaP by the tissue. This DNA binding was associated with BaP-induced CYP1B1 as well as CYP1A1 expression, as evidenced by mRNA measurements. Co-treatment of the oral tissue with dietary polyphenols, including resveratrol and quercetin, and BaP, resulted in significant inhibition of the BaP-DNA binding. Using fluorescence microscopy as well as simultaneous autoradiography, we also demonstrated that quercetin indeed penetrates the entire stratified tissue layer, but that quercetin was also oxidized within the cells. Thus, this bioengineered oral tissue construct opens up improved ways of understanding and preventing/treating smoking-induced oral cancer.

The increased presence of tobacco-related carcinogen-DNA adducts in oral squamous cells of smokers compared with nonsmokers has demonstrated a strong link between smoking and the development of oral cancer (Hsu et al., 1997; Romano et al., 1999; Besarati Nia et al., 2000). Tobacco smoke components, including polycyclic aromatic hydrocarbons (PAHs), N-nitrosamines, and aromatic amines, are all thought to be potent carcinogens. Metabolic activation of tobacco-associated PAHs like benzo[a]pyrene (BaP) to reactive metabolites and the DNA adducts formed have been postulated to be central to the carcinogenic process of PAH-induced cancers (Hecht, 2002). However, how the carcinogens arrive at the critical cells from which the oral cancers develop, i.e., the basal cells of the highly stratified oral epithelium, is not clearly understood, mainly because of lack of a suitable biological model to study this question. Epidemiological studies have demonstrated a protective role of fruits and vegetables in oral cancers, presumably mediated by their content of polyphenols, particularly flavonoids (Block et al., 1992; Levi et al., 1998; Sakagami et al., 1999). However, how these potentially protective dietary compounds may get to the critically important basal cells in the complex stratified oral epithelium is not well understood, mainly, again, because of lack of a suitable biological model. Limited studies in this area using epidermis and oral mucosa from pigs (Squier and Lesch, 1988; Du et al., 2000) and humans (Bánóczy et al., 2003) have suggested that small organic molecules indeed can penetrate these highly complex stratified cell layers. However, the mechanisms involved are poorly understood.

In a recent study, we examined the bioactivation of BaP and its binding to cellular DNA in a human oral epithelial cell line cultured as monolayers, as well as the protective role of several polyphenols (Wen and Walle, 2005). This study explored the molecular mechanisms of BaP bioactivation and its inhibition by polyphenols. Highly specific for oral cells, BaP induced CYP1B1 mRNA and protein expression and, to a much lower extent, CYP1A1 expression. Selected polyphenols blocked this induction. It now became important to determine whether these molecular events may occur in the physiologically more relevant stratified oral epithelium, using a novel bioengineered human tissue construct closely resembling the normal gingival tissue. The main hypothesis under test was that both carcinogens and potentially protective polyphenols can reach the basal cells of this complex multilayer tissue.

Materials and Methods

Materials. EpiOral tissue and assay medium were supplied by MatTek Corporation (Ashland, MA; www.mattek.com). The EpiOral tissue model consists of normal human oral keratinocytes cultured to form three-dimen- sional differentiated tissue, which histologically and biochemically adopts a buccal phenotype. The cells are seeded in coated inserts. After several days of submerged culture, the culture inserts containing the developing tissues are elevated to the air-liquid interface, which induces stratification and differentiation. [14C]Quercetin (53 mCi/mmol) and [14C]resveratrol (61 mCi/mmol) were obtained from the National Cancer Institute Radiochemical Repository at San Diego CA, April 2–6, 2005: Walle T, Walle UK, Sedmera D, and Klausner M (GM55561) and the Department of Defense (N6311602MD200 and GC-3532-03-MK543). M.K. is an employee of the MatTek Corporation.

