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ORIGINAL RESEARCH

Innovative study on lactoferrin in periodontal disease

Papel de la lactoferrina en enfermedades periodontales

Laura Estela Castrillon Rivera,* Alejandro Palma Ramos,* Susana Macin Cabrera§

ABSTRACT

Lactoferrin (LF) is a protein present in secretions and is a component of neutrophil specific granules released when these cells are activated by periodontal pathogenic agents during the course of inflammatory processes like gingivitis or periodontitis. LF levels are related to the number of neutrophils present in lesions so that this molecule has been proposed as a biochemical marker of periodontal activity. Due to LF biological activities such as cytotoxic ability and its interference in biofilm development, its use can be considered useful in the prevention and treatment of periodontal diseases.

RESUMEN

La lactoferrina (LF) es una proteína presente en secreciones y es un componente de los gránulos específicos del neutrófilo que se libera cuando estas células son activadas por agentes periodontopatógenos en el caso de los procesos inflamatorios de gingivitis y periodontitis. Se ha correlacionado el nivel de LF con el número de neutrófilos presentes en las lesiones y se propone a esta molécula como un marcador bioquímico de actividad periodontal. Debido a la diversidad de actividades biológicas de la LF como su capacidad citotóxica y su interferencia en el desarrollo de biopelículas entre otras, se propone su uso en la prevención y tratamiento en enfermedades periodontales.

Palabras clave: Lactoferrina, periodontitis, gingivitis, periodontopatógenos.
Key words: Lactoferrin, periodontitis, gingivitis periodontal pathogens.

INTRODUCTION

Lactoferrin (LF) is a multifunctional glycoprotein originally isolated in 1939 from bovine milk. In 1960 it was isolated from human milk. It is produced by exocrine glands and is widely distributed in human body fluids such as tears, saliva, bile and pancreatic juice. Furthermore, LF is the main component of neutrophil polimorphonuclear leukocytes which, when activated during inflammatory processes, release the contents of their secondary granules (or specific granules) directly into the plasma.[1,2]

Lactoferrin belongs to the non hemic protein family known as transferrins, due to their ability to capture iron, copper, zinc, manganese and gallium.[3] Lactoferrin is produced by various mammal species, and fulfills diverse functions *(Figure 1)* like antimicrobial, anti-inflammatory, immuno-modulating, antioxidant and anti-tumor activities. For all these reasons, this protein is considered an important component for the innate defence response.[3]

Human LF is a 80 kDa glycoprotein with a 8.7 isoelectric point. It possesses one single chain of 692 to 703 aminoacids, with intramolecular disulfide links forming two terminal N- and C- globular lobes, with 37% homology between them. This protein is synthesized as a 711 amino acid molecule, with a sequence of 19 amino acids corresponding to the peptide signal that, when removed, generates mature protein.[4] In each lobe resides a site where the ferric ion ($Fe3^{+}$) synergistically joins the carbonate ion (CO 3 2-). When lactoferrin joins Fe it is known as hololactoferrin (holoLF), when it is free of this ion it is called apolactoferrin (apoLF).

LF ANTIMICROBIAL PROPERTIES

Transferrin/lactoferrin are proteins with bacteriostatic properties; due to their great affinity for iron they

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* Biological Systems Department, Immunology Laboratory, Universidad Autonoma Metropolitana Unidad Xochimilco (Metropolitan Autonomous University, Xochimilco Campus, Mexico City, Mexico).
§ Health Care Department, Universidad Autonoma Metropolitana, Unidad de Xochimilco (Metropolitan Autonomous University, Xochimilco Campus, Mexico City, Mexico).

