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Pectin Nanocoating Reduces Proinflammatory Fibroblast Response to Bacteria

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ABSTRACT

Implant failures are primarily related to bacterial infections and inflammation. Nanocoating of implant devices with organic molecules is a method used for improving their integration into host tissues and limiting inflammation. Bioengineered plant-derived rhamnogalacturonan-Is (RG-Is) from pectins improve tissue regeneration and exhibit anti-inflammatory properties. Therefore, the aim of this study is to evaluate the *in vitro* effect of RG-I nanocoating on human gingival primary fibroblast (HGF) activity and proinflammatory response following *Porphyromonas gingivalis* (*P. gingivalis*) infection. Infected HGFs were incubated on tissue culture polystyrene (TCPS) plates coated with unmodified RG-I isolated from potato pectin (PU) and dearabinanated RG-I (PA). HGF morphology, proliferation, metabolic activity, and expression of genes responsible for extracellular matrix (ECM) turnover and proinflammatory response were examined. Following the *P. gingivalis* infection, PU and PA significantly promoted HGF proliferation and metabolic activity. Moreover, gene expression levels of *IL1B*, *IL8*, *TNFA*, and *MMP2* decreased in the infected cells cultured on PU and PA, whereas the expression of *COL1A1*, *FN1*, and *FGFR1* was upregulated. The results indicate that RG-Is are promising candidates for nanocoating of an implant surface, can reduce inflammation, and enhance implant integration, particularly in medically compromised patients with chronic inflammatory diseases such as periodontitis and rheumatoid arthritis.

Keywords: dental implants, rhamnogalacturonan-I, nanocoating, inflammation, fibroblast

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1. INTRODUCTION

The mechanism of progressive bone and soft connective tissue loss, as seen in rheumatoid arthritis (RA) and periodontal disease (PD) is associated with the host immune response.[1,2] The most recent studies confirm the relationship between RA and PD caused by similar risk factors and mechanism related to *Porphyromonas gingivalis* (*P. gingivalis*) infection. Soft and mineralized tissue destruction in RA and PD appears to be a result of a complex interaction between the *P. gingivalis* infection and the host immune response.[3-6] As a consequence of bacterial stimuli, the host cells synthesize and secrete a great number of mediators attracting inflammatory cells to the infected area. This contributes to bone destruction by induction of osteoclast formation and release of proteolytic enzymes such as matrix metalloproteinases (MMPs) and apoptosis of cells.[7] Therefore, in immunocompromised patients with an impaired healing capacity and greater risk of implant failure, improvement of bone tissue regeneration is required.

A number of studies have shown that plant-derived polysaccharides are immunomodulatory agents.[8-11] Recently, it was reported that pectin polysaccharide rhamnogalacturonan-I (RG-I) can prevent inflammation,[8,9] and improve tissue regeneration.[12-16] Our previous studies also showed that nanocoated RG-I pectins enhance bone tissue regeneration *in vitro* through upregulation of the gene expression of osteogenic markers in human and murine osteoblasts.[17-19] Moreover, pectin polysaccharides have been reported to affect the behavior of fibroblasts, the most common cells of connective tissue, which play a critical role in the soft and bone tissue healing process.[20] RG-I is able to induce adhesion, proliferation and survival of fibroblasts and may stimulate soft and hard tissue regeneration as a result.[16] Its ability to modulate the host's inflammatory response as well as stimulate tissue repair makes RG-I a

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promising candidate for biomaterial applications in tissue engineering and dental implantation, particularly in RA and PD.

The success of implant therapy depends on the outcome of the bone healing processes following implant placement.[20,21] According to the concept of the wound healing process, bone healing is divided into four phases: hemostasis, inflammation, proliferation, and remodeling (Figure 1).[22] Fibroblasts are a key cellular player in this process and act at the early stage of bone healing after implantation. Fibroblasts activated by growth factors migrate from the surrounding healthy tissue toward the implant surface, and after cellular attachment, they begin to synthesize new collagen, elastin, and fibronectin, inducing the formation of granulation tissue and angiogenesis.[20-22] However, as a consequence of bacterial invasion, the peri-implant bone healing process is disturbed. During infection, bacterial virulence factors stimulate immune and periodontal tissue-derived cells to release a very large number of proinflammatory cytokines, resulting in reduced bone formation.[23]

