Extracellular DNA as an essential component and therapeutic target of microbial biofilm

Zewnątrzkomórkowy DNA jako istotny składnik oraz cel terapeutyczny biofilmu bakteryjnego

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Słowa kluczowe: zewnątrzkomórkowy DNA, biofilm, DNaza I.

Abstract

The dominant part of human infections is associated with biofilm formations. Biofilm represents structured communities of bacterial or fungal cells enclosed in self-produced polymeric matrixes adherent to supporting surfaces. Microbial DNA and the host cell DNA, after their release at the infection site, show the ability to promote biofilm formation. Between the different constituents of biofilm matrixes, extracellular DNA (eDNA) may be the only component indispensable for the initial attachment and early biofilm formation through an enhanced matrix structural integrity. The effect of DNA on bacterial/fungal attachment is non-specific, as indicated by the stimulatory effect of plasmid, chromosome, or eukaryotic DNA. DNase I impaired bacterial biofilm growth and the targeting eDNA were recently proposed to eliminate and/or prevent different microbial infections associated with biofilm formations.

Streszczenie

Znaczna część infekcji występujących u ludzi jest związana z powstawaniem biofilmu. Biofilm stanowi złożoną strukturę składającą się z drobnoustrojów oraz wyprodukowanej przez nie polimerowej macierzy, umożliwiającej adhezję do różnych powierzchni. Drobnoustroje oraz ludzkie DNA, pochodzące odpowiednio z mikroorganizmów i komórek gospodarza, uwalniane w miejscu infekcji działają jako czynnik promujący rozwój biofilmu. Uważa się, że zewnątrzkomórkowe DNA (eDNA), dzięki zwiększeniu integralności konstrukcji macierzy, jest niezbędnym elementem biorącym udział w adhezji bakterii do podłoża i wczesnym formowaniu się biofilmu. Wyniki badań wykorzystujących plazmidy, chromosomy oraz eukariotyczne DNA wykazały, że wpływ eDNA na rozwój biofilmu jest niespecyficzny i niezwiązany bezpośrednio ze źródłem materiału genetycznego. DNaza I hamuje powstawanie biofilmu. Postuluje się, że wykorzystanie eDNA jako celu terapeutycznego może mieć istotne znaczenie w zapobieganiu i leczeniu zakażeń związanych z formowaniem się biofilmu.

Introduction

The process of bacterial biofilm development consists of an initial adhesion and the aggregation of dispersal cells from mature biofilm, which are encased in self-produced extracellular polymeric substances (EPSs) [1, 2]. The polysaccharides, host F-actin, and extracellular DNA (eDNA) are the major components present in a biofilm matrix [3]. The majority of these molecules are recognised by the innate immune system via toll-like receptors (TLR), a family of membrane proteins [4, 5]. Extracellular polymeric substances can interact with antibiotics, which decreases their antibacterial potential [6, 7]. eDNA is a crucial component of the biofilm matrix during the first stages of biofilm formation [8, 9]. In some cases, eDNA might represent...
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the only source of carbon and energy for microbial growth [10]. The DNA network within a biofilm is also involved in gene transfers [11] and the development of antimicrobial resistance [12, 13]. Many microorganisms release DNA during lysis, which occurs as part of the quorum sensing mechanism and initiates the process of biofilm formation [14] (Figure 1). Large amounts of DNA are released from neutrophils during necrosis or as extracellular traps (NETs), resulting in the accumulation of this polymer at the infection sites, such as those observed in cystic fibrosis (CF) airways [15]. eDNA contributes significantly to sputum viscosity, which can be reduced following the addition of recombinant DNase I [16–18]. Interestingly, eDNA, reduces the biofilm’s susceptibility to some antiseptic and disinfectant agents [12, 13].

