Expression of Clusterin in the Salivary Gland under Restraint Stress

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The belief that stress leads to illness has a long history. A number of the orofacial disease are also closely associated with stress. Despite research in the relationship of stress and the orofacial diseases leading to statistically significant correlations, the pathology remains vague. In the present study, the expression of clusterin, a stress protein responsible for antiapoptosis and cytoprotection, under restraint stress condition was observed in the submandibular gland, one of the major salivary glands.

Sprague-Dawley rats were divided into 2 groups: normal group and restraint stress group. The rats of restraint stress group were placed in the stress cages and then sacrificed at day 0, 3 and 5 day of the experiment. After that, the submandibular glands of all the rats were excised immediately. The levels of clusterin proteins and mRNA in the tissues were measured by immunohistochemistry and Northern blot analyses, respectively. The results were as follows:

1. In the immunohistochemistry, clusterin protein was detected only immediately after the application of restraint stress.
2. In the restraint stress group, at day 3 and 5, histologically apoptosis was induced with karyorrhectic and pyknotic changes.
3. By the restraint stress, acinic cells were destructed earlier than ductal cells.
4. In the Northern blot, mRNA of clusterin was expressed only immediately after the application of restraint stress.

The overall results suggest that as an early response to stress, clusterin is expressed in the glands to protect the glandular cells from the stress. But if stress is so strong and prolonged that it can exceed the stress adaptability of the cells, then the cells may undergo apoptosis instead of producing clusterin. An Epidemiologic Study of Symptoms of Temporomandibular Disorders in Korean College Students.

Key words : Restraint Stress, Salivary Gland , Clusterin, mRNA

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Ⅰ. INTRODUCTION

Today, stress, the nonspecific response of the body to any demand,[1] and other psychological factors are assumed to play an important role in the major health problems in several countries, and increasing attention has been addressed to the relationship of stress and diseases during the past several decades. Biologists and medical scientists tend to be concerned with sources of stress that are concrete and observable and can otherwise consider that as causes of diseases and injury. In contrast, social and behavioral sciences are inclined to regard sources of stress as information that arises from outside the person mediated by higher centers of the central nervous system.[2]

The physiologic stress reaction involves a complex interaction of the nervous and endocrine systems, and so affects various organs.[3,4] Virtually various organ injuries by all forms of stress or some pathologic stimuli start with molecular or structural alterations in cells; cellular adaptive response, cell stress response (production of stress protein), reversible cell injury, and irreversible cell injury such as apoptosis (programmed cell death) and necrosis.[5,6] All such cellular defensive responses to injury not only play an important role in physiological situations but also limit damage in response to disease processes. Thus it is clear that stress ca lead to alterations of internal functions down to the cellular and the molecular level, and stress is the potential cause of disease.

There are many stress-related symptoms and diseases in the orofacial tissue such as lichen planus, aphthous stomatitis, geographic tongue, recurrent herpes labialis, xerostomia, halitosis, burning mouth syndrome, temporomandibular disorders, muscle tension headache, and atypical odontalgia.[7] And that is the reflection of the fact that the orofacial tissue is emotionally charged or highly reactive to psychologic influences, representing directly or symbolically our major instincts and desires-food, sex, hostility and so on.

Despite extensive and intensive research in the relationship of stress and the orofacial diseases leading to statistically significant correlations, however, the pathological evidence remains vague. Especially, xerostomia and halitosis are closely related to the function of the salivary glands. Therefore, it is important to study the stress-induced functional changes of the salivary gland and the mechanisms involved in the changes. To have a better understanding of the relationship between stress and the salivary gland dysfunction, we have developed an experimental model in which expression of clusterin in the salivary gland can be altered by stress.