Here, we hypothesize that RG-I pectin polysaccharides coated on implants in nanoscale may modulate the behavior of human gingival fibroblasts (HGFs) and their response to *P. gingivalis* infection at an early stage of peri-implant bone healing. The impact of bioengineered RG-Is is considered to limit inflammation and improve the integration of dental implants, particularly in patients with a compromised healing capacity such as that observed in PD and RA. Further, the structure of RG-I pectins can be enzymatically modified, which results in different physicochemical properties, such as total charge, side chain branching, and molecular weight. The possibility of controlling the structure with a set of specific enzymes widens the applicability of RG-I as a biomaterial coating.[13,15] Therefore, the aim of our study was to evaluate the effect of potato dearabinanated RG-I (PA) and potato unmodified RG-I

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(PU) on *in vitro* HGFs' behavior and modulation of proinflammatory response stimulated by

P. gingivalis infection.

2. MATERIALS AND METHODS

2.1 Isolation, modification and nanocoating of RG-I

RG-I was isolated and prepared by the enzymatic treatment of potato pulp (P) as previously

published.[18,19] Two different pectin RG-Is, PA and PU, were used in this study. The chemical

properties, monosaccharide composition, and linkage analysis of PU and PA have been

presented in our previous works.[18,19] In this study, PU and PA RG-Is (128 µg/mL) were

adhered to the surface of 6-well, 24-well, and 96-well tissue culture polystyrene (TCPS)

plates (Techno Plastic Product) as described earlier.[14]

2.2 *In vitro* studies

2.2.1 Cell culture

Fibroblast primary cells were isolated from human gingival leftovers after surgery (ethical

number: EA1/081/14) as described[24] and cultured in a DMEM medium (Gibco) supplemented

with 10% fetal bovine serum (FBS) (Biochrom), 1% non-essential amino acids (Biochrom),

1% antibiotic (streptomycin and penicillin) (Biochrom), 1% amphotericin (Biochrom), and

1% L-Glutamine (Biochrom). The cells were incubated at 37°C with 5% CO_2 (Heraeus). The

cell morphology was monitored and documented before and after infection with *P. gingivalis*

by light microscopy (Leitz).

2.2.2 *Porphyromonas gingivalis* cultivation

The *P. gingivalis* strain ATCC 33277 (Manassas) was used in this study. *P. gingivalis* bacteria

examined by the commercial biochemical test kit ID 32A (API BioMérieux) were grown at

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37°C under anaerobic conditions on Columbia agar (Sifin Diagnostics GmbH) supplemented with 5% sheep blood (Acila Sarl), 0.1% vitamin K (Sigma-Aldrich), and 0.25% hemin (Fisher Scientific GmbH) for 3–5 days.

2.2.3 *Porphyromonas gingivalis* infection assay

For the infection assay, the *P. gingivalis* strain was firstly resuspended in the DMEM medium containing no antibiotics. The number of bacteria was determined by measuring the optical density (OD) using a UV-visible spectrophotometer (Shimadzu Co.) at a wavelength of 520 nm, based on a standard curve established by the colony formation on bacterial plates. The confluent HGF monolayers were infected with *P. gingivalis* at a multiplicity of infection of 100 and incubated for 2 hours (at 37°C in 5% CO_2). After co-incubation of the HGFs with the bacteria, the supernatant was replaced with a fresh medium containing 0.5 mg/mL of gentamicin (Biochrom) and 0.1 mg/mL of metronidazole (Sigma-Aldrich) for 1 hour to remove the extracellular bacteria. After 1 hour the HGF cultures were washed twice with sterile phosphate buffered saline (PBS). Finally, the fresh DMEM medium supplemented with fetal bovine serum and antibiotics was applied to each well. The infected HGFs were incubated at standard conditions (at 37°C in 5% CO_2) for the *in vitro* experiments.

2.2.4 Proliferation

Cell proliferation was assessed using bromodeoxyuridine (BrdU) on the 96-well plate with 1 $\times$ 10^5 cells/mL seeded and measured at 12, 24, 48, and 72 hours after infection. The proliferation was performed using commercially available BrdU kit (Roche Diagnostics GmbH). The cells were processed according to the recommendations in the manufacture's protocol.

