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ORIGINAL ARTICLE

Bromelain down-regulates myofibroblast differentiation in an in vitro wound healing assay

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Abstract Bromelain, a pineapple-derived enzyme mixture, is a widely used drug to improve tissue regeneration. Clinical and experimental data indicate a better outcome of soft tissue healing under the influence of bromelain. Proteolytic, antibacterial, anti-inflammatory, and anti-oedematogenic effects account for this improvement on the systemic level. It remains unknown, whether involved tissue cells are directly influenced by bromelain. In order to gain more insight into those mechanisms by which bromelain modulates tissue regeneration at the cellular level, we applied a well-established in vitro wound healing assay. Two main players of soft tissue healing—fibroblasts and microvascular endothelial cells—were used as mono- and co-cultures. Cell migration, proliferation, apoptosis, and the differentiation of fibroblasts to myofibroblasts as well as interleukin-6 were quantified in response to bromelain (36×10^{-3} IU/ml) under normoxia and hypoxia. Bromelain attenuated endothelial cell and fibroblast proliferation in a moderate way. This proliferation decrease was not caused by apoptosis, rather, by driving cells into the resting state G0 of the cell cycle. Endothelial cell migration was not influenced by bromelain, whereas fibroblast migration was clearly slowed down, especially under hypoxia. Bromelain led to a significant decrease of myofibroblasts under both normoxic (from 19 to 12 %) and hypoxic conditions (from 22 to 15 %), coincident with higher levels of interleukin-6. Myofibroblast differentiation, a clear sign of fibrotic development, can be attenuated by the application of bromelain in vitro. Usage of bromelain as a therapeutic drug for chronic human wounds thus remains a very promising concept for the future.

Keywords Bromelain · Wound · Endothelial cell · Fibroblast · Myofibroblast · Co-culture · Hypoxia · Interleukin-6 (IL-6)

Introduction

Due to the current population development in western countries, healing of skin and soft tissue wounds is an important clinical, scientific, and economic issue. Within the German population, the number of people who will be older than 80-years is increasing from 4.5 million in the year 2013 to more than 9 million in the year 2060 (Statistisches Bundesamt 2013). The increasing mean age will lead to an increase of age-related risk factors of wound healing as well as of different concomitant diseases. At present, about 100 million euro per year are necessary for therapy of about 4 million people in Germany suffering from chronic wounds (Dill-Müller and Tilgen 2005). Chronic wounds develop as a consequence of a disturbed healing progression, which might be caused by a variety of different reasons such as concomitant diseases like diabetes mellitus (Lebrun et al. 2010), peripheral vascular diseases (Franz et al. 2009; Eneroth and Persson 1993), chronic venous insufficiency (Pappas et al. 2005), the post-thrombotic syndrome and chronic renal insufficiency (Kannon and Garrett

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1995) or neoplasms, malnutrition, pharmaceutics, and excessive cigarette smoking (Silverstein 1992).

A large fraction of these chronic wounds is characterized by a disturbed healing sequence on the basis of periwound and local fibrosis (Enoch and Price 2004a; Pappas et al. 2005; Smith 2006; Schreml et al. 2010; Borthwick et al. 2013), often manifesting as a consequence of persistent inflammation in the form of chronic ulcers (Smith 2001; Diegelmann and Evans 2004). Fibrosis occurs when excess amounts of extracellular matrix (ECM) proteins are produced, with collagens playing a major role (Kisseleva and Brenner 2008). The main cell type producing ECM proteins is the wound myofibroblast (MF), an α-smooth muscle actin (α-SMA) positive subtype of the normal tissue fibroblast (Powell et al. 1999). Enhanced MF differentiation is clearly linked to fibrosing conditions (Wynn 2007; Duffield et al. 2013) and an increased presence of MF in cell cultures from chronic wound tissue explants compared to cultures from regularly healing wounds has recently been shown (Schwarz et al. 2013).

In the case of persisting fibrosis, an abnormal wound closure will be achieved in the form of a hypertrophic scar or a keloid. The processes occurring within chronic ulcers, which are characterized by a persisting inflammation accompanied by a triggered neutrophil infiltration, can be interpreted as an attempt to terminate enhanced fibrosis. Neutrophil derived collagenases are able to digest excess ECM and elastase might decrease excess cytokine levels (Diegelmann and Evans 2004). The latter seems to be important especially in the context of transforming growth factor-β (TGF-β), an inducer of MF differentiation, which might be limited after a neutrophil mediated TGF-β decrease.

In addition, hypoxia is known to be a crucial factor influencing wound healing in general (Tandara and Mustoe 2004). Although ischemia/hypoxia is triggering the healing process in the initial phase (Hunt 1988; Trabold et al. 2003) it inhibits healing when it persists (Enoch and Price 2004b; Hopf and Rollins 2007). Enhanced fibrosis (Yarnold and Brotons 2010; Wynn 2007) and development of ulcers (Schreml et al. 2010) might, at least in part, be correlated to chronic hypoxic conditions. Not only fibroblasts, but also endothelial cells as major cell type in charge of neoangiogenesis during wound healing, are regulated by hypoxia. However, the response of endothelial cells to hypoxia is quite heterogeneous according to the literature (Tucci et al. 1997; Iida et al. 2002).

The pineapple plant (*Ananas comosus*) derived enzyme mixture bromelain, mainly containing cysteine proteases, is a well-established therapeutic drug due to its anti-inflammatory effect, e.g., for the treatment of angina pectoris, bronchitis, and sinusitis. Side-effects are rare, so that it is furthermore applied in oncology, odontology, and in a wide range of sports injuries. Several controlled clinical studies with bromelain showed its efficacy (reviewed by Maurer 2001). In general, the application of bromelain in the context of wound healing is beneficial (reviewed by MacKay and Miller 2003). Experimentally induced firearm wounds showed improved healing in an animal model (Hu et al. 2011). In human, accelerated healing was demonstrated when bromelain was applied during the treatment of burn wounds (Königs and Jester 2008; Krieger et al. 2012). Although its anti-inflammatory and cleaning properties are obvious, a direct impact of bromelain at the cellular level was not identified until now with respect to soft tissue regeneration. Therefore, we used a well-established in vitro model (Oberringer et al. 2007) to verify the general impact of bromelain on the wound healing protagonists endothelial cells, fibroblasts, and MF.

