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THE ROLE OF NITRIC OXIDE SYNTHASE IN THE MODULATION OF THE IMMUNE RESPONSE IN ATOPIC DISEASE

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Abstract

Present study aimed to determine the effect of endothelial and inducible nitric oxide synthase on the inflammatory process of soft tissues in the oral cavity of experimental animals in the modulation of atopic disease. To simulate the atopic process, young experimental animals (three-month-old male rabbits) were sensitized by intraperitoneal injection of ovalbumin and aluminum hydroxide during the first 3 days of the experiment. Twice lower dose of ovalbumin was instilled intranasally under local anesthesia five days later (Day 8) with repeated intranasal administration of ovalbumin on the 16th, 17th, 20th and 21st day of the experiment. Obtained specimens of oral cavity were examined histologically, and immunohistochemical study was performed to determine the immunoreactivity of eNOs, iNOs, CD23, CD20.

Histological investigation of obtained microslides detected that atopic modeling process is implemented by a complex of pathological changes of oral mucosa with the presence of intraepithelial lymphocytes, eosinophils, focal erosive lesions, signs of proliferation of the basal cell layer, moderately expressed papillomatosis. Such histological picture can be interpreted as the development of inflammatory, degenerative, dyscirculatory process. It was found that such changes are accompanied by disturbance of nitric oxide synthase metabolism, characterized by increased activity of inducible nitric oxide synthase more than twice and endothelial synthase in the extravascular space. Inflammatory infiltrate in atopic process is presented by B-lymphocytes, activated macrophages, eosinophils both in the lamina propria and epithelium that is indicated by a sharp increase in the immunoreactivity of CD23 and CD20. Accumulation of these cells has strong correlation dependence with nitric oxide synthase. The most pronounced correlation has been detected between CD23 and CD20 (r=0.89), iNOs and CD23 (r=0.85), iNOs and CD20 (r=0.87) while comparing the results of immunohistochemical study with eNOs, iNOs, CD23, CD20. The obtained data can be used as a basis for the development of preventive measures in patients with atopic diseases, based on the correction of disturbed nitric oxide metabolism.

Keywords: *atopy, nitric oxide synthase, experiment, oral cavity, inflammation.*

Introduction

Atopic conditions are characterized by inherited peculiarity to produce immunoglobulin E antibodies as a response to small amounts of common environmental proteins such as pollen, house dust mite and food allergens [*Thomsen S, 2015*]. Today, many aspects of the development of bronchial asthma, allergic rhinitis and atopic dermatitis remain unexplored, and there is a necessity for further experimental studies to clarify the pathogenesis of atopic diseases and creation of primary prevention and pathogenetically based treatment of patients suffering from atopic diseases including their clinical manifestations in oral cavity [*Staab D et al., 2006; Thomas M et al., 2010*]. As the frequency of atopic dermatitis in children is about 15% [*Scharschmidt T, Segre J, 2008; Krivenko L, Nazaryan R, 2015*], and the presence of this pathology entails the increase of carious lesions [*Bezruk V et al., 2015*], the study of the pathogenesis of atopic changes becomes a goal not only for allergists, internists, pediatricians, but also for dentists.

One of unclear aspects in the pathogenesis of

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atopic processes remains the disturbance of nitric oxide metabolism with its involvement in the immune processes, including the processes in the oral cavity [*Evereklioglu C et al., 2002; Yildirim M et al., 2004*].

Present study aimed to determine the effect of endothelial and inducible nitric oxide synthase on the inflammatory process of soft tissues in the oral cavity of experimental animals in the modulation of atopic disease

Material and methods

In order to study the morphofunctional state of the tissues of the oral mucosa in atopic disease, an experimental study was carried out, that allows to eliminate the influence of somatic pathology and social factors. Ovalbumin was used to simulate atopic process according to the previously proposed and widely used scheme [*Yoshida M et al., 2002; Cho S et al., 2008; Kim H et al., 2014*]. To simulate the atopic process, young experimental animals (three-month-old male rabbits) were sensitized by intraperitoneal injection of ovalbumin and aluminum hydroxide during the first 3 days of the experiment. Twice lower dose of ovalbumin was instilled intranasally under local anesthesia five days later (Day 8) with repeated intranasal administration of ovalbumin on the 16th, 17th, 20th and 21st day of the experiment. Doses of used medicine were determined according to animal body weight. We formed two groups with 8 animals each – intact animals and group of animals with simulated atopy. Two groups with 8 animals each were formed – group of intact animals and group of animals with simulated atopy.