2.2.5 Cell metabolic activity

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Cell metabolic activity was analyzed on the 24-well plate with 2×10^4 cells/mL seeded and measured at 3, 24, and 72 hours after infection by a tetrazolium salt (WST-1) colorimetric assay (Roche Diagnostics GmbH). Briefly, the culture medium was removed, and the experimental medium containing a 10% (100 µL) WST-1 reagent was applied (250 µL/well). The plates were incubated at 37°C with 5% CO_2 for 1 hour, and 100 µL/well was transferred to a 96-well plate (Nunc). The plate was shaken before the absorbance measurement at 450 nm and 650 nm (baseline correction) by a Microplate Reader 500. Wells containing the WST-1 medium without cells were used as the background and were subtracted from all the measurements.

2.2.6 Reverse transcription and real-time polymerase chain reaction

The RNA isolations were conducted after 3, 7, 14, and 21 days using an RNeasy mini kit (Qiagen) as previously described.[18] Isolated RNA was reverse transcribed using a one-step high-capacity cDNA reverse transcription (RT) kit (Applied Biosystem). The level of gene expression was calculated using the comparative C_t method ($\Delta\Delta C_t$). Results of the beta actin (*ACTB*) expression were used as the C_t reference for each of the samples, and results of the investigated genes obtained for 3-day HGFs were used as the ΔC_t calibrator sample. LightCycler 480 SYBR Green I Master (Roche Diagnostics GmbH) and specific oligonucleotide primers (Sigma-Aldrich) were used for examining the expression of target genes: collagen type 1 (*COL1A1*), fibronectin 1 (*FN1*), matrix metalloproteinase 2 (*MMP2*), fibroblast growth factor receptor 1 (*FGFR1*), tumor necrosis factor-alpha (*TNFA*), interleukin-1 beta (*IL1B*), interleukin-8 (*IL8*), and beta actin (*ACTB*) as the endogenous control gene. Manually prepared cDNA of 2-µL and 8-µL reaction mixes were pipetted to each well of the 96-well plate (Roche Diagnostics GmbH), according to the manufacturer's instructions. The polymerase chain reaction (PCR) conditions were 95°C for 5 min, followed by 40 cycles of 10 s at 95°C, 15 s at 62.3°C, 20 s at 72°C, and a final cycle of 20 min with increasing

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temperature from 60°C to 95°C, followed by the standard denaturation curve. The analyzes were performed with the CFX96 Real-Time PCR Detection System (Bio-Rad).

2.2.7 Statistical analyzes

Descriptive statistics were used, and mean values were calculated. Data are shown as mean ± standard error of the mean (SEM) and were analyzed using one-way analysis of variance (ANOVA) and post hoc Bonferroni test (IBM SPSS Statistic 22). As the significance level, a p-value (probability value) of 5% was used throughout the study.

3. RESULTS

3.1 PU and PA coatings do not influence the infected HGF morphology

First, we analyzed the impact of the RG-I nanocoating on the *P. gingivalis*-infected HGFs in terms of morphological differences. As shown in Figure 2, neither the nanocoating with PU nor that with PA influenced the morphology of the infected HGFs as compared to the corresponding TCPS control. No differences in cells shape, size, and spreading were found between the HGFs grown on the different surfaces (PU, PA, and TCPS).

3.2 PU and PA coatings enhance the infected HGF proliferation and metabolic activity

To determine the influence of the RG-I nanocoating on the fibroblast proliferation after the *P. gingivalis* infection, we analyzed the BrdU incorporation rate of these cells on the different coatings (PU, PA, and TCPS as the control) by using ELISA. The proliferation rate of the infected HGFs increased over time irrespective of the different culture surfaces/coatings used (Figure 3). But, the proliferation rate was significantly higher in the infected HGFs cultured on the PU- and PA-coated than that of the cells grown on TCPS as observed at each period being analyzed (12, 24, 48, and 72 hours). Moreover, the cellular metabolic activity increased over time in HGFs after the *P. gingivalis* infection (Figure 4). Compared with the infected

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HGFs seeded on TCPS, significantly higher cellular metabolic activity was detected in cells grown either on the PU-coated surfaces at 3 hours or on the PA-coated surfaces at 3 hours and 24 hours post infection.

3.3 PU and PA coatings considerably influence the infected HGF gene expression

To investigate the influence of the RG-I nanocoating on the proinflammatory response of *P. gingivalis*-infected HGFs, we first analyzed the gene expression of *IL1B*, *IL8*, *TNFA*, and *MMP2* (Figure 5). In general, the *IL1B*, *IL8*, *TNFA*, and *MMP2* expression decreased over the time from 3 to 21 days and was the lowest in the infected HGFs cultured on the PA-coated surface and the highest in cells on the control TCPS surface.