We were especially interested in whether we were able to show an effect of bromelain on basic cellular parameters like migration, proliferation, cell cycle regulation, MF differentiation, and cytokine release (Gharaee-Kermani and Phan 2001). One focus was on interleukin-6 (IL-6), which is one of the pro-inflammatory cytokines in the course of wound regeneration and fibrosing conditions (O'Reilly et al. 2012) and which was shown to be regulated by bromelain in other cell types (Rose et al. 2005).

Material and methods

Cells and cell culture

Normal human dermal fibroblasts (NHDF) (PromoCell, Heidelberg, Germany; cell culture passage (P)7) were cultured in Quantum 333 medium (PAA-laboratories, Pasching, Austria). Human dermal microvascular endothelial cells (HDMEC) (PromoCell; P8) were grown separately in endothelial growth medium (Endothelial Growth Medium MV, PromoCell). Both cell types were grown under standard conditions (37 °C, 5 % CO_2/95 % air) and passaged by trypsination (Trypsin-EDTA, PAA-laboratories).

Co-cultures (n=6), mono-cultures of HDMEC (n=6), and NHDF (n=6) were produced (for details see (Oberringer et al. 2007)) in Quadriperm dishes (Greiner Bio-One, Frickenhausen, Germany) with each culture comprising five microscopic glass slides, resulting in a total of 90 specimens. For the co-culture experiments, mono-cultured cells were pooled in order to obtain an approximate 50:50 ratio of HDMEC and NHDF in co-cultures during the experiments. The same medium was used for the mono- and the co-cultures: it was composed of 75 % Endothelial Growth Medium MV and 25 % Quantum 333 medium. Normal cell morphologies during expansion of the different cultures are shown in the Online Resource 1a–c.

In vitro wounds

A central element of the study was a well-established wound assay termed *co-culture scratch wound migration assay*

(CCSWMA) (Oberringer et al. 2007), which was developed on the basis of protocols introduced by Todaro et al. (1967) and Gotlieb et al. (1979). Therefore, 24 h after cell seeding, a sterile 1,000-µl pipette tip (SARSTEDT, Nümbrecht, Germany) was used to induce a straight-lined in vitro wound by scratching the cell layer manually, producing cell-free wound areas. Cells are able to migrate into the wound from both edges. The migration is measured by quantitative microscopy (for details see "Data acquisition").

The distance between the edges of a wound depends on the cell types and ranged between 1.4 and 1.5 mm in this study. Figure 1a and b and Online Resource 2 illustrate the assay. Two wounds were created per slide to obtain two areas for the subsequently performed different immunocytochemical staining. Cell layer-scratching was the start of the experiment, referred to as 0 h. One slide of every culture was fixed immediately in order to determine initial values of all analytical parameters.

Culture conditions

Normoxic controls were represented by slides cultured for 18 h under standard incubator conditions. In addition to the normoxic control slides, one slide was exposed to hypoxic conditions for 18 h. For hypoxia, a sealed chamber at constant gas-flow (37 °C, 5 % CO_2/95 % N_2) was used (Breit et al. 2011). It assures an O_2-saturation of <5 mmHg in the culture medium, which is comparable to an O_2-atmosphere of 0.5 % (Purins et al. 2010). The hypoxic slides were fixed after 18 h without any reoxygenation.

In order to investigate the effects of bromelain on mono- and co-cultures, the cell culture medium was supplemented with bromelain (bromelain from pineapple stem, enzyme Commission no. 3.4.22.32, CAS no. 37189-34-7, B4882, Sigma, Saint Louis, USA) and cultured under hypoxic as well as under normoxic conditions for 18 h.

Terms for the five slides were: 0 h [0 h N], 18 h normoxia [18 h N], 18 h normoxia/bromelain [18 h N/B], 18 h hypoxia [18 h H], and 18 h hypoxia/bromelain [18 h H/B]. All slides were fixed as follows: Cells were washed twice in 5 ml of phosphate buffered saline (PBS), incubated in 0.05-mol/l KCl and fixed by addition of $methanol_{abs}$ at −20 °C. Afterwards, the slides were covered with PBS/glycerine (1:10) and stored at −20 °C.

Due to the fact that data on experimentally applied bromelain concentrations vary enormously in the literature, we performed a preliminary dose-dependence experiment, where both NHDF and HDMEC were incubated for 48 h with seven different concentrations of bromelain (1, 3, 6, 9, 18, 36, and 72×10^{-3} IU/ml) prepared from the stock (1 IU/ml) by dissolving in cell culture medium and sterile filtration. A concentration of 72×10^{-3} IU bromelain/ml seemed to be toxic for the cells, nearly all cells detached from the surface. Up to a concentration of 36×10^{-3} IU/ml for

