

The Efficacy of Imiquimod on Dysplastic Lesions of the Oral Mucosa: An Experimental Model

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Abstract. *Aim: To study the potent efficacy of the immunomodulatory agent imiquimod when applied on dysplastic lesions of the oral mucosa. Materials and Methods: Carcinogen (DMBA) was applied to the mucosa of the left buccal pouch of 26 male Wistar rats for 8 weeks, until dysplastic lesions were observed and histologically diagnosed. At the second phase of the experiment, 5% imiquimod cream was applied to these dysplastic lesions for 16 weeks. Biopsies were taken before and after treatment. Results: The histological effect of imiquimod was the regression of mild dysplasia to hyperplasia for all the samples. In one case, a well-differentiated squamous cell carcinoma was converted to a papilloma-like squamous neoplasm with a benign morphology. Conclusion: Our results indicate that imiquimod may be effective in treatment of precancerous lesions of the oral mucosa and thus inhibit the progress of carcinogenesis.*

Dysplasia is of critical importance in the field of cancer chemoprevention field, as any degree of dysplasia may progress to severe dysplasia or carcinoma in situ. In fact dysplasia is a precancerous lesion, that is: “a morphologically altered tissue in which cancer is more likely to occur than in its apparently normal counterpart” (1). Hence, it always remains a challenge to discover new therapeutic agents that can prevent the transformation of precancerous lesions into cancer, or even cause their regression. As far as the oral cavity is concerned, it is widely accepted that dysplastic lesions are considered to be precancerous lesions with a certain potential for developing a carcinoma *in situ* and more invasive forms of cancer.

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The most common precancerous lesion of the oral mucosa is leukoplakia, which in fact is a clinical term only. “Oral leukoplakia is a predominant white lesion of the oral mucosa that cannot be characterized as any other definable lesion. Some oral leukoplakias will transform into cancer” (1). Histological examination of leukoplakia is always necessary, in order to determine the exact grade of histological lesion, if present. Hence a white lesion of the oral mucosa can either be dysplastic or non-dysplastic. This is why the term leukoplakia remains strictly a clinical term only, and after a biopsy is performed, the term leukoplakia is replaced by the diagnosis obtained histologically (2).

The management of oral dysplasia consists first of all of the elimination of the causative factor, which is usually the use of tobacco. According to most published studies, elimination of the cause leads to regression of the lesion. However, when no possible cause can be found, or when the lesion does not disappear, it is necessary to proceed to other therapeutic procedures: surgical excision, cryosurgery, CO₂-laser ablation and application of: vitamin E, vitamin A, retinoids, beta-carotene, bleomycin, alpha-tocopherol, and calcipotriol (2).

Imiquimod is a novel synthetic compound that belongs to the class of ¹H-imidazo-[4,5-c]quinolines, a nucleoside analogue of the imidazoquinoline family known for its competence as immune response modifiers and stimulators (3). Its main biological effect is the induction of endogenous antiviral pro-inflammatory mediators. Besides its antiviral activity, imiquimod has shown a very interesting efficacy against several types of human malignancies, thus increasing the research about its exact mechanism of action and its potential antitumoral applications

The Food and Drug Administration of the USA approved imiquimod for the treatment of external anogenital and perianal warts in 1997. In 2004, imiquimod received FDA approval for the treatment of actinic keratosis (AK) and superficial basal cell carcinoma (SBCC).

Imiquimod acts on both the major divisions of the immune system: the innate and the cell-mediated system.