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remove it from the extra cellular environment, thus depriving microorganisms of this nutrient vital for their growth. It has been suggested that lactoferrin contributes to defence mechanisms in mucosa, since it delays *in vitro* growth of bacteria and fungi and can show antimicrobial activity over a broad spectrum of pathogenic agents among which, bacteria, yeast, fungi protozoa and viruses can be found.[5,6] Iron excess can reverse the inhibitory effect of LF microbial growth, this suggests its bacteriostatic effect; nevertheless, if the capacity to join with iron is saturated, this potential no longer operates.[7]

Lactoferrin multifunctional ability has shown that the antimicrobial action can be explained through several mechanisms besides its ability for chelation, which results in the inhibition of microbial growth, another can be consequence of the formation of the peptid called lactoferricine as well as lactoferrin s capacity to interfere colonizing factors to the tissues.[8]

Lactoferrin digestion produces potent bactericidal peptides, of which several loops-segment have been identified. The active peptide is known as lactoferricin (LFcin or lactoferricin B). Initially it was identified as an antimicrobial peptide derived from lactoferrin digestion through pepsin. This peptide is active against Gram positive bacteria, Gram negative bacteria, yeasts, filamentous fungi and protozoa parasites.[9] LFcin is a low molecular weight cationic peptide, composed of 25 amino acid residues, corresponding to the N-terminal segment, and is a different region to the site of iron binding; therefore its cytotoxic mechanism is independent from this ion. Of the 25 LFcin amino acid residues, eight are basic. This allows them to join the bacterial lipopolysaccharide molecules freeing them from the membrane, in a way similar to that accomplished by native protein. The membrane potential and integrity results consequently collapsed, and the microorganism cellular membrane permeability increases, causing ruptures, and finally lysis and death of the bacteria or fungi *(Figure 1)*.[10,11] Bovine lactoferricine has shown higher antimicrobial power than human, goat or mouse lactoferricine, when facing reference strains of *E. coli* and *S. aureus*.[12] The appearance of synthetic peptides containing both cationic lactoferrine N-terminal segments have demonstrated their activity over *Candida albicans* and *Aspergillus fumingatus*.[13]

LF ACTIVITY OVER PERIDONTIUM PATHOGENIC AGENTS

Periodontal disease is an infectious process characterized by the destruction of connective tissue, accompanied by loss of periodontal insertion and alveolar bone resorption. Gram negative anaerobic bacteria like lipopolysaccharides (LPS) along with their products and components are responsible for these processes.

In periodontal disease, neutrophil granulocytes play an important role in the maintenance of host-bacteria homeostasis. After bacterial invasion, neutrophils and other cells migrate towards the swollen gingival tissue, and predominate in the connective tissue adjacent to the periodontal pocket. Chemotactic factors are synthetized and released in the swollen area; these factors can be derived from periodontal pathogens of the host.[14]

Since lactoferrin is the main component of neutrophil granules, and is present in saliva and crevicular gingival fluid (CGF) and interacts with microorganisms pathogenic to the periodontium, it can be an important element for host defence against periodontal disease. This reason has led to the study of its cytotoxic capacity over some microorganisms. There are reports of activity over *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia* and *Prevotella nigrescens* in physiologic concentrations in the secretory environment of the oral cavity.[15,16] *S. mutans* is highly susceptible to LF bactericidal action, in concentrations well within the physiological range of many secretions and over the number of bacteria commonly found *in situ*.[17] Later studies have determined the adhesion inhibitory capacity of this free bacteria, either aggregated or in biofilms over abiotic surfaces.[18]

Over *P. gingivalis* an LF alternative bacteriostatic mechanism has been described, since this bacteria has the ability to adsorb lactoferrin through its binding to the haemoglobin receptor (HbR), removing it from the cell surface and consequently altering its iron uptake system through (via) haemoglobin, this latter being its only source of this ion.[19] Other reports on LF direct bactericidal potential are associated to its ability to bind to porins and the lipid A of the lipopolysaccharide (LPS) present in the external membranes of Gram negative bacteria like *A. actinomycetemcomitans* as well as *P. gingivalis* altering their function.[16,20] This fact enhances the action of antibiotics since it facilitates their access to the interior of the bacteria, as proven in the case of *Salmonella* strains with erythromycin.[21]

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LF ANTIOXIDANT ACTIVITY

Destruction of support tissue of the tooth is associated with liberation of many proteolytic enzymes and oxygen reactive species, predominantly activated neutrophils[22] as well as leukocyte production of Interleukin-1 β (IL-1β). This suggests that periodontitis patho-

genesis and etilogy is associated to the production of proinflammatory cytokines such as IL-1β[23] which stimulate radicals as tissue damage elements.[24]