The specimens of soft tissues of the oral cavity were stained with hematoxylin and eosin, picrofuxine according to van Gieson after the routine proceeding. Immunohistochemical study was performed by indirect immunoperoxidase reaction with monoclonal antibodies to endothelial and inducible NO-synthase fractions (eNOs and iNOs, respectively), CD23 (detected on mature B cells, activated macrophages, eosinophils, has affinity with immunoglobulin E [*Kijimoto-Ochiai S, 2002*]), CD20 (coreceptor located on the surface of all B-lymphocytes [*Janas E et al., 2005*]). All used monoclonal antibodies are manufactured by Thermo Scientific (USA). The reaction was visualized using a set of UltraVision LP Detection System HRP Polymer & DAB Plus Chromogen (Thermo Scientific, USA). All microspecimens were performed in the Department of Pathological Anatomy of the Kharkiv Medical Academy of Postgraduate Education.

The microslides were studied using "Olympus BX-41" microscope (Japan) with subsequent processing by "Olympus DP-soft version 3.2" software (Japan), which was used both for definition of the intensity of immunohistochemical reactions and for morphometric study. The intensity of immunohistochemistry was analyzed by detecting the optical density of relevant morphological structures in conventional unit. Morphometric study was performed by overlaying a grid with a square cell (side 10^{-4} *m*) and detecting the density of cellular elements of inflammatory series, including the immunopositive staining of CD23, CD20.

Statistical analysis of the study results was performed on a personal computer using Microsoft Excel and Statistica-10 database software. The criteria of non-parametric statistics were used in order to assess the significance of differences in sample populations. Statistical comparison was performed using Mann-Whitney test for statistical analysis. Spearman's rank correlation coefficient (r) was counted for measure of the strength of a relationship between paired data. The accepted level of significance was $p<0.05$.

The study was approved by Institutional Bioethics Committee and conforms to the principles of the Guide for the Care and Use of Laboratory Animals published by US NIH (No 85-23, revised in 1985).

Results

Histological investigation of obtained microslides detected that atopic modeling process is implemented by a complex of pathological changes of oral mucosa. Squamous epithelium is characterized by uneven thickness with the presence of intraepithelial lymphocytes, eosinophils, focal erosive lesions (Fig. 1), signs of proliferation of the basal cell layer, moderately expressed papillomatosis.

Perivascular inflammatory infiltrates, diffuse distribution of eosinophils, swelling of connective tissue fibers have been revealed in the lamina propria. Microcirculation is characterized by uneven blood supplying with the presence of predominantly dilated vessels.

Immunopositive tissue to eNOs is detected primarily in the wall of the microvasculature both in the study group and the group of intact animals. Whereby, longitudinal and transverse sections of vessels in both groups of animals are characterized by eNOs accumulation primarily in the endothelium. At the same time the group of animals with simulated atopy, eNOs immunoreactivity is detected between the vessels, while such pattern is observed in the lamina propria and underlying muscle plate (Fig. 2).

Determination of the optical density of the dyestuff accumulation indicates that the activity level of eNOs did not significantly differ in the vascular wall of experimental animals of both groups, while extravascular localization of eNOs is significantly higher in the group of animals with simulated atopy (Table).

More significant differences are identified while comparing the results of peroxidase reaction with iNOs. So, more pronounced rate has been revealed in the group of rabbits with the modeling of atopy that is morphometrically confirmed by almost double growth (Table). In this case there are areas both with diffuse and focal immunopositive amplification of stained tissues. The presence of local zones of increased immunoreactivity led us to assume that such changes could be the result of the activation of inflammatory cells. The fact of most active iNOs localization around the inflammatory cells in epithelial layer (Fig. 3) and in the lamina propria could be used as evidence of such immunomodulatory interactions.

Most pronounced activity of iNOs had been detected in the affected areas of the lamina propria and was associated with perivascular inflammatory microinfiltration, and the level of immunoreactivity intensity was associated with the quantitative and qualitative composition of the cellular infiltration into tissues in atopy.