Second, we analyzed the expression of genes coding the proteins important for the HGF function such as the extracellular matrix (ECM) proteins *COL1A1* and *FN1* as well as the fibroblast survival and growth-related receptor *FGFR1*.

In contrast to the proinflammatory gene expression analyzed, the *COL1A1, FN1,* and *FGFR1* expression increased over time. The HGFs grown on TCPS decrease the expression of *FN1* on day 21. But, the *COL1A1, FN1* and *FGFR1* expression at different endpoints (3, 7, 14, and 21 days) was the highest in the infected HGFs grown on the PA-coated surface and the lowest in the cells cultivated on the TCPS control (Figure 5). The statistically significant differences in the *IL1B*, *IL8*, *TNFA, MMP2, COL1A1, FN1*, and *FGFR1* gene expression between the TCPS control and PU and, PA were found at different endpoints (3, 7, 14, and 21 days), as illustrated in Figure 5.

4. DISCUSSION

Periodontal pathogens, such as *P. gingivalis*, colonize and invade the host tissue, causing inflammation and periodontal tissue destruction. Most studies investigating periodontal and

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peri-implant diseases indicate a loss of soft connective tissue and bone integrity as a result of the host inflammatory response to the bacterial infection.[1,25,26] Pectin polysaccharides, including RG-I, have been shown to have immunomodulatory properties, which may reduce the risk of peri-implantitis.[8,9]

The aim of our study was to investigate the capability of modified (PA) and unmodified (PU) RG-Is to modulate function and the proinflammatory response of HGFs induced by *P. gingivalis*. Our data showed for the first time that pectin RG-Is influence the proinflammatory cytokine response and promote the proliferation and metabolic activity of HGFs as well as the expression of selected genes required for extracellular matrix (ECM) turnover following *P. gingivalis* infection. In response to the bacterial infection, cell morphology remained unaffected in terms of the spreading and the shape of the infected HGFs by the different surfaces analyzed (Figure 2).

The virulence factors of *P. gingivalis* have an inhibitory effect on HGF proliferation,[27] which is particularly noticeable in chronic inflamed tissues observed in medically compromised patients with inflammatory diseases such as rheumatoid arthritis and periodontitis.[28] According to our results, RG-Is, particularly PA, significantly inhibited the decrease in HGF proliferation and metabolic activity induced by the bacteria *P. gingivalis* as compared to that on the control surface without pectins (Figures 3 and 4). These findings are supported by results obtained with murine macrophage cell line J774.2 cultured on modified hairy region-α (MHR-α) from apple pectin in the presence of the bacterial virulence factor lipopolysaccharide (LPS). The MHR-α nanocoating prevents the decrease in the proliferation and cell viability of LPS-activated macrophages.[9] A number of studies investigating the destructive mechanisms of periodontal pathogens have shown that immune response is directly responsible for connective tissue breakdown and bone loss.[1,2,25,26] Immune response

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cells as well as periodontal tissue-derived cells stimulated by bacteria release proinflammatory cytokines such as IL-1β, IL-8, and TNF-α. The proinflammatory cytokines stimulate fibroblasts, macrophages, and epithelial cells to synthetize proteolytic enzymes, leading to ECM degradation.[26] We examined the proinflammatory gene expression at the RNA level to evaluate the *in vitro* response of HGFs to bacterial stimuli with and without the pectin nanocoating. The levels of the proinflammatory gene expression of *IL1B*, *IL8*, and *TNFA* were significantly lower in cells grown on PA than on the TCPS control at different time points, while those grown on PU did not show significant differences (Figure 5). Our findings indicate that the sugar composition and structure of RG-Is are responsible for the proinflammatory cytokine response of HGFs following the *P. gingivalis* infection. Enzymatically modified RG-I pectin with a lower content of arabinose and a higher amount of galactose seemed to have the ability to modulate the inflammation. In accordance with these results, a number of other studies demonstrated that pectin polysaccharides are immunomodulatory agents.[8-11] Our results also demonstrate that the content of arabinose and galactose in RG-Is influences the inflammatory response of the infected HGFs. It has been recently reported that modified citrus pectin (MCP) attenuates the inflammatory response in human synovial fibroblasts (SF) obtained from rheumatoid arthritis and osteoarthritis patients through a reduction of the cytokine *IL6* levels. MCP binds to the galectin-3 receptor, an important player in the initiation of the inflammatory response, and inhibits the inflammatory response of SF.[29] This finding is in line with our results, and therefore, it can be speculated that the anti-inflammatory effect of RG-I pectin is mediated through the galectin-3 receptor. Experimental evidence indicates that the region of RG-I with a higher galactose content binds specifically and inhibits the function of galectin-3 found on the cell surface.[30] But, the molecular mechanism of the interaction between the RG-I structure and the cellular galectin-3 receptors under bacterial infection is still poorly defined and needs further investigation.