A direct cytotoxic mechanisms over the action of periodontopathogens consists in the formation of oxygen reactive species such as free radicals (O_2, OH) as well as hydrogen peroxide (H_2O_2) and hypochloric (HOCI) produced by the activation of breathing bursts of phagocytic cells such as neutrophils when they are activated either by their receptor Fcγ, directly by microorganisms or by proinflammatory cytokines such as interleukine 1β (IL-1β).[25] These metabolites can cause damage through varied mechanisms like lipid peroxidation, protein oxidation, proteases inhibitors inactivation, direct damage to DNA and activation of transcription signals which modify cellular function and can range from metabolic alterations up to the demise of the patient.

To avoid toxicity towards healthy tissue of these strongly reactive mediators, the body counts with antioxidant mechanisms. The peroxidase glutathione enzyme can be counted among these, which through the use of gluthatione as reducing agent detoxifies hydrogen peroxide as well as several hydroperoxides. The reaction of hydrogen peroxide with superoxide, forms highly reactive hydroxyl radicals (-OH^-) and needs to be catalyzed by iron or copper ions. Therefore, the lactoferrin capture of these ions determines its role in the defence against the aggression of these reactive agents. The imbalance between levels of myeloperoxidase/IL-1β and peroxidase/lactoferrin glutathione will result in tissue damage performed by reactive oxygen species (ROS) in periodontitis, which is initiated and perpetuated by agents pathogenic to the periodontium.[26]

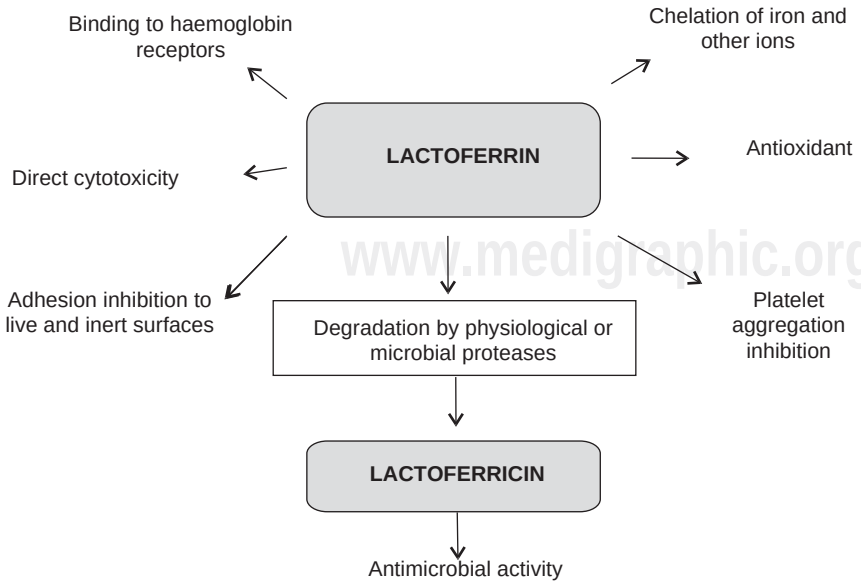
LF DEGRADATION

Certain bacteria colonizing the periodontal pocket can degrade lactoferrin. This activity is quite evident in *P. gingivales and C. sputigena,* slow in the case of *C. orchracea, A. actinomycetemcomitans and P. intermedia* and very slow or absent in *P. nigrescens, C. rectus, C. sputorum*, *F. nucleatum* ssp, *C. gingivalis*, *T. forsytia* and *M. micros*.[27]

Enzymes which degrade LF and are probably derived from microbial sources, cause a decrease in LF concentration in saliva, thus enhancing growth of *A. actinomycetemcomitans* and this can increase the risk of oral infections.[28] Recently, *in vitro* studies have demonstrated that the decline of LF antibacterial activity after long periods of incubation with *P. gingivalis* and *P. intermedia* can be due to the degradation of this protein.[29]

