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Neutrophil elastase, CD68, HLA-DR and CD105 immunohistochemical expressions of oral verruciform xanthoma

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Abstract
Verruciform xanthomas (VXs) typically present in the oral cavity as a solitary, painless mucosal lesion with a verrucoid surface, and it was considered to be reactive. The etiology and pathogenesis of VXs remains unclear, though many researchers had discussed the mechanism of foam cells accumulation in the subepithelial tissue. The purpose of this study was to investigate the characteristics of VXs and related foamy cells deposition. The average age of all the 28 subjects used in this study was 53.8 ± 17.0 years old, and the most predominant site of location was gingivae. In histopathological and immunohistopathological aspects, the VXs appeared as a nodular mass of macrophage marker CD68 positive foamy cells deposition covered with acantholytic stratified epithelium. Intraepithelial NP57 positive neutrophils infiltration was observed in VXs. HLA-DR positive rate of epithelial cells and micro vessel density in VXs were statistically significant than those of normal mucosae. Consequently, complicate interaction may form a unique histopathological architecture; the persistence of epithelial hyperplasia, neutrophil infiltration in the epithelium, chronic inflammation in the subepithelium and foamy cells deposition in VX.

Key Words: Verruciform xanthoma, Etiology, Immunohistochemical study

Introduction
Xanthomas represent the accumulation of lipid-rich histiocites (macrophages) known as foam cells. Shafer W firstly reported this lesion in 1971 1). Verruciform xanthomas (VXs) typically present in the oral cavity as a solitary, painless mucosal lesion with a verrucoid surface, and was considered to be reactive 2). The original pathogenic mechanism of VXs was proposed by Zegarelli et al. in 1975 3). He speculated that an inciting agent initially damaged the keratinocytes, and leading to degeneration...
and macrophages response. The macrophages distributed in the VXs indicated that >50% of them were of the reparative and matured macrophage phenotypes. Concerning about macrophages filling the papillary mucosa, Positive cytokeratin staining of faint cytoplasmic granules has been observed in these foamy cells, and these granules were surmised fragmented keratin filaments. Helm provided additional evidence that the source of lipid was epidermal origin in the skin. Most immunohistochemical and electron microscopic studies suggested that foam cells represented macrophages, but their origin had been regarded as fibroblasts or melanocytes, but was also controversial. Ji-an Hu proposed that accumulation of macrophages in VXs might be pathogenetically similar to that of atheroma, in which foam cells express CD68. The etiology and pathogenesis of VXs remains unclear.

Therefore, the purpose of this study was to investigate the clinico-, histo-pathological and immunohistochemical characteristics of VXs and related foamy cells deposition.

Materials and methods

1. Subjects
The study comprised 28 cases of resected VXs which diagnosed histopathologically by 3 oral pathologists between 2001 and 2013 at the Department of Diagnostic Pathology in the Nihon-University School of Dentistry at Matsudo Hospital. The resected specimens had been immediately fixed in 10% neutral formalin solution and paraffin embedded blocks had then been prepared according to the standard method. Serial sections (4 μm thick) were prepared from the paraffin blocks for histopathological and immunohistochemical observation.

The present study performed in the patient’s agreement, and paid the sufficient consideration to privacy, the diagnostic outcome, and the management. Informed consent was obtained from all patients before retrieving the pathological specimens.

2. Light microscopy with histochemical and immunohistochemical stainings
Sections were stained with hematoxylin-eosin (H.E.) staining. All 28 cases were observed their histological appearance in sections stained with H.E. Immunohistochemical staining was performed for all cases. Immunohistochemical studies were also conducted using 10% neutral formalin solution-fixed, paraffin-embedded tissue from all cases.
Sections (4 μm thick) were deparaffinized in xylene, and hydrated in graded ethanol solution. The secondary antibody was ChemMATE Envision (DakoCytomation, Glostrup, Denmark), was used for antigen detection. Primary antibodies used were directed against the following antigens: Neutrophil elastase (NP57, 1:100; Dako); CD68 (KP1, 1:100; Dako); HLA-DR Antigen (Tal.1B5, 1:1,000; Dako) and CD105 (SN6h, 1:10; Dako). Antigen retrieval was performed in a pressure pot with citrate buffer solution (pH 6.0 for NP57, CD68 and HLA-DR for pH 9.0, respectively). The sections were developed in a solution of 3, 3′-dianibobenzidine tetrahydrochloride (DAB). Finally, all sections were counterstained with Mayer’s hematoxylin. Positive control for HLA-DR was specimen of the lymphoid tissue that for NP57, CD 105 and CD68 was specimens of inflammatory granulation tissue of the oral mucosa. For evaluation of the immunohistochemical staining technique, as a negative control, mouse IgG1 (dilution: 1: 200, DakoCytomation, Glostrup, Denmark) was used in place of the primary anti-CD105, anti-CD68, anti-NP57 and anti-HLA-DR antibodies was used in place of the primary antibodies. After dehydration and clearing in a xylene-alcohol series, the slides were mounted.

