Different wound healing properties of dermis, adipose and gingiva mesenchymal stromal cells

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Abstract

Oral wounds heal faster and with better scar quality than skin wounds. Deep skin wounds where adipose tissue is exposed, have a greater risk of forming hypertrophic scars. Differences in wound healing and final scar quality might be related to differences in mesenchymal stromal cells (MSC) and their ability to respond to intrinsic (autocrine) and extrinsic signals, such as human salivary histatin, epidermal growth factor and transforming growth factor beta1. Dermis-, adipose- and gingiva derived MSC were compared for their regenerative potential with regards to proliferation, migration and matrix contraction. Proliferation was assessed by cell counting and migration using a scratch wound assay. Matrix contraction and alpha smooth muscle actin was assessed in MSC populated collagen gels, and also in skin and gingival full thickness tissue engineered equivalents (reconstructed epithelium on MSC populated matrix). Compared to skin derived MSC, gingiva MSC showed greater proliferation and migration capacity, and less matrix contraction in full thickness tissue equivalents, which may partly explain the superior oral wound healing. Epidermal keratinocytes were required for enhanced adipose MSC matrix contraction and alpha smooth muscle actin expression, and may therefore contribute to adverse scarring in deep cutaneous wounds. Histatin enhanced migration without influencing proliferation or matrix contraction in all three MSC, indicating that salivary peptides may have a beneficial effect on wound closure in general. Transforming growth factor beta1 enhanced contraction and alpha smooth muscle actin expression in all three MSC types when incorporated into collagen gels. Understanding the mechanisms responsible for the superior oral wound healing will aid us to develop advanced strategies for optimal skin regeneration, wound healing and scar formation.

Introduction

Immediately after injury to the skin or oral mucosa, the body responds with a series of overlapping phases to repair and regenerate the damaged tissue, i.e. the inflammatory phase, proliferative phase and remodeling phase. Wound healing is initiated by immediate contraction of the wound margins. In the inflammatory phase bacteria and debris are removed and factors are secreted to attract and activate cells that are involved in the proliferative phase. During the proliferative phase multiple processes take place, such as angiogenesis, cell migration, granulation tissue formation and re-epithelialization. Transforming growth factor beta1 (TGFβ1) is involved in regulating angiogenesis and deposition of extracellular matrix (ECM), such as fibronectin and collagen and is also important in wound contraction (1,2). Mesenchymal stromal cells (MSC) (also known as fibroblasts in dermis and gingiva) play an important role by depositing ECM. In the remodeling phase, ECM is further synthesized, remodeled and realigned. The quality of the final scar is dependent on the interplay between all above mentioned phases. Excessive or abnormal ECM deposited by MSC can lead to abnormal scar formation (e.g. hypertrophic scar).

Even though the general processes leading to wound healing described above are comparable in skin and oral mucosa, clear clinical differences have been reported. Wounds in the oral mucosa heal faster and with a far better scar quality than wounds in the skin (3,4). It is currently unknown why these differences exist. Most likely both intrinsic properties of the cells within the oral mucosa as well as interactions with the surrounding tissue environment are involved, leading to the superior oral wound healing. For example, we have previously shown that using identical methods to culture organotypic tissue engineered skin and oral mucosa, the difference in epithelial keratin expression observed in these native tissues is due only to properties of the keratinocytes and/or fibroblasts. This is in contrast to the increased proliferation observed in oral epithelium compared to skin, which is not due to keratinocyte-fibroblast interactions, and therefore must be regulated by other external factors such as cross-talk with additional neighboring cells or saliva (5,6). The increased proliferation in native oral epithelium leads to a thicker (more cell layers) epithelium, as well as a higher baseline turnover than skin, both of which are not observed in in vitro tissue equivalent culture (5-7).