Clusterin is a puzzling, multifunctional and ubiquitous sulphated glycoprotein. This protein is known to cause cell aggregation, lipid transport, sperm maturation, regulation of the complement cascade, membrane recycling, and apoptosis.[8-10] A number of studies have focused on the particular close relationship between clusterin expression and apoptosis,[11-14] then they have provided a possible explanation for high sensitivity of clusterin expression to environmental changes and allowed the classification of clusterin as an extracellular version of a stress protein (heat shock protein).[11] Clusterin, expressing at the various membrane boundaries between fluid and tissue compartment, is likely to serve protecting barrier cell membranes under noxious conditions. Therefore, aberrant expression of clusterin could be deleterious.[15]

The present study using the animal model under restraint stress condition was performed to observe the expression of clusterin in the submandibular gland, one of the major salivary glands by immunohistochemical and Northern blot analyses.

Ⅱ. MATERIALS AND METHODS

1. Experimental animals and tissue preparation

Sprague-Dawley, 10-week-old rats were purchased from Dae-Han Experimental Animal Research Center, Seoul, Korea. They were maintained at 20-23℃ and fed *ad libitum* on a

normal laboratory diet. The rats were divided into 2 groups: normal group and restraint stress group. The rats of restraint stress group were placed in the stress cages throughout the period of the experiment. All the rats were sacrificed at day 0, 3 and 5 day of the experiment and the submandibular glands were excised immediately. The tissues were either fixed and processed for immunohistochemistry or rapidly frozen by storing at -70℃ for Northern blot analysis. After fixation in Bouin solution overnight, the tissues were embedded in paraffin resin for immunohistochemistry. Serial paraffin sections (4~6㎛) were cut, placed on poly-L-lysine coated slides, and stored at -70℃ until use.

2. Preparation of clusterin antibody

Prior to raising antibody against clusterin, a synthetic peptide corresponding to the sequence of 144-158 amino acids (NH_2-GDRIDSLMENDRQQS-COOH;1865.9 atomic mass units) from the porcine clusterin α-subunit was prepared by Fmoc peptide synthesis procedure and purified by repeated HPLC. The α-peptide (2.2mg) was conjugated to 2mg of cathionized BSA using SuperCarrier EDC system(Pierce Rockford, IL, USA). A New Zealand white rabbit was injected at multiple sites subcutaneously with the conjugated peptide-carrier in complete Freund's adjuvant. Starting one week after the first injection, the rabbit was boosted weekly for 2 weeks with the same conjugate in incomplete Freund's adjuvant. Ten days after the last third injection, the rabbit was bled and antiserum was collected.

3. Immunohistochemistry

Immunohistochemical analysis was performed on the paraffin section by ABC (avidin-biotin-peroxidase complex) method as described previously using the anti-clusterin α-peptide antiserum[16]. Briefly, the sections were deparaffinized in xylene, hydrated, washed in phosphate buffered saline (PBS), and incubated twice in methanol containing 0.5% H_2O_2 for 5 min each at room temperature. After rinsing with PBS, slides were incubated in PBS containing 0.1% Triton X-100 for 10 min, and then immersed in normal goat serum diluted with rabbit IgG anti-clusterin peptide for 24 hr at 4℃. After washing with PBS, biotinylated goat anti-rabbit immunoglobulin was applied to the slides for 1 hr at room temperature. The slides were then washed in PBS and incubated with avidin-biotin-peroxidase complex (ABC kit ; Vector Lab., Burlingame, CA, USA) for 1 hr. Thereafter, immunohistochemical reactions were detected by color development using diaminobenzidine solution (100mM Tris, pH 7.4, 0.01% H_2O_2, 0.05% diaminobenzidine hydrochloride).