Study of the density of cellular elements with immunoreactivity to monoclonal antibodies of CD23 protein was interesting as it is protein, which, as stated above, is detected on mature B-cells, activated macrophages, eosinophils, and that is important for studying atopic process, has affinity to immunoglobulin E. It is established that such elements in the group of intact animals are found as single cells in the lamina propria. In the group

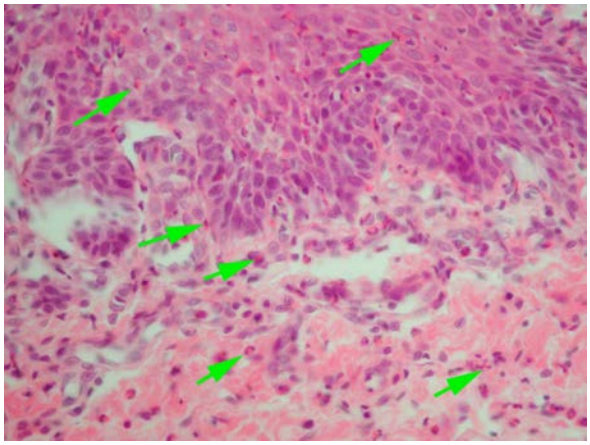

***FIGURE 1**. Uneven thickness of squamous epithelium with the presence of intraepithelial lymphocytes, eosinophils (indicated by arrows), proliferation of basal layer cells. The presence of perivascular inflammatory infiltrates with eosinophils and their diffuse distribution in the lamina propria, swelling of connective tissue fibers. Hematoxylin and eosin stain. Objective ×40*

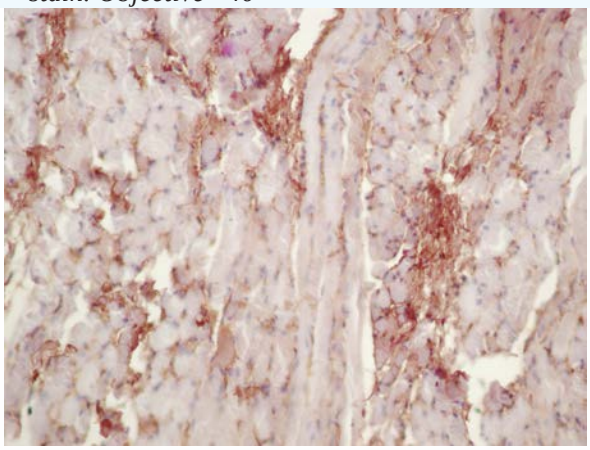

***FIGURE 2.** Localization of eNOs is observed not only in the microvasculature, but in the perivascular space of lamina muscularis mucosae. Peroxidase reaction with monoclonal antibodies to eNOs. Objective x20*

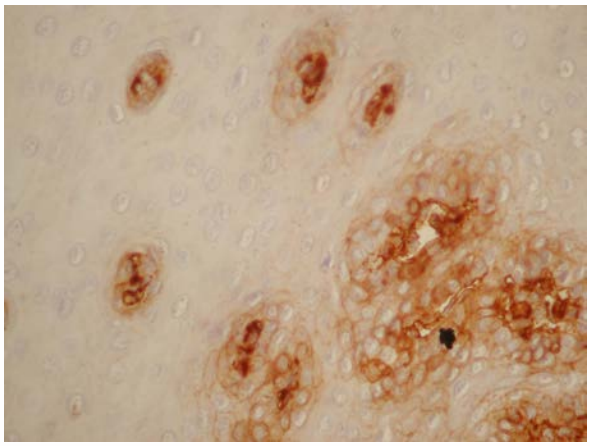

***FIGURE 3.** Pronounced activity of iNOs around the inflammatory cells including those located intraepithelially. Peroxidase reaction with monoclonal antibodies to iNOs. Objective ×40*

Table

Morphometric indicators of the activity of immunohistochemical reactions

Groups	eNOs in vascular wall (*c.u.*)	eNOs of extravascular localization (*c.u.*)	iNOs (*c.u.*)
Intact animals	0.899±0.061	0.193±0.05	0.241±0.052
Animals with simulated atopy	0.793±0.112 1	0.271±0.041*	0.499±0.073*

Note: ** – p<0.05 compared to the intact animals*

of animals with simulated atopy their distribution differs both qualitatively and quantitatively. First of all, almost all intraepithelial inflammatory cells were positive to CD23. Majority of the cellular elements were also proved to be immunopositive to CD23 in the lamina propria, where the density of cells with CD23 nuclear staining was 7.7 times higher than in intact animals.