The major extrinsic difference between skin and oral mucosa is the presence of saliva. In addition to being rich in proteins, saliva creates a humid environment which has been shown to be beneficial for wound healing (7,8). Wound licking promotes cutaneous wound healing in mice (9) and in rats it was shown that desalivation delays oral wound healing (10). The proteins within human saliva include classical growth factors, as well as defensins, cathelicidin and trefoil factors known for their positive effect on wound healing (11-13). Epidermal growth factor (EGF) plays an important role in re-epithelialization, by increasing proliferation and migration (1); it also promotes granulation tissue formation (14). EGF is found in rodent saliva as well as human saliva, but one of the great differences between rodent and human saliva is the amount of EGF, which is found abundantly in rodent saliva,
but only in trace amounts in human saliva (15). This suggests that other factors are present in human saliva which may stimulate wound closure. Importantly, we have shown that human saliva contains histatins, which are the predominant peptides responsible for enhanced oral keratinocyte and fibroblast migration (16). This strongly implicates their role as an extrinsic factor in oral wound healing. Histatins are a family of peptides which are specifically produced in salivary glands of higher primates and are encoded by two genes, \textit{HTN1} and \textit{HTN2}. The latter encodes for histatin 3 and derivatives which possess antimicrobial activities (17,18). \textit{HTN1} encodes for histatin 1 and 2 that have been found to enhance keratinocyte and fibroblast migration \textit{in vitro} (15,16,19,20).

In superficial wounds mostly dermal MSC are involved in wound healing. However, in deep cutaneous wounds the MSC within the exposed underlying adipose tissue may also play a role in the healing process. Previously we have shown that both dermal and adipose derived MSC express typical stem cell surface markers, as well as show multi-lineage (adipogenic, chondrogenic and osteogenic) differentiation capacity (21). Others have shown that gingival MSC also have typical stem cell characteristics (22,23). In wound healing, dermal MSC are associated with normotrophic scar formation and adipose MSC with hypertrophic scar formation, whereas gingival MSC are associated with superior scar quality (24-26). However, the underlying mechanism for the differences in quality of the final scar is still largely unknown. Therefore, in this study we compared the intrinsic capacity of the three different MSC populations to proliferate, migrate and contract a matrix, since these are major events resulting in wound healing and tissue regeneration. Additionally, matrix contraction and the expression of a smooth muscle actin (\alpha SMA), which represent scar forming myofibroblasts, was investigated in full thickness skin and gingiva equivalents which consisted of a fully differentiated epithelium on a MSC populated connective tissue matrix. Finally, the extrinsic effect of human salivary histatin on these MSC was investigated in comparison to EGF and TGF\textbeta 1. EGF is reported to influence migration and proliferation and TGF\textbeta 1 is reported as pro-fibrotic and increases matrix contraction (1). Furthermore, TGF\textbeta 1 expression is lower in fetal skin (which is associated with scarless healing) compared to postnatal skin. Understanding the mechanisms responsible for the superior oral wound healing will aid us to develop advanced strategies for optimal skin wound healing and scar formation.

\textbf{Methods}

\textbf{Isolation and culture of mesenchymal stromal cells from human skin and gingiva}

Human abdominal skin was obtained after informed consent from healthy adult donors undergoing corrective abdominal plastic surgery; gingiva was obtained after informed consent from healthy adult donors after molar tooth extraction or implant surgery. Tissue was used in an anonymous fashion in accordance with the "Code for Proper Use of Human Tissues" as formulated by the Dutch Federation of Medical Scientific Organizations (www.fmwv.nl) and following procedures approved by the institutional review board of the VU University medical centre.

Dermal MSC: After removal of all adipose tissue and large vessels, dermis (same size as gingiva tissue, see below) was washed in phosphate buffered saline (PBS) (B. Braun, Melsungen, Germany) and incubated overnight on dispase II solution (Roche, Mannheim, Germany) at 4 °C. The next day epithelial sheets were separated from the dermis and the dermis was incubated in collagenase type II (Gibco, Life Technologies, Grand Island, USA) / dispase II in Hanks balanced salt solution (HBSS)(Gibco) at 37 °C for 2-3 h. Enzymatic activity was neutralized by adding PBS containing 0.1% bovine serum albumin (BSA)(Sigma-Aldrich, St. Louis, USA). Tissue debris was removed by filtration (filter chamber: NPI, Emmelo-Compascuum, the Netherlands). MSC medium, consisting of Dulbecco’s modified Eagle medium (DMEM) (Lonza, Verviers, Belgium) containing 1% ultroserG (UG) (Biosepra, Cergy-Saint-Christophe, France) and 1% penicillin-streptomycin (P/S) (Gibco) was added. Dermal MSC were harvested by centrifugation (300 x g, 5 min) and suspended in MSC medium. The suspension was passed through a 100 \mu m and 40 \mu m cell strainer (Becton Dickinson Falcon, Erembodegem, Belgium).