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ABBREVIATIONS: PAH, polycyclic aromatic hydrocarbon; BaP, benzo[a]pyrene; RT-PCR, reverse transcription-polymerase chain reaction.
Chemsys Science Laboratories (Lenexa, KS). [14C]Mannitol (55 mCi/mmol) and [3H]benz(o)pyrene (50 Ci/mmol) were purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). [14C]Ellagic acid (20 mCi/mmol) was a gift from Dr. Gary Stoner (Ohio State University Comprehensive Cancer Center, Columbus, OH). Quercetin, resveratrol, chrysirin, and BaP were bought from Sigma-Aldrich (St. Louis, MO), and 5,7-dimethylxlfavone was obtained from Indofine Chemical Co. (Hillsborough, NJ).

BaP was used as the model carcinogen, and the radiolabeled forms of quercetin, resveratrol, and ellagic acid, common dietary polyphenols present, for instance, in onions and apples, grapes and peanuts, and berries, respectively, were used in the transport studies. For the chemoprevention studies, the choice of polyphenols in this study was mainly based on their abilities to affect CYP1A1/1A2/1B1 activities (Wen et al., 2005).

Permeability Experiments. EpiOral tissues grown in inserts were equilibrated in a 37°C humidified incubator (5% CO₂) with 0.3 ml of assay medium in 24-well plates for 1 h; i.e., only the basolateral side of the tissue was exposed to medium. Assay medium (0.5 ml) containing the various radiolabeled test compounds (quercetin, resveratrol, ellagic acid, mannotol, and BaP) was added to the apical side of the tissues. At specified intervals, each tissue insert was moved to another well containing 0.3 ml of assay medium. Basolateral and apical solutions were assayed for total radioactivity. The tissues were digested with 0.5 M NaOH and assayed for radioactivity.

BaP DNA Binding. For measurements of DNA binding, the generally preferred method is the 32P-postlabeling assay (Reddy, 2000). However, because BaP has been well established to bind covalently to DNA after enzymatic bioactivation (Xue and Warshasky, 2005), we used the simpler radio-labeling assay, which has been modified and refined (Shibutani et al., 1999; Walle et al., 2003). EpiOral tissues were incubated with 1 μM [3H]BaP alone for specified times or for 24 h together with 25 μM quercetin, resveratrol, chrysirin, or 5,7-dimethylxlfavone. The tissue inserts were washed, and the tissues peeled off the membranes and homogenized with a Polytron homogenizer in 1 ml of 1% SDS/10 mM EDTA/20 mM Tris-HCl (pH 7.4). The homogenate was then incubated at 37°C for 30 min with 0.2 mg of RNase A and 0.5 mg of proteinase K (Shibutani et al., 1999). After extraction with phenol/chloroform/isoamyl alcohol (four times) and precipitation of DNA with sodium acetate and ethanol, the pellets were washed twice with ethanol and dissolved in water. The DNA purity (260/280 nm ratio ~1.7) and concentration were determined by UV, and [3H]BaP bound to DNA was determined by scintillation counting.

Expression of CYP1 Isoforms after BaP Treatment. EpiOral tissues were treated for 24 h with 1 μM BaP or dimethyl sulfoxide in medium. The tissues were transferred to microtubes and homogenized in lysis buffer. RNA was isolated using an RNAeasy Mini kit (QIAGEN, Valencia, CA). Typical yields were 10 to 15 μg/tissue. RNA (1 μg) was reverse-transcribed using an Omniscript RT kit (QIAGEN). CYP1B1 expression was then determined using semiquantitative PCR with normalization to 18S rRNA. CYP1B1 mRNA was clearly induced (Fig. 2A). The accumulation was very slow, with maximum tissue levels reached at 24 h.

We next determined whether the metabolic activation pathways required for DNA binding are present in the EpiOral tissue. Significant DNA binding occurred at 6 h, with a further increase at 24 h (Fig. 1B). When comparing the tissue uptake at 3.5 h with the transport, the compounds studied behaved quite differently (Table 1). As expected for a paracellular transport marker, mannotol showed minimal tissue uptake. In contrast, the tissue uptake of resveratrol and quercetin was substantial. This was also true for ellagic acid, which had very low transepithelial flux. Not unexpectedly, the highly lipophilic BaP demonstrated very large tissue accumulation with little transepithelial transport.