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The primary function of fibroblasts such as HGFs is to maintain the ECM turnover by the deposition of ECM components and the secretion of ECM-degrading MMPs. The balance between the deposition and the degradation of ECM is essential for the structural integrity of the tissue. The inflammation process interferes with the bone homeostasis, induces higher MMP secretion, and leads to an excessive degradation of ECM.[31,32] Previous studies intend to use the ECM proteins as implant coatings to improve the bone healing, but they can denature and thereby lose the ability to bind cells. Additionally, the proteins can be subjected to the enzymatic degradation, particularly those stemming from the mammalian origin that has classically been used due to their involvement in the bone formation (i.e. collagen). The plant-derived polysaccharides, mainly represented by the RG-Is have the advantage of being relatively inexpensive and easy to obtain than the commonly used proteins. Furthermore, the RG-I molecules are not enzymatically degraded in the body and their structure is relatively easy to control.[12,13,33] Our results showed that the RG-I properties may have a great effect not only on osteoblasts,[14,17,18] but also on fibroblasts during the bone healing process due to increasing expression levels of *COL1A1* and *FN1* (Figure 5). These findings are of great importance since collagen and fibronectin are the main components of ECM.[31]

Recent clinical investigation has demonstrated the important role of the FGF/FGFR signaling in the promotion of bone regeneration. FGFR1 is known as a major factor affecting cell proliferation and differentiation.[34] Thus, the enhanced *FGFR1* expression in the presence of RG-I could contribute to greater bone regeneration following the *P. gingivalis* infection. The present study also demonstrated the ability of RG-Is, particularly PA, to suppress the *MMP2* expression in the presence of *P. gingivalis*. A lower *MMP2* expression may lead to the inhibition of the excessive degradation of ECM following the *P. gingivalis* infection (Figure 5). To summarize, the RG-I coating on surfaces does not only seem to decrease the proinflammatory response of HGFs through a downregulation of *IL1B*, *IL8*, and *TNFA*, but

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might also induce ECM formation through the upregulation of *COL1A1*, *FN1*, and *FGFR1*, and the suppression of *MMP2*. These studies may have a large implication for clinical use as nanocoating of implant surface, but also modification of bone substitute materials to stimulate bone growth and modulate inflammatory response. However, due to sparse data concerning pectin nanocoatings more *in vitro* as well as *in vivo* studies need to be performed to provide valuable data of the RG-I utility in surface modification of dental implants.

5. CONCLUSION

Nanocoatings with plant-derived RG-Is affect HGF proliferation and metabolic activity following *P. gingivalis* infection. Further, pectin RG-I, particularly with a high content of galactose and a low amount of arabinose, has the capacity to reduce the inflammatory response of the infected HGFs and stimulate the ECM formation through a downregulation of *IL1B*, *IL8*, *TNFA*, and *MMP2* and an upregulation of the *COL1A1*, *FN1*, *FGFR1* gene expression.

Our findings are of great importance for biomaterial applications and therapeutic approaches. The present study indicates the potential role of RG-Is in limiting inflammation and promoting implant integration in immunocompromised patients with chronic inflammatory diseases such as rheumatoid arthritis and periodontitis.