Adipose MSC: Adipose tissue was carefully dissected from skin and large vessels were removed. Adipose tissue was washed several times with PBS, cut into small pieces and incubated overnight at 4 °C in HBSS. The next day it was incubated in collagenase type II/ dispase II in HBSS at 37 °C for 2 h and then treated the same as dermis (see above).

Gingival MSC: Gingiva biopsies (8-15 mm²; material of multiple donors was pooled if pieces were too small) were washed with PBS + 1% P/S and incubated overnight on dispase II. The next day, epithelial sheets were separated from the lamina propria and incubated in collagenase type II / dispase II in HBSS at 37 °C for 2-3 h. After this, MSC medium was added and the solution was passed through a 100 \mu m and 40 \mu m cell strainer.

Single-cell suspensions derived from the three different MSC types were seeded at approximately 3.5 x 10^4 cells/cm². Cells were cultured in MSC medium for the proliferation assay, the migration assay and prior to constructing the tissue equivalents or in DMEM containing 1% F/S and 5% fetal bovine serum (FBS) (Thermo Fischer Scientific, Waltham, USA) for the collagen contraction gels. Medium was changed twice a week and cultures were passaged when 90% confluent using 0.5 mM EDTA/ 0.05% trypsin (Gibco). The cells were too small) were washed with PBS + 1% P/S and incubated overnight on dispase II. The next day, epithelial sheets were separated from the lamina propria and incubated in collagenase type II / dispase II in HBSS at 37 °C for 2-3 h. After this, MSC medium was added and the solution was passed through a 100 \mu m and 40 \mu m cell strainer. This shows the experimental set-up and the different donors used in the experiments.
Isolation and culture of keratinocytes from human skin and gingiva

After overnight incubation on dispase II (see dermal and gingival MSC), epithelial sheets were separated from the dermis and lamina propria. The epithelial sheets were incubated in 0.05% trypsin for 10 min, keratinocyte medium was added and cell suspension was passed through a 100 μm and 40 μm cell strainer. Keratinocytes were then seeded in tissue culture dishes coated with 0.5 μg/cm² human placental collagen IV (Sigma-Aldrich) in a density of approximately 4 x 10⁴ cells/cm², cultured in keratinocyte medium and maintained at 37˚C in a humidified atmosphere containing 7.5% CO₂. Keratinocyte medium consisted of DMEM/ Ham’s F-12 (Gibco) (3:1, v/v), 1% UG, 1% P/S, 1 μM hydrocortisone (Sigma-Aldrich), 1 μM isoproterenol (Sigma-Aldrich), 0.1 μM insulin (Sigma-Aldrich) and 2 ng KGF for skin keratinocytes and 1 ng/ml rhEGF for gingival keratinocytes (both Sigma-Aldrich). First passage keratinocytes were stored in liquid nitrogen until further use in constructing skin and gingiva equivalents.

Peptide synthesis

Histatin 2 (Hst2) (RKFHEKHHSHREFFFPPFYGDYGSNYLYDN) was synthesized by solid-phase peptides synthesis using Fmoc chemistry with a Syro II synthesizer (Biotage, Uppsala, Sweden). Purification was conducted by ultimate 3000 RP-HPLC (Thermo Scientific) and authenticity was confirmed by mass spectrometry (MALDI-TOF) (Bruker Daltonik GmbH Germany) as previously described (27).

