Oxidative DNA damage in pterygium

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Purpose: Epidemiological evidence suggests that UV irradiation plays the most important role in pterygial formation. The noxious effects of UV irradiation are either directly by a UV phototoxic effect or indirectly by formation of radical oxygen species (ROS). ROS are very harmful to cells, because they injure cellular DNA, proteins, and lipids (called oxidative stress). Among numerous types of oxidative DNA damage, the formation of 8-hydroxydeoxyguanosine (8-OHdG) presents only a minor fraction of UV induced DNA damage, but it is a ubiquitous marker of oxidative stress. If pterygium is related to UV, we surmised oxidative stress exists in pterygium. To provide the molecular evidence of UV radiation, 8-OHdG was detected in pterygium. Moreover, human 8-oxoguanine glycosylase (hOGG1) is the key component responsible for the removal of 8-OHdG. To determine whether the hOGG1 was expressed in pterygium, this enzyme was also evaluated.

Methods: Immunohistochemical staining using a monoclonal antibody to 8-OHdG and hOGG1 were performed on 52 pterygial specimens and 6 normal conjunctiva.

Results: There were 12 (23.1%) pterygial specimens positive for 8-OHdG staining, limited to the nuclei of the epithelial layer. No substantial staining was visible in the subepithelial fibrovascular layers. In pterygium with 8-OHdG staining, there were 4 (4/11, 36.4%) specimens with hOGG1 expression. However, in pterygium without 8-OHdG staining, there were only 3 (3/41, 7.3%) specimens with hOGG1 expression. hOGG1 expression was significantly associated with 8-OHdG positive staining. All normal controls were negative for 8-OHdG and hOGG1 staining.

Conclusions: Our study demonstrated for the first time 8-OHdG in pterygium, which represented oxidative stress in pterygium. The increased level of 8-OHdG in pterygium is not due to decreased expression of hOGG1, while increased levels of 8-OHdG induced the expression of hOGG1.

Pterygium is a chronic condition characterized by the encroachment of a fleshy triangle of conjunctival tissue into the cornea. The pathogenesis of pterygium is under investigation and several factors including ultraviolet radiation, immunoinflammatory process, virus infection, and genetic factors were reported to be related to pterygial formation [1]. Epidemiological evidence suggests that UV irradiation plays the most important role [1-3].

The noxious effects of UV irradiation are either directly by UV phototoxic effects or indirectly by formation of radical oxygen species (ROS) [4-6]. ROS is very harmful to cells, because they injure cellular DNA, proteins, and lipids, called oxidative stress [4-7]. Among numerous types of oxidative DNA damage, 8-hydroxydeoxyguanosine (8-OHdG) has received considerable attention because of its demonstrated mutagenic potential and is a ubiquitous marker of oxidative stress [7,8].

If pterygium is related to UV, we surmised oxidative stress exists in pterygium. To provide the molecular evidence of UV radiation, 8-OHdG was studied immunohistochemically in pterygium and normal conjunctiva. Moreover, there is a base excision repair system to correct 8-OHdG in the human genome. 8-Oxoguanine glycosylase (hOGG1) is the key component responsible for removal of 8-OHdG [8]. To determine whether the hOGG1 was expressed in pterygium, hOGG1 was also detected by immunohistochemistry in both pterygium and normal conjunctiva.

METHODS

Pterygium samples were harvested from 52 patients undergoing pterygium surgery. Our technique of pterygium excision was described previously [9]. Briefly, under topical and subconjunctival anesthesia, the head of the pterygium was first separated at the apex of pterygium, lifted off the corneal surface, and excised. Then, after most of the body of the pterygium and subconjunctival Tenon’s tissue being excised, 0.02% mitomycin C was used for 30 s and the wound was managed by simple surgical closure by suture. Only the head of primary pterygium was used as pterygium sample in this study. Normal conjunctiva samples were collected from medial conjunctiva in 6 patients without pterygium and pinguecula while undergoing cataract or vitreoretinal surgery. This study was carried out with approval from the Human Study Committee of the China Medical University Hospital and National Cheng Kung University Hospital. Informed consent was obtained from all individuals who participated in this study.