Imiquimod exerts its predominant effect *via* its binding to the toll-like receptors (TLR) 7 and 8. TLR-7 and TLR-8 belong to the family of ten currently known TLRs which are crucially involved in the innate immune system's recognition of various microbial antigens (4). TLR-7 and TLR-8 are present on dendritic cells, macrophages, and monocytes. Their activation leads to the stimulation of the nuclear factor κ B (NF- κ B), which is a central transcription factor. NF- κ B is normally inactive because of its binding to inhibitory κ B (I κ B). After TLR-mediated stimulation, NF- κ B is dissociated from I κ B. This leads to the entrance of NF- κ B into the nucleus, where it induces the transcription of a large number of genes. Products of these genes include an important number of pro-inflammatory mediators (cytokines): tumor necrosis factor α (TNF- α), interferon α (IFN- α), interleukin-1 (IL-1), (IL-6), (IL-8), (IL-10) and (IL-12). These cytokines up-regulate cell-mediated Th-1 responses, which have antitumoral and antiviral effects, while they down regulate Th-2 responses (5).

Independently from TLR-7 and TLR-8, imiquimod interacts with adenosine receptor (especially A2A) and down regulates adenylyl cyclase. Both these effects result in suppression of a negative feedback mechanism which normally limits inflammatory reactions. The final result is again the increase of pro-inflammatory activity. Furthermore, imiquimod induces 2'5'oligoadenylatesynthetase, thus stimulating natural killer (NK) cells and enhances the maturation of epidermal Langerhans cells and their migration to regional lymph nodes, promoting a specific T-cell response.

The antitumoral activity of imiquimod is not only attributed to its numerous stimulating effects on the immune system, but also on the induction of apoptosis of tumour cells. Activation of transcription-1 signaling pathway favors the secretion of pro-inflammatory cytokines, and takes part in the apoptosis of tumour cells. Moreover, topical imiquimod increases the expression of the death receptor CD 95 (Fas) and its binding to the Fas-ligand (5), resulting in the apoptosis of tumour cells. In addition, imiquimod reduces the expression of the anti-apoptotic Bcl-2 protein. This results in release of mitochondrial cytochrome *c* and activation of caspase-9 and terminal caspases, mainly caspase-3, thus causing apoptosis of the malignant cells (6). It is evident that apoptosis exerts an important role in the overall antitumoral activity of imiquimod, which, on the other hand, is not totally understood yet.

Imiquimod's small size ($M_r=240.3$) and hydrophobicity allow it to penetrate the epidermal barrier and be used as a topical agent. The area of application should be cleaned and dried with sterile gauze and the cream is then rubbed in well with a cotton bud. The cream needs to be left on for 6 to 10 hours (this is why it is usually applied before night sleep), while the appearance of side-effects may impose limitation on this duration. The most common side-effects of

imiquimod are topical pain, burning, itching, erythema, erosions, edema and ulceration. These local side-effects cause re-determination of the application dosage (7).

The aim of the present study was to evaluate any possible regression of premalignant lesions after local administration of imiquimod.

Materials and Methods

The animal that served as animal model in our experiment was the Wistar rat. Thirty-six male Wistar rats that were 60 days old and weighed about 350-400 g each at the beginning of the experimental period were used. The animals were housed two per cage and were given standard laboratory food and water *ad libitum*. They were maintained in a controlled environment, under standard laboratory conditions of temperature and humidity.

The carcinogen used was 7,12-dimethylbenz[*a*]anthracene (DMBA; Sigma Chemical Co, S. Louis MO, USA), an aromatic polycyclic hydrocarbon. DMBA powder (1g) was dissolved in 200 ml acetone, in order to produce a solution of 0.5% DMBA, which was kept in a brown bottle in the refrigerator through the time of the experiment (8, 9).

All the treatments on the animals were carried out under general anesthesia with isoflurane. Isoflurane was considered to be suitable for this experiment because it achieves the depth and duration of anesthesia necessary for our treatments, while it causes only slight side-effects. This was very important for our experiment, as it assured the survival of the animals for the duration of long period that lasted the experiment (24 weeks). After the inhalation of the anesthetic, the animal was held in a supine position and the mouth was opened with a pair of forceps. The right buccal pouch served as a control and was not treated. The mucosal surface of the left buccal pouch was cleared of food debris and dried with sterile gauze. Then it was painted with 0.5 ml of DMBA with the help of a hairbrush, in a circular motion 10 times. The application was carried out 3 times per week for 8 weeks (10). The mouth was kept opened for about 2 minutes after each application, to ensure that DMBA was absorbed into the buccal pouch epithelium and not lost or swallowed. Food and drink were not held from the animals for about 3 hours after each application.

