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Potential periodontal regeneration by application of monoclonal antibodies against integrin-subunits α6 and β1

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Summary

Successful guided tissue regeneration (GTR) should result in a functional attachment apparatus of the periodontium. The crucial points in the healing process are potential microbiological colonization of mechanical barrier membranes, lacking contact to the connective tissue and apical growth of the gingival epithelium. The membranes' success might be improved by equipping them with antibiotics, specific inhibitors for the epithelial growth and growth factors for periodontal ligament cells (PLC) or progenitor cells. Monoclonal antibodies (mAb) directed to integrin subunits α6 and β1 were tested for their specificity to epithelial growth. In vitro assays were performed as direct and indirect contact by seeding the human HaCaT-cell-line and gingival fibroblasts.

Keywords: Periodontal diseases and therapy; Periodontal regeneration; Gingival epithelial cells and fibroblasts; Integrins; Monoclonal antibodies

Introduction

Inflammation of the marginal periodontium (Parodontitis marginalis), caused by plaque accumulation on the tooth surface and in the adjacent soft tissue, leads to a gradual loss of periodontal tissue (alveolar bone, root cement and desmodontal fiber apparatus) in case of insufficient oral hygiene. As a physiological counter-reaction on the inflammatory processes, the proliferation of affected tissue cells is initiated. Along with an apical migration and lateral extension, the cells of the gingival junctional epithelium proliferate more quickly than the cells of the lower-level desmodontal fiber apparatus. As a consequence, deep gingival pockets and bone pockets develop, that prevent a physiological regeneration of the tooth supporting apparatus. In the long run, this will mean for the patient that the teeth in the jaw areas that are affected by the inflammation will loosen or even get lost. In progressive periodontal therapy the strategy is to inhibit the deep growth of the epithelium mechanically on the basis of Guided Tissue Regeneration by artificial barriers. This way periodontal regeneration shall be improved [1,2]. However, from the histological and clinical perspectives, this therapy alone could achieve only partly satisfying results with clearly restricted indication [3,4,5].The option of affecting the growth processes of cells and tissues by applying specific substances provides an alternative to the mechanical therapy [6]. The principles of a future innovative approach to the therapy of parodontal diseases which are presented here, describe the growth retardation of the gingival epithelium by using antibodies against certain cellular adhesion molecules (CAM) on the surface of these cells. This method generates a barrier on molecular level,

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which is to enable the cells of the periodontal ligament to regenerate physiologically [7].

Precondition for the postperiodontal re-epithelisation is the closed contact between cells of the gingival junctional epithelium and their extracellular matrix (ECM). It is provided by interactions between specific receptors on the surface of the epithelium cells – the so called integrins and adhesive components of extracellular matrices such as collagen, laminin or fibronectin. The integrins have extracellularly oriented epitopes that consist of α- and β-subunits. Their specificity for different ligands – such as the mentioned ECMs, immunoglobins or the Willebrand-factor - results from the different combinations of subunits [8-15]. If the cell-matrix-interactions between the integrins and the ECM are interrupted, the migration of the gingival epithelium should be prevented. Actually, the first studies of our laboratory carried out with organ cultures of marginal gingival biopsies could show that a blockage of the integrin-subunits α6 and β1 through monoclonal antibodies (mAb) leads to a significant inhibition of the epithelium's growth [16,17]. However, the proven inhibition of expression and growth of the human gingival epithelium through anti-α6-, and anti-β1-antibodies was not quantified in the past examinations. Furthermore, in the organ assay it remained unclear whether the applied antibodies will suppress both the migration and the proliferation of epithelium cells. In the present study, these parameters have, therefore, been inspected at a human permanent keratinocyte cell line HaCaT (German Cancer Research Center (DKFZ), Heidelberg), that exprimate the integrin subunits α6 and β1, too. Compared to the gingival epithelium, HaCaT-cells are of homologous histogenetic origin. In addition to this, they show in-vitro growth characteristics, which are close to those of the native epithelium cells [18]. Another important aspect for assessing the possible clinical use of the tested antibodies is their effect to the cells of the periodontal ligament. If the growth of even these cells was inhibited, this would counteract against the postperiodontal physiological regeneration of the periodontium, so this would cause a contra-productive undesired effect. Therefore, the effect of the used antibodies especially on the proliferation behavior of this cell type should be examined. A primary fibroblasts' culture from human gingiva was selected as model. Fibroblasts are the main population in the desmodontal cells and are evenly distributed in the desmodont because of their function – producing the collagen fiber apparatus. So, they are a cell faction which is representative for the intended studies.