NEUTROPHIL MEDIATORS AS RESPONSIBLE AGENTS FOR TISSUE DAMAGE

Analysis of leukocyte proportions in healthy and chronically inflamed crevices has shown an increase in number, but proportions remain constant, polimorphonuclear neutrophils form 95-97%, lymphocytes 1-2% and monocytes 2-3%, it therefore can be concluded that the differences between healthy and inflamed



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Iron chelating activity as well as direct citotoxicity are associated with its antibacterial and antitumor capacity, Its protective role to tissue damage can be related through its antioxidant activity, the inhibition to adhesion to surfaces, which prevents the development of biofilms as well as the inhibition of its plaque aggregation. The degradation from lactoferrin to lactoferricine retains its antimicrobial activity

Figure 1. Lactoferrin biological activities.

gingival tissue are only quantitative,[30] for this reason it is important to recognize the role of the neutrophil as mediator of inflammatory responses, and even as the cell responsible for tissue damage, as long as there is hyperstimulation of this cellular population.

In periodontal disease, an increase in the number of neutrophils in the gingival groove and adjacent connective tissue has been detected, once these neutrophils are activated they experience degranulation of granules containing proteases carbohydrases, and antimicrobial substances like lactoferrin, lysozime, myeloperoxidase as well as other mediators, increasing thus inflammatory mediators which consequently will define the destination of the response towards resolution or tissue damage. The aberrant activity of the hosts response to plaque build up in the gingival crevice causes degradation of collagen fibres and apical migration of the binding epithelium. Neutrophils, which form the greater percentage of leukocytes in healthy and inflamed tissues, play an important role. They represent the first line of cellular defence against bacterial invasion, nevertheless they are also involved in tissue damage. To determine damage caused by this cell to the basal membrane, concentrations of laminin and lactoferrin have been correlated. Hyperreactivity has been found during transmigration process through endothelium and epithelium.[31]

In adult periodontitis peripheral neutrophil hyperreactivity has been demonstrated, and the possibility has been sought of an aberration in the expression of molecules related to this activity. For this purpose, crevicular neutrophils were collected from several inflammation sites with or without tissue damage as well as in inflamed sites in control patients with gingivitis. Neutrophil activation markers such as CD15, CD11a, CD11b and CD16 were determined by flow cytometry. No differences were found in the expression of membrane molecules in the case of neutrophils of periodontitis lesions when compared with neutrophils of lesions due to gingivitis.[32] Elastase is an endopeptidase of the neutrophil which can degrade proteins of the extracellular fibrillar and non fibrillar matrix The levels of this enzyme in CGF increase in experimental gingivitis, as well as in sites with well established periodontal lesions. It is also known that this enzyme decreases its production with conventional treatment of affected sites.[33] Therefore, the significant increase of free elastase in patients with untreated periodontitis, when compared with gingivitis patients, is associated with tissue damage.[34,35] For this reason quantification of this enzyme has been proposed as a promising diagnostic biomarker of active periodontal disease.[36]

Other neutrophil derivated mediators associated with clinical parameters of periodontal disease are beta-glucuronidase in the CGF which have a strong association with probing bleeding tests, and are proposed as a diagnostic method of active disease in periodontitis,[37] as well as free proteases levels and alfa1-antitripsyn in inflamed tissues with or without tissue damage. An imbalance between proteases and anti-proteases has been found.[38]

LF AS PERIODONTAL INFLAMMATION MARKER

Components of CGF are used to identify or diagnose active disease, to anticipate the risk of contracting it, as well as to determine its progression. The response of neutrophil granulocytes plays an important role in periodontal disease. CGF non-specific defence system can be determined through cytokines and neutrophil lysosomal enzymes, proteases like collagenases or intracytoplasmic enzymes such as dehydrogenase lactate aspartate amino transferase which allow to monitor progression of periodontal disease.[14]