At the end of 8 weeks, the lesions were photographed and biopsy specimens were taken from the left buccal pouch mucosa of all the animals. The piece of the tissue was taken under general anesthesia with intraperitoneal injection of ketamine (200 mg/kg of body weight) and xylazine (10 mg/kg of body weight).

The specimens were fixed in formalin 10%, then embedded in paraffin and finally cut into sections and stained with hematoxylin and eosin in order to be studied histologically.

The second phase of the experiment, where the therapeutic agent was used, was then carried out. The procedure was practically the same: under general anesthesia with isoflurane, the mouth was opened, the left buccal pouch mucosa was cleaned and dried with sterile gauze and imiquimod was rubbed in well with a cotton bud. Food and drink were not held from the animals for about 3 hours after each application. Imiquimod was applied 3 times per week for a period of 16 weeks. After the 16 weeks, the lesions were photographed again, then blood samples were taken and the animals were sacrificed. The area of the lesion of the left buccal pouch was fully excised, as was the mucosa of the right (healthy) buccal pouch.

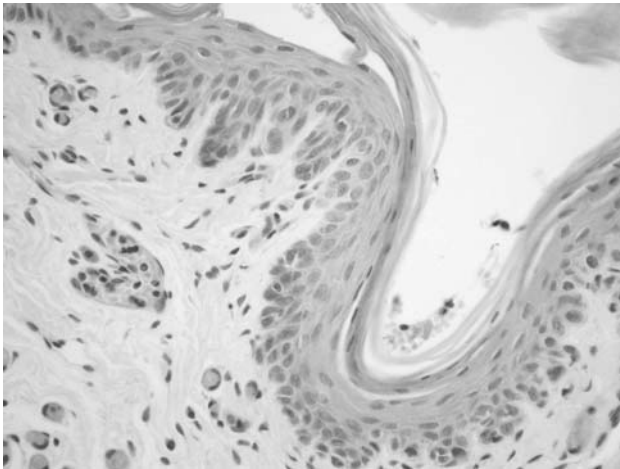


Figure 1. Mildly dysplastic squamous keratinized epithelium (H-E, $\times 400$).

The specimens were again fixed in formalin 10%, embedded in paraffin and finally cut into sections and stained with hematoxylin and eosin in order to be studied histologically.

Results

After the first phase of the experiment the specimens were studied histologically in order to confirm the presence of dysplastic lesions (which, according to the current literature, are achieved after 6 to 8 weeks of DMBA application). Indeed, low-grade (mild to moderate) dysplasia developed in all these specimens and was characterized by slight nuclear abnormalities in size and shape, mostly marked in the basal third of the epithelium, with occasionally noticeable nucleoli in the parabasal cells. In the upper layers, the cells showed maturation and stratification. Abnormal mitoses were absent. Hyperkeratosis was frequently present. (Figures 1 and 2).

The specimens that were taken after the application with imiquimod were also histologically studied. The main histological alteration after the application with imiquimod was hyperplasia of the oral mucosal squamous epithelium. Inflammation was occasionally noticed and insignificant. (Figures 3 and 4). Low-grade dysplasia had regressed in all samples.

Interestingly, in only one case in which a well-differentiated squamous cell carcinoma (SCC) had developed after the painting with DMBA, after the administration of imiquimod, a more benign morphology, equivalent to a squamous cell papilloma, was observed (Figures 5-7).

