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Review

Extracellular Vesicles in Periodontal Medicine: The Candidates Linking Oral Health to General Health

Kaya YOSHIDA[1), Kayo YOSHIDA[2), Mariko SEYAMA[2), Kazumi OZAKI[2), Hirohiko OKAMURA[3)

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Abstract : The term, periodontal medicine is used to describe the multitude of systemic diseases which are regarded to link periodontal disease. The concept of periodontal medicine has been widely accepted today, however, the molecular mechanisms which periodontal diseases impact general health in whole body are not elucidated in detail. Extracellular vesicles (EVs) and outer membrane vesicles (OMVs) are the nano-sized particles released from mammalian cells and bacterial cells resectively, which influence the health and various disease by transporting biological factors to the neighbor and distant cells. In this review, we will discuss whether EVs and OMVs produced in periodontal diseases could be implicated in periodontal medicine.

1. Introduction

The links between periodontal diseases and various systemic diseases, including diabetes mellitus, cardiovascular diseases, rheumatoid arthritis and chronic obstructive pulmonary disease, have been well accepted today[1-4]. "Periodontal medicine" is a general term for these systemic diseases, and is used to describe how periodontal condition impact extraoral and systemic health[5]. It has been suggested that the induction of inflammatory cytokines and immune responses by oral bacterial infection in periodontal tissue may be risk factors for periodontal medicine[6]. However, the molecular mechanisms how these factors in the oral cavity can reach to the distant organs, and how these factors impact general health in whole body are not elucidated in detail.

Extracellular vesicles (EVs) are the nano-sized particles released from cells, which regulate cell-to-cell and intracellular communications by transporting their cargoes to the neighbor and distant cells. EVs derived from pathogenic bacteria, as known as outer membrane vesicles (OMVs), are also affect the host immune systems and disease development[7-9]. Moreover, EVs derived from host cells infected with pathogenic bacteria have been reported to induce diseases in the distant organs[10, 11].

In chronic periodontitis region, the abundant of EVs appear to be produced by various types of cells, including cells that form periodontal tissue, periodontal bacteria and periodontal bacteria-infected host cells (Fig.1). The released EVs into extracellular environment, then, might be implicated in periodontal health and diseases. The part of released EVs are likely to be taken up into body fluids such as saliva and blood, and then be delivered to the distant organs, and there affect systemic health. We, therefore, hypothesized that EVs may be the candidates which connect the oral cavity to the whole body, and play important roles in pathogenesis of periodontal

1) Department of Oral Healthcare Education, Institute of Biomedical Sciences, Tokushima University Graduate School
2) Department of Oral Healthcare Promotion, Institute of Biomedical Sciences, Tokushima University Graduate School
3) Department of Oral Morphology, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University

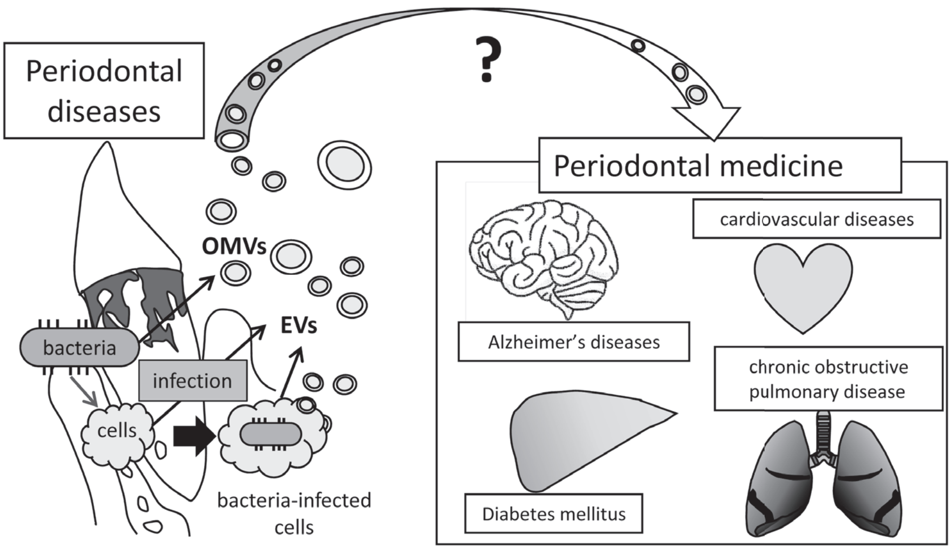

Fig. 1 Extracellular vesicles (EVs) observed in region of periodontal diseases
In chronic periodontitis region, EVs appear to be produced by various types of cells as follows: i) EVs derived from cells that form periodontal tissue, ii) OMVs of periodontal bacteria and iii) EVs derived from periodontal bacteria-infected host cells. The released EVs into extracellular environment, then, are implicated in periodontal health and diseases. The part of released EVs are possible to be taken up into body fluids, and then be delivered to the distant organs, and there affect systemic health.

medicine.