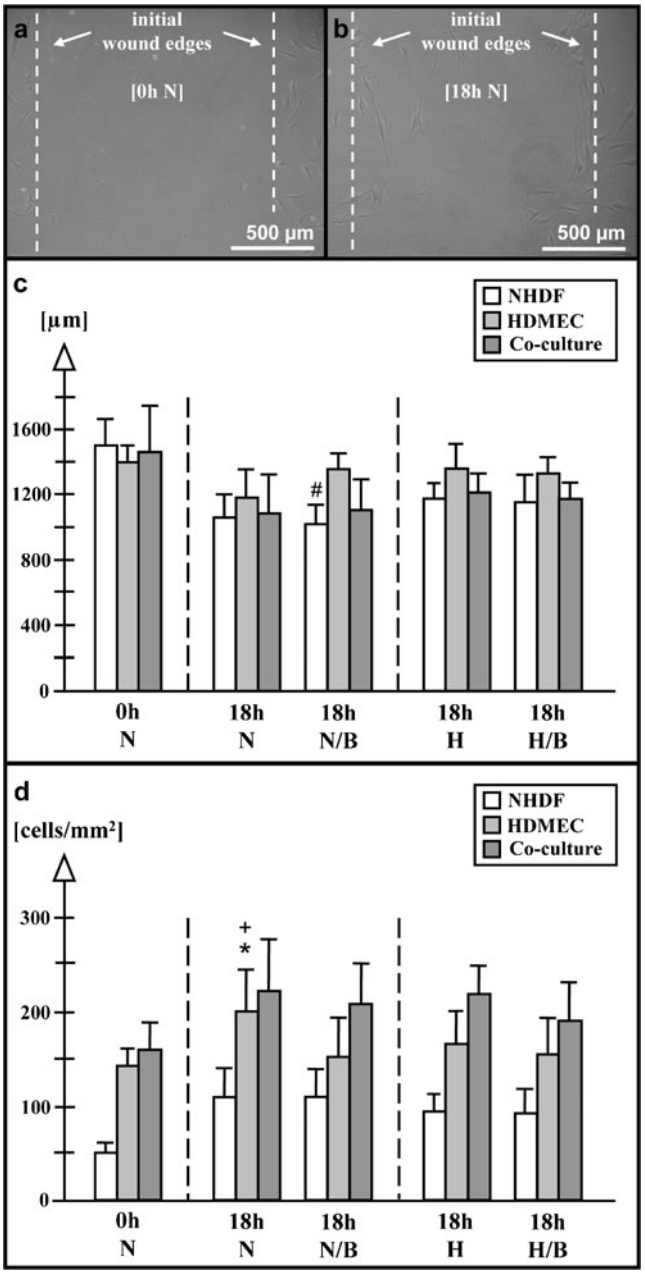

Fig. 1 Migration and proliferation in the scratch wound migration assay. **a** Wound of a normal human dermal fibroblast (NHDF) monoculture immediately after scratching at [0 h N] and **b** the referring wound after 18-h culture time under standard incubator conditions [18 h N] showing cell migration into the wound. **c** Mean wound-edge distance (in micrometer, with SD) and **d** cell density (mean cell number per square millimeter, with SD) of the cell cultures (n=6 each) in dependence on culture time, oxygen conditions, and bromelain supplementation. In the mono-cultures one cell type, either NHDF or human dermal microvascular endothelial cells (HDMEC), was cultured individually, whereas both cell types were cultured together in the co-cultures. In all cultures the same medium was used. Time: 0 and 18 h; *N* normoxia, *H* hypoxia, *B* bromelain supplementation ($c=36\times10^{-3}$ IU/ml). $^{+}p<0.05$ vs. the referring culture of the same cell type after [18 h N/B], $^{*}p<0.05$ vs. the referring culture of the same cell type after [18 h H], $^{\#}p<0.05$ vs. the referring culture of the same cell type after [18 h H/B]

both NHDF and HDMEC showed slightly higher cell numbers compared to the control, which was not supplemented with bromelain. This preliminary result enabled us to exclude any cytotoxic effect of bromelain on the cells up to a concentration of 36×10^{-3} IU bromelain/ml, which consequently was used for the study.

Immunocytochemistry

In order to enable discrimination and quantification of all present cell populations in co-cultures, namely HDMEC, NHDF, and MF, we applied cell type specific antibodies: von Willebrand factor (vWF), (rabbit polyclonal anti-von Willebrand factor Ab-1, 1:500 in PBS/bovine serum albumin 0.1 %, no. 12881, Dianova, Hamburg, Germany) was used for HDMEC and α-SMA (mouse monoclonal anti-α-SMA, 1:800, no. A2547, Sigma, St. Louis, USA) was used for MF. Cells neither expressing vWF nor α-SMA were NHDF (Online resource 3a). All nuclei were counterstained by 4′,6-diamidino-2-phenylindole (DAPI, VECTASHIELD, Vector Laboratories, Burlingame, USA).

Immunocytochemical staining was carried out according to a standardized protocol (for details see (Schwarz et al. 2013)). In brief: Specimens were washed in PBS/0.5 % Tween® 20 and incubated with the vWF antibody. A Cy3™-labeled antibody (Cy3™-conjugated goat anti-rabbit, 1:200, no. 111-166-045, Dianova) was used to detect HDMEC. Subsequently one half of the area of the slide was used for detection of the α-SMA antibody via adding a fluorescein isothiocyanate (FITC)-conjugated antibody (FITC-conjugated goat anti-mouse, 1:100, no. 115-095-003, Dianova). The other half area of the slide was covered with a Ki-67/MIB-1 antibody (mouse anti Ki-67/MIB-1, no. M7240, Dakocytomation, Glostrup, Denmark) and the same FITC-conjugated antibody. Ki-67/MIB-1 is a proliferation associated antigen, which is expressed in all cell cycle stages from G1 to M and which is absent in G0 (Online resource 3b) (Gerdes et al. 1984). After washing, fixation in 4 % paraformaldehyde (in PBS) and a last washing step, an ascending ethanol series (70, 80, and 96 % (*v*/*v*)) was performed and slides were air-dried and finally cover-slipped using mounting medium containing DAPI.

Interleukin-6 enzyme linked immunosorbent assay

A commercially available enzyme linked immunosorbent assay (ELISA) (Quantikine® ELISA, human IL-6, D6050, R&D Systems, Minneapolis, USA) was used to determine IL-6 concentrations in cell culture supernatants of all cultures (n=90). Therefore, all culture media were collected before fixation of the cells and were frozen at −80 °C. Tests were carried out in accordance to the manufacturer's instructions. All assays were run with dilution of the supernatants. The minimum detectable dose of IL-6 was 0.70 pg/ml. The optical density of each sample was determined with a microplate reader (Infinite 200, Tecan Group Ltd, Männedorf, Switzerland) and the calculation of the sample concentration was performed by a standard curve. IL-6 values could be expressed in picogram per 10^6 cells, because accurate cell numbers of each culture were determined by quantitative microscopy.

Data acquisition

A Zeiss Axioskop 2 (Carl Zeiss Microscopy GmbH, Jena, Germany) and the appropriate software (Axiovision 4.2., Carl Zeiss) were used for microscopic analysis. Cell migration is represented by the remaining width (distance) between the edges of the in vitro wounds after 18 h. Subsequent to the measurement of the wound distance in 10 consecutive high-power fields along the scratch (for details see (Oberringer et al. 2007)), the mean wound distances were determined. Cell proliferation in both the preliminary dose-dependence experiment and in the final study was quantified by inspecting 20 high-power fields. Subsequently, cell densities per square millimeter were determined. Percentages of all three cell types in co-cultures—HDMEC, NHDF, and MF—were determined after inspection of a minimum of 600 cells located in eight different areas.