Recently, lactoferrin has been used as a biochemical marker owing to its ability to participate in several biological processes associated with periodontal disease. From 1993 onwards studies have achieved to correlate lactoferrin in CGF as an effective marker for the number of polymorphonuclear neutrophils present in periodontal disease.[39]

Lactoferrin released by polimorphonuclear leukocytes into the CGF is a good indicator of periodontal inflammation, since a strong relationship has been demonstrated among clinical parameters like CGF volume, depth when probing, levels of epithelial insertion and plaque index. Lactoferrin levels in CGF (ng/site) increase up to 20 μM in the crevicular gingival fluid of patients affected with juvenile periodontitis, gingivitis and adult periodontitis in relation to inflammation severity, therefore, its quantification detects the degree of periodontal inflammation.[40] It has been shown there are differences in inflammatory response among periodontally healthy individuals during the course of experimental gingivitis.[41] In the search for markers that warrant the differentiation of inflammatory response between cases of gingivitis as compared to periodontitis, levels of elastase , (azurophilic granules) and lactoferrin (specific particles) in CGF of three different sites have been determined. The sites were the following: inflamed sites in gingivitis cases as well as inflamed sites in periodontitis cases, with and without tissue damage. It was found that elastase levels increase only in periodontitis cases, in contrast with lactoferrin

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which increases in both diseases, this suggests that patients afflicted with periodontitis experience a greater rate of cell release and a specific response of the host associated to granulocytes.[25] Another study reports no significant differences in absolute values and elastase concentration, and correlation of lactoferrin levels with activation of polymorphonuclear cells was achieved.[42] It is important to note that in both studies it is suggested that release of components of primary and secondary granules indicate alterations in the polymorphonuclear cells in different sites of both diseases.

In 2009, a research has been conducted achieving an experimental gingivitis longitudinal study to assess efficiency of lactoferrin as possible marker of the disease progression. This study assesses lactoferrin levels in CGF and blood. The study showed that local inflammation created by the accumulation of dental plaque causes increase of LF levels in blood and in CGF, and re-establishing oral hygiene decreases LF levels, although alterations are statistically insignificant.[43]

Lactoferrin bacteriostatic and bactericidal activity in saliva and crevicular gingival fluid is considered as part of mucosa defence mechanisms. It has been observed that its levels increase in periodontitis afflicted sites when compared to healthy ones.[44] Its concentration after surgical treatment has been assessed associating changes in stimulated and non stimulated saliva. It has been found that LF is appropriate to monitor periodontal treatment results since high LF concentrations in parotid gland and saliva in patients suffering aggressive periodontitis decrease in the CGF after surgical treatment in both salivas.[45]

BIOFILM FORMATION INHIBITION

Biofilms (BF) are microbial communities which represent the most successful colonization configuration among microorganisms, they are ubiquitous in nature and are responsible for many diseases. They are considered microorganisms communities and grow embedded in a self-produced exopolysaccharide matrix, they adhere to inert surfaces or live tissues. Formation of biofilms occurs as a continuous process according to the following development phases: a) conditioning, b) adhesion, c) extracellular matrix synthesis d) maturation and e) dispersion. After the biofilm maturation, dispersion sets in, either of isolated cells or in conglomerates, which colonize new surfaces initiating thus a new cycle for the formation of biofilms. There are differences in the characteristics in detaching cells; since sessile (adhered) cells can keep the biofilm functionality (e.g. resistance to antibiotics), isolated cells, instead, can present a planktonic (free) phenotype and be susceptible to the hosts defence mechanisms.

Bacterial biofilm is the primary etiological factor for periodontal disease. Gram negative and Gram positive bacteria possess virulence factors among which are the following: lipopolysaccharide (LPS), peptidoglycans, lipoteichoic acids, fimbriae, formyl-methionine peptids, thermal shock proteins, proteases, and toxins among others which can cause direct or indirect damage on periodontal tissues, stimulating the host cells to activate the onset of inflammatory response causing gingivitis and in some cases periodontitis.[46]