In each case, hyperplasia was detected by comparison to the mucosal epithelium of normal thickness present in the same section and it was found to be mild in all specimens. Consequently, there was no need to perform a statistical analysis to confirm its presence. As far as dysplasia is

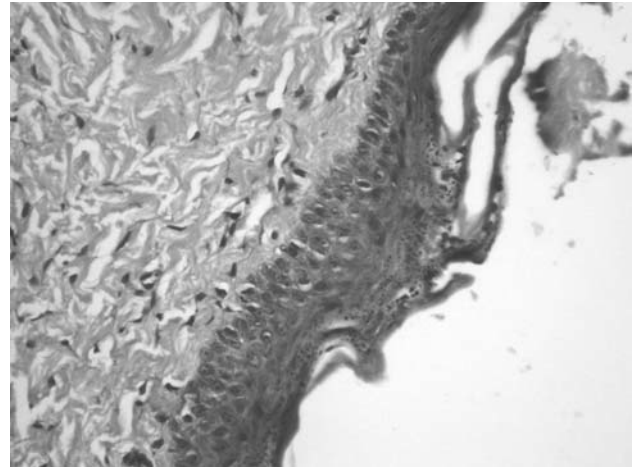


Figure 2. Presence of mild dysplasia in squamous epithelium. Note the absence of any inflammatory reaction (H-E, $\times 400$).

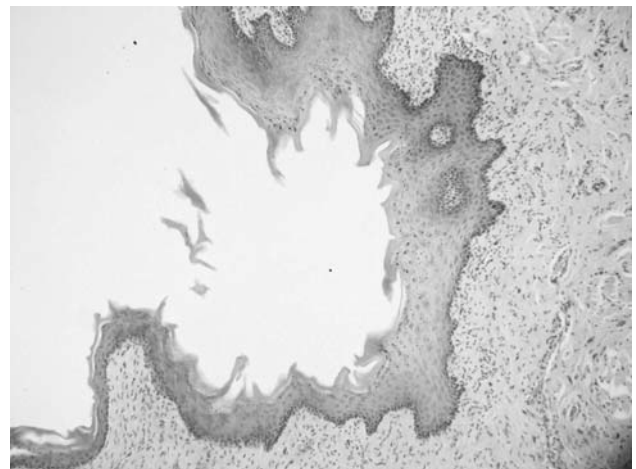


Figure 3. Hyperplastic, mainly acanthotic squamous epithelium. Note the thickness of the normal squamous epithelium on the left (H-E, $\times 100$).

concerned, statistical analysis was not performed because this lesion always was of low grade and occurred in all cases except for the one with SCC.

Discussion

Anogenital and perianal warts (condylomata acuminata) are a very common sexually transmitted disease with a high prevalence rate especially in developed countries (11) and are the result of infection with human papillomavirus (HPV). To date, 20 HPV subtypes have been recognized (12) and types 16 and 18 represent the high-risk variants capable of inducing dysplasia and squamous intraepithelial neoplasia. Among several therapies for genital warts imiquimod represents a well-established therapeutic agent with an acceptable safety profile.

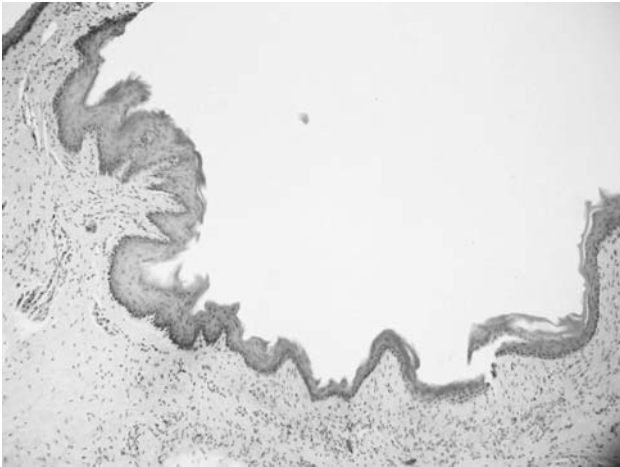


Figure 4. Hyperplastic squamous epithelium on the left, by comparison to squamous epithelium of normal thickness on the right (H-E $\times 100$).