The important role of eDNA in biofilm architecture, the development of resistant strains, and the host immune response

The accumulation of eDNA in microbial biofilm plays different functions [19]. Whitchurch et al. reported that the formation of a stable biofilm and the attachment of bacterial cells to culture flow-chambers are prevented by the addition of DNase I to the culture medium [20]. Additionally, the ability of DNase I to reduce mature biofilm masses suggests that eDNA is critical for the integrity of these bacterial communities. This role, in particular, of eDNA in the formation of biofilm structures is supported by an increasing number of studies, reporting the inhibitory effects of numerous antibiotics and DNase I on the growth of established biofilms of various bacteria, used separately or together. Treatment of the biofilms with DNase I significantly reduces neutrophil activation markers. DNase I treatment might dissolve an established 72-hour biofilm; however, much older 84-hour biofilm was more resistant to the addition of DNase I, suggesting that the matrix in mature biofilm may contain substances other than eDNA or that mature biofilms may produce proteolytic exoenzymes to locally deactivate the DNase I [4]. Two mechanisms were proposed to describe the potential of DNase I to inhibit microbial biofilm formation. DNase I might shorten the nucleic acids linked to bacteria surfaces that are involved in bacterial adhesions to environmental components where the biofilm forms. The digestion of eDNA mainly in young biofilms, eliminates the major cell-to-cell adhesion and interconnecting aggregates of microbial cells [4, 21]. Interestingly, it
was recently shown that the combination of poly (aspartic acid) with DNase I resulted in a synergistic increase in biofilm disruptions [22]. eDNA is involved in horizontal gene transfers (HTG), which mainly occur through the transformation, conjugation, or transduction of bacterial cells. Transformation requires the availability of eDNA within the environment [23, 24]. eDNA present in bacterial biofilm communities constitutes a dynamic gene pool from which bacteria can derive genetic information by HTG. Acquired antibiotic resistance may be due to a mutation or an acquisition of resistant genes. Biofilm production and eDNA released synergistically, contribute to the development and spread of an antibiotic resistance of HTG [4]. Neutrophils form the first line of defence against invading microbial pathogens – the essential effectors that mediate the phagocytosis and destruction of bacteria through oxygen-dependent and oxygen-independent mechanisms [25]. TLRs recognise pathogen-derived ligands and follow cell activation via the Toll/IL-1R (TIR) signal pathway [26]. TLR9, as an intracellular receptor, is required for a response to the unmethylated CpG motifs of bacterial DNA [27]. TLR2 is critical for the recognition of several pathogens as a Gram-positive bacteria, including bacterial lipoproteins, peptidoglycan (PGN), and lipoteichoic acids [4]. eDNA activates neutrophils through CpG- and TLR9-independent mechanisms [25]. eDNA also plays a significant role in neutrophil activation by bacterial biofilms, because the treatment of lasI rhlI mutant biofilm with DNase I does not modify its ability to stimulate neutrophil IL-8 and IL-1β [25]. Upon phagocytosis and the digestion of Staphylococcus aureus in the phagosome, bacterial DNA is liberated, and it engages TLR9. TLR9-dependent activation can be triggered not only by phagocytosis of whole S. aureus cells, but also by eDNA [19].

**Biofilm formation of Pseudomonas aeruginosa strains**

The role of eDNA in bacterial infections has been reported mainly with *P. aeruginosa* infections [12, 28, 29]. During *P. aeruginosa* infections, eDNA is released mostly by lysis that is regulated by the quorum sensing process [28]. Recent data demonstrates that eDNA from *P. aeruginosa* biofilm matrixes plays a critical role in activating neutrophil proinflammatory responses (Figure 2). Moreover, the data shows that the degradation of eDNA significantly reduces the release of proinflammatory cytokines by neutrophils added to established biofilms, as well as bacterial phagocytosis [25]. Allesen-Holm et al. suggest that extracellular DNA is analogous with chromosomal DNA in *P. aeruginosa*, which serves as a cell-to-cell interconnecting component in biofilm matrixes [28]. They also presented out that eDNA is primarily located in high concentrations within the stalks of mushroom-shaped microcolony structures. Montanaro et al. suggest that extracellular DNA in *Pseudomonas* biofilms probably has a stabilising role [4]. The stabilisation and structural development of *P. aeruginosa* biofilms depend on the quorum sensing (QS) systems lasRI and rhlRI [30, 31]. In some, the extension of the rhlRI systems is controlled by the lasRI system [31]. Through QS mechanisms, bacteria can monitor their density in cell populations through extracellular signalling molecules [32]. In the case of the