4. Northern blot analysis

Total RNA was isolated from the submandibular and the parotid glands by the acid guanidium thiocyanate and phenol/chloroform extraction method as described by Chomezynski & Sachci.[17] Total RNA (10㎍) was denatured and fractionated in a 1.2% agarose gel containing 0.67M formaldehyde, and then transferred to positively charged Hybond-N^+ nylon membrane (Amersham, Arlington Heights, IL, USA) by capillary action with 10×SSC. The membranes were baked at 120℃ for 30 min and prehybridized in hybridization buffer (50% formamide, 1.5×SSC, 5×Denhardt's reagent, 1.0% SDS, 20mM sodium phosphate, pH 7.0, and 100㎍/ml yeast RNA) at 55℃ for 2 hr. T7 RNA polymerase was used to generate [α-^{32}P] UTP-labeld cRNA antisense probe (2×106 c.p.m./ml). After hybridization with the probe at 55 ℃ for 16 hr, the membranes were washed twice in 0.5×SSC/0.1% SDS at room temperature for 16 min each, and more stringently once in 0.2×SSC/0.1% SDS at 65℃ for 30-45 min. The membranes were then exposed to X-ray film with an intensifying screen at -80℃ for 2 days.

Ⅲ. RESULTS

1. Immunohistochemistry of clusterin protein in the submandibular glands

To localize the expression of clusterin protein in the submandibular glands, the tissues of each group were fixed and processed for immunohistochemistry using the specific antibody against a clusterin α-subunit peptide.

In the normal control group, clusterin immunoreactivity was detected slightly in the ductal cells (Fig. 1).

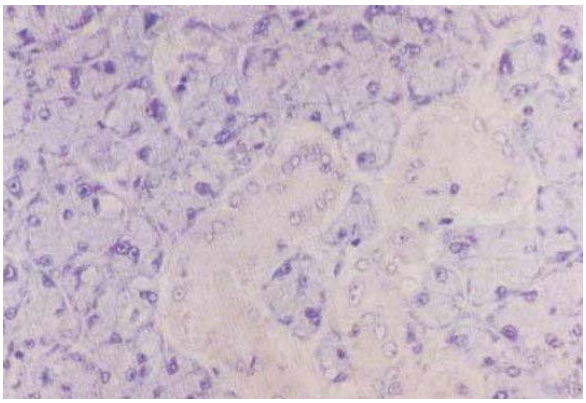

Fig. 1. Immunolocalization of clusterin protein in the submandibular gland of normal rat.

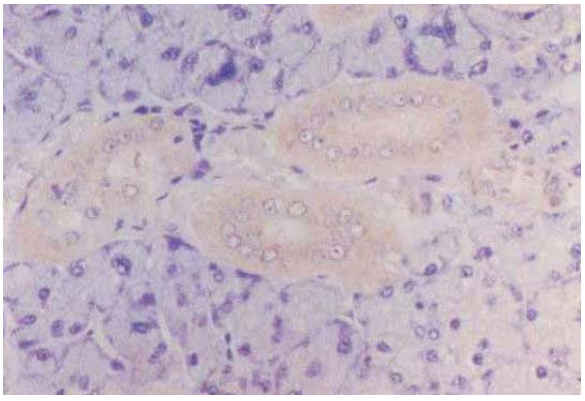

Fig. 2. Immunolocalization of clusterin protein in the submandibular gland of rat under restraint stress immediately after the application of the stress.

In the restraint stress group, clusterin was observed immediately after the application of the stress and then disappeared. However, the clusterin expression was found to be weak. By the restraint stress, histologically, apoptosis was observed, showing karyorrhectic and pyknotic changes. Besides, acinic cells were destructed earlier than ductal cells (Fig. 2~4).

2. Northern blot analysis of clusterin mRNA in the submandibular glands

Northern blot analysis of clusterin mRNA

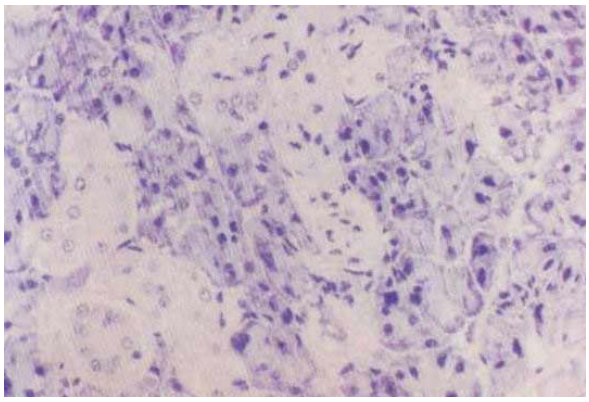

Fig. 3. Immunolocalization of clusterin protein in the submandibular gland of rat under restraint stress at day 3 of the experiment.