Periodontal disease, of great incidence humans, is characterized by an inflammatory process which results in loss of support for the tooth. The process begins with the formation of a glandular origin film (saliva, mucus) which covers mucosa and dental and epithelial surfaces of the gum. The first colonizers arrive shortly afterwards and offer the means for the retention of other microorganisms, this allows for the formation of a diverse cellular community or biofilm.[47] Uncontrolled development of resident microbes in theses communities can contribute to the development of oral diseases.[48]

Lactoferrin ability to chelate ions as well as to interact with microbial components enables the modification of microorganisms interactions with tissue surfaces. This is the reason why LF interference capacity in biofilms development phases has been explored. It has been reported that LF suppresses the initial union of *S. gordonii*, as well as the coaggregation of this bacteria through iron abduction. This finding leads to the inhibition of the initial phase and development of the oral biofilm. It nevertheless has been shown there is no effect on *P gingivalis* or *F nucleatum.*[49]

Moreover, LF prevents biofilm formation on bacteria who have escaped initial death. In the presence of subinhibitory concentrations of LF (20 µg/mL) *P. aeruginosa* bind and multiply but fail in the formation of microcolonies or biofilms differentiated structures. This is due to the fact that LF stimulates a special type of bacterial locomotion which puts distance between daughter cells from the site of parental division. Due to these reasons, microcolonies can not be established, this then demonstrates interference in early phases of biofilm development[50,51] and allows for a strategy to prevent the antibiotic resistance which is related to the formation of these structures.

P. gingivalys and *P. intermedia* reside as biofilms in subgingival plaque. Recent studies have determined that allocating bovine lactoferrin to patients afflicted with chronic periodontitis decreases the number of

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these bacteria in the plaque. For these reasons, biofilm formation ability of several forms of lactoferrin has been explored: human and bovine apo (iron free) native and holo (iron saturated) as well as lactoferricin (LFcinB). Inhibition of biofilm formation activity has been found in low concentrations. It has also been found that use combined with antibiotics enhances the effect. For all the aforementioned reasons lactoferrin use is proposed in prevention and treatment of periodontal diseases.[29]

TREATMENT

Due to its multiple functions, lactoferrin use has been proposed for therapy. It has been reported that use of lactoferrin, xilitol or the combination of both in *in vitro* studies on the structure of *P. aeruginosa* biofilms has resulted in a viability reduction > 2 log, when used in combination. This is due to the disintegration capacity in the biofilm structure effected by the xilitol, and to the bacterial permeabilization preformed by lactoferrin. For these reasons, the combined use of both molecules is currently proposed for biofilm elimination treatment.[52]

On account of lactoferrin antimicrobial activity, formulae have been developed using activated lactoferrin (ALF)[53] which prevent interaction of bacteria with tissue. These preparations will generate benefits for human health, among them oral health (plaque control, mouthwashes, denture cleansing, etc.) as well as wound protection care, food protection, and in the avoidance of biofilm formation in catheters, which has caused many hospital related diseases.

CONCLUSION

The search for periodontal activity molecular markers which allow, besides emission of diagnosis, to determine severity and progression of the disease has allowed to contemplate the use of a great amount of molecules coming from serum or from crevicular gingival fluid as well as of cells locally participating in the inflammatory process. Lactoferrin can be a useful candidate as biochemical/immunological marker since its presence in biological samples correlates clinical parameters with the number of *in situ* activated neutrophils, reflecting activity in the affected tissue.

Moreover, due to the inverse relationship between lactoferrin with elastase in immune responses, it is very important to take this fact into account when appraising evolution of periodontal lesions. It is important then to consider this index as a maker for oral health, since high elastase concentrations are associated with tissue aggression mechanisms in contrast with lactoferrin's protective response.

Lactoferrin plays a protective role in inflammation due to its antioxidant capacity, direct cytotoxic action and in the inhibition of biofilm formation. These findings permit to propose its use in prophylactic preparations to avoid oral microflora colonization. The near future will probably see the use of this type or formulae.

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Mailing Address:
Dr. Susana Macin Cabrera
Calz. Del Hueso Núm. 1100,
Col. Villaquitud,
Delegación Coyoacán,
México D.F. 04960.
E-mail: macinsu@prodigy.net.mx