Proliferation assay

Freshly isolated MSC were grown to max 90% confluency in DMEM containing 1% P/S and 1% UG, counted in duplicate using an Accu Chip, (Digital Bio, Sedul, Korea) and seeded in 6-well plates (passage 1) with a cell density of approximately 4 x 10⁴ cells/cm². The cells were left to attach for 4 h and were then exposed to different concentrations of Hst2 (0.05, 0.5, 5 and 50 μM), 10 ng/ml rhEGF and 10 ng/ml rhTGFβ1 (R&D Systems, Minneapolis, USA). Vehicle (H₂O) supplemented medium was used as negative control. After 4 days of culture, cells were trypsinized, and reseeded at a dilution of 1:4 (passage 2). Culture medium was supplemented with Hst2, rhEGF and rhTGFβ1 as described above. After an additional 4 days (8 days total), cells were harvested and counted in duplicate dishes (max 90% confluency). This number was corrected for the dilution factor 1:4 and divided by the starting number of cells, to give a fold increase in proliferation after 8 days of culture.

Migration assay

The migration assay was performed exactly as described earlier (21). In brief, confluent MSC monolayers in a 48-well plate were incubated with serum free medium (DMEM containing 1% P/S and 0.1% BSA) for four days to ensure growth arrest. After four days a scratch was drawn with a plastic disposable pipette tip. The cultures were washed twice with PBS to remove detached cells and then exposed to different concentrations of Hst2 (0.05, 0.5, 5 and 50 μM) in serum free medium. Medium supplemented with 10 ng/ml rhEGF was used as a positive control, vehicle supplemented medium was used as negative control. Phase contrast micrographs were taken after drawing the scratch and after four days of exposure. Data were analyzed using an image processing algorithm which has been described in detail in our previous work (28-30). This algorithm is designed to non-destructively and quantitatively determine the scratch area. Specifically, micrographs were segmented into two regions: denuded areas and cell-populated areas. The segmentation process automatically distinguishes between the two regions based on local texture homogeneity measures, where any denuded areas are characterized by higher local texture homogeneities with respect to cell-populated areas. Standard deviation (SD) of pixel intensities is used as the measure of texture homogeneity; lower SD values correspond to higher texture homogeneities, and vice versa.

Table 1: Experimental set-up and donors used in experiments

<table>
<thead>
<tr>
<th>Experiment type</th>
<th>Experiment</th>
<th>Dermal MSC</th>
<th>Adipose MSC</th>
<th>Gingival MSC</th>
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<td>Donor 1</td>
<td>Donor 2</td>
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<td></td>
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<td>Donor 5</td>
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<td>Donor 7</td>
<td>Donor 8 + 9</td>
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<td></td>
<td>2</td>
<td>Donor 10</td>
<td>Donor 10</td>
<td>Donor 11</td>
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<tr>
<td></td>
<td>3</td>
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<td>Donor 12</td>
<td>Donor 13 + 14 + 15</td>
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<tr>
<td></td>
<td>4</td>
<td>Donor 16</td>
<td>Donor 16</td>
<td>Donor 17 + 18 + 19</td>
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<tr>
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<td>3</td>
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<td></td>
<td>4</td>
<td>Donor 26</td>
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<td>Donor 45</td>
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All donors were consenting adults (age 18 – 65) and biopsies were obtained from healthy abdominal skin or gingiva with no signs on inflammation. The donors used within the experiments are indicated. In some cases when gingiva biopsies were small, donors were pooled as indicated.
**Contraction assay**

Contraction was measured using two different methods, the first method used only MSC in collagen gels; the second method used human skin and gingiva equivalents. Collagen matrix: Collagen gels were prepared using 4 mg/ml collagen in 0.1% acetic acid isolated from rat tails. The collagen solution was mixed on ice with MSC (2 x 10^5 cells/gel). Subsequently, 1 ml of collagen suspension was pipetted into each well of a 12-wells plate (Costar, Corning Incorporated, New York, USA). After one hour of incubation at 37°C in a humidified atmosphere containing 5% CO₂, to allow gel polymerization, the gels were gently detached with the aid of a spatula. They were then exposed to different concentrations of Hst2 (0.05, 0.5, 5 and 50 μM) in DMEM containing 1% P/S and 5% FCS. Medium supplemented with 10 ng/ml TGFβ1 was used as a positive control, vehicle supplemented medium was used as negative control. Medium was changed three times a week. Photographs were taken with the Canon Powershot G12 digital camera at the beginning, before each medium refreshment and at the end of the experiment. The diameter of the cultures was measured using NIS-Elements AR 3.2 software. All cultures were formalin-fixed and paraffin embedded using standard methods.