DNA Binding of BaP and Inhibition by Polyphenols. The carcinogenic process is thought to involve metabolic activation of BaP and binding to DNA. To examine this process in the EpiOral tissue, we first determined the accumulation of BaP over time (Fig. 1A). The accumulation was very slow, with maximum tissue levels reached at 24 h.

The sections were examined on a compound Leica DMLB microscope (Leica Microsystems GmbH, Wetzlar, Germany) in phase contrast and epifluorescence mode. A 4’,6-diamindino-2-phenylindole filter set was used (excitation band pass 340–380 nm, emission long pass 430 nm). Fluorescence images obtained with a 3CCD Hamamatsu camera (Hamamatsu Photonics, Hamamatsu City, Japan) were superimposed on the phase-contrast images, and depth of penetration into the multilayered tissue construct (in micrometers) was measured based on the drop in fluorescence intensity (Martí et al., 1999). To measure the penetration of total radioactivity (quercetin + metabolites and degradation products), slides with mounted sections were exposed for 48 h at 4°C to Kodak autoradiographic film (Eastman Kodak, Rochester, NY). Pictures of the developed film were taken on the same microscope and matched to phase contrast and fluorescence images of the same sections. The depth of radiolabel penetration was determined in the same fashion as for the fluorescence.

Results

Uptake and Transepithelial Transport. Our first experiments examined the ability of selected chemicals to traverse the multilayered EpiOral tissue simply by measuring the radioactivity in the basolateral compartment in short-term (3.5-h) studies. All five compounds examined demonstrated an essentially linear time-dependent flux. Mannotol, the paracellular transport marker, and the two polyphenols resveratrol and quercetin all had quite high fluxes, whereas the fluxes for the carcinogen, BaP, and the polyphenol ellagic acid were very low (Table 1).

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Induction of BaP-Activating Cytochrome P450 Isozymes. Previous studies have indicated that CYP1A1 and CYP1B1 can oxidize BaP, eventually leading to covalent binding of BaP to cellular DNA (Kim et al., 1998; Hecht, 1999; Nebert et al., 2004). When EpiOral tissue was treated with 1 μM BaP as compared with dimethyl sulfoxide for 24 h, CYP1B1 mRNA was clearly induced (Fig. 2A). CYP1A1 and 1A2 mRNAs were less induced. A semiquantitative comparison is given in Fig. 2B. Thus, CYP1B1 is the isoform most effectively induced in the epithelial tissue. Attempts to measure protein expression by Western blotting, as previously done in SCC-9 cell monolayers (Wen and Walle, 2005), failed because of very low expression levels.

Cell-to-Cell Transport of Quercetin by Microscopy. Although extensive epithelial tissue uptake of BaP and several of the dietary

### Table 1

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Added Concentration</th>
<th>Transport</th>
<th>Cellular Uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannitol</td>
<td>20</td>
<td>1.96 ± 0.15</td>
<td>0.15 ± 0.07</td>
</tr>
<tr>
<td>Quercetin</td>
<td>25</td>
<td>1.96 ± 0.18</td>
<td>5.26 ± 0.23</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>10</td>
<td>3.33 ± 0.20</td>
<td>4.47 ± 0.30</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>10</td>
<td>0.20 ± 0.09</td>
<td>2.44 ± 0.34</td>
</tr>
<tr>
<td>Benz(o)pyrene</td>
<td>1</td>
<td>0.19 ± 0.02</td>
<td>31.3 ± 4.6</td>
</tr>
</tbody>
</table>