Non-apoptotic cells in all cell cycle stages with the exception of G0 which are positive for Ki-67/MIB-1 were expressed in percent of all cells. The same was done for cells exhibiting a sign of late apoptosis: the separation of the nucleus into multiple (at least three) micronuclei (Online resource 3c). Micronuclei assessment to identify apoptotic cells was done following Fenech et al. (2003), who developed an accurate scheme on the basis of lymphocyte cultures. All analytical parameters were expressed as mean values with standard deviation (SD).

Statistical analysis

We analyzed all data concerning migration, proliferation, cell cycle (*cycling cells not being in G0* and *apoptosis*), MF differentiation, and IL-6. Statistically significant differences were examined by applying a one-way repeated measures analysis of variance (ANOVA). In the case of non-given normality or equal variance, an ANOVA on ranks was performed. Post-hoc multiple comparisons were done by Student–Newman–Keuls method. P values below 0.05 were considered statistically significant. All significances concerning the comparison of hypoxia vs. normoxia groups and of bromelain supplementation vs. non-supplementation groups are given in the results figures. Significant developments from 0 h to 18 h are omitted due to figure clarity, but are always mentioned in the text.

Results

Migration

Figure 1c depicts the results of the migration analysis: NHDF mono-cultures showed an initial wound edge distance of 1,505 μm which was significantly reduced to 1,060 μm at [18 h N], to 1,020 μm at [18 h N/B] and to 1,174 μm at [18 h H] and 1,154 μm at [18 h H/B]. Hypoxia caused a significant migration decrease when NHDF were bromelain supplemented.

HDMEC mono-cultures, starting with an initial wound-edge distance of 1,400 μm, were only able to reduce the wound edge distance by migration under normoxia [18 h N] to 1,181 μm, whereas distances were not significantly reduced under normoxic bromelain supplementation: 1,351 μm [18 h N/B] and under hypoxia 1,368 μm [18 h H] and 1,328 μm [18 h H/B]. However, hypoxia and bromelain supplementation did not affect HDMEC migration significantly.

Co-cultures (initial wound-edge distance of 1,456 μm) showed a migration response which was similar to that of NHDF mono-cultures, namely, significantly reduced wound distances of 1,090 μm at [18 h N]; 1,101 μm at [18 h N/B]; and of 1,210 μm at [18 h H] and 1,176 μm at [18 h H/B]. Hypoxia and bromelain supplementation did not cause a significant migration response among co-cultures.

Proliferation

Figure 1d shows the results of the proliferation analysis: Mean initial cell densities [0 h N] of NHDF mono-cultures were 51 cells/mm^2. Cell densities significantly increased to 110 cells/mm^2 after [18 h N], and to 109 cells/mm^2 after [18 h N/B], representing almost equal levels. Hypoxia also allowed a significant proliferation to 96 cells/mm^2 [18 h H], and to 93 cells/mm^2 [18 h H/B] representing a weak, but not significant, NHDF reduction under the influence of bromelain.

In HDMEC mono-cultures, the mean initial density at [0 h N] was 143 cells/mm^2 and increased to 200 cells/mm^2 at [18 h N]. Hypoxia [18 h H] caused a significant inhibition of proliferation (166 cells/mm^2) compared to normoxic cultures [18 h N], but bromelain supplementation did not result in any significant change (155 cells/mm^2) under hypoxia [18 h H/B]. However, bromelain [18 h N/B] significantly reduced the HDMEC number to 152 cells/mm^2 under normoxia.

The mean cell density of the co-cultures was 160 cells/mm^2 at [0 h N]. Co-cultured cells were able to proliferate under each condition to 221 cells/mm^2 at [18 h N], to 208 cells/mm^2 at [18 h N/B], and to 219 cells/mm^2 at [18 h H] and 190 cells/mm^2 at [18 h H/B]. Hypoxia and bromelain supplementation did not result in a significant proliferation change here, indicating that co-culturing of NHDF and HDMEC abrogates those effects of hypoxia and bromelain, appearing in the mono-cultures, to a certain extent.

Cell cycle (cycling cells/cells not in G0)

The results of the cell cycle analysis are displayed in Fig. 2a: The different experimental groups of mono-cultured NHDF exhibited Ki-67/MIB-1 rates in a range from 84 to 96 %, equivalent to a very small number of cells being at rest in G0 in general. At 18 h, Ki-67/MIB-1 rates were significantly higher than at 0 h. Nevertheless, hypoxia caused a significant decrease in cycling cells from 96 % at [18 h N] to 92 % at [18 h H] and from 92 % at [18 h N/B] to 88 % at [18 h H/B] when bromelain supplemented. In addition, bromelain supplementation also led to a significant decrease in cycling cells under both normoxic and hypoxic conditions.

In co-culture, the population of NHDF showed slightly lower amounts of cycling cells as in mono-culture, ranging from 79 to 92 %. Although the general response to hypoxia and bromelain was similar to the mono-cultured NHDF, a significant decrease of cycling cells was only detectable during normoxia coincidental with bromelain supplementation [18 h N/B] with 88 % Ki-67/MIB-1 positive cells.

Mono-cultured HDMEC showed Ki-67/MIB-1 rates between 34 and 50 %, illustrating that a larger proportion of HDMEC is at rest compared to the NHDF cell type. After 18 h normoxia, Ki-67/MIB-1 rates were significantly higher than at 0 h. Rates under hypoxia were significantly lower compared to the rates under normoxia. In addition, bromelain caused a significant decrease of the Ki-67/MIB-1 rate to 34 % under hypoxia [18 h H/B].

In co-culture, the population of HDMEC generally showed lower amounts of cycling cells than in mono-culture, ranging from 22 to 26 %. Stable Ki-67/MIB-1 rates did not result in any significant differences.