Materials and methods

HaCaT-cell line: The HaCaT cell line is a human, spontaneously transformed permanent keratinocyte cell line [19]. HaCaT-cells are routinely cultivated in DMEM, adding penicillin (400 U/ml), streptomycin (50 µg/ml) and 10 % fetal calf serum at 37°C and 7,5 % CO_2.

Fibroblasts-primary culture: Biopsies of human gingiva are cultivated in 6-corrugated boards at 37°C and 7,5 % CO_2 in DMEM, adding penicillin (400 U/ml), streptomycin (50 µg/ml) and 10 % fetal calf serum for 14 days. Both after 7 and 10 days, the culture medium is exchanged. During the second week, the fibroblasts grow out of the biopsies as cellular lawn. After 14 days, the pieces are removed from the culture, the fibroblasts are taken off by using Trypsin/EDTA and sawed in cell culture bottles for another cultivation [7]. The fibroblasts of the 3rd to 7th passage are used in proliferation-assays

Rat-Anti-Mouse-α6: CD49f (Immunotech, Marseille, F); Mouse-Anti-Human- β1: P4C10 (Biomol, Hamburg, G); Anti-Rat-IgG FITC-conjugated (Sigma, Deisenhofen, G); Anti-Mouse-IgG Cy3-conjugated (Biomol, Hamburg, G).

Expression evidence of the integrin subunits α6 and β1: Anti-α6-FITC-marking [20]. 500 µl of a cell suspension (45.000 cells/ml) are given in each whole of a 8-corrugated flexiperm board. 24 h later, the cells are fixed with acetone/ethanol (1:1) at -20°C for 10 minutes. After rinsing, blocking is effected with 2 % BSA in PBS for 20 minutes. Incubation with primary antibody (dilution: 1:40) at 37°C for 45 minutes. Afterwards, the cells are three times washed with Tween 20 followed by incubation with the secondary antibody (dilution: 1:128) at 37°C for 45 minutes. After this, the cells are washed again three times with Tween 20 and incubated with 60 % glycerol in 0,1 M Tris-buffer, pH 9,3. Finally, the cells are washed three times again and the fluorescence is detected in the confocal laser-scan-microscopy at 488 nm. Anti-β1-Cy3-conjugation [21]. 500µl of a cell suspension (45.000 cells/ml) are given into each whole of a 8-corrugated flexiperm board. 24 h later the medium is sucked off. The cells are fixed with 70 % ethanol at room temperature for 15 minutes. After rinsing, incubation with 0,1 M glycine in 0,05 M Tris-HCl (pH 7,4) for 20 minutes, followed by blocking with 3 % BSA in PBS for 30

minutes. Incubation with the primary antibody (dilution 1:1500) at 37°C for 45 minutes. After washing, incubation with the secondary antibody (dilution: 1:40) at 37°C for 60 minutes. Finally the cells are washed three times with PBS and detected in the confocal laser-scan-microscopy at 565 nm.

Colorimetric cell proliferation test with Bromo-2-deoxy-Uridine (BrdU-Labeling-Kitt, Boehringer, Mannheim, D): The antibodies‘ effect on the proliferation of the used HaCaT cell line is inspected in the BrdU-test. A 96-multi corrugated board is colonized with cells (30.000 cells/ml, 100 µl per corrugation). After 24 and 48 hours, antibodies (anti-α6: 5 µg/ml, anti-β1: 0,56 µg/ml) are added to the cells. The antibodies are used both individually and in combination. Control groups remain free of antibodies. 72 hours later, the effect on cell proliferation is inspected in the BrdU-test [22-26]. In another test series, antibodies are added to the HaCaT-cells after 24, 48 and 72 hours. After 96 hours, the effect of the antibodies is checked once again in the BrdU-test. In addition, the antibodies‘ effect also on the proliferation behavior of the fibroblasts is inspected in the BrdU-test. Cells of a subconfluent culture are taken off and suspended to $3x10^4$ cells/ml. 100 µl of the suspension are given into each corrugation of a 96 multi corrugated board. 48 hours later, the antibodies are added to the cells in the above given concentration. Negative tests remain free of antibodies. The influence on proliferation is measured after 72 hours in the BrdU-test (BrdU-incorporation 3 h, substrate reaction 20 min.). For the positive test, cells in all test series are incubated with 10 % ethanol in the medium.

Results and discussion

To prove the integrin expression, the integrin subunits α6 and β1 were detected both on the cell line HaCaT and on the used primary culture of human gingival fibroblasts through immunohistochemical fluorescence marks. The corresponding incubation with the primary monoclonal antibodies anti-α6 and anti-β1 respectively was followed by another incubation with the relevant secondary antibodies which are linked with fluorescence. Both the FITC-conjugated α6 and the Cy3-conjugated β1-subunits are apparently distributed over the entire surface of the cells. Control group cells which have been incubated with the secondary antibodies only, were not visibly colored in fluorescence-microscopic reference. Contrary to the positive integrin evidence on the permanent cell line HaCaT, the same methods did not succeed in specifically proving the expression of the integrin subunits α6 and β1 with the used fibroblast primary culture from biopsies of human gingiva.