In this review, we aim to discuss whether EVs produced in periodontal diseases could be strong candidates for regulator of periodontal medicine. We will first explain the general properties of EVs, then introduce the current insights about the role of EVs in periodontal diseases, as well as periodontal medicine. Finally, we will propose the required further research in the fields of EVs.

2. Properties of extracellular vesicles

2.1. EVs from mammalian cells

EVs are the general term of membrane-enclosed vesicles that are released from various mammalian cell types to body fluids in normal physiology and abnormalities. EVs are divided into two categories, ectosomes and exosomes according to their size and origins[12]. Ectosomes including microvesicles, microparticles and large vesicles, are vesicles generated by budding of plasma membrane and in the size range of 50 nm to 1 μm in diameter. Exosomes are generated by exocytosis of intracellular multivesicular bodies such as endosomes, and have a size range of 40 to 160 nm in diameter.

EVs include many kinds of cellular constituents as called as “cargoes”, for example, nucleic acids (e.g., double-stranded DNA, single-stranded DNA, mRNA, micro RNA (miRNA)), proteins (e.g., cytosolic proteins, nuclear proteins and cell-surface proteins), lipids and metabolites[13-15]. Because EVs are made from cellular lipid bilayers, EVs can deliver their cargoes into recipient cells through body fluids excepting degradation and immune clearance, leading to effective alteration of biological responses in health and disease. Indeed, EVs miRNA are delivered to recipient cells and suppress the expression of target genes[16]. The membrane-associated receptor proteins in EVs are transferred to recipient cells, lead to activation of signaling pathways[17].

Accumulating evidence has revealed the contribution of EVs to health and various disease such as immune responses, pregnancy, cancer progression, metabolic and cardiovascular diseases. For example, breast milk-derived EVs enhance the number of T-regulatory cells, suggesting that EVs can regulate postnatal health and immune tolerance[18]. In obese mice fed a high fat diet, EVs miRNAs promote insulin resistance through downregulation of *Ppara (peroxisome proliferator-activated receptor alpha)* in white adipose tissue[19]. The reports showing that heat shock proteins in EVs promote survival of metastatic oral cancer cells[20], and that noncoding RNA in EVs can increase growth and spread of hepatocellular cancers[21] provide the potential of EVs as cancer diagnostic tools.

2.2. OMVs from bacteria

Like mammalian cells, prokaryotic microorganisms such as gram-positive bacteria, gram-negative bacteria, mycobacteria and fungi can produce and release EVs into extracellular environment[22-24]. EVs produced by microorganisms are often called as outer membrane vesicles (OMVs), therefore, the term "OMVs" will be used to refer bacterial EVs in this review. The studies about OMVs from gram-negative bacteria have been progressed since they were first observed in the 1960s, but there was little known about OMVs from gram-positive bacteria, mycobacteria or fungi. Some reviews on OMVs from gram-positive bacteria are available[24, 25], then, we will focus on gram-negative bacterial OMVs in this section.

The gram-negative cell envelope consists of two membrane (e.g., outer membrane and cytoplasmic membrane) and periplasmic space which in between them[26]. The cytoplasmic membrane is typical phospholipid bilayer, while the outer membrane is composed of interior leaflet of phospholipids and exterior leaflet of lipopolysaccharide (LPS). In outer membrane, there are many kinds of integral outer membrane proteins and lipid-anchored lipoproteins. The periplasmic space is consists with the net-like peptidoglycan (PG) layer and periplasmic proteins.

OMVs released from various gram-negative bacteria are spherical blebs in a diameter of 20 to 200 nm which are derived from bacterial envelope[27]. It has been regarded that OMVs are generated by "vesiculation" occured by the loss of interaction between outer membrane and the underlying PG layer without cell lysis[25]. On the other hand, OMVs could be generated by the turgor pressure of outer membrane caused by the accumulation of periplasmic proteins and PG fragment in periplasmic space[28]. Recently, it was also observed that explosive cell lysis produces OMVs through the vesiculation of shattered membrane fragments[29].