All specimens were formalin fixed and paraffin embedded. Sections were cut at a thickness of 3 µm, mounted on
glass, and dried overnight at 37 °C. All sections were then deparaffinized in xylene, rehydrated through a series of alcohol, and washed in phosphate buffered saline. This buffer was used for all subsequent washes. Sections for 8-OHdG detection were heated in a microwave oven twice for 5 min in citrate buffer (pH 6.0). Mouse anti-8-OHdG (Trevigen, Gaithersburg, MD) and anti-OGG1 (Alpha Diagnostic International, San Antonio, TX) monoclonal antibody was used as the primary antibody and the incubation time was 60 min at room temperature followed by a conventional streptavidin peroxidase method (LSAB Kit K675; DakoCytomation, Glostrup, Denmark). Signals were developed with 3, 3’-diaminobenzidine for 5 min and counterstained with hematoxylin. Negative controls were obtained by leaving out primary antibody. The results were evaluated independently by three observers and scored for the percentage of positive nuclei. A score of 0 indicates no positive staining, a score of +1 indicates from 1% to 10% positive, a score of +2 indicates from 11% to 50% positive, and a score of +3 indicates more than 50% positive cells. In this study, scores of +1, +2, and +3 were considered to be positive immunostaining, and a score of 0 was seen as a negative immunostaining.

RESULTS

The study was comprised of 28 males and 24 females in the pterygium group (age range of 53-80 years, mean of 64.2 years), and 3 males and 3 females in the control group (age range of 57-81 years, mean of 68.3 years).

IHC of 8-OHdG: In the pterygium group, 40 (76.9%) of the specimens were negative (0), 1 (1.9%) were +1, 11 (21.2%) were +2, and 0 (0%) was +3. Overall, 12 (23.1%) were positive for 8-OHdG. 8-OHdG staining was distributed in the epithelium, and limited to the nuclei of the epithelial cells (Figure 1A-C). No substantial staining was visible in the subepithelial fibrovascular layers. In normal conjunctiva group, all specimens were negative (Figure 1D).

IHC of hOGG1: In the pterygium group, 45 (86.5%) specimens were negative (0), and 7 (13.5%) were +2 for hOGG1 staining. hOGG1 staining was distributed in the epithelium, and limited to the nuclei of the epithelial cells (Figure 2A-C). No substantial staining was visible in the subepithelial fibrovascular layers. In normal conjunctiva group, all specimens were negative (Figure 2D).

Relationship between 8-OHdG and hOGG1: The relationship between 8-OHdG and hOGG1 was shown in Table 1. In pterygium with 8-OHdG staining, there were 4 (4/11, 36.4%) specimens with hOGG1 expression. However, in pterygium without 8-OHdG staining, there were only 3 (3/41, 7.3%) specimens with hOGG1 expression. hOGG1 expression was significantly associated with 8-OHdG positive staining.

Figure 1. 8-OHdG staining. 8-OHdG positive staining is shown in the nucleus of the epithelium, and distributed in the basal (A,B), middle (B), and superficial (B,C) layers of the epithelium. Arrows indicate the immunostained nuclei. No substantial staining is visible in the subepithelial fibrovascular layer or in normal control (D).
DISCUSSION
UV radiation has been proven to produce a wide range of DNA damage, such as 8-OHdG, protein-DNA crosslinks, single strand breaks, pyrimidine dimer, and thymine glycol [6]. Among them, 8-OHdG is considered to be a marker of oxidative stress, though it represents only a minor fraction of UV induced DNA damage [7,8]. Hence, in our study, 8-OHdG was found in only 23.1% of pterygial specimens, but it supported the hypothesis that there was oxidative stress in pterygium and could provide molecular evidence of UV radiation in pterygium.

Pterygium has long been considered a chronic degenerative condition. However, abnormal expression of the p53 gene being found in epithelium, some researchers feel that pterygium is a tumor rather than degenerative while others still believe that pterygium is degenerative in nature [10-14]. 8-OHdG is highly mutagenic because it causes a G to T transversion mutation [8]. The increase in 8-OHdG content in DNA has been reported to increase cancer risks [15]. Hence, the detection of 8-OHdG in pterygium suggests that gene mutations may exist in pterygium and supports the hypothesis that pterygium may be a tumor.