This study was conducted after obtaining patient consent, and attention was paid to maintaining patient privacy (Ethics Committee Approval No. EC14-033).

3. Quantitative morphological study

For determination of new blood vessels, the cell membrane of vascular endothelial cells was stained a dark reddish-brown using the anti-CD105 antibody, and vessels forming a luminal structure were examined under an optical microscope at 200 × magnification.

Quantitative analysis of the microvessel density (MVD) was performed based on the criteria of Weidner et al. using an optical microscope at 200 × magnification. To determine the MVD, slides were screened and three areas with the highest number of stained microvessels (hotspots) were selected. Immunohistochemical results were assessed by combining quantitative concentration analysis, which was performed using Win ROOF Version 3.4 (Mitani Corporation) image analysis software, with microscopic observation. The protein expression level of HLA-DR, NP57 and CD68 was judged as follows, +++: strong positive (20 or less), ++: moderately

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**Fig.2 Immunohistochemical staining for NP57**

The NP57 positive cells were observed in subepithelial connective tissue and epithelial cells of VXs.

**Fig.3 Immunohistochemical staining for HLA-DR**

a: × 10, The HLA-DR positive cells were observed in subepithelial connective tissue and epithelial cells of VXs.

b: × 40, Many HLA-DR positive cells were observed in the epithelium and subepithelial connective tissue of VXs.
positive (21-50), +: weak positive (51-136), ±: very weak (137-178), and -: negative (179 or more).

4. Statistical study
Statistical analysis was performed by using SPSS 11.0J software. For MVD and HLA-DR positive rates, Student t test was performed to check for significant differences in mean values between types. The level of statistical significance was set as less than 5%.

Results
1. Clinico-pathological results
The average age of all subjects was 53.8 ± 17.0 years old, and the range was from 25 to 87 years old consisted 11 male and 17 female. The sites of locations were 20 gingivae, 5 palates, 2 tongues and 1 cheek. The clinical diagnoses were 11 benign tumors, 8 papillomas, 5 squamous cell carcinomas, 3 verruciform xanthomas, 1 leukoplakia.

2. Histopathological results
The histopathological figures were shown in Figs.1. Microscopically, the VXs appeared as a nodular mass of foamy cells deposition covered with stratified epithelium. There is hyperkeraosis, focal parakeratosis, verrucous acanthosis without atypia and elongation of rete ridges in the epithelium and vascular ectasia in the subepithelial tissue of VXs. The accumulation of foam cells or xanthoma cells has numerous vacuoles that contain lipid in the connective tissue papillae between the epithelial ridges. A slight to moderate degree of chronic inflammatory cell infiltration consisting mainly of lymphocytes was often observed in the subepithelial connective tissue where hyalinizations of collagen fibers were occasionally seen. Neutrophils were present sporadically within the epithelium of VXs.

3. Immunohistochemical results
The figures of immunohistochemical results were normal mucosa and the boundary portion of VXs were shown in Figs.2~5. As for NP57 (Fig.2) and HLA-DR (Figs.3), the positive findings were observed in subepithelial connective tissue and epithelial cells of VXs. And in normal mucosa, a positive finding for NP57 was slightly shown. Slight degree of HLA-DR expression by keratinocytes and Langerhans cells in the normal mucosa adjacent to the VXs. Concerning about positive ratio of HLA-DR of epithelial cells, VX was 6.6 ± 2.5%, normal mucosa was 0.7 ± 0.5%, respectively (P<0.01).

As for CD68, it was slightly observed in normal subepithelial connective tissue of the although cluster of many positive cells was appeared in the those of VXs (Fig.4). From the result of CD105 (Fig.5), neovasculation and extension of many small blood vessels were observed by the subepithelial connective tissue of VXs. The MVDs of normal and VXs were 14.1 ± 5.2 and 45.4 ± 8.9, respectively (P<0.05).

Fig.4 Immunohistochemical staining for CD68, × 10
The cluster of many positive cells was appeared in the subepithelial connective tissue of VXs.

Fig.5 Immunohistochemical staining for CD105, × 20
The neovasculation and extension of many small blood vessels were observed by the subepithelial connective tissue of VXs.
Discussion

VXs are an uncommon benign lesion firstly described in 1971\(^2\). To clarify the mechanism of pathological characteristics of VXs, the clinico- and histopathological studies and immunohistochemical analysis were performed. It occurs mainly in the oral cavity, although any mucosal site can be involved. The gingiva is the most predominant location\(^9\). Macroscopically, VXs were presented as solitary, asymptomatic, slow-growing, flat or slightly raised, papules with a papillary or verrucoid surface, with a yellow or whitish color, and this observation was similar to the previous report\(^4\).