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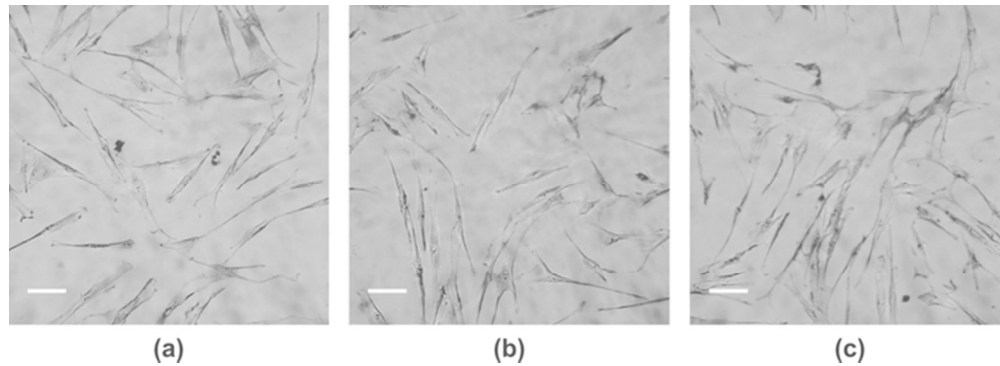
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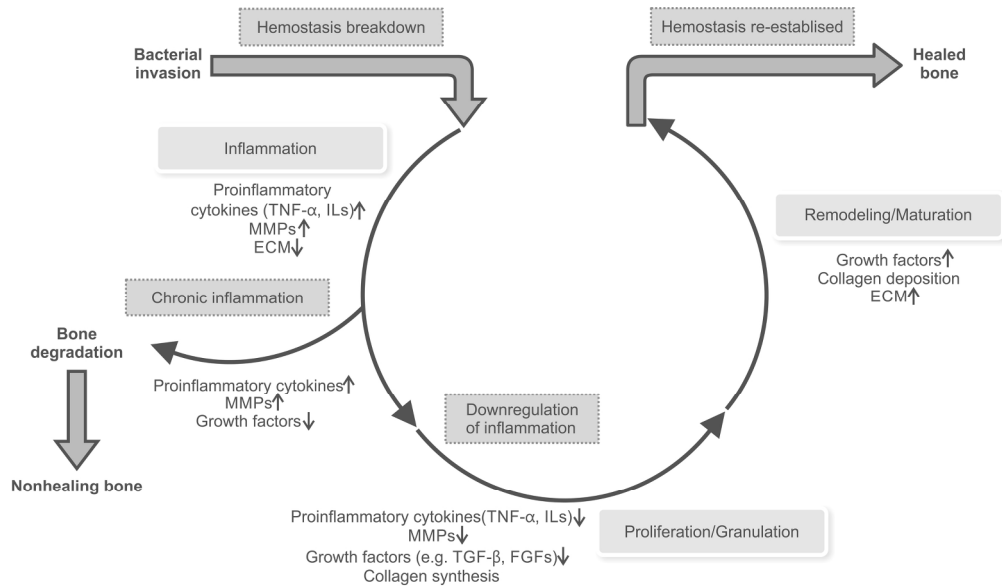
Schematic illustration of normal and abnormal bone healing process in terms of the bacterial colonization and invasion. ECM: extracellular matrix; FGFs: fibroblast growth factors; ILs: interleukins; MMPs: matrix metalloproteinases; TGF-β: transforming growth factor-beta; TNF-α: tumor necrosis factor-alpha.

