Abstract. Background: Cyclooxygenase (COX) is the key regulatory enzyme in prostaglandin (PG) synthesis and is upregulated in many premalignant and malignant lesions. The aim of this study was to investigate the in vitro DNA protective or damaging effects of COX-2 inhibitors using the single-cell gel electrophoresis (Comet) assay. Materials and Methods: Cells from miniorgan cultures of pharyngeal mucosa from 30 patients were incubated once or five times with the COX-2 inhibitors celecoxib and rofecoxib. After treatment with H$_2$O$_2$, DNA fragmentation was determined. Results: DNA strand-breaks were significantly reduced in cells pre-incubated with COX-2 inhibitors. Repeated incubation with celecoxib showed the strongest effect. This direct influence on DNA repair could be excluded by implementing DNA repair steps into the Comet assay. Conclusion: The findings suggest that, in addition to the known influence of COX-2 inhibitors on immune surveillance, neo-angiogenesis and cell proliferation, these substances may express a direct antimutagenic effect in conditions of oxidative stress.

Cyclooxygenase (COX) is a key regulatory enzyme in the production of prostaglandins (PGs) from arachidonic acid. PGs are synthesized in most tissues and act through autocrine or paracrine mechanisms influencing cell signalling (1, 2). COX-1, the constitutive isoform, is present in most tissues; it mediates the synthesis of PGs required for physiological functions, including the production of protective mucous by the gastrointestinal mucosa and platelet aggregation. Conversely, the enzyme COX-2 is not detected in most tissues. It is induced by a variety of pathophysiological conditions, including growth factors, inflammatory stimuli, cytokines, oncogenes, tumour promoters and tobacco carcinogenesis (2, 3). Increased amounts of COX-2 are commonly found in premalignant and malignant conditions, including oral leukoplakia and head and neck squamous cell carcinoma (HNSCC) (4, 5). The levels of COX-2 are also increased in normal-appearing mucosa adjacent to HNSCC (5). The functions of COX-2 may include PG-mediated increases in cell proliferation, increased angiogenesis, enhancement of cell migration and invasiveness, and impaired immune surveillance (6-12). Thus, COX-2 inhibitors have been implicated in the prevention of cancer development and progression. They have been recognised to have chemopreventative effects in several tumour models and were successfully used in clinical trials, especially in colorectal cancer, but also in head and neck cancer (1, 13). In addition to COX-2 inhibition, further COX-2-independent mechanisms, such as phase 1 mono-oxygenase activity in xenobiotic detoxification, were implicated in the anticarcinogenic effect of these substances (3, 14). Recent reports on carcinoma prevention in xenobiotic-induced animal head and neck carcinoma models suggest the relevancy of additional early preventative effects in the carcinogenic process (15, 16).

The aim of this study was to further investigate the in vitro DNA protective or damaging effects of the COX-2 inhibitors, celecoxib and rofecoxib, using the single-cell microgel electrophoresis (Comet) assay.

Materials and Methods

Macroscopically healthy pharyngeal mucosa biopsies from 30 patients, suffering from premalignant lesions in the upper aerodigestive tract (chronic cigarette smoking-induced laryngitis, leukoplakia), were taken during routine endoscopic investigations after informed consent and ethical approval by the ethics committee of Ludwig-Maximilians-University, Munich, Germany. Miniorgan cultures were maintained to allow repetitive incubations with the agents under investigation. The influence of these xenobiotics on human mucosal cells determined by the Comet assay is a reliable method of detecting genotoxicity (17, 18). Additionally, the DNA repair capacity was measured at different end-points (30 and 60 min) following genotoxic incubation.
Miniorgan cultures. The specimens were dissected into cubes of 1 mm³, using only the mucosa and excluding any deeper layers. The specimens were washed in bronchial epithelial cell growth medium (BEGM; Promocell, Heidelberg, Germany) three times and placed in 24-well plates, one fragment in each well, and were coated with 0.75% agar noble dissolved in Dulbecco’s modified eagle medium (DMEM; Gibco, Eggenstein, Germany), 10% foetal calf serum (FCS; Gibco) and non-essential amino acids, streptomycin and amphotericin B. The miniorgan cultures floated in their medium and did not adhere to the plates. BEGM (250 µl) served as the medium for each mucosal fragment. The cultures were subjected to a temperature of 37°C, in an atmosphere of 5% CO₂ with 100% relative humidity. The BEGM was renewed every day, and the multiwell plates were replaced after incubations on days 7 and 10. After 11 days, the initial oropharyngeal mucosa fragments had become completely coated with epithelium.