Gram-negative bacterial OMVs have reported to include LPS, lipids, proteins, nucleic acids, and virulence factors. Because OMVs originate from bacterial envelope, it seems to be reasonable that the components of outer membrane such as particular lipid species, LPS and outer membrane proteins (Omps) and lipid-anchored proteins are enriched in OMVs[30-32]. In most case, inner-membrane and cytoplasmic components were also detected in OMVs[33, 34]. High-throughput proteomic analysis have identified more than 100 vesicle proteins in OMVs derived from various kinds of Gram-negative bacteria[25]. Two types of vesicular DNAs (luminal and surface-associated DNA) and RNA were identified in EVs derived from *E. coli* and *Neisseria gonorrhoeae*[35, 36], however, the mechanisms how specific DNA/RNA are sorted into EVs remain unclear.

Importantly, OMVs can play physical and pathological roles in bacteria-bacteria (quorum sensing, biofilm formation, bacterial survival and antibiotic resistance) and bacteria-host interactions (virulence factor delivery, host cell modulation and immune evasion) by directly activation or by delivering cargoes to the recipient cells. The reviews on the functions of general bacterial OMVs were available[25, 37], then, we will focus on OMVs derived from periodontal bacteria and overview its functions in the next section.

3. The roles of EVs/OMVs in periodontal diseases and periodontal medicine

It is possible that both cellular EVs and bacterial OMVs are released into the region of chronic periodontitis (Fig. 1). In this section, we will discuss about the involvement of these EVs/OMVs in etiology of periodontal diseases as well as periodontal medicine.

3.1. EVs in periodontal diseases

Some reports have revealed that EVs derived from cells in periodontal tissue might be implicated in pathology and progression of periodontal diseases. The protein-enriched EVs derived from keratinocytes were transferred to fibroblasts, and there increase fibroblast migration by regulating gene and protein expression, suggesting that keratinocytes EVs can modulate wound healing[38]. Gingival epithelial cells (GECs) also produce EVs under infection with or without oral bacterium biofilm. Interestingly, the infection with oral bacteria biofilms increased EVs production, whereas the 80% of identified cargo proteins in EVs were common between control and biofilm-treated GECs. EVs derived from control and biofilm-treated GECs increase expression of genes associated with inflammation and matrix degradation[39]. These observation suggested that EVs derived from dental pocket epithelium affect the progression of periodontal diseases by modulating gene expression in the underlying fibroblasts to control inflammation.

The human periodontal ligament fibroblast (hPDLFs) were also observed to produce EVs which affect osteogenic activity[40]. EVs isolated from cultured media of hPDLFs were rich in CD9, CD63 and TSG101 proteins, and treatment with *P. gingivalis* LPS significantly increase the protein concentration in EVs. EVs of *P. gingivalis* LPS-treated hPDLFs were incorporated into human osteoblast cell line MG-63, and there inhibited osteoblastogenesis by decreasing expression of bone-related genes such as alkaline phosphatase (ALP), type I collagen and runt-related transcription factor-2 (Runx2). In addition, many kinds of miRNA included in EVs were associated with alveolar bone loss in periodontal diseases[41], suggesting that localized periodontal infection and inflammation may influence bone remodeling by release of EVs.

Indeed, EVs isolated from human saliva were observed to include protein cargoes and influence biological responses. The levels of CD9 and CD81-positive EVs isolated from

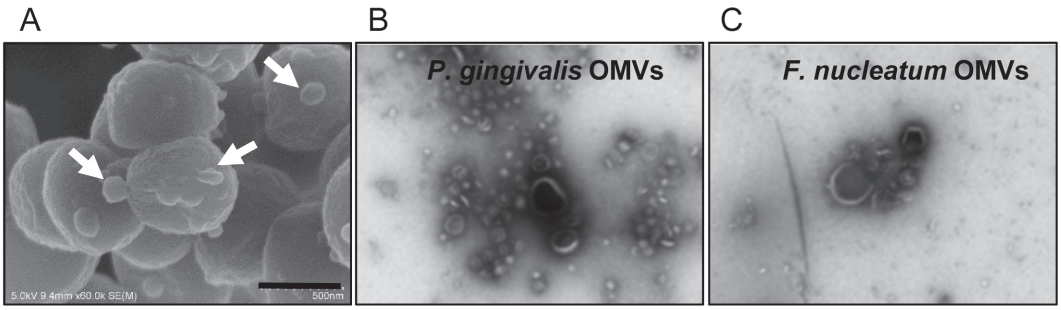

Fig. 2 Outer membrane vesicles (OMVs) of periodontal bacteria
The images of OMVs through scanning electron microscopy (SEM) (A) or transmission electron microscopy (TEM) (B, C). (A) Cultured *Pg* observed through SEM. The arrows indicate OMVs derived from bacterial cells. The bars indicate 1000 nm. (B), (C) The shape of OMVs isolated from the *Pg* and *Fn* culture media, respectively (×20,000).