Cell cycle (apoptosis)

Figure 2b shows the results of the apoptosis evaluation: Between 0.90 and 2.13 % of the NHDF in mono-culture were characterized by micronuclei, a clear sign of apoptosis. The quite low apoptosis rate under normoxia was significantly triggered by hypoxia, resulting in 7.31 % at [18 h H] and 6.94 % in [18 h H/B]. A similar behavior was detected for NHDF in co-culture, with the exception of slightly higher numbers of apoptotic cells in hypoxia: 8.84 % at [18 h H] and 9.15 % in [18 h H/B].

In the different experimental groups of mono-cultured HDMEC, between 2.34 and 3.25 % of the cells showed micronuclei under normoxic conditions and increased values of 4.73 % at [18 h H] and 4.20 % at [18 h H/B]. The apoptotic response of HDMEC was less intensive under hypoxia compared to NHDF, which showed higher levels of apoptosis under this condition.

In co-culture, hypoxia also tended to induce a slight increase of apoptosis among co-cultured HDMEC. In general,

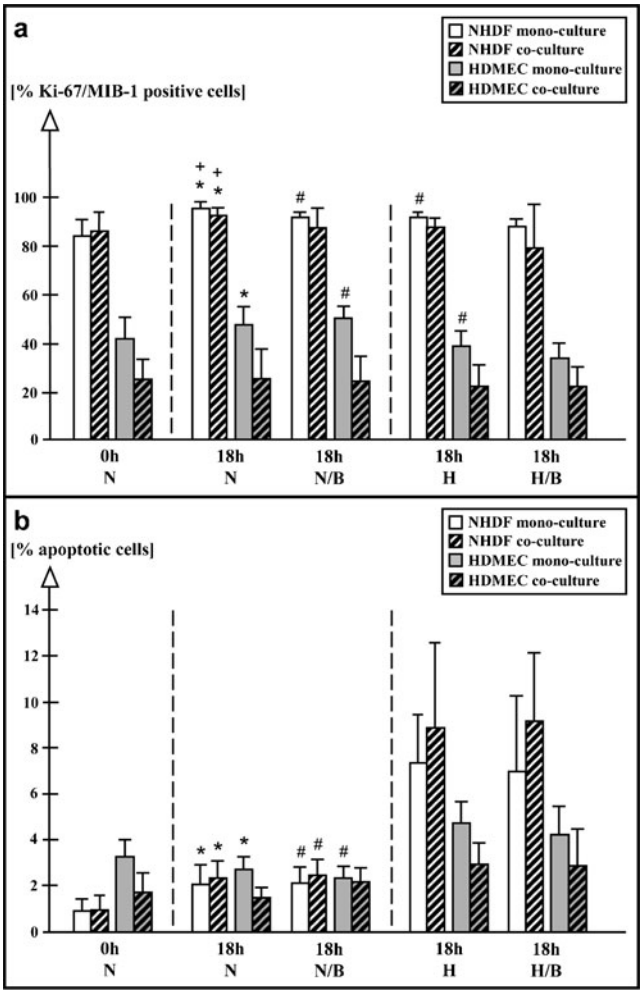

Fig. 2 Proliferation and apoptosis in the assay determined after immunocytochemical staining. **a** Proliferation as indicated by Ki-67/MIB-1 positive nuclei (mean number of cells with Ki-67/MIB-1-positive nuclei in percent of all cells, with SD) and **b** apoptosis rate as indicated by cells with micronuclei (mean number of cells with micronuclei in percent of all cells, with SD) of the cell cultures (n=6 each) in dependence on culture time, oxygen conditions, and bromelain supplementation. In the mono-cultures one cell type, either normal human dermal fibroblast (NHDF) or human dermal microvascular endothelial cells (HDMEC), was cultured individually, whereas both cell types were cultured together in the co-cultures. In all cultures, the same medium was used. Time: 0 and 18 h; *N* normoxia, *H* hypoxia, *B* bromelain supplementation ($c=36\times10^{-3}$ IU/ml). ^{+}p <0.05 vs. the referring culture of the same cell type after [18 h N/B], *p <0.05 vs. the referring culture of the same cell type after [18 h H], $^{\#}p$ <0.05 vs. the referring culture of the same cell type after [18 h H/B]

we detected lower apoptotic rates among HDMEC compared to their mono-culture, indicating a general benefit from culturing HDMEC together with NHDF. In contrast, co-culturing triggered NHDF apoptosis when compared to the mono-cultures.

Myofibroblast differentiation

Figure 3 displays the results of MF differentiation: In NHDF mono-cultures, the MF population, expressed in % of all fibroblasts, decreased with cultivation time from 28 % at the beginning [0 h N] to significantly lower levels after 18 h (19 %), which is the regular behavior of fibroblasts in vitro. Hypoxia [18 h H] induced a slight increase of MF (22 %), when compared to the normoxic cultures [18 h N]. In this setup, the MF increase during hypoxia was not significant as expected from previous data; this may be caused by the lower starting NHDF cell number.

Here, bromelain showed a clear and significant effect: During normoxia, the MF population was reduced from 19 % [18 h N] to 12 % [18 h N/B] by bromelain supplementation. The same effect was detectable during hypoxia: the MF population was reduced from 22 % [18 h H] to 15 % [18 h H/B] by bromelain.

Among the co-cultures, the MF population within the fibroblasts was generally smaller compared to the mono-cultures indicating an ameliorating effect of HDMEC on MF differentiation. The effect of hypoxia in co-culture was not that distinct. Bromelain did not cause a significant MF reduction in normoxia, but there was at least a slight MF reduction in response to bromelain during hypoxia, presenting values of 17 % [18 h H] and 13 % [18 h H/B], respectively.