Incubation. The miniorgans were incubated with 0.1 or 1.0 µg/ml celecoxib or 0.0125 µg/ml rofecoxib, for 30-min periods, either daily on the seventh to eleventh days or once on day 11. The substances were washed out twice using BEGM and the cultures were subsequently damaged with 1 mM H₂O₂ for 15 min. Earlier incubations did not appear to be appropriate since it takes approximately 10-11 days for the miniorgans to be fully-coated by superficial epithelial cells. After a 15-min incubation period, the cultures were washed in BEGM twice and placed onto new plates to exclude further genotoxic reactions. DMSO was used as the negative control (166 µmol/ml). The concentrations of the substances had been chosen according to pilot tests on miniorgans, dose-response relationships and prior studies. The cultures were either directly transferred to the Comet assay or left to repair for 30 or 60 min at 37°C.

Comet assay. The first portion of miniorgans underwent enzymatic digestion (10 mg hyaluronidase, 10 mg collagenase, 50 mg protease) for 45 min at 37°C (19). The viability was tested by Trypan blue staining. The cells were resuspended in 0.7% low-melting agarose and applied to slides, frosted at the long edges and covered with 0.5% normal-melting agarose, to provide stability of the agarose layers. The slides were placed into a solution of 10% DMSO, 1% Triton-X, 2.5 M NaCl, 10 mM Trizma-Base, 100 mM Na₂EDTA and 1% N-lauroylsarcosine sodium salt for 1 h. The slides were placed into a horizontal gel electrophoresis chamber (Renner, Dannstadt, Germany), positioned close to the anode and covered with alkaline buffer solution containing 300 mM NaOH and 1 mM Na₂EDTA at pH 13.2. After a 20-min DNA "unwinding" period, electrophoresis was started at 0.8 V/cm and 300 mA for 20 min, followed by neutralisation (400 mM Trizma base, pH 7.5; Merck).

Staining and analysis. Ten microliters of DAPI (42 ng/ml) with antifade were applied after air-drying of the slides, followed by storage protected from light at –20°C. The DNA fragmentation was visualised using a fluorescence microscope and digital analysis (Comet++, Kinetic Imaging®) (12). (Figure 1). Twenty cells per slide were analysed.

Analysis of data. Statistical analysis was performed using SPSS 12.0®. The Olive tail moment (OTM) values of all groups were compared (Mann-Whitney U-Test, Wilcoxon test). The general level of significance was p≤0.05.

Table I. DNA fragmentation in the different study groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>OTM* deviation</th>
<th>Cell vitality**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>1.08 0.28</td>
<td>97</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>19.42 4.10</td>
<td>86</td>
</tr>
<tr>
<td>H₂O₂ + repair 30 min</td>
<td>12.99 2.93</td>
<td>90</td>
</tr>
<tr>
<td>H₂O₂ + repair 60 min</td>
<td>12.80 6.02</td>
<td>90</td>
</tr>
<tr>
<td>Celecoxib 0.1 µg</td>
<td>1.28 0.35</td>
<td>100</td>
</tr>
<tr>
<td>Celecoxib 1.0 µg</td>
<td>1.48 0.43</td>
<td>93</td>
</tr>
<tr>
<td>Celecoxib 0.1 µg + H₂O₂</td>
<td>9.80 3.20</td>
<td>93</td>
</tr>
<tr>
<td>Celecoxib 1.0 µg + H₂O₂</td>
<td>9.49 1.99</td>
<td>90</td>
</tr>
<tr>
<td>Celecoxib 0.1 µg + H₂O₂ + repair 30 min</td>
<td>5.87 2.66</td>
<td>88</td>
</tr>
<tr>
<td>Celecoxib 0.1 µg + H₂O₂ + repair 60 min</td>
<td>5.16 1.64</td>
<td>86</td>
</tr>
<tr>
<td>Celecoxib 0.1 µg 1x (day 11) + H₂O₂</td>
<td>9.80 3.20</td>
<td>93</td>
</tr>
<tr>
<td>Celecoxib 0.1 µg 5x (days 7-11) + H₂O₂</td>
<td>7.29 2.60</td>
<td>91</td>
</tr>
<tr>
<td>Rofecoxib 1x (day 11) + H₂O₂</td>
<td>10.08 2.61</td>
<td>91</td>
</tr>
<tr>
<td>Rofecoxib 5x (days 7-11) + H₂O₂</td>
<td>9.76 3.44</td>
<td>90</td>
</tr>
</tbody>
</table>