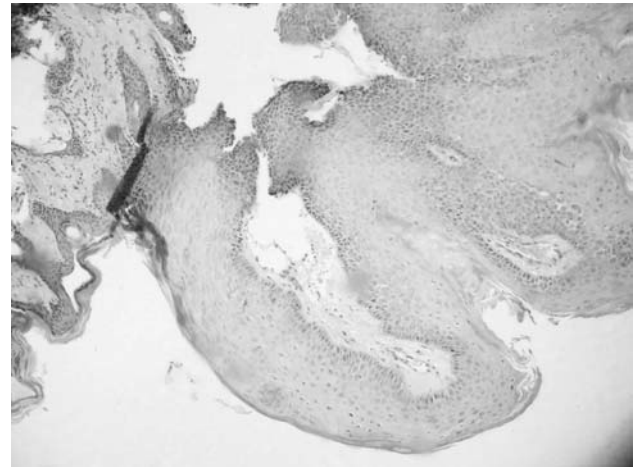


Figure 6. Squamous cell papilloma with keratinization. Note its exophytic growth pattern, in relation to normal squamous epithelium on the left (H-E $\times 100$).

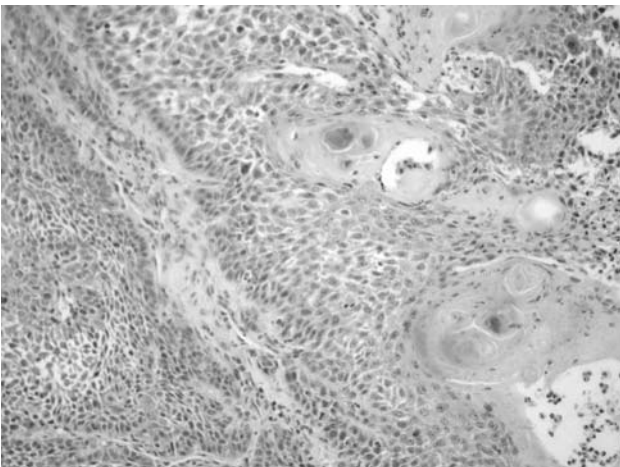


Figure 5. Well-differentiated SCC. Increased keratin production by the malignant cells. (on the right)(H-E $\times 400$).

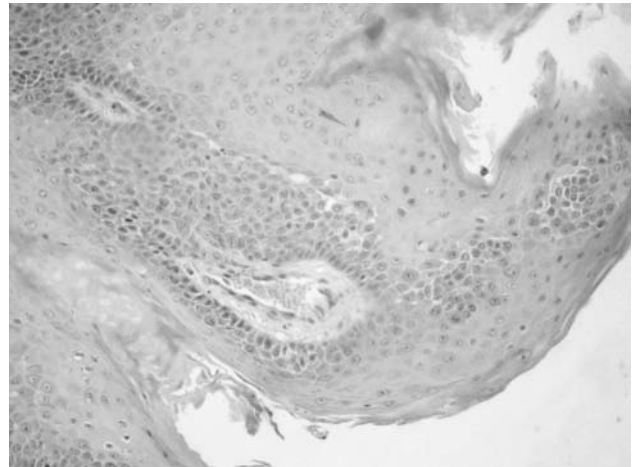


Figure 7. The papilloma structure of the previous neoplasm and the normal pattern of maturation in most squamous cells (H-E $\times 200$).

Many randomized clinical trials, case reports, non- randomized clinical trials, progressive and retrospective studies have been published, as well as review papers. According to one of the latest review papers (2009), (13) imiquimod should be applied 3 times a week for 16 weeks and achieves complete clearance of warts in more than 50% of patients. Recurrence rates are 19% after 3 months and 23% after 6 months of therapy (13). More studies are needed to compare the efficacy of combination therapy vs. monotherapy. Longer follow-up is also needed to evaluate recurrence rates after monotherapy as well as in combination with other treatments for anogenital warts.