Skin and gingiva equivalents: Skin equivalents (containing dermal or adipose MSC) and gingiva equivalents were created using the same method as previously described (31). In short, mesenchymal cells (4 x 10^5) were seeded into 2.2 x 2.2 cm collagen-elastin-matrix (Matriderm®, Dr. Suwelack Skin & Health Care, Billerbeck, Germany) and cultured submerged in culture medium containing DMEM /Ham's F-12 (3:1), 2% UG, 1% P/S, 5 μg/ml insulin, 50 μg/ml L-ascorbic acid (Sigma-Aldrich), and 5 ng/ml rhEGF in a 0.4 μm pore size transwell (Cat nr 3450, Costar) in a 37°C, 5% CO₂ atmosphere. After three weeks of culturing the connective tissue equivalents were harvested. For the full thickness tissue equivalents, keratinocytes (5 x 10^5 cells/culture) were seeded onto the MSC-populated matrix. After three days of submerged culture, equivalents were further cultured at the air-liquid interface in DMEM/ Ham's F-12 (3:1), 0.2% UG, 1% P/S, 1 μM hydrocortisone, 1 μM isoproterenol, 0.1 μM insulin, 10 μM L-carnitine (Sigma-Aldrich), 10 mM L-serine (Sigma-Aldrich), 1 μM dl-α-tocopherol acetate (Sigma-Aldrich), enriched with a lipid supplement, for another ten days until a total of five weeks culture had been reached. The lipid supplement contained 25 μM palmitic acid, 15 μM linoleic acid, 7 μM arachidonic acid and 24 μM BSA, all obtained from Sigma-Aldrich. The cultures received new culture medium twice a week. Photographs were taken with a Canon Powershot G12 digital camera at the first medium refreshment (four days after seeding MSC in the matrix), after three weeks of culture resulting in connective tissue MSC equivalents or after five weeks of culture resulting in full thickness tissue equivalents. The surface area of the cultures was measured using NIS-Elements AR 3.2 software. All cultures were formalin-fixed and paraffin embedded using standard methods.

**Histological and immunohistochemical analysis**

Paraffin embedded sections (5 μm) of the connective tissue equivalents, as well as the full thickness equivalents were either stained with haematoxylin and eosin for morphological analysis, or with an αSMA antibody (clone IA4; 1:200, Dako, Glostrup, Denmark), followed by Envision (Dako) for immunohistochemical analysis, essentially as described by the supplier.

**Statistical analysis**

All data are presented as mean ± standard error mean. Differences were considered significant when *P < 0.05, **P < 0.01, ***P <0.001. Statistics were calculated in GraphPad Prism (San Diego, CA, USA) and method used is indicated in figure legends and text.

**Results**

Adipose derived MSC show less proliferation than dermal and gingival MSC

In order to close a wound, MSC need to proliferate to generate cells which can in turn migrate into the wound bed. Therefore, we first determined whether the three types of MSC showed differences with regards to proliferation (Figure 1). Whereas no statistically significant difference was found between the fold increase in number of cells after an 8 day study period for dermal and gingival MSC, adipose MSC clearly showed a lower proliferation compared to either dermal (significant) or gingival (trend) MSC.

Next, it was determined whether the wound closing factor histatin was able to influence proliferation. Supplementation of culture medium with histatin did not influence the proliferation of any of the MSC subtypes (data not shown).

**Figure 1:** Intrinsic proliferation of dermis-, adipose and gingiva-derived MSC

Fold increase in total cell count of MSC after 8 days of culture relative to the cell number at the start of the experiment is shown. Cells were passaged (1:4 dilution) once after four days (see Materials and Methods). Symbols represent three different experiments. Filled symbols represent donor matched dermal and adipose MSC, open symbols represent non donor matched gingiva. See table 1 for details. *P < 0.05 (ordinary one-way ANOVA with Tukey’s multiple comparisons test).
Gingival MSC migrate faster than dermal and adipose MSC

During wound healing, MSC migrate from the healthy tissue at the wound boundaries into the wound bed to form granulation tissue. To determine whether MSC derived from dermis, adipose tissue and gingiva showed different intrinsic migration rates, a scratch assay was performed (Figure 2A). All MSC types migrated as individual cells, rather than as a front in the 4 day study period. Under culture conditions where serum was absent to ensure that no proliferation occurred, the intrinsic migration rate of both dermis- and adipose-derived MSC was similar during the four day study period, whereas gingival MSC migrated twice as fast as either dermal or adipose MSC (Figure 2B).

