Reflectance Confocal-Laser-Scanning Microscopy In Vivo Assessments of Cigarette-Induced Dynamic Alterations of Cutaneous Microcirculation on Histomorphological Level

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ABSTRACT Objective: Until now, high resolution reflectance confocal-laser-scanning microscopy (CLSM) was used for observation of cutaneous morphology in vivo and in real time. We hypothesized that CLSM also allows observation of dynamic processes of cutaneous microcirculation. Methods: Reflectance CLSM (Vivascope1500; Lucid, Rochester, NY) was performed in 24 young male habitual smokers (23 years, range: 19–26, body mass index 23.9 ± 4.04) with relatively limited cigarette exposure (mean: 3.1 ± 2.4 pack-years). Eight matched nonsmokers served as controls. The quantitative blood cell flow and the diameter of capillary loops were determined prior (baseline), during, as well as 5 and 10 min after smoking. Results: Baseline value for blood cell flow was 55.50 ± 2.33 cells/min, and decreased over 45% during smoking (30.43 ± 3.76/min; $P = 0.02$). They were still 22% lower (43.33 ± 2.45/min; $P = 0.01$) 5 min after smoking and exceeded baseline values 10 min after smoking by 13% (63.00 ± 3.10/min; $P > 0.05$). The baseline values for capillary loop diameter (9.03 ± 0.22 μm) decreased by 21% (7.18 ± 0.28 μm; $P = 0.03$) during smoking, remained about 9% (8.23 ± 0.18 μm; $P = 0.01$) lower 5 min after smoking and exceeded baseline values insignificantly by 4% (9.38 ± 0.28 μm; $P > 0.05$) 10 min after smoking. There were no significant differences to the controls. Conclusion: Reflectance CLSM enables qualitative and quantitative observation of dynamic processes of cutaneous microcirculation on histomorphological level. *Microsc. Res. Tech. 72:347–350, 2009.*

KEY WORDS noninvasive imaging; blood flow; dermal capillaries; histomorphology

INTRODUCTION

Reflectance confocal-laser-scanning microscopy (CLSM) has been introduced as a technique to qualitatively and quantitatively assess the epidermal-dermal pattern in human skin up to a depth of 350 μm (Aghassi et al., 2000; Gonzalez et al., 1999a; Rajadhyaksha et al., 1999). Until now, this high resolution technique was used for observation of cutaneous morphology on cellular and subcellular levels in vivo and in real time. Whereas this technique was validated in the burn care arena for burn depth determination and in dermatology to visualize healthy skin morphology, photoadaptive processes, inflammatory conditions, and histomorphological patterns of skin lesions suspected of malignancy (Aghassi et al., 2000; Altintas et al., 2008a,b in press; Gonzalez et al., 1999a,b; Rajadhyaksha et al., 1999; Sauermann et al., 2002, Gambichler et al., 2006), it has not been used for the determination of dynamic processes of the microcirculation. The effects of smoking on microvascular network have been examined in a number of papers with both negative (Cunningham et al., 2005; Dalla Vecchia et al., 2004; Forrest et al., 1991; Riefkohl et al., 1986; Rossi et al., 2007) as well as no clear proof of smoking on microcirculation (Mehrara et al., 2006; Parsa et al., 2006; Steenvoorde et al., 2007). Most of these studies were carried out using various invasive (Forrest et al., 1991; Riefkohl et al., 1986) and noninvasive (Dalla Vecchia et al., 2004; Rossi et al., 2007) approaches. Although invasive methods not only alter the original morphology but also always necessitate an iatrogenic trauma, other methods allow noninvasive investigation of the microcirculation. Whereas laser Doppler flowmetry is the most commonly described noninvasive technique to measure cutaneous blood flow, various other studies report the use of a plethora of other modalities such as thermography, pulse plethysmography, photoplethysmography, calorimetry, multilead plethysmography, pulse oximetry, and transcutaneous oxygen electrode measurements (Dalla Vecchia et al., 2004; Mehrara et al., 2006; Parsa et al., 2006; Rossi et al., 2007; Steenvoorde et al., 2007). None of these methods, however, allows observation of the microcirculation on a histomorphological level. In the present preliminary study, we present the novel application of high resolution in vivo reflectance CLSM to characterize and quantify the dynamic and acute effects of cigarette consumption on cutaneous microcirculation.

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PATIENTS AND METHODS

Volunteers

Twenty-four healthy male smokers (age: 23 years, range: 19–26, mean body mass index 23.9 ± 4.04) with relatively limited cigarette exposure (