ORAL CARCINOGENESIS: BIOENGINEERED TISSUE MODEL

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polyphenols was observed, it was not clear whether the whole tissue layer was penetrated. We examined this question for quercetin using both fluorescence microscopy and autoradiography, because quercetin has strong native fluorescence. After incubating the tissue with \[^{14}C\]quercetin for up to 8 h, the tissues were fixed and sectioned, and visualized by fluorescence microscopy or exposure to X-ray film (Fig. 3, A and B, respectively, at the 4-h time point). The time course of penetration is shown in Fig. 3C. On the basis of both techniques, quercetin was already detected in the tissue at 15 min, with maximum penetration is shown in Fig. 3C. On the basis of both techniques, the extent and site of penetration of BaP as well as the polyphenols compared with the tissue construct in this study may be due to differences between tongue (SCC-9) and gingival (EpiOral) cells or cancer (SCC-9) versus normal (EpiOral) cells. The present study in the oral tissue construct is probably more physiologically relevant than the SCC-9 cell monolayer study.

When the tissue was coincubated for 24 h with BaP and four different polyphenols, all proposed in various studies to be chemopreventive, there was a significant reduction in the DNA binding (Fig. 1A). Also, DNA binding of BaP occurred in the tissue. Statistically significant binding occurred after 6 h of exposure to BaP (Fig. 1B), which further increased after 24 h of exposure. Still, the DNA binding of BaP in the tissue was low compared with that of monolayers of human oral epithelial cells (Wen and Walle, 2005), probably because of the lower bioavailability of BaP and/or lower bioactivating enzyme levels.

The findings in Fig. 2 clearly indicated that the enzymes required for bioactivation of BaP were expressed in the oral tissue construct. Because both CYP1A1 and 1B1 are capable of metabolizing BaP (Kim et al., 1998; Hecht, 1999; Nebert et al., 2004), it was important to identify which isozymes were expressed and whether they were inducible by BaP in this tissue in a manner similar to that in human oral epithelial SCC-9 cells grown as monolayers (Wen and Walle, 2005). Interestingly, both CYP1A1 and 1B1 mRNA were expressed in uninduced tissue. As shown in Fig. 2, a 24-h exposure to a low (1 μM) concentration of BaP induced all CYP1 mRNAs, but primarily CYP1B1 and CYP1A1, with a very modest expression of CYP1A2. This is similar to recent observations in SCC-9 cells (Wen and Walle, 2005). The relatively higher expression level of CYP1B1 in SCC-9 cells compared with the tissue construct in this study may be due to differences between tongue (SCC-9) and gingival (EpiOral) cells or cancer (SCC-9) versus normal (EpiOral) cells. The present study in the oral tissue construct is probably more physiologically relevant than the SCC-9 cell monolayer study.

The extent and site of penetration of BaP as well as the polyphenols in the oral tissue construct with respect to time of exposure could not be determined from these experiments. We therefore used microscopy coupled with the fluorescence of the polyphenol quercetin (Fig. 3A), as well as microautoradiography, when using \[^{14}C\]quercetin in our incubation experiments (Fig. 3B). After 2 h, the native fluorescence of quercetin had penetrated about one third of the oral epithelial tissue construct. Conversely, at that time, the radioactivity had penetrated the entire tissue layer (Fig. 3C), i.e., reached all the way down to the DNA binding. C, inhibitory effect of polyphenols (25 μM) on the 24-h BaP DNA binding. Q, quercetin; RV, resveratrol; 5,7-DMF, 5,7-dimethoxyflavone. +, significantly higher than 0.5 h (P < 0.05); ++, significantly lower than control (P < 0.01).

**Discussion**

The short-term experiments with bioengineered human oral tissue demonstrated clear transport of all five molecules tested through the entire tissue layer. The mechanism of this transport may be mainly paracellular for mannitol, a commonly used paracellular transport marker with very low cell membrane penetration. Although the extent of transport of quercetin was identical to that of mannitol, this may probably be more the result of transcellular transport (Walgren et al., 1998). The higher value for resveratrol may also be due to transcellular absorption, which is highly efficient for this lipid-soluble polyphenol, at least in cell monolayers (Kaldas et al., 2003). However, this does not exclude the possible involvement of specific transporters for these molecules. The low value for ellagic acid is consistent with the previous finding of a basolateral transport barrier for this polyphenol (Whitley et al., 2003). All three polyphenols showed considerable cell uptake, consistent with their relatively lipophilic nature, in contrast to mannitol. The very low transepithelial transport for BaP is most likely due to the extensive cellular uptake of this highly lipophilic compound. The cell uptake of BaP is thus about 10-fold higher than that for the polyphenols (Table 1).