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Representative images of the infected HGF morphology (a) on control TCPS surface, (b) on TCPS surface coated with PU, (c) on TCPS surface coated with PA investigated at 24 hours post infection by light microscopy. The bar is a scale of 10 µm. HGF: human primary gingival fibroblast; TCPS: tissue culture polystyrene; PU: potato unmodified rhamnogalacturonan-I; PA: potato dearabinanated rhamnogalacturonan-I.
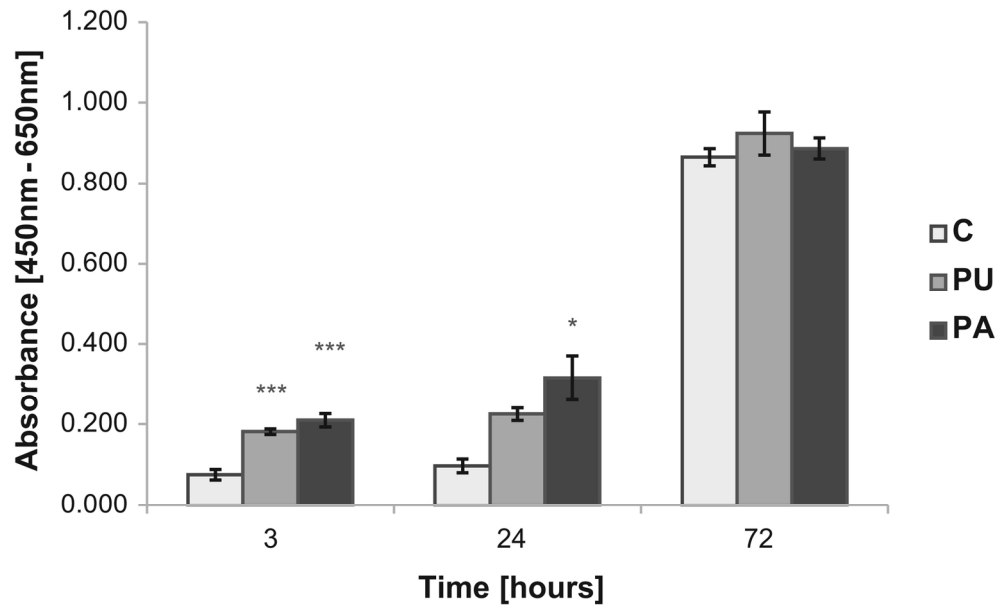
61x21mm (300 x 300 DPI)
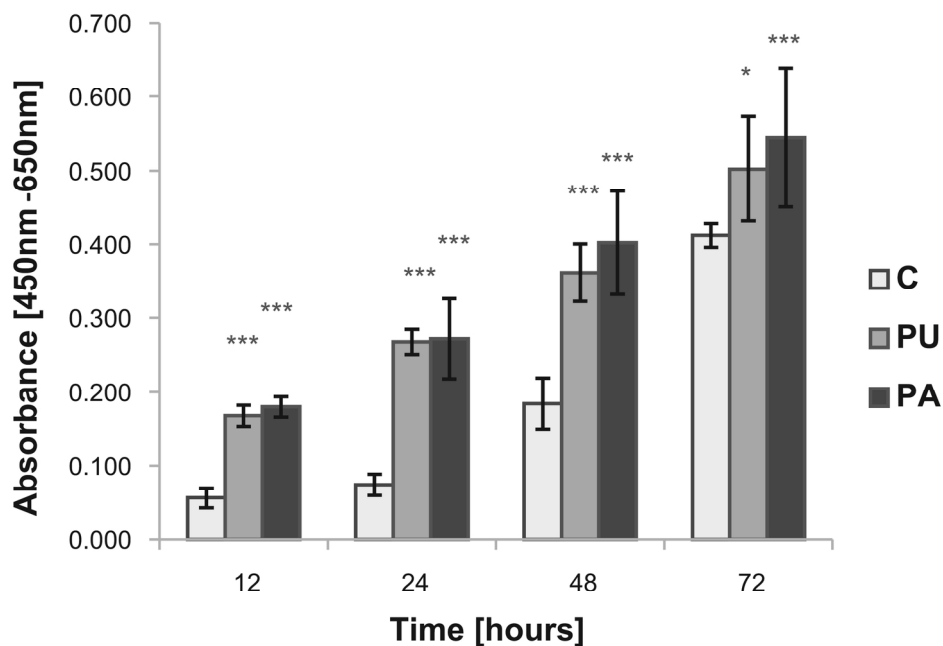
Proliferation of the infected HGFs cultured on TCPS surface without coating (C), on TCPS coated with PU, and on TCPS coated with PA as assessed by BrdU incorporation after 6, 12, 24, 48, and 72 hours using colorimetric assay. Data are given as means +/- SEM (n = 6) and were statistically analyzed using one-way ANOVA with Bonferroni corrections for multiple comparisons. (p-values: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). HGFs: human primary gingival fibroblasts; TCPS: tissue culture polystyrene; PU: potato unmodified rhamnogalacturonan-I; PA: potato dearabinanated rhamnogalacturonan-I; BrdU: bromodeoxyuridine; SEM: standard error of the mean; ANOVA: analysis of variance.
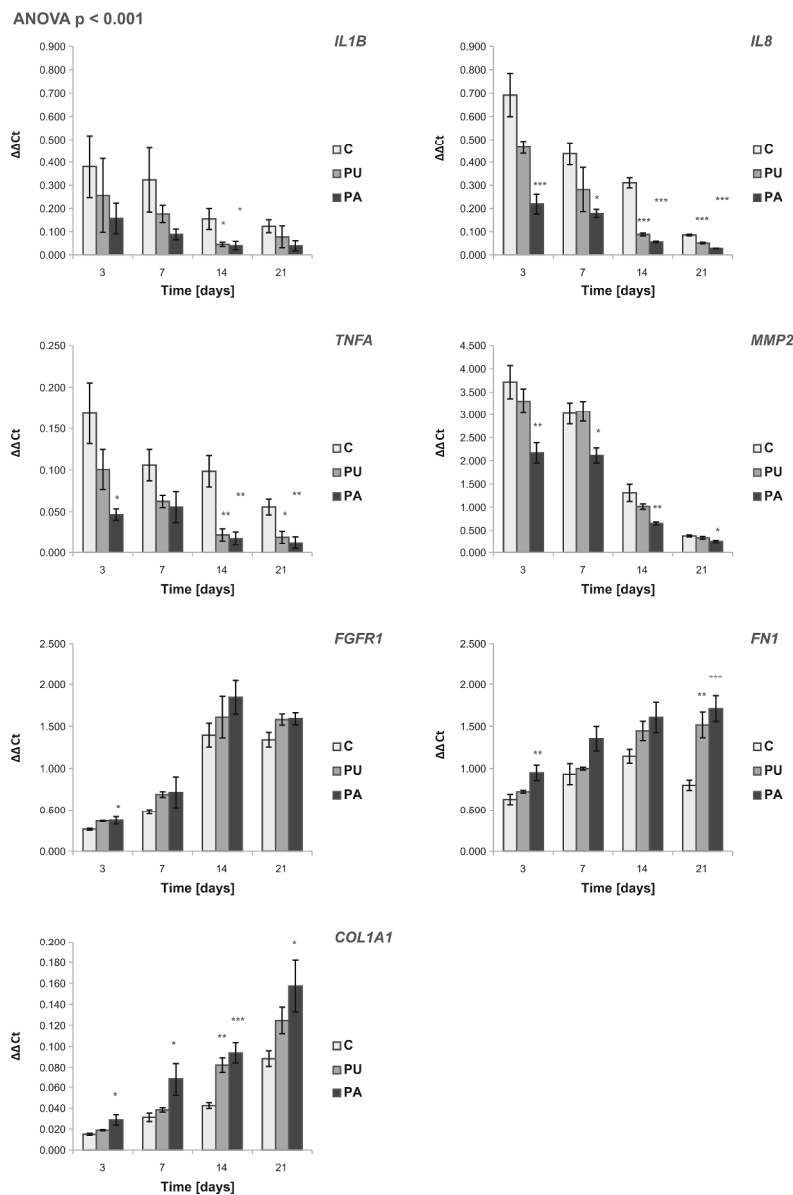
81x65mm (600 x 600 DPI)