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**Figure 2.** Major functions of extracellular DNA (eDNA) consist of activation or pro-inflammatory responses that involve the activation of TLR9 pathways and the initiation of bacterial biofilm formation.
las system, gene products, lasI, control the synthesis of the actively secreted, extracellular signalling molecule 3-oxo-C12-homoserine lactone (3-oxo-C12-HSL). When the concentration of lactone increases within the environment of cells, following its penetration into a microorganism, the production of the transcriptional activator LasR increases. This complex has the ability to activate the expression of many genes, including enzymes lasA, lasB, and the rhlR-rhlI system [31, 33, 34]. The signal molecules involved are primarily C4-HSLs, in the case of the rhlRI system. The second QS system in P. aeruginosa is associated with the regulatory protein RhlR and the C4-AHL (acylated homoserine lactone) molecule, which is the product of the rhlI gene. The C4-AHL molecule connects with the Rhl, which is the transcription activator. This complex (Rhl-C4) regulates the operon responsible for the synthesis of some factors, including rhlAB [31, 35]. The Pseudomonas quinolone signal (PQS), a third signalling system based on 2-heptyl-3-hydroxy-4-quinolone, is part of the quorum-sensing regulatory network [36]. Allesen-Holm et al. suggest that QS-controlled factors, which might play a regulatory role in biofilm development by P. aeruginosa, are associated with programmed DNA releases [28].

Biofilms formed by lasI rhlI mutant strains stimulate lower levels of cytokine production [25]. On the other hand, DNase I treatment inhibits P. aeruginosa biofilm growth in vitro [28, 29]. It was recently shown that the destruction of eDNA could modify the properties of biofilms formed by P. aeruginosa and Streptococcus pneumoniae [20, 37, 38]. Is it known that P. aeruginosa encodes PEL, PSL, and alginate extracellular polysaccharides. A new report presented that pel-, psl-, and alg-independent biofilms are also regulated by the release of eDNA [39].

Biofilm formation of Staphylococcus aureus strains

Biofilm formation in S. aureus consists of a two-step process. The initial phase consists of the attachment of cells to a surface, and the second phase consists of cell–cell aggregation and the formation of a multi-layered architecture [40]. In S. aureus, the CidA/LrgAB system regulates cell lysis, eDNA releases, and biofilm development by possibly regulating access of murein hydrolases to cell wall substrates [41]. The lrg operon reduces extracellular murein hydrolase activity and increases penicillin tolerance, whereas the cid operon increases extracellular murein hydrolase activity and decreases penicillin tolerance [4]. Furthermore, Rice suggests that eDNA has a biological role in bacterial programmed cell death and cidA mediated lysis. Some results have shown that the treatment of streptococcal biofilm with DNase I inhibits biofilm formation [37, 42]. Houston et al. presented a production of eDNA in S. aureus suspension during cell lysis mediated by the autolysin AtlA [43]. The authors also demonstrated the essential role of eDNA in the primary attachment and early stages of Atl-dependent, FnBP-mediated MRSA biofilm. Kaito et al. demonstrated that S. aureus colony spreading requires the digestion of eDNA by nucl1 and nucl2 secretory nucleuses [44]. They believe that two possible mechanisms are involved in blocking the effect of eDNA on S. aureus colony spread. The first is associated with increased viscosity of the extracellular matrix that inhibits colony spread. The second is based on the recognition of eDNA by S. aureus, resulting in inhibition of the expression of some genes, which leads to the inhibition of colony spread. Kaplan et al. assessed S. aureus biofilm formation in the presence of a sub-minimal concentration of β-lactam antibiotics. They observed that low level concentrations of β-lactam induce the release of autolysin-dependent eDNA and induce biofilm formation of the S. aureus strain [45]. This data remains consistent with other studies demonstrating that eDNA form a major biofilm matrix in S. aureus biofilms cultured in a deficiency of antibiotics [38, 41, 43, 46]. The model of the autolysis of S. aureus involved an altruistic suicide (Figure 1). Staphylococcus aureus cells may be divided into altruists and survivors, and in the environment that activates biofilm formation, the altruists commit suicide by programmed cell death [4].

Biofilm formation of Staphylococcus epidermidis and Streptococcus pneumoniae strains