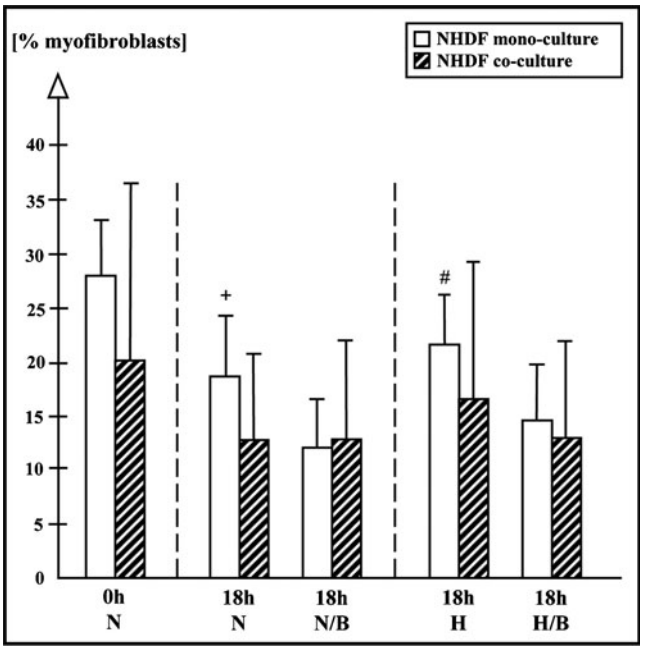

Fig. 3 Fibroblast response in terms of differentiation to myofibroblasts (MF) in the scratch wound migration assay determined after immunocytochemical staining of α-smooth muscle actin (α-SMA). Percentage of α-SMA-positive MF relative to all fibroblasts in mono- and in co-culture (n=6 each) in dependence on time, oxygen condition and bromelain supplementation (means with SD). In the mono-culture normal human dermal fibroblasts (NHDF) were cultured individually, whereas NHDF were cultured together with human dermal microvascular endothelial cells (HDMEC) in the co-cultures. In both culture types, the same medium was used. Time: 0 and 18 h; *N* normoxia, *H* hypoxia, *B* bromelain supplementation ($c=36\times10^{-3}$ IU/ml). ^{+}p <0.05 vs. the referring culture of the same cell type after [18 h N/B], $^{\#}p$ <0.05 vs. the referring culture of the same cell type after [18 h H/B]

Interleukin-6

The results, with respect to the analysis of IL-6 in the medium supernatant of the mono-cultures, are shown in Fig. 4a: The maximum IL-6 value of NHDF in mono-culture was 19,107 pg/10^6 cells at [0 h N], may be giving a hint to a general "stress" situation after cell seeding among fibroblasts. IL-6 decreased to significantly lower levels after 18 h. Hypoxia causes a slight, but not significant, increase of IL-6 levels compared to the normoxic cultures: 1,908 pg/10^6 cells [18 h H] vs. 1,265 pg/10^6 cells [18 h N] and 2,242 pg/10^6 cells [18 h H/B] vs. 1,477 pg/10^6 cells [18 h N/B]. Bromelain supplementation led to a significant increase of the IL-6 values during hypoxia from 1,908 pg/10^6 cells [18 h H] to 2,242 pg/10^6 cells [18 h H/B].

HDMEC in mono-culture showed a more homogenous IL-6 level, starting with 3,624 pg/10^6 cells [0 h N]. During 18-h normoxia, the IL-6 value increased slightly to 4,038 pg/10^6 cells [18 h N] and significantly in the case of bromelain supplementation: 4,899 pg/10^6 cells [18 h N/B]. The hypoxia response of HDMEC was contrary to that of NHDF as indicated by a significant decrease compared to normoxia: 2,888 pg/10^6 cells [18 h H] vs. 4,038 pg/10^6 cells [18 h N] and 2,588 pg/10^6 cells [18 h H/B] vs. 4,899 pg/10^6 cells [18 h N/B]. During hypoxia, we detected a decreased IL-6 release of HDMEC by trend after bromelain supplementation: 2,588 pg/10^6 cells [18 h H/B] vs. 2,888 pg/10^6 cells [18 h H].

In co-culture, as shown in Fig. 4b, the IL-6 value measured was much higher than expected on the basis of the values of the mono-cultures. The starting value of 39,767 pg/10^6 cells [0 h N] significantly decreased during 18 h culturing to a minimal value of 13,654 pg/10^6 cells [18 h N]. A significant effect of bromelain on co-cultured cells was not detectable. However, comparable IL-6 release levels in normoxia and hypoxia in co-culture might be explained by the combination of the cellular responses of mono-cultured NHDF (increased IL-6 values during hypoxia) and of mono-cultured HDMEC (decreased IL-6 values during hypoxia).