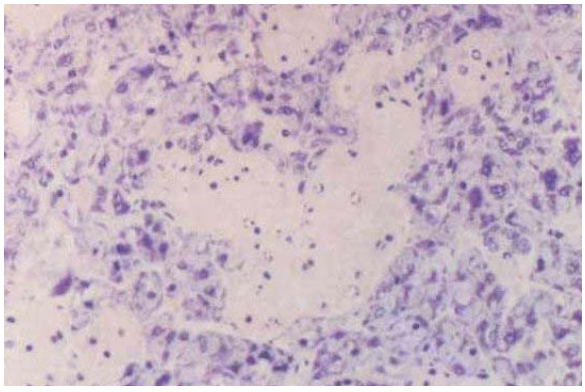

Fig. 4. Immunolocalization of clusterin protein in the submandibular gland of rat under restraint stress at day 5 of the experiment.

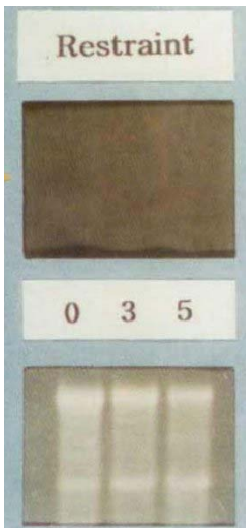

Fig. 5. Northern blot analysis of clusterin mRNA in the submandibular gland of rat under restraint stress.

isolated from the submandibular glands showed a band of approximately 2.0kb in size and the equality of RNA loading was demonstrated by the ethidium bromide staining of 18S rRNA.

In the restraint stress group, mRNA of clusterin was detected only at the day of the application of stress, although the level of the expression was very low (Fig. 5).

Ⅳ. DISCUSSION

The belief that stress leads to illness has a long history.[18,19] Stress is the nonspecific response of the body to any demand[1] and can also be defined as an interactional process between the individual and the environment.[3]

The stress response system, as a vital mediator of the individual's health/illness dynamics at physical, psychological, and social levels, is modeled through a systemic approach. The underlying physiological stress response comprises three main mechanisms[20] such as autonomic nervous system, hormonal system, and immune response system, and affects various organs.[3,4] Virtually various organ injuries by all forms of stress or some pathologic stimuli start with cellular responses; cell adaptive response, cell stress response (production of stress protein), reversible cell injury and ultimately cell death such as apoptosis (programmed cell death) and necrosis.[5,6] These responses enable the cell not only to survive and recover from stressful conditions but also to limit damage in response to disease processes.

It has been shown that stress is strongly related to apoptosis which is a process of genetically programmed alteration of cell structure that leads to failure of proliferation and eventual cell death, and induced by a variety of injurious stimuli.[5,6] The chief morphologic features of apoptosis are cell shrinkage, chromatin condensation, formation of apoptotic bodies and phagocytosis of them by adjacent healthy cells. This morphologic pattern of cell death should be differentiated from the necrosis, the more common type of cell death after exogenous stimuli, occurring after hypoxia and chemical injury, and manifested by severe cell swelling or cell rupture, denaturation and coagulation of cytoplasmic proteins, and breakdown of cell organelles. In contrast, apoptosis is a more regulated event without eliciting inflammation and is induced by various stimuli that are capable of producing necrosis, but when given in low doses induce apoptosis.[5,6] Tomei et al.[21] suggested that psychological stress may impair the function of the immune system through alteration in the ability of cells to initiate apoptosis, which is likely to be mediated by stress-related changes in endocrine function since glucocorticoids are effective inducers of apoptosis in lymphocytes.[22]