Qin et al. showed that eDNA is a major component, essential for the initial attachment of S. epidermidis to surfaces, as well as for the subsequent early phase of biofilm development by this bacteria [47]. Using polymerase chain reaction (PCR), the authors showed that extracellular DNA is similar to genomic DNA. The activity of the autolysin AtlE causes a release of eDNA from S. epidermidis. The eDNA was also found in a wild-type of S. epidermidis biofilm. The established biofilms were associated with AtlE-mediated cell lysis [47]. Heilmann et al. investigated another autolysin protein associated with DNA releases in the S. epidermidis. It was named Aae, and it is characterised by bacteriolytic activity and adhesive properties [48]. Interestingly, Vuong et al. reported that the expression of AtlE and the biofilm formation in S. epidermidis were increased in an agr quorum sensing mutant, and it can be linked to DNA releases [47, 49]. Moreover, Izano et al. demonstrated that S. epidermidis biofilms were not inhibited and/or detached during DNase I treatment [38]. Streptococcus pneumoniae are characterised by a high prevalence of lysogenic bacteriophages existing in their host chromosome, and the researchers proposed that prophage impulsive activation results in bacterial lysis that provides eDNA. eDNA enhances pneumococcal biofilm development [50]. Streptococcus
pneumoniae contains a major autolysin LytA, an N-acetyl-muramyl-L-alanine amidase [51]. Moreover, DNA release is associated with cell lysis, dependent upon LytA [52]. The observation that S. pneumoniae biofilm formation depends upon the presence of eDNA and that LytA mutants have a reduced capacity to form biofilms suggests that LytA-induced pneumococcal lysis could be similar with biofilm formations and the release of eDNA [21, 37, 50].

Fungal biofilms

The life-threatening infections caused by Candida albicans are often associated with biofilm formation [53, 54], and the mainstream displays of candidiasis, at both mucosal and systemic sites, are related to the formation of biofilms [54–56]. The first step of biofilm formation (mainly in Candida glabrata biofilm) is initially conditioned by the activity of adhesion, which is required for surface attachment. Their presence is also necessary for cell-cell stabilisation in the form of a biofilm structure. It was discovered that their expression is strictly correlated with the degree of biofilm development [57]. The stage of fungal biofilm formation is clearly related to the eDNA level. Various studies have shown that eDNA is an important factor in Candida biofilms, including those of C. albicans, C. tropicalis, and C. parapsilosis [58–61]. This is consistent with previous research demonstrating that eDNA is a substantial component of mature C. albicans biofilm [58]. Interestingly, studies conducted by Rajendran et al. revealed that the eDNA released during Candida biofilm formation depends upon the fungal species and is connected to biofilm heterogeneity. It was hypothesised that the diversity of biofilms is conditioned upon the difference in the amount of released eDNA – it was assumed that isolates, characterised by their greater ability to form biofilms, liberate more eDNA in comparison with isolates developing structurally simple biofilms. Importantly, eDNA-mediated heterogeneity of fungal biofilm significantly affects the pathogenicity and sensitivity of these pathogens to antifungal agents. Given the above, a detailed investigation of the mechanism of such a phenomenon is required. To date, studies have revealed that the differential degree of biofilm mass formations are associated with chitinase regulated autolytic events [62]. Growth in the pathogenicity of fungi may also be conditioned by morphological transitions of fungi to more invasive forms during biofilm development. Recent studies indicate that eDNA may play a key role in the transition from yeast to hyphal growth in C. albicans cultures. It is worth noting that a low concentration of eDNA is able to induce the transformation of a yeast form [63]. Since hyphal forms are characterised by increasing resistance to antifungal treatment and they play a significant role in C. albicans pathogenicity, the targeting of eDNA is gaining importance [64]. It was shown that increases in a biofilm mass is induced by both homogenous and heterogeneous eDNA, which takes on particular significance in light of the studies reporting a major occurrence of polymicrobial infections [63, 65]. An intrinsic resistance of C. albicans biofilms against most antifungal agents like fluconazole and amphotericin was reported [66–72]. The detailed mechanism of antifungal resistance of fungal biofilms remain unclear. It is known that C. albicans biofilm cells display reduced susceptibility to polyenes and azoles as compared to planktonic cells [8]. Improved efficacy of amphotericin B in combination with DNase I, which degrades eDNA in C. albicans biofilm matrixes, was observed [8]. Some results have shown that caspofungin is highly active against isolates demonstrating high levels of fluconazole resistance [73–75]. Rajendran et al. presented that Aspergillus fumigatus releases eDNA during autolysis in a phase-dependent manner. This is in agreement with previous studies conducted on Candida species, showing that the level of eDNA strictly correlates with the maturity of biofilm [58]. They also showed that DNase I treatment destabilised biofilm integrity in A. fumigatus [61]. Moreover, the authors demonstrated that the combination of DNase I with amphotericin B and caspofungin significantly improved antifungal activity.

Conflict of interest

The authors declare no conflict of interest.

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