Several factors other than UV have been reported to be related to pterygium, including immunoinflammatory process, virus infection, and genetic factors [1]. The detection of 8-OHdG in pterygium does not allow us to rule out the role of other factors in pterygium formation because oxidative stress also can be induced by these factors [16]. Further study on the presence of C to T transitions or CC to TT tandem mutations on dipyrimidine sites, which are the UV molecular signature [17], is suggested to investigate the role of UV in pterygium formation. On the basis that oxidative stress is a common link in all of the suspected risk factors of pterygium [16,18,19], we suggest oxidative stress indeed plays a causative role in the development of pterygium.

hOGG1 is a specific DNA glycosylase/apurinic lyase that is responsible for the removal of 8-OHdG from DNA [8]. The hOGG1 molecule can recognize 8-OHdG, and then catalyze both the release of 8-OHdG and the cleavage of DNA at the resulting apurinic site [8]. Hence, accumulation of 8-OHdG will result in expression of hOGG1, and 8-OHdG and hOGG1 expression was reported to be significantly up regulated in cancer [20]. Inactivation of hOGG1, however, also resulted in the accumulation of 8-OHdG, and mutations in the hOGG1 gene has been associated with an increased risk of cancer [7,21,22]. Our present study explored whether the high expression of 8-OHdG in pterygium was related to a lack of expression of the hOGG1 gene. We found that the expression of hOGG1 protein was higher in pterygium than in controls, and higher in pterygium with 8-OHdG expression than in pterygium without 8-OHdG expression. Therefore, the increased

Figure 2. hOGG1 staining. hOGG1 positive staining is shown in the nucleus of the epithelium, and distributed in the basal (A,B), middle (B), and superficial (B,C) layers of the epithelium. Arrows indicate the immunostained nuclei. No substantial staining is visible in the subepithelial fibrovascular layer or in normal control (D).
level of 8-OHdG in pterygium was not due to decreased expression of hOGG1. We suggest that the increased level of 8-OHdG in pterygium is induced by the reported risk factors, such as UV. The accumulated 8-OHdG induces expression of hOGG1.

Our study concurs with two previous studies that polymorphisms of hOGG1 and glutathione S-transferase M1, an antioxidative enzyme, were reported to be associated with pterygial formation [18,19]. The two studies and ours provide the evidence that oxidative stress plays an important role in pterygium formation. Further studies via quantitative evaluation of 8-OHdG itself and other oxidative stress markers (e.g., 4-hydroxy-2-nonenal, malondialdehyde, and thioredoxin) might help to address the importance of oxidative stress in pterygium.

There were two limitations in our study. Firstly, we did not check the level of Glucose-6-phosphate dehydrogenase (G6PD) in all patients, though no one reported to have a G6PD deficiency. The major role of G6PD is to generate NADPH to protect cells from oxidative stress [23]. G6PD deficiency results in an impaired ability to deal with oxidative stress and brings about high levels of 8-OHdG in cells [24]. The prevalence of G6PD deficiency in Taiwan is estimated to be around 2.1% (male 3.1%, female 0.9%) [25]. No patients reported a G6PD deficiency in our series, and the positive staining of 8-OHdG in our study was up to 23.1%, the chance of 8-OHdG in pterygium is due to the prevalence of G6PD deficiency is slim. However, we suggest if G6PD deficiency shows a significant prevalence in the geographical area where the patients originated, their enzymatic condition should be known. Secondly, diet and drug were not evaluated in patients and controls. Both factors were reported to play a role in antioxidant protection and formation of ROS [26,27]. However, they are difficult to evaluate by questionnaires. In this study, all patients and controls were Taiwanese from the same city whose habit of diet were not different. Among drugs, several antibiotics were reported to participate in the formation of ROS [27].

Because no pterygium patients were known to receive topical antibiotics before surgery from reviews of their chart, the chance of 8-OHdG in pterygium is due to the use of eye drops is also slim.

In conclusion, our study demonstrated for the first time that there was 8-OHdG in pterygium, which represented that there is oxidative stress in pterygium. Increased levels of 8-OHdG in pterygium induces the expression of hOGG1. Further study on the role of oxidative stress and antioxidant in pterygium formation or recurrence is suggested.

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REFERENCES

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