human saliva were decreased in patients with periodontitis compared with healthy controls[42]. CD9 and CD81, known as general marker for EVs, are tetraspanins which interact with transmembrane molecules, including integrins and receptors, and affect biological responses by organization of molecules in tetraspanin-enriched microdomain[43]. The dysfunction of these proteins resulted in induction of systemic inflammation and delayed wound healing[44, 45]. These reports proposed that the alteration of CD9 and CD81-including EVs in saliva may play important roles in progression of periodontal diseases.

3.2. EVs in periodontal medicine

As mentioned above, it has been widely accepted that EVs are associated with various kinds of systemic diseases, therefore, the cargoes of EVs isolated from peripheral blood (e.g. microRNA and proteins) are used as biomarker and diagnostic tools. EVs are also detected in saliva, and the correlation between salivary EVs and systemic diseases has been reported[46]. Based on these observations, it is possible that EVs might be produced in periodontal region and then released into saliva or blood circulation, and impact systemic diseases. However, there is little evidence indicating that EVs produced by inflammation or infection during periodontitis can directly influence systemic diseases.

On the other hand, some bacteria, but not periodontal bacteria, can modulate systemic diseases in the distant organs by delivering their own virulence factors packaged in the host cell EVs. Like the infection with periodontal bacteria, *Helicobacter pylori (H. pylori)*, a gram-negative bacteria colonize in the epithelium of stomach and cause gastric inflammation and cancer, could also contribute to various extragastrointestinal diseases such as cardiovascular diseases, Alzheimer's diseases and diabetes mellitus[47]. Indeed, EVs isolated from *H. pylori*-infected host cells contain abundant of cytotoxin-associated gene A (CagA) proteins, a major *H. pylori* virulence factor[48]. These EVs-packaged CagA can be taken-up into blood circulation and delivered to the distant organs, and influence extra-gastric diseases[49, 50]. It is worth investigating whether EVs from periodontal bacterial-infected cells could have similar mechanisms in the development of extra-oral diseases.

3.3. OMVs in periodontal diseases

Like other bacteria, periodontopathic bacteria, including *Porphyromonas gingivalis (Pg)*, *Fusobacterium nucleatum (Fn)* and *Aggregatibacter actinomycetemcomitans (Aa)*, are able to produce OMVs (Fig. 2). Analysis using liquid chromatograpy-tandem mass spectrometry (LC-MS/MS) revealed that these OMVs contained numerous putative virulence-related proteins[51-53].

Among periodontal bacterial OMVs, *Pg* OMVs are well-known to impact periodontal diseases by regulating the environment of bacteria such as bacterial plaque formation, aggregation and attachment. *Pg* OMVs have capable to facilitate the aggregation of nonaggregating species such as *Treponema denticola*, *Eubacteium saburreum* and *Capnocytophaga ochracea*[54]. *Pg* OMVs enhance the invasion of *Tannerella forsythiainto* epithelial cells[55].

Pg OMVs were also reported to affect the host cell functions. Because *Pg* OMVs are enriched in gingipains. the cysteine proreases regarded as *Pg* major virulence compared to *Pg* bacterial surface, they allows *Pg* to spread throughout periodontal tissue and invade into the host cells[53, 56]. Moreover, OMV-bound gingipains are more stable to heat if compared to soluble gingipains[57], suggesting that stabilization of gingipains by packaging OMVs may be helpful for *Pg* to survive in periodontal pockets and to progress periodontal disease.

Pg OMVs themselves are also considered to be internalized by gingival epithelial and endothelial cells via caveolindependent or lipid raft-mediated endocytosis, and cause the destruction of gingival tissue[58]. For example, *Pg* OMVs within host cells can cause functional impairment of cellular migration and detachment in epithelial cells[59, 60]. *Pg* OMVs also inhibited wound repair by attenuating the proliferation and of fibroblasts and endothelial cells[61].

Moreover, many studies revealed that *Pg* OMVs can promote inflammatory cytokines through by pattern recognition receptors (PPPs) in gingival epithelial cells, fibroblasts, endotherial cells and macrophages[62]. The proinflammatory cytokines are considered to activate the neutrophils, T and B lymphocytes, macrophages, natural killer cells and osteoclasts, resulting in the destruction of connective tissue and alveolar bone in chronic periodontitis.