By blocking the integrin subunits α6 and β1 with monoclonal antibodies, the proliferation of the HaCaT-cells which represent gingival epithelium, should be suppressed. In this process, the inhibition of the proliferation of the subepithelial periodontal ligament was absolutely undesired for the purpose of the intended complete postperiodontal regeneration. To find out, whether the applied antibodies really inhibited the proliferation of the cells, it is necessary to catch the cells in an exponential growth phase, i.e. during the S-phase with active DNA-replication. Therefore, growth charts where recorded from the cell cultures

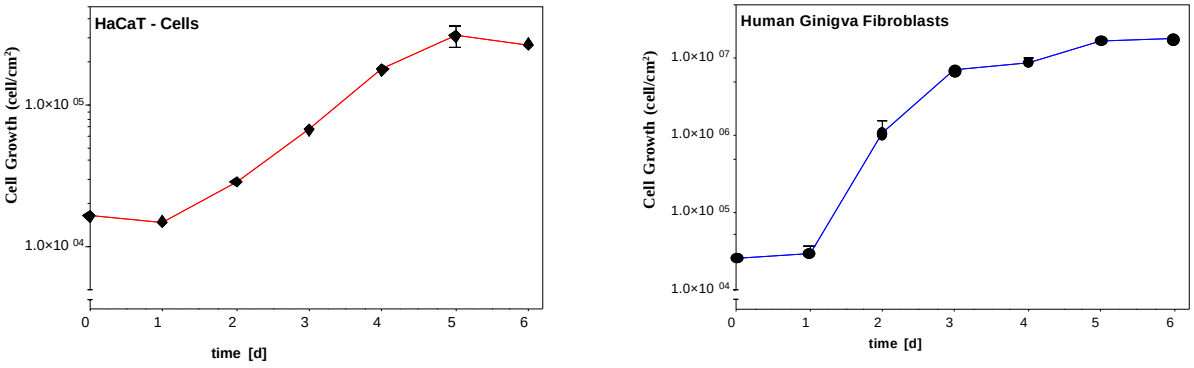

Figure 1: Growth charts of the permanent cell line HaCaT (left hand side), and of primary cultures of human gingival fibroblasts (right hand side). Trypsinated cells from previous passages of the corresponding cultures were seeded in a density of 17.000 c/cm² (HaCaT) and 25000 c/cm² (fibroblasts) on polystyrene boards and incubated at 37°C. In intervals of 24 hours, the cell number was counted in the hemocytometer. Results arise from n=3 independent experiments; data ± SEM.

(HaCaT cell line and human fibroblast primary culture) in their native status, before using them for the proliferation test. After passaging the HaCaT-cells the initial cell concentration is 17.000 c/cm^2. After a lag-phase of one day, the exponential growth phase starts for a period of more than 72 hours (Figure 1, left hand side). The initial cell concentration of the gingival fibroblasts after seeding is 25.000 c/cm^2. Similar to the HaCaT-cells, the logarithmic growth phase starts after 24 hours at the latest and ends after about 48 hours (Figure 1, right hand side). But even after 72 hours of incubation, a subconfluent monolayer can still be recognized in the microscopic picture, where the majority of cells is not yet inhibited by contacts from cell to cell. The conditions for the proliferation tests were derived on the basis of the growth parameters which were found out. Two different periods were selected for the antibody incubation of the HaCaT cells - a) 48 hours and b) 72 hours. For the control proof of the antibodies' effects on the fibroblasts' culture, we limited the procedure to 72 hours of incubation. The influence of the used mAbs on the mitotic activity of the cells was ascertained in the BrdU-test. In a first dose-response-screening with HaCaT-cells, antibody concentrations of 5 µg/ml for anti-α6 and 0,56 µg/ml for anti-β1 proved to be effective amounts. In comparison to the untreated cells (negative control), the proliferation of the cells was inhibited significantly by using both individually and commonly applied antibodies (Figure. 2). Besides the used concentration, the incubation time is decisive for the extend of inhibition. Compared to the 48 hours of incubation (Figure 2, top) the proliferation rates of the cells with 72 hours of incubation were still reduced by half (Figure 2, bottom). In contrary to this, the antibodies did not show any inhibiting effect on the proliferation of the fibroblasts' primary culture, even after 72 hours of incubation. (Figure 3). Applying mAb against the integrin subunit α6 even resulted in a slight increase of the proliferation.