As stated above, it is clear that stress can lead down the physiological function even at the cellular and molecular level, so stress can be the potential cause of disease. The evidence that stress may cause disease is established for a large number of factors and diseases.[3] For example, it is assumed that the psychobiological stress responses, such as elevated blood pressure, catecholamine and cortisol levels, accelerate the atherosclerotic process and influence cardiac functioning and thus, contribute to

hypertension, stroke and myocardial infarction.[20,23,24)]

Especially, there are many stress-related symptoms and diseases in the orofacial tissue,[7)] and that is the reflection of the fact that the orofacial tissue is emotionally charged or highly reactive to psychologic influences, representing directly or symbolically our major instincts and desires-food, sex, hostility and so on.

The relationship of stress to the orofacial tissue can be considered in four aspects:[3)] normal physiological and psychological functions of the mouth, stress-relieving orofacial activities, stress-related orofacial disorders and diseases, and stress and dental treatment.

The mouth is partly or completely concerned with normal human physiological and psychological functions like breathing, eating, chewing, drinking, talking, and so on. The stress-relieving orofacial activities include eating, chewing, sucking, talking, shouting, singing, kissing, smoking, medicating and oral placement of foreign objects.

Recently, quite a few studies have suggested that stress is strongly associated with orofacial diseases. Chun and Hong[25)] indicated that stress causes various forms of diseases in the region including orofacial psychosomatic diseases in which emotional stress appears to play a major role (lichen planus, aphthous stomatitis), orofacial diseases in which psychologic factors appear to play a role (erythema multiforme, benign mucous membrane pemphigoid, geographic tongue), orofacial infections where emotional stress is a significant predisposing factor (recurrent herpes labialis, acute necrotizing ulcerative gingivitis), orofacial lesions induced by neurotic habits inflicting trauma (biting of oral tissues, physical trauma with foreign objects, leukoplakia due to smoking, bruxism and clenching), neurotic orofacial symptoms (xerostomia, halitosis, burning mouth syndrome, altered or loss of taste perception, pain or discomfort with no tissue change), and orofacial pain induced by emotional stress (temporo-mandibular disorders, muscle tension headache, atypical odontalgia.

As mentioned, the orofacial area is a primary pleasure zone and that is one reason why some patients and potential patients become upset in the dental situation. The dentist is not only invading this pleasure zone, but he or she is doing it to inflict pain.[26)]

In these days, clinicians confront many patients complaining xerostomia, or halitosis, or the symptoms of burning mouth syndrome, which are common stress-related orofacial symptoms.[27)] Xerostomia is the subjective feeling of dry mouth which may or may not be caused by salivary gland hypofuction. Saliva is an important body fluid which plays several vital roles. In addition to moistening and protecting the oral tissue, it acts as an aqueous solvent necessary for taste and aids oral function as well as the digestion of food. Also, saliva has an antibacterial action which inhibits or prevents the onset of dental caries and inflammation.[27,28)]

Xerostomia is rarely seen in isolation, but with time, the changes of quantity and quality of saliva can be devastating to oral health and may severely affect the general well-being and lifestyle of the patient. In deed, the oral tissues become susceptible to infection, and the ability for oral function may be disturbed. In addition, a number of pathologic conditions can develope, such as halitosis, burning mouth syndrome, gustatory changes and other oral inflammatory diseases.[29,30)]

The salivary glands are controlled by the autonomic nervous system. Parasympathetic stimulation increases salivary flow, while anticholinergic drugs inhibit secretions. However, the sympathetic influence on the salivary gland function is more complex. Generally, sympathetic activity inhibits salivation, but the opposite case is also observed. Emotional stress induces a variety of responses in the body. In most instances alarm-like reactions and depression and anxiety inhibit, while pleasurable, relaxed sensations promote increase salivation.[27,31)] Xerostomia can be caused by various factors that can affect salivation, including the use of xerogenic drugs, aging, radiation therapy,

systemic or local diseases such as Sjögren syndrome and diabetes, dehydration, and psychogenic factors such as emotional stress.[29,32]

Such xerostomia can induce many complications, and so can be regarded as a very important condition in clinic. In spite of studies on the relationship between stress and diseases, the mechanism of the stress-related orofacial diseases remains vague. In this study, to provide it, we have researched stress response of the salivary gland by the expression of clusterin, known as a stress protein, under restraint stress condition.