*OTM: Olive trail moment measuring amount of DNA fragmentation. **Cell vitality was measured before microgel electrophoresis.
Results

The results of the Comet assay were determined as OTMs, reflecting the amount of DNA fragmentation (Table I, Figure 2). The statistical analysis is presented in Table II. As expected, mucosa cells damaged with H2O2 alone showed the highest level of DNA fragmentation (mean OTM 17.23), while untreated cells and cells treated only with the COX-2 inhibitors celecoxib and rofecoxib showed no significant DNA fragmentation (OTM<2). Cells simultaneously incubated with H2O2 and the COX-2 inhibitors showed significantly reduced DNA fragmentation (celecoxib 0.1 ìg/ml, OTM 9.80 (50.46%); celecoxib 1.0 ìg/ml, OTM 9.49 (48.87%); rofecoxib 0.0125 ìg/ml (50.26%)) compared to cells treated with H2O2 alone (mean OTM 19.42). Interestingly, the cells damaged with H2O2 in the presence of the lower celecoxib concentration (0.1 ìg/ml) showed the same degree of DNA fragmentation as with H2O2 alone, reflected in the mean OTMs of 9.80 and 9.76, respectively (Figure 2). Further investigation of the DNA repair capacity revealed only minor differences in the proportion of DNA repair at 30 min and 60 min between cells damaged by H2O2 alone (repair 30 min, 33.11%, repair 60 min, 33.37%) or in the presence of celecoxib (repair 30 min, 40.10%, repair 60 min, 47.35%, Table I).

Discussion

Genetic changes, that lead to the initiation and progression of HNSCC, are dependent on exogenous and endogenous factors (17). The exogenous factors include xenobiotics, as well as physical and biological hazards, which can all cause DNA strand-breaks, leading to chromosomal aberrations and non-homologous recombination, thus resulting in apoptosis or the modification of cell differentiation. The single-cell gel electrophoresis, or Comet, assay is a well validated test for detecting DNA strand-breaks in single cells (17-19). Loops of DNA-containing breaks are pulled out of the nucleus in the direction of the anode, forming a 'comet tail'. The relative density of DNA in the tail is related to the degree of DNA damage.

In the present study, the DNA damage in genotoxically-stressed cells was markedly reduced in cells pretreated with the COX-2 inhibitors celecoxib and rofecoxib. This effect was strongest with repeated incubation (5 x 0.1 ìg/ml of celecoxib on days 7-11, oxidative DNA damage on day 12) compared to single application. Recent studies showed that pre-malignant lesions in the upper aerodigestive tract (UADT) produce more PGs than the normal tissues from which they arise (20, 21). Increased synthesis of PGs in transformed premalignant cells and tumours can be a consequence of the enhanced expression of COX-2. COX-2 may play a role in the progression of UADT cancer through a number of mechanisms, which include PG-mediated increases in cell proliferation, increased angiogenesis, the enhancement of cell migration and invasiveness, impaired immune surveillance and apoptosis (6-10, 22). COX-2 is induced, however, by factors implicated in HNSCC, including oncogenes, growth factors, cytokines and tobacco carcinogenesis. The stimulation of Ras and protein kinase C signalling pathways, known to be dysregulated in HNSCC, induces COX-2 in oral epithelial cells (23). In contrast, wild-type but not mutant p53 suppresses COX-2 transcription (11). p53 mutations are detected in both pre-cancer and cancer lesions of the head and neck (24). This raises the possibility that the p53 status could be a determinant of COX-2 expression in HNSCC, as well as in precursor lesions.

There is, however, epidemiological and experimental evidence that the inhibitors of COX (and thereby PG formation), particularly COX-2, protect against a variety of

Table II. Proportional OTMs and significance level of compared study groups.