ANOVA p < 0.001

Cell metabolic activity of the infected HGFs cultured on TCPS surface without coating (C), on TCPS coated with PU, and on TCPS coated with PA measured by the cleavage of a WST-1 after 1, 3, and 7 days. Data are given as means +/- SEM (n = 6) and were statistically analyzed using one-way ANOVA with Bonferroni corrections for multiple comparisons. (p-values: * p < 0.05; ** p < 0.01; *** p < 0.001). HGFs: human primary gingival fibroblasts; TCPS: tissue culture polystyrene; PU: potato unmodified rhamnogalacturonan-I; PA: potato dearabinanated rhamnogalacturonan-I; WST-1: tetrazolium salt; SEM: standard error of the mean; ANOVA: analysis of variance.

76x56mm (600 x 600 DPI)

Gene expression of the infected HGFs cultured on TCPS surface without coating (C), on TCPS coated with PU, and on TCPS coated with PA as assessed by real-time PCR measurements of the expression of IL1B, IL8, TNFA, MMP2, COL1A1, FN1, and FGFR1 after 3, 7, 14, and 21 days. Data are given as means +/- SEM (n = 6) and were statistically analyzed using one-way ANOVA with Bonferroni corrections for multiple comparisons. (p-values: * p < 0.05; ** p < 0.01; *** p < 0.001). HGFs: human primary gingival fibroblasts; TCPS: tissue culture polystyrene; PU: potato unmodified rhamnogalacturonan-I; PA: potato dearabinanated rhamnogalacturonan-I; PCR: polymerase chain reaction; SEM: standard error of the mean; ANOVA: analysis of variance.

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