Integrins are cellular adhesion molecules, which connect different structures of extracellular matrices such as laminin, collagen or fibronectin. The integrins' interactions serve to transmit signals and

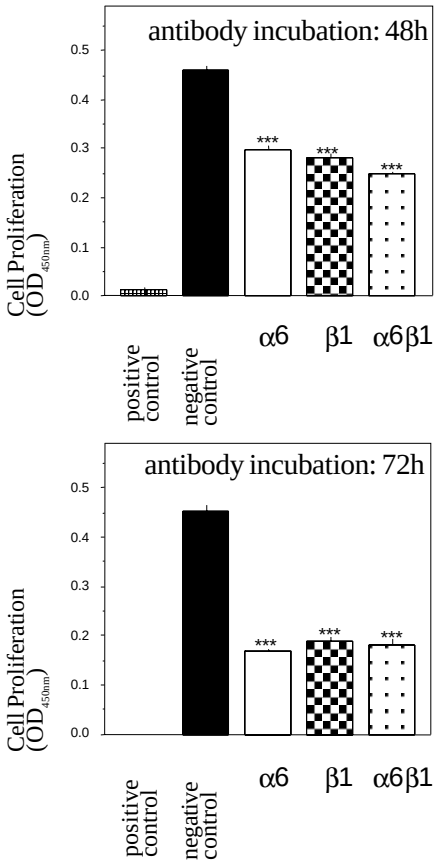

Figure 2: Effects of anti-α6- and anti-β1-antibodies on the proliferation of the human permanent cell line HaCaT. Log-phase-cells were incubated for 48 h (top) and 72 h (bottom) with and without the mentioned mAbs or with 10 % (v/v) ethanol (positive control). Consequently, the mitotic activities of the cells were ascertained by using the BrdU-test in ELIZA at λ=450 nm. Statistical evaluation: the data originate from n=48 independent measurements; data ± SEM; (***): p<0,001 compared to the negative control (ANOVA for paired samples).

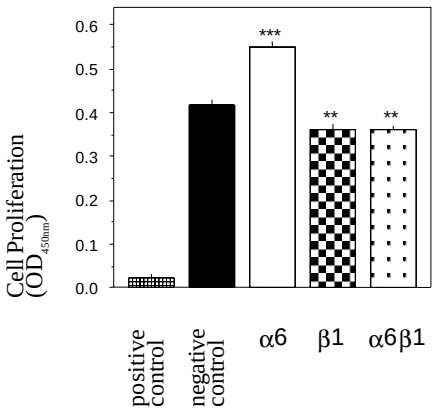

Figure 3: Effects of anti-α6- and anti-β1-antibodies on the proliferation of human gingiva fibroblasts. Log-phase-cells were incubated for 72 h with and without the mentioned mAbs or with 10 % (v/v) ethanol (positive control). Consequently, the mitotic activities of the cells were ascertained by using the BrdU-test in ELIZA at λ=450 nm. Statistical evaluation: the data originate from n=48 independent measurements; data ± SEM; (***): p<0,001 and (**): p<0,01 compared to the negative control (ANOVA for paired samples).

control different processes in and on the cells, on molecular level [27]. In epithelial cells these are in particular growth processes during the wound healing periods [28-33]. Earlier investigations could show, that in humans integrins are exprimated in all epithelial cells [34]. In the gingival epithelium, especially the integrin subunits α6 and β1 are co-exprimated [35]. So, this is the case on the human keratinocyte line HaCaT, too. Growth inhibiting effects of monoclonal antibodies against β1-integrin subunits could be proved for the first time by Hergott et. al., in 1993.

In the present study, we used monoclonal antibodies against both integrin subunits, and examined their specific effectiveness with reference to the proliferation of epithelial keratinocytes. Both, isolated application and combined application of antibodies lead to a significant reduction of the cell proliferation. For an intended clinical effect in the treatment of periodontal diseases, it will be of particular importance that the mAb do not have any inhibitory effects on the proliferation of the remaining gingival tissue. In our tests, it was not observed that the proliferation of human gingiva fibroblasts was inhibited by either of the used antibodies, anti-α6 and anti-β1. So, the undesired deep growth of the gingival junctional epithelium can be suppressed in a controlled and directed way on the molecular level. At the same time this allows to provide the deeper fiber generating cells of the connective tissue with the possibility to refill the periodontal defect completely. The in vivo effectiveness on the postperiodontal regeneration will be inspected in a planned animal experiment in the dog model by releasing mAbs against the integrin subunits α6 and β1 in a drug release system.

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