Significantly more migration response to 5 μM Hst2, and dermal MSC (but not adipose MSC) showed significantly more migration to rhEGF compared to gingiva MSC (P < 0.05). However, when taking into account that intrinsic migration of gingival MSC was twice that of dermal and adipose MSC, Hst2 and rhEGF supplementation of gingival MSC cultures showed a similar net coverage of the scratch area compared to that observed for dermis- and adipose-derived MSC cultures.

Dermal, adipose and gingival MSC contract collagen gels to a similar extent

Contraction of a wound is part of the healing process and results in a decreased wound area. To explore possible differences in the ability of dermis-, adipose- and gingiva-derived MSC to contract a connective tissue matrix, the MSC were seeded into a collagen gel (Figure 3A). Collagen gel contraction was measured as the reduction in diameter of the gels over a period of 13 days, expressed as percentage of the original gel diameter. All three MSC types induced comparable levels of contraction of the collagen gels: gingival MSC: 61% reduction; adipose MSC: 56% reduction; and dermal MSC: 52% reduction in collagen gel diameter (Figure 3B).

Next the influence of histatin on migration of the three MSC types was determined. Stimulation of MSC derived from dermis, adipose tissue and gingiva with different concentrations of Hst2 (0.05, 0.5, 5 and 50μM) enhanced the migration of all three MSC types (Figure 2C). Using a two-way ANOVA with Tukey’s multiple comparison test, adipose (but not dermal) MSC show significantly more migration response to 5 μM Hst2, and dermal MSC (but not adipose MSC) showed significantly more migration to rhEGF compared to gingiva MSC (P < 0.05). However, when taking into account that intrinsic migration of gingival MSC was twice that of dermal and adipose MSC, Hst2 and rhEGF supplementation of gingival MSC cultures showed a similar net coverage of the scratch area compared to that observed for dermis- and adipose-derived MSC cultures.

Figure 2: Migration properties of dermis-, adipose- and gingiva-derived MSC
A: Representative photographs showing intrinsic dermal MSC migration in a scratch assay directly after introducing the scratch at day 0 (left) and after four days of migration (right).
B: Intrinsic MSC migration measured as extent of closure of the area of the scratch introduced into a confluent layer of MSC derived from dermal, adipose and gingiva tissue, after four days. Symbols represent four different experiments. Filled symbols represent donor matched dermal and adipose MSC, open symbols represent non donor matched gingiva. See table 1 for details. *P < 0.05 (ordinary one-way ANOVA with Tukey’s multiple comparisons test).
C: Extrinsic MSC migration. At time of introduction of the scratch cultures were supplemented with Hst2, rhEGF (10ng/ml: positive control), or vehicle (H2O, negative control). Four days later the extent of migration (area covered) was determined with an image processing algorithm (see Materials and Methods). *P<0.05, **P<0.01 (repeated measures one-way ANOVA with Dunnett’s multiple comparison test).
Bars represent means ± SEM of three independent experiments in quadruplicate relative to vehicle exposed control cultures.

Figure 3: Contraction properties of dermis-, adipose- and gingiva-derived MSC in collagen gels
A: Representative photographs showing dermal MSC-mediated contraction of collagen gels exposed to control or TGFβ1 treatment for 0, 8 and 13 days.
B: Contraction of collagen gels populated with dermal, adipose and gingival derived MSC, exposed to control medium and TGFβ1 for up to 13 days. *P < 0.05 (two-way ANOVA with Tukey’s multiple comparisons test). Bars represent means ± SEM of four independent experiments in duplicate.
Addition of TGFβ1, known for its capacity to enhance dermal matrix contraction, resulted in a further 20% contraction of collagen gels, independent of the type of MSC they contained (Figure 3). In contrast histatin did not influence contraction of the MSC populated collagen gels (Figure 4A). Furthermore histatin did not influence the TGFβ1 mediated enhanced contraction of MSC populated collagen gels in any way (Figure 4B).