AK is a dermatological lesion that is considered to be premalignant with a risk of evolution to SCC, being part of the continuum of a multi-step carcinogenesis process (14). The rate of A.K. that may transform to SCC varies in the

literature from 0, 1% to 5%. Since 2004, imiquimod has been used to treat AK with a therapeutic rate for complete clearance of about 70%. There are several randomized clinical trials, review papers and three meta-analysis studies to our knowledge that support imiquimod as a reliable treatment option among others including cryotherapy, topical 5- fluorouracil, photodynamic therapy, retinoids, curettage, surgical excision, laser-ablation, chemical peels/resurfacing procedures . In AK treatment, imiquimod is used 3 days per week for a period of 4 to 8 weeks (14, 15).

SBCC is treated successfully with imiquimod, according to randomized clinical trials that report even 100% cure rate (16). Nodular BCC is more difficult to treat with imiquimod, most likely because of the skin barrier and the deeper localization of tumour cells (17).

As imiquimod appears to be efficient in many dermatological disease entities in which the immune system is involved, it has been tried in numerous uses besides its licensed indications ('off label' uses). These include: invasive SCC (6 studies) (18), Bowen's disease (SCC *in situ*), extra mammary Paget's disease, metastatic melanoma, lentigo maligna, mycosis fungoides, erythroplasia of Queyrat, vulvar intraepithelial neoplasia (16), keratoacanthoma. For most of them there are only case reports in the literature, while for vulvar intraepithelial neoplasia and lentigo maligna there are non-randomized clinical trials. Randomized clinical trials are published for genital and perianal warts, actinic keratosis, SBCC and Bowen's disease (17).

As far as our field of interest is concerned, which is the oral mucosa and its dysplastic lesions, the current literature is very poor. More specifically, there are only six published studies to our knowledge concerning oral mucosa. One of them reports the malignant conversion of florid oral and labial papillomatosis during immunotherapy with imiquimod (19). The second reports oral bowenoid papillosis in an HIV-positive male, where no resolution of the lesions was observed after imiquimod was applied (20). The effectiveness of imiquimod in treating viral infections and among them oral hairy leukoplakia (Epstein-Barr virus) in HIV-positive patients was estimated in another paper (21). But the most encouraging studies are the following: In a case report of a man with intraepithelial melanoma of oral mucosa with many *in loco* recurrences, the application of imiquimod on a new recurrence proved efficacious after six months of therapy (three times a week) (22). In another study, 3 cases of focal epithelial hyperplasia (rare disorder caused by specific types of HPV) involving oral mucosa were successfully treated with imiquimod (23). And finally a case report of two women with HPV-associated oral leukoplakia reported effective therapy with imiquimod applied three times per week for 6 and for 12 weeks (24).

Our study is thus the first experimental attempt to use imiquimod as potent therapeutic agent in oral mucosal dysplastic lesions. Our experiment is original for the use of imiquimod in an experimental oral carcinogenesis model. Our aim was to study any possible regression of premalignant lesions after the administration of imiquimod. Our results are encouraging, as the hyperplastic lesions observed after imiquimod was applied represent a non-malignant transformation of oral mucosa, compared to those observed after the application of DMBA. We even observed the transformation of a SCC to a papilloma, a lesion of benign morphology with significantly less malignant potential. These results imply that imiquimod may actually be efficacious on oral mucosal dysplasia, and thus inhibit the progress of carcinogenesis. Of course, more clinical and experimental studies are necessary before this use can be established.

Our study is still in progress, as immunochemical examination of the tissues is being performed, with an ultimate goal of elucidating the exact mechanism of action of imiquimod and in particular, its role in apoptosis.

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