Clusterin is an intriguing, ubiquitous, and highly conserved glycoprotein. It is secreted as a heterodimer of 70~80kDa glycoprotein, comprising α and β subunits.[33,34] It has potential amphipathic helical domains that allow this protein to bind to hydrophilic molecules, as well as potential heparin-binding domains responsible for interaction with the cell membranes and extracellular matrices.[35]

Although the function of clusterin remains unclear, there has been a speculation that it may play a role in cell aggregation, lipid transport, sperm maturation, regulation of the complement cascade, membrane recycling, and apoptosis.[8-10]

A notable property of clusterin is its massive induction during apoptosis[11-14] in castration-mediated prostatic involution,[36] in several models of kidney injury,[37,38] and in neuronal development.[39] On the basis of its elevated expression in apoptotic tissues, it was originally proposed that the protein might be causally involved in apoptosis.[36] In contrast to earlier notion, however, it has been recently suggested that clusterin expression is not enhanced but rather is down-regulated in the cells undergoing apoptosis,[40] and that its expression in the apoptotic tissue is restricted to the vital neighboring cells.[40,41] Sensibar et al.[42] also provided evidence that clusterin plays a role in the protection of TNF-induced cell death. Therefore, it is likely that clusterin overexpression is a reactive antiapoptotic and cytoprotective response to environmental changes rather than a causative factor in cell death. The cytoprotective mechanism of clusterin has been proposed based on the findings that it inhibits complement-mediated lysis, binds to surface active toxic hydrophobic compounds and neutralize at or near the cell membranes by formation of soluble complex, and preserves the integrity of the barrier as a potent cell aggregation and adhesion molecule.[15,43,44] Since clusterin is associated with various forms of diseases in response to different stress, it should be considered as a nonspecific sensitive cellular response to various tissue insults and a marker of pathological injury. In addition to its cytoprotective functions, therefore clusterin has been thought of as an extracellular version of a stress protein.[11,45]

Clusterin is also observed in the various membrane boundaries between fluid and tissue compartment and thought to play an important role in protecting barrier cell membranes under noxious condition.[15] Therefore, it is conceivable that increased expression of it to some stimuli may represent the cell stress response that enables cells to survive environmental insults. Hong et al.[46] reported that clusterin is expressed in the salivary gland of the rat in the early stage of cold stress but disappeared since then. And they suggested that clusterin is not expressed by prolonged cold stress, because the cells of the tissue may survive and recover from stressful condition owing to clusterin's cytoprotection.

The salivary glands, the major salivary glands including the parotid, the submandibular and the sublingual glands, and the several minor glands, produce and secrete saliva. Saliva is formed by units of secretory cells of which there are 3 types, serous, mucous and seromucous, and is modified by cells of the ductal system as it passes toward the oral cavity.[31] The composition of the final saliva, as well as the primary saliva, is quite possibly unique for each salivary gland and is determined by the nature of the secretory endpiece cells and ductal elements of that gland.[27]

Therefore, when salivary glands are affected by a variety of stimuli, the cells of the salivary glands

may adapt to them by eliciting various cellular responses, such as structural and biochemical transformations. But if the stimulus is lying outside an acceptable range of normality, it may cause such changes at the clinical and subclinical level as alteration in the production, flow rate, the response to stimuli, the components and the immune function of saliva.

In the present study, we examined the expression of clusterin in the salivary glands, the orofacial tissue-fluid interface, under restraint stress condition by immunohistochemistry and Northern blot analyses, in order to inquire the relationship between stress and orofacial diseases.