With the focus of our study on the carcinogen BaP, we observed that its cellular uptake progressed very slowly in the oral tissue construct, with a maximum uptake achieved in about 24 h (Fig. 1A). Also, DNA binding of BaP occurred in the tissue. Statistically significant binding occurred after 6 h of exposure to BaP (Fig. 1B), which further increased after 24 h of exposure. Still, the DNA binding of BaP in the tissue was low compared with that of monolayers of human oral epithelial cells (Wen and Walle, 2005), probably because of the lower bioavailability of BaP and/or lower bioactivating enzyme levels.

**Fig. 1.** Accumulation of and DNA binding of BaP (1 μM) in the EpiOral tissue. A, the tissue uptake curve, obtained by nonlinear regression analysis. B, effect of time on the DNA binding. C, inhibitory effect of polyphenols (25 μM) on the 24-h BaP DNA binding. Q, quercetin; RV, resveratrol; 5,7-DMF, 5,7-dimethoxyflavone. +, significantly higher than 0.5 h (P < 0.05); ++, significantly lower than control (P < 0.01).
basal cell layer from which the oral cancer develops. The reasons for this discrepancy can only be speculated on. Quercetin has previously been shown to be oxidized intracellularly, presumably by hydrogen peroxide and peroxidases, and to bind covalently to proteins (Wall et al., 2003). When quercetin is oxidized, multiple reactions occur, including disruption of the C-ring, most likely resulting in loss of the fluorescence (Nordström, 1968; Nishinaga and Matsuura, 1973; Nishinaga et al., 1979). Figure 3 gives further strong evidence that once quercetin enters cells, it is effectively altered by cellular oxidation. Figure 3 also shows that quercetin metabolites/degradation products reach the basal layer of the oral epithelium. Previous studies (Boulton et al., 1999) have indicated that degradation products of quercetin may have beneficial health effects.

The time for maximum induction of CYP1A1/1B1 by BaP was not
determined in these experiments. It is clear that the present study only provides a limited view of transport as well as metabolic processes in this highly complex oral epithelial tissue. Paracellular versus transcellular transport has not been clearly addressed. Also, the role of possible transporters in this tissue, as well as metabolic enzymes other than CYP1A1/1B1/1A2, is unknown.

It should be emphasized that for some of the polyphenols, the concentrations used in this study can easily be achieved in the diet. For example, resveratrol concentrations can reach 35 μM in red wine and 8 μM in peanuts (Sanders et al., 2000), and total quercetin concentrations in cooked onions can reach 1000 μM (Walle et al., 2000). Other polyphenols are present in the diet at lower levels.

In summary, in a bioengineered construct of the multilayered stratified human oral epithelial tissue, the data generated is consistent with the notion that smoking-derived carcinogens, e.g., BaP, are bioactivated by BaP-induced CYP1B1/1A1 to bind to DNA and to initiate the carcinogenic process. We have also shown that multiple polyphenols believed to be cancer-chemopreventive agents inhibit this DNA binding in this tissue. Using microsurgical techniques, we generated evidence that these compounds indeed will penetrate this complex tissue and thus reach the highly essential basal cell layer. It can be concluded that bioengineered human oral tissue can serve as a novel tool in studies pursuing improved prevention/treatment of oral cancer. It is also clear that this complex, multilayered tissue will require more in depth studies to better understand both transport, bioactivation, and chemoprevention mechanisms.

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Walle T, Otake Y, Walle UK, and Wilson FA (2000) Quercetin glucosides are completely


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