Contraction of full thickness skin and gingiva equivalents
Because the margins of wounds are epithelialized during wound contraction, we next determined whether differences in contraction occurred in full thickness skin and gingiva equivalents (Figure 5). The full thickness tissue equivalents consisted of a fully differentiated epithelium on a MSC populated matrix (Figure 5B) while the connective tissue equivalents consisted only of a MSC populated matrix (Figure 5A). Full thickness skin equivalents constructed with adipose derived MSC showed more contraction than skin equivalents constructed with dermal MSC (both constructed with epidermal keratinocytes). Full thickness gingiva equivalents (constructed with gingiva keratinocytes) showed a similar degree of contraction as full thickness skin equivalents containing dermis MSC (Figure 5C). In order to confirm that the differences observed in contraction between the full thickness tissue equivalent model and the collagen gel model described above were not due to differences in methodology, contraction of connective tissue equivalent matrices (in the absence of keratinocytes) was also determined. In line with collagen gel contraction experiments, no difference in contraction was observed between the three MSC types in the absence of keratinocytes, indicating that keratinocytes are required to enhance adipose MSC mediated contraction (Figure 5C).

Absence of αSMA expression in full thickness gingival equivalents
As αSMA expressing myofibroblasts are involved in wound contraction, we determined whether there was a correlation between the degree of matrix contraction and αSMA expression. Immunohistochemical staining showed absence of αSMA expression in the connective tissue equivalents, independent of the type of MSC they contained (Figure 5A). This is in line with our observation that the three types of MSC caused a similar degree of matrix contraction (collagen gels and connective tissue equivalents) in the absence of keratinocytes. However, the full thickness skin equivalent, containing adipose MSC and a differentiated epidermal layer, showed strong αSMA expression (Figure 5B). The αSMA expressing myofibroblasts were located mainly underneath the epidermis. Less αSMA expression was observed in skin equivalents containing dermal derived MSC and very little αSMA expression was observed in gingival equivalents. Gingiva MSC were capable of expressing αSMA, since when collagen gels were exposed to TGFβ1, αSMA was strongly expressed in the tissue sections (data not shown). Histatin had no influence on αSMA expression in this experiment in any MSC type.
Discussion

In this study we show that migration, proliferation and matrix contraction characteristics of dermal, adipose and gingival MSC differs, and may in part explain the differences observed between skin and oral wound healing. Gingiva MSC showed a greater proliferative and migration capacity than adipose derived MSC, and less matrix contraction in full thickness tissue equivalents. These properties could be related to the enhanced scar free healing observed in the oral cavity. Furthermore, epidermal keratinocytes are required for enhanced adipose MSC matrix contraction and αSMA expression, and may therefore contribute to adverse scar formation in deep cutaneous wounds. Histatin enhanced migration without influencing matrix contraction or proliferation in all three MSC, indicating that saliva peptides may have a beneficial effect on wound closure in general.

Cell proliferation is an essential part of wound closure. It is well recognized that gingival epithelium is highly proliferative compared to skin epidermis (5), but studies directly comparing skin and gingival MSC proliferation are scarce. In our study, the proliferation rate of dermal and gingival MSC was similar. These results are in line with Lee et al., who described that oral mucosal MSC proliferate slightly (not significantly) faster than dermal MSC (32). However, we observed a lower proliferation rate for adipose MSC compared to either gingival or dermal MSC, the latter of which has already been described in literature (33). This finding is in line with the clinical observation that a decreased rate of wound closure in deep cutaneous wounds (where adipose tissue is exposed) correlates to a greater chance of developing a hypertrophic scar (34).