3.4. OMVs in periodontal medicine

In contrast, the roles of OMVs derived from periodontal bacteria on systemic diseases has been less understood. A study indicated that *Pg* OMVs were reported to induce the calcification of vascular smooth muscle cells and mouse aortas by promoting the activity of key osteogenic transcription factor, runt-related transcription factor 2 (Runx2) via ERK signaling[63]. This observation may suggested that OMVs derived from periodontal bacteria are involved in the pathogenesis of atherosclerosis.

In addition, the relationship between citrullinated proteins and periodontal medicines has been noticed. *Pg* is the only human pathogen expressing the citrullinated proteins by peptidyl arginine deiminase (PAD), which catalyzes modification of peptidyl-arginine to peptidyl-citrulline with ammonia. PAD were proposed to link periodontal diseases and systemic diseases such as rheumatoid arthritis, atherosclerosis and Alzheimer's diseases[64]. Indeed, a recent study demonstrated that 51 citrullinated proteins were identified in *Pg* OMVs[65]. These important findings proposed that citrullinated surface proteins in *Pg* OMVs could link periodontal diseases to systemic diseases.

Notably, we have recently reported that *Pg* OMV alteres glucose metabolisms in the liver and contributes to the progression of diabetes mellitus[66]. In agreement with other studies, we also identified various *Pg* virulence factors such as gingipains (Kgp, RgpA, RgpB) and components of fimbriae (FimA, MFAs) in *Pg* OMVs by LC-MS/MS analysis. The intraperitoneal injection of *Pg* OMVs attenuated insulin sensitivity in the liver cells, leading to the suppression of hepatic glycogen synthesis in mice. This attenuation of the glycogen synthesis actually increased blood glucose level in *Pg* OMV-treated mice. In addition, *Pg* OMVs also attenuated the insulin-induced Akt/ glycogen synthase kinase-3β (GSK-3β) signaling in hepatic HepG2 cells. Importantly, the analyze by IVIS spectrum imaging system indicated that *Pg* OMVs which were injected into abdominal cavity or tail vein can transfer and accumulate in the liver through blood circulation *in vivo*. This means that the delivery of bacterial virulence by OMVs might be important mechanism which induce periodontal medicines.

Consistent with our result, Han EC *et al*. also indicated that OMVs derived from periodontal bacteria can influence systemic diseases by delivering their cargoes to the distant organs *in vivo*[67]. They purified OMVs from gram-negative periodontal bacteria, *Aggregatibactor actinomycetemcomitans (Aa)*, and treated human macrophage cell line U937 with *Aa* OMVs. *Aa* OMVs contained miRNA and deliver them to host U937 macrophages, and there, induced TNF-α expression through TLR-8 and NF-κB signaling. Interestingly, the cardiac injection of *Aa* OMVs were successfully delivered to the brain after crossing the blood-brain barrier. These miRNA cargoes in *Aa* OMVs actually promoted TNF-α expression in the mouse brain, suggesting that miRNA in *Aa* OMVs regulated host gene expression. It was also proposed that the transfer of periodontal bacterial miRNA by OMVs to the brain may cause neuroinflammatory diseases likes Alzheimer's.

4. Conclusion

Due to numerous studies, it is clear that both cellular EVs and bacterial OMVs are responsible for the etiology of periodontal diseases. In contrast, little is known about the effects of EVs and OMVs on periodontal medicines, whereas the ability of EVs/OMVs seems to be advantageous in mechanism by which periodontal disease progresses systemic diseases.

The further research is warranted to clarify whether and how EVs/OMVs contribute to the development of periodontal medicine. Especially, the further studies to address the following points will be important in understanding of periodontal medicine: i) Analysis conducted by *in vivo* is essential. The present data showing relationshp between EVs/OMVs and host are mainly provided by *in vitro* models as such as cultured cells or bacteria. Therefore, we should confirmed whether EVs/OMVs are actually functional in animal models, and whether EVs/OMVs can go to the distant organs in mice by using *in vivo* imaging systems, ii) The research field combining mammalian cells and oral bacteria is required. To date, the biogenesis and biological roles of EVs and OMVs in physical conditions have been well documented, respectively. However, little is known about EVs/OMVs derived from manmmalian cells infected with periodontal bacteria. Because periodontal medicine might be followed by periodontal bacterial infection, EVs which are released from host cells infected with bacteria is more likely to impact on periodontal medicine.

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