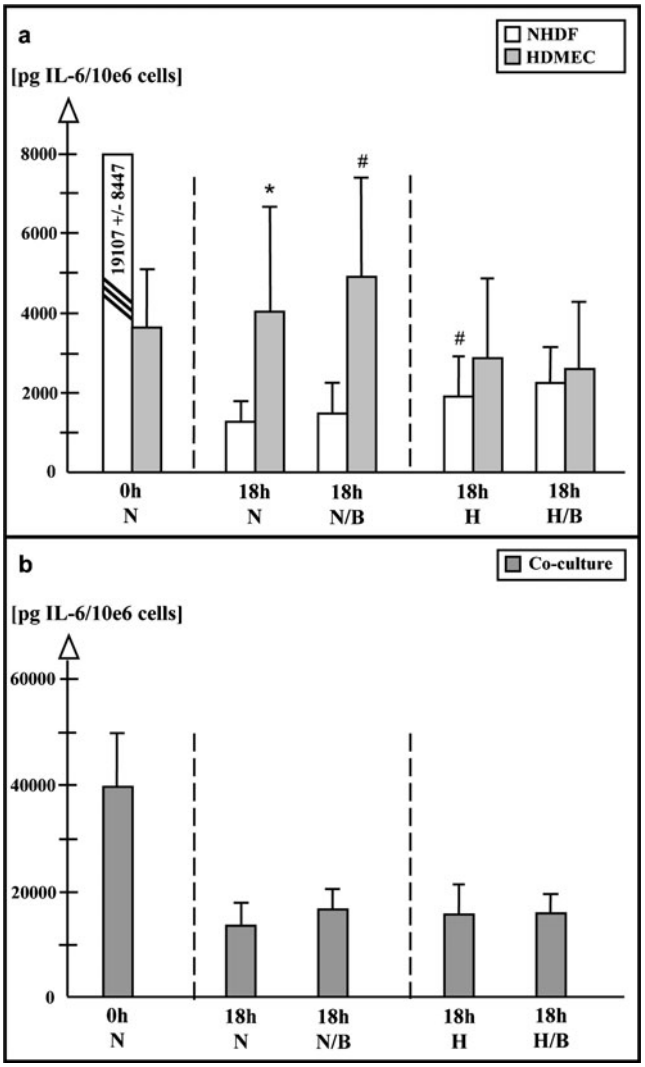

Fig. 4 Cytokine release into the culture medium supernatant in the scratch wound migration assay determined by enzyme linked immunosorbent assay (ELISA). Interleukin (IL)-6 values by mono- (**a**) and co-cultured (**b**) normal human dermal fibroblasts (NHDF) and human dermal microvascular endothelial cells (HDMEC) (n=6 each) in pg/10^6 cells in dependence on time, oxygen conditions and bromelain supplementation (means with SD). In the mono-cultures one cell type, either NHDF or HDMEC, was cultured individually, whereas both cell types were cultured together in the co-cultures. In all cultures the same medium was used. Time: 0 and 18 h; *N* normoxia, *H* hypoxia, *B* bromelain supplementation (c=36×10^{-3} IU/ml). *p <0.05 vs. the referring culture of the same cell type after [18 h H]; $^{\#}p$ <0.05 vs. the referring culture of the same cell type after [18 h H/B]

Discussion

The application of bromelain in order to prevent extreme scarring during burn-wound healing has been shown to be a very promising approach: first, clinical results using the bromelain-based Debrase® gel emphasized the effectivity of this enzyme mixture (Königs and Jester 2008; Krieger et al. 2012). This product also accelerated wound healing in different experimental studies (Singer et al. 2011; Rosenberg et al. 2012). Although bromelain is able to degrade ECM proteins, mainly necrotic tissue is attacked, whereas healthy tissue seems to be unaffected by the treatment (Rosenberg et al. 2012). Bromelain was also used to accelerate healing of shotgun wounds of pig (Hu et al. 2011). Again, there was no negative influence on the healthy granulation tissue of the wound observed (Hu et al. 2011; Wu et al. 2012). Bromelain, in combination with further drugs, produced a better healing outcome in a clinical trial on skin wounds (Brown et al. 2004).

By the present approach applying bromelain to a well-established co-culture setup, the so called co-culture scratch wound migration assay (CCSWMA) (Oberringer et al. 2007), we tried to find out, whether it is possible to detect

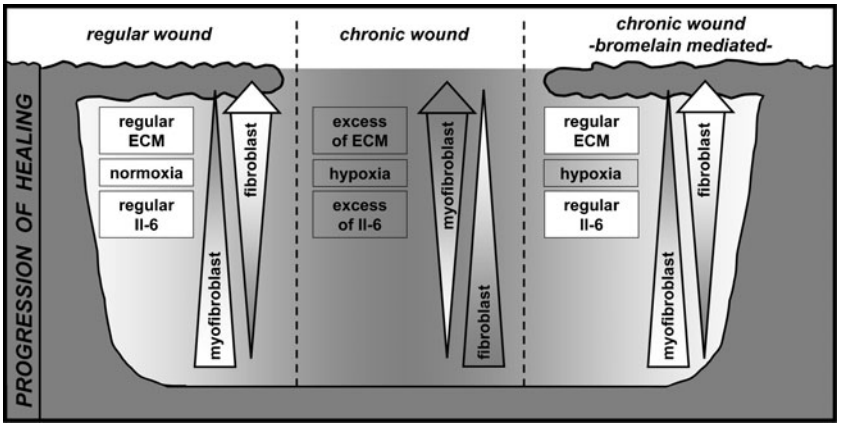

Fig. 5 Relationship of oxygen conditions, matrix deposition, IL-6 release, and the healing tendency in the course of soft tissue healing. Excess extracellular matrix production, elevated IL-6 levels and hypoxia occur in chronic wounds and are accompanied by a shift towards enhanced MF differentiation. On the basis of the results it is indicated that bromelain adjusts imbalanced IL-6 levels and ameliorates MF differentiation in order to achieve the re-establishment of tissue homeostasis despite persisting hypoxia, finally succeeding healing improvement

effects of bromelain on basic cellular parameters like migration, proliferation, cell cycle, cytokine release, and MF differentiation. Should we be able to detect positive effects on the wound healing protagonists endothelial cells and fibroblasts in this setup, the application of drugs based on the bromelain enzyme might be expanded to those chronic wounds, which show a delayed healing due to an excess amount of MF and excessive fibrosis.

Focusing on cell migration, we, on one hand, did not detect a positive effect of bromelain in HDMEC and NHDF mono- or co-cultures. On the other hand, there was only a statistically significant negative influence of bromelain detected among NHDF under hypoxia, which indicates that migration is not the main parameter to be affected by bromelain in general. However, the migration decrease of NHDF under hypoxic conditions and bromelain supplementation in vitro might lead to the consideration that bromelain could especially be helpful to prevent a massive presence of fibroblasts in wounds, which are accompanied by hypoxic conditions, for example burns and radiation wounds (Yarnold and Brotons 2010) as well as ulcera developing on the basis of peripheral vascular disease and chronic venous insufficiency (Eneroth and Persson 1993; Pappas et al. 2005; Franz et al. 2009). Furthermore, decreased fibroblast migration in vivo could lead to a shift towards the epithelial cell population, enabling a better migration of epithelial cells from the wound edge into the center with the consequence of accelerated wound closure.

With respect to cell proliferation in vitro, several studies demonstrated inhibiting effects of bromelain on tumor cells (reviewed by Chobotovaa et al. 2010). In contrast, Zetter et al. (1976) were able to show both an increased DNA synthesis and an enhanced mitotic rate of chicken fibroblasts in response to bromelain.

The response, with respect to cell proliferation in our setup, was different within the cell populations of NHDF and HDMEC: NHDF and the co-cultures showed a proliferation increase up to 18 h. Hypoxia was only slightly reducing proliferation. Bromelain supplementation did not produce any significant effect. In contrast, HDMEC were, as expected, more affected by hypoxia, but bromelain did not ameliorate the HDMEC proliferation decrease under this condition. It was even more interesting that HDMEC under normoxia responded to bromelain with a proliferation decrease compared to the non-supplemented cultures. This decrease was clearly produced by attenuated proliferation itself and not by increased HDMEC apoptosis: There were approximately the same amounts of HDMEC in cell cycle phase G0. In this context, Bahde et al. (2007) showed endothelial cell damage in vivo after bromelain application to be more intensive in the normoxic controls compared to the ischemia/reperfusion animals, indicating the oxygen dependence of the bromelain activity.