<table>
<thead>
<tr>
<th>Groups: a vs. b</th>
<th>% OTM</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Celecoxib 0.1 ìg 1x (day 11) + H2O2 vs. H2O2 alone</td>
<td>50.46</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Celecoxib 0.1 ìg 5x (day 7-11) + H2O2 vs. H2O2 alone</td>
<td>40.78</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Rofecoxib 1x (day 11) + H2O2 vs. H2O2 alone</td>
<td>51.91</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Rofecoxib 5x (days 7-11) + H2O2 vs. H2O2 alone</td>
<td>50.26</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Celecoxib 0.1 ìg 5x (days 7-11) + H2O2 vs. celecoxib 0.1 ìg 1x (day 11) + H2O2</td>
<td>80.82</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Rofecoxib 1x (day 11) + H2O2 vs. rofecoxib 5x (days 7-11) + H2O2</td>
<td>98.38</td>
<td>p=0.85</td>
</tr>
</tbody>
</table>

OTM: Olive tail moment.
human malignancies including HNSCC (25-30). The antimitagenic effects of celecoxib and rofecoxib were unknown up to now, but are most probably unrelated to a COX-2 effect on PG metabolism. More recently, PG-independent mechanisms have been implicated in the anticarcinogenic effects of this class of agents (12). Hence, COX is a bifunctional enzyme that has both peroxidase and cyclooxygenase activities. The peroxidase activity catalyzes the conversion of procarcinogens to carcinogens (14). In the liver, these oxidative reactions are principally catalyzed by cytochrome P450s. Extrahepatic tissues, such as those of the head and neck, however, frequently have low concentrations of P450 and, therefore, significant amounts of xenobiotics might be co-oxidised to mutagens by the peroxidase activity of COX. In addition to potentially catalyzing the synthesis of active mutagens from procarcinogens, COX-2 can be induced by procarcinogens. For example, benzo(a)pyrene BaP, a procarcinogen in tobacco smoke, stimulates COX-2 transcription. In turn, COX-2 catalyzes the oxidation of B(a)P-7,8-diol to B(a)P-7,8-diol-9,10-epoxide, a highly reactive and strong mutagenic compound (3). This raises the possibility that B(a)P-mediated induction of COX-2 facilitates its own conversion to B(a)P-7,8-diol-9,10-epoxide, thereby amplifying the effect of a given dose of B(a)P on tumour initiation (31). Although speculative, these findings suggest a potential role for COX-2 inhibitors in preventing tobacco smoke-related DNA damage. In contrast to cigarette smoke-related carcinogens, H2O2, which does not need peroxidase activity to induce oxidative DNA damage, was used. Thus, the inhibition of COX peroxidase activity by celecoxib and rofecoxib is very unlikely to be the underlying mechanism.

The measurement of mutagen sensitivity modifications by the Comet assay allow for the quantification of DNA repair (17). Using this procedure, it was shown that the antimitagenic effect resulted only to a minor extent from influences on DNA repair mechanisms, particularly when comparing the overall repair (33.57% H2O2 alone, compared to 47.35% celecoxib pretreatment + H2O2). At present, selective COX-2 inhibitors are being evaluated as chemopreventive agents in many precancerous conditions, including oral leukoplakia and Barrett’s oesophagus. Because chemopreventive agents may require prolonged use, the safety profiles of selective COX-2 inhibitors need to be established in each of these patient populations. Although COX-2, in contrast to the COX-1 isofrom, is regarded as an inducible enzyme that only has a role in pathophysiological processes, such as pain, inflammation and malignant transformation, recent studies have shown that it executes important physiological functions in the kidney and in the vascular epithelium (32).

Evidence supports an association between adverse cardiovascular effects and the use of celecoxib and rofecoxib, which resulted in the withdrawal of rofecoxib from the market. However, this risk was not equally distributed among the drugs and celecoxib seemed to be one of the safest within this group (33). Since celecoxib expresses its antimitagenic effect at low concentration, its use should be further evaluated in chemoprevention trials. Patients with a history of HNSCC are at increased risk of second primary tumours and increased amounts of COX-2 have been detected in premalignant lesions throughout the UADT, thus the potential role of selective COX-2 inhibitors in decreasing the incidence of HNSCC and second primary tumours should be further investigated. While the antimitagenic effects of COX-2 inhibitors on pharyngeal cells have not been elucidated in detail, such knowledge could be extremely important in the development of strategies to prevent tobacco-induced carcinogenesis.

References

Received December 5, 2005
Revised March 24, 2006
Accepted April 10, 2006