When studying the distribution of CD20 (the co-receptor located on the surface of B-lymphocytes), changes are found that are qualitatively similar to those observed in the study of CD23 (the appearance of immunopositive cells in the epithelium and a sharp increase in their number in the propria of the mucosa) with membrane localization of the dye.

Studying of CD20 distribution (co-receptor located on the surface of B-lymphocytes) revealed qualitatively similar changes to those observed while studying CD23 (appearance of the immunopositive cells in the epithelium and a sharp increase in the number of lamina propria) with membrane substance localization. Comparison of the CD20 cell density of intact animals and animals with atopy showed an increase of 8.9 times.

Comparison of the immunohistochemistry results for eNOs, iNOs, CD23, CD20 revealed the most pronounced correlation between CD23 and CD20 (r=0.89), iNOs and CD23 (r=0.85), iNOs and CD20 (r=0.87).

Discussion

Increased activity of nitric oxide synthase in oral mucosa in atopic process noted in our work is combined with previously published data about increasing NOs in the skin in atopic dermatitis [*Kubo M et al., 2005*], allergic conditions [*Ten Broeke R et al., 2006*]. Thus, our Japanese colleagues [*Kubo M et al., 2005*] indicate an increase of both fractions of nitric oxide synthase in the skin in atopic dermatitis, including eNOs in the vascular wall, whereas in our study, we observe a slight decrease of this parameter. At the same time, M. Kubo and co-authors [*Kubo M et al., 2005*] hypothesize about metabolic pathways of NO in atopic skin lesions, in case of hyperplasia of the epidermis, the suppression of neuronal NOs expression realized in reducing NO production. In the dermis, activity of eNOs and iNOs is increased with NO production mainly due to the formation of reactive nitrogen forms, which leads to the formation of nitrotyrosine. In general, it can be argued on a similar picture of changes in nitric oxide synthesis in atopic states by activating primarily iNOS, in particular, the same pattern as in this study in the oral mucosa, as in the cited above work in the skin, in nasal mucosa in allergic rhinitis [*Kawamoto H et al., 1999; Oh S et al., 2003*]. Considering the identified strong correlations between CD23 and iNOS, iNOS and CD20, it can be argued on the active participation of nitric oxide in the modeling of humoral inflammatory response, which is the main process in the atopic process development [*Thomsen S, 2015*]. A special feature of present study is identification of NOs not only in mast cells, which are considered the main place of production of nitric oxide synthase [*Gilchrist M et al., 2004; Yip K et al., 2008*].

Simultaneously, there are available data about enhancing communication of iNOs activity not only with inflammatory cells, but also with cytokeratin-positive cells [*Kawamoto H et al., 1999*]. Immunoreactivity of iNOs occasionally was detected in relatively large mononuclear cells for non-epithelial populations staining with cytokeratin negative, while the majority of other cells including neutrophils and small lymphocytes are characterized by the absence of iNOs reaction [*Kawamoto H et al., 1999*]. The last one, taking into account the distribution of the CD20 and CD23 proteins, is also combined with obtained results.

Thus, we have described the morphological changes in the tissues of the oral mucosa which are usually regarded as a manifestation of atopic process with the development of inflammatory, degenerative, dyscirculatory processes, metabolic disorders, development of which has been involved actively disturbance of nitric oxide metabolism and which can serve as a basis for the development of preventive measures in patients with atopic diseases based on the correction of violations of nitric oxide.

Conclusion

Atopic process in the oral cavity is characterized by morphological picture with inflammatory, degenerative, dyscirculatory changes which are accompanied by disturbance of nitric oxide synthase. The activity of inducible nitric oxide fraction is increased more than twice in the oral mucosa.

In case of atopic processes in the oral cavity, the morphological picture is characterized by inflammatory, degenerative, dyscirculatory changes accompanied by disturbance of nitric oxide synthase. The activity of inducible nitric oxide fraction in the oral mucosa is increased more than twice.

Accumulation of inflammatory infiltrate in atopic process presented by B-lymphocytes, activated macrophages, eosinophils, has strong correlation dependence on the activity of inducible nitric oxide fraction.

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