During wound healing, cell proliferation goes hand in hand with cell migration in order to close the wound. We found that gingival MSC had a two-fold higher intrinsic migration capability compared to either dermal or adipose MSC. This is in line with the clinical observation that oral wounds heal faster than skin wounds (3,25) and indicates not only enhanced re-epithelialization, but also enhanced MSC migration plays a role in the better oral wound closure. Surprisingly, the saliva derived histatin peptide had a greater effect on adipose MSC migration and rhEGF on dermal MSC migration than on gingival MSC migration. However gingival MSC had a notably higher intrinsic migration, which is probably the reason why little additional migration upon stimulation was observed with gingiva MSC. Our findings indicate that a high rate of MSC migration in the oral cavity is intrinsically regulated by the MSC and therefore there is a smaller effect of environmental factors. Furthermore, our results indicate that in particular an enhanced MSC migration as well as MSC proliferation may contribute to enhanced oral wound closure compared to skin.

In addition to cell proliferation and migration, faster wound closure is associated with contraction from the wound margins in order to decrease the size of the wound. The final scar quality of the closed wound is determined in part by the extent of tissue contraction. We found that in the absence of keratinocytes, the extent of matrix contraction and number of αSMA expressing myofibroblasts in the presence or absence of Hst2 was virtually similar for all three MSC types. However, when MSC were cultured together with keratinocytes, for example in the full tissue equivalent, skin equivalents constructed with adipose derived MSC showed...
significantly more contraction than gingiva equivalents or skin equivalents constructed with dermal MSC and surprisingly high amounts of αSMα expressing myofibroblasts underneath the epidermis. Our results indicate that a very specific crosstalk occurs in our model between epidermal keratinocytes and adipose MSC, which occurs to a lesser extent between dermal MSC and is absent between gingiva keratinocytes and MSC, which may play a pivotal role in hypertrophic scar formation. Further research is required to identify the soluble mediators involved. Clearly the MSC respond differently to keratinocyte soluble mediators with only adipose MSC undergoing a strong phenotypic change to a αSMα expressing myofibroblasts which can enhance matrix contraction. Our observations, although unexplainable at present, are in line with the clinical observation that a higher risk of hypertrophic scar formation exists after deep cutaneous wounding and that there is rarely a scar visible after wounding of oral mucosa (4,34). We do realize though that our model is relatively simple, including only keratinocytes and MSC, and that other skin residential cells most likely influence these processes as well.

Our study showed no significant differences in contraction between the three MSC types (in the absence of keratinocytes), although there was a trend towards more contraction of gingival MSC than dermal MSC in our free-floating collagen gels. This is in line with other studies with human tissue that show that oral mucosal MSC contract collagen gels more than dermal MSC (35-38), while Lee et al. stated that dermis contracts more than oral mucosa (32).

Interestingly, the contraction observed in all three types of MSC in either collagen gels or connective tissue equivalents (without keratinocytes) was independent of αSMα expression since negligible αSMα expression was observed. Literature is conflicting on this subject. While some authors found αSMα expression higher in skin than in oral mucosa, in monolayers and free floating as well as attached-relaxed collagen gels (37,38), Lygoe et al. showed greater basal αSMα expression in monolayers of oral mucosa MSC than in dermal MSC (36). In an in vivo study with red Ducoc pigs, in both the unwounded situation as well as at 14, 28 and 60 days after wounding there was more αSMα expression found in the oral mucosa than in the dermis (39). It was also shown that desalivated mice show less wound contraction (9) and there were less myofibroblasts found in desalivated rats (10). Whether saliva also enhances αSMα expression in the oral mucosa is currently unknown. Stephens et al. suggested that contraction may be caused by increased movement of the oral fibroblasts through the collagen gel, coupled with intimate association with collagen fibrils (35). However, we did find that gingival MSC, similar to dermis and adipose MSC, were able to produce αSMα when stimulated with TGFβ1 (data not shown).

Taken together our results indicate that intrinsic properties of MSC together with crosstalk with epithelial cells result in differential proliferative, migratory and matrix contraction properties of dermal, adipose and gingival MSC which may in turn influence wound closure and the quality of the final scar. Since the saliva peptide Hst2 stimulates skin MSC migration to an even greater extent than gingiva MSC migration, without influencing matrix contraction or αSMα expression, Hst2 may possibly be a novel skin wound healing therapeutic.

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**Reference List**