Concordance rate of clinical diagnoses was 10.7% with VXs, and 17.9% cases were macroscopically judged squamous cell carcinomas in this study. The malignant-looking feature of VXs was surmised from their papillary surface with acceleration of keratinization. The pathological process of VXs might be based on an inflammatory response. However, the true pathogenesis remains to be recognized. Oliveira et al. found that 70% of VXs reported in the oral mucosa were localized in the masticatory mucosa, an anatomic region continuously exposed to trauma\(^10\). Inflammatory and immunologic etiologic factors have been proposed as pathogenic mechanisms of VX\(^9,11\). On the basis of ultrastructural findings, a hypothesis of the etiology proposed that VXs was inflammatory in origin\(^3,12\). Many reports had suggested that VXs were induced either by bacterial colonies or by HPV\(^3\), but Ersahim C denied these associations\(^13\). In addition, numerous studies have failed to detect HPV DNA\(^3,5,7,14,15,16\). In this study, the gingiva of occupied 73.3% (P<0.01), and the relation between the masticatory stimuli and VXs occurrence was concordance with the previous reports\(^2,17\). After attacked by stimuli, some investigators speculated that inflammatory cells might initiate the lesion\(^3,10\). Intraepithelial neutrophilic infiltration presented in VXs, and the positive findings of NP57 were shown in the epithelial cells and subepithelial connective tissue of VXs in this observation. These neutrophils may be attracted toward the intraepithelial compartment by chemotactic factors released by previously damaged keratinocytes in situations with inflammatory response\(^3\). Human neutrophil elastase (NP57) is an anti-microbial enzyme contained within azurophilic granules in neutrophils\(^18\), and release by acute neutrophil-mediated inflammation. During inflammatory reaction, activated endothelial cells release may signaling cytokines and present adhesion molecules that induce functional changes in neutrophils. And platelet activating factor and interleukin-8 are expressed by active endothelial cells allowing paracrine activation of neutrophil degranulation\(^19\). The numbers of neovascularization in the stroma in VXs were significantly higher than that in the normal mucosa in the present study.

Cluster of cells with positive reaction for anti-macrophage antigen CD68 was observed in the subepithelial connective tissue in VXs in these cases, and this result was concordance with the previous reports\(^10,20,21\). Concerning about deposition of foam cells, Zagarelli et al. proposed that initial damage to keratinocytes by an inciting agent and subsequent keratinocyte degeneration release of lipid material that is ultimately scavenged by the macrophages\(^3\). The neutrophils then accelerate the keratinocyte damage, resulting in the release of phospholipids, which components of cytoplasmic and organelle membranes, and formation of foamy cells by macrophage phagocytosis\(^22\), and this phenomenon might contribute to the cluster of macrophages in adjacent stroma.

In addition, the possibility that VXs might represent a local cell-mediated immunologic disorder had also proposed\(^16,21\). In this study, the foam cells intensely positive for CD68 and infiltrated lymphocytes in VX suggested an immunological response in the tissue. Further, S-100 protein-positive denderitic cells were detected in regional connective tissue. The presence of neutrophils in the epithelial cells alone appears insufficient to induce accumulation of foam cells\(^7,23\). Foam cells in VXs belong to the monocyte-macrophage lineage degrades matrix proteins of the basal membrane of...
the epithelium that promotes the reciprocal induction between epithelium and mesenchyme \(^7\), which may play a key role in the development of VXs\(^9\). In normal tissue, Class II major histocompatibility complex antigen HLA-DR attained Langerhans cells were only rarely observed towards the tips of the connective tissue papillae. Concerning about anti-CD1a, HLA-DR and S-100, only the anti-HLA-DR antibody detected Langerhans cells in both frozen and wax-embedded sections \(^20\). Keratinocytes expression of the HLA-DR, were seen in several inflammatory disorders of skin and mucosa \(^25\), and some previous studies \(^25\) \(^{26}\) described that the distribution of Langerhans cells are related to keratinocyte expression of HLA-DRs. As for positive ratio of HLA-DR of epithelial cells, VX was 6.6 ± 2.5%, normal mucosa was 0.7 ± 0.5%, respectively (p<0.01) in the present study. The relation between foamy cells deposition and the immunoresponse was suggested.

Concerning about the mechanism of verruciform epithelial hyperplasia, Travis et al. also supported the view that the xanthoma cells are macrophages responsible for removal of lipid that accumulated in the subepithelial tissues and that epithelial hyperplasia is a secondary event \(^8\). Mohsin et al. added to this hypothesis, speculating that the degenerating keratinocytes release chemotactic cytokines that attract neutrophils and stimulate rapid epidermal growth \(^9\). Nowparast et al. suggested that the exspholic architecture, affected by the nutrient and metabolism of the epithelial cells, might be leading to a hyperkeratotic change \(^8\).

HLA-DR expressed keratinocytes under inflammation induce hyperplasia of epidermal keratinocytes followed by degenerative changes and accumulation of macrophages. Finally accumulated macrophages scavenge lipids and are converted into xanthoma cells \(^8\).

This complicate interaction may form a unique histopathological architecture; the persistence of epithelial hyperplasia, neutrophil infiltration in the epithelium, chronic inflammation in the subepithelium and foamy cells deposition in VX.

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