NHDF responded to bromelain with significantly enhanced numbers of cells in G0 under both normoxic and hypoxic conditions, but without showing any differences with respect to late apoptosis. A future therapeutic application of bromelain in order to slow down the fibroblast cell cycle in fibrotic wounds seems therefore to be independent of the oxygen content in the wound.

Enhanced fibrosis within a given wound is always correlated to a healing delay. The main factors impeding the regular healing sequence are persistent inflammation and excess production of ECM, the latter mainly caused by the differentiated form of the normal tissue fibroblast, the MF. Although the MF is important to trigger wound healing in the early phase by wound contraction and matrix and cytokine supply, its chronic persistence is counterproductive for healing (Powell et al. 1999).

Our model clearly showed that bromelain is a very effective drug to reduce the population of MF in vitro: In the NHDF mono-cultures under both normoxic and hypoxic conditions, MF were reduced significantly compared to controls. The decrease of MF in response to bromelain was not correlated to enhanced myofibroblast apoptosis, because the apoptosis response of undifferentiated NHDF and of MF was not significantly different (data not shown). This indicates that the MF population is diminished under bromelain supplementation due to a slower proliferation of MF compared to undifferentiated NHDF. To our knowledge, a de-differentiation of existing MF, which may also explain a decrease under bromelain, has not been shown so far for fibroblasts in culture.

Focusing onto the ameliorated MF differentiation by bromelain in more detail, we decided to quantify IL-6 as one of the most important cytokines during inflammation, regeneration, and MF differentiation (Liechty et al. 2000; O'Reilly et al. 2012). Within a wound, epidermal cells, Langerhans cells, as well as endothelial cells and dermal fibroblasts are producers of IL-6 (Paquet and Piérard 1996) in a time-dependent manner (Mateo et al. 1994). The efficacy of bromelain to modulate the cytokine release of tissue cells was already shown (Onken et al. 2008), but it was quite unclear whether endothelial cells and fibroblasts are also affected.

During hypoxia HDMEC cultures exhibited lower IL-6 values compared to the normoxic controls. Bromelain supplementation led to a significant increase of IL-6 in the supernatant only under normoxia. This was just the condition where HDMEC cell numbers were lowest, indicating, that low cell numbers are correlated to high IL-6 values.

NHDF cultures started with excessive amounts of IL-6 in the supernatant at 0 h N; this may be caused by a kind of "adhesion stress" and is coincidental with the highest numbers of MF. Here, high IL-6 levels are likely the driving force of early MF differentiation in our setup. IL-6 values decreased within 18 h, the hypoxia values ranging lower in general. Bromelain led to a significant increase of IL-6 in hypoxia when compared to the non-supplemented cultures. At first view, it is somehow confusing that these high IL-6 values occur simultaneously with a significant MF decrease, as we have shown before. In general, IL-6 in tissue regeneration and fibrosis is regarded as a trigger of MF differentiation (O'Reilly et al. 2012). However, several in vivo and in vitro data suggest a more sophisticated regulation of MF differentiation by IL-6, namely a possible inhibitory role of IL-6 with respect to MF differentiation and α-SMA expression. There seems to be a rather variable regulation, where low levels of IL-6 increase the expression and higher levels inhibit the expression (Gallucci et al. 2006). With respect to MF differentiation a dose- and time-dependent role of IL-6, also mediated by TGF-β, was suggested by Vyas et al. (2010), too. Indeed, TGF-β and its receptor TGF-βR2 play a key role in IL-6 modulated MF differentiation, because both are regulated by IL-6 (Luckett-Chastain and Gallucci 2009).

With respect to the expression of TGF-β in response to bromelain, the literature provides heterogeneous results. Whereas Wu et al. (2012) showed an increased TGF-β expression in wound tissue after bromelain supplementation, there was an evident down-regulation of TGF-β in cases, where it was highly expressed before, which was strongly dependent on the tissue situation (Paczek et al. 2001; Desser et al. 2001).

High IL-6 values coincidental with decreased MF differentiation subsequent to bromelain supplementation under hypoxia in our setup might also be explained by a selective degradation of the IL-6 receptor, which is likely to be present in large amounts on the MF surface. Low numbers of MF with degraded IL-6 receptors would lead to a situation, where the consumer of IL-6 is lacking, with the consequence of IL-6 accumulating in the supernatant, as detected in our setup. The ability of bromelain to selectively degrade surface receptors was already shown for the example of the IL-8 receptor (Fitzhugh et al. 2008).

Furthermore, it seems to be possible that a proliferation inhibition by IL-6 in a dose- and time-dependent manner, which was shown to occur among lung fibroblasts (Moodley et al. 2003), especially involves the MF type in our setup and undifferentiated fibroblasts to a lesser degree. However, we could clearly show that bromelain leads to a MF reduction correlated to increased IL-6 levels.

Our results indicate that negative effects of bromelain on basic cell parameters were rare, whereas bromelain was able to modulate critical parameters of chronic soft tissue healing like IL-6 and MF differentiation. Figure 5 reflects the in vitro results in the context of human soft tissue healing in vivo. Besides sufficient revascularization regular healing needs the re-establishment of the oxygen supply and the cytokine homeostasis. In addition, the reduction of MF and ECM proteins has to occur to finalize the healing process. In the case of delayed or chronic healing, one or several of these parameters are deregulated due to different reasons. Bromelain seems to be able, at least to a certain extent, to ameliorate these disadvantageous conditions, especially when hypoxia is persisting in a wound.

With respect to the application of bromelain in the context of chronic human wound healing, our results clearly indicate, that it is worthy to examine its effects in terms of clinical trials, not only involving burns (Königs and Jester 2008; Krieger et al. 2012), but extended to various chronic and fibrotic soft tissue disorders like ulcers, hypertrophic scars, and keloids. In addition to the established and well-known anti-bacterial, anti-inflammatory, anti-oedematogenic, and debridement/cleaning effects of bromelain (Maurer 2001), we showed the efficacy of bromelain to modulate relevant basic parameters. Thus, from the biological point of view, the approach of using bromelain in the context of chronic human wound healing remains a promising future concept.

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