It was found that clusterin was expressed in the submandibular glands immediately after the application of the stress. However the expression of clusterin was weak. It was also observed that the glandular cells were apparently apoptotic. Interestingly, the ductal cells, that expressed clusterin died later than acinar cells that failed to produced clusterin. The overall results suggest that as an early response to stress, clusterin is expressed in the glands to protect the glandular cells from the stress. But if stress is so strong and prolonged that it can exceed the stress adaptability of the cells, then the cells may undergo apoptosis instead of producing clusterin. In summary, stress may cause apoptosis depending upon degree and duration of stress applied. Clusterin can be expressed as a stress protein to minimize cell damage and ensure cell viability, probably, only if the stress falls within the bearable range for the cells. If the stress far exceed the range, this is not the case. A strong stress may suppress the production of clusterin by the cells but induce apoptosis of the cell. Since stress-induced apoptosis alter the normal salivary physiology, we propose that pathogenic change of salivary gland can be induced by stress and subsequently by apoptosis. It is also proposed clusterin expressed as a part of physiologic stress response can be used as a stress marker.

Since stress-induced apoptosis alters the normal salivary physiology, we propose that pathologic change of salivary gland can be induced by stress and subsequently by apoptosis. It is also proposed that clusterin expressed as a part of physiologic stress response can be used as a stress marker.

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국문요약

구속 스트레스에 의한 타액선 조직내의 Clusterin 발현

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일상생활에서 우리는 항상 스트레스에 노출되어 있으며, 스트레스에 대한 반응은 우리 삶의 질과 건강에 중요한 영향을 미친다. 특히 정서적으로 중요한 구강안면영역에도 다양한 스트레스성 질환이 존재한다. 그러나 스트레스와 질병에 관한 오랜 연구에도 불구하고 스트레스성 구강안면질환의 병인은 아직 명확하지 않다. 이에 저자는 최근 임상의학에서 관심이 집중되어지고 있는 스트레스와 질병에 대한 분자생물학적 접근의 일환으로 스트레스성 구강안면질환의 병인을 규명하고자 본 연구에 임하였다. 즉, 스트레스 단백질로 알려진 clusterin이 스트레스의 생리적 반응으로서 세포보호작용 결과 발현된다는 사실에 기초하여, 스트레스 부여 후 타액선 조직 내에서의 그 발현변화를 추적하였다.

Sprague-Dawley계 웅성 백서 (200-230g/bw)를 구속 스트레스 부여군 (구속장치에 구속한 후 0, 3, 5일에 희생) 및 정상군으로 나누고, 각각 악하선을 적출하였으며, 면역조직화학법 및 Northern Blot을 이용하여 Clusterin의 변화를 관찰하였다. 그 결과는 다음과 같다.

1. 구속 스트레스 부여군의 clusterin 단백질은 실험 즉일군에서 증가되었고, 그 이후에는 감소되었다.
2. 구속 스트레스 부여 3일군과 5일군에서 핵붕괴 및 핵농축 등의 핵변화를 동반한 apoptosis가 유도되었다.
3. 구속 스트레스 부여 결과, 선포세포가 도관세포보다 일찍 세포사하였다.
4. 구속 스트레스 부여군의 clusterin mRNA는 실험 즉일군에서만 미약하게 관찰되었다.

즉, 타액선 조직은 스트레스 단백질인 clusterin을 생산하여 세포를 보호함으로써 스트레스 상황에 적응하지만, 생리적 적응한계를 넘는 스트레스에 노출될 때에는 조직이 apoptosis됨이 확인되었다. 따라서, 본 연구결과는 구강건조증등 스트레스성 타액선관련 증상 및 질환의 병리적 기전에 대한 규명에 도움이 되리라고 생각하며, 또한 clusterin은 향후 생체에 가해진 스트레스에 대한 표식자(marker)로 사용될 수 있을 것으로 사료된다.

주제어: 구속 스트레스, 타액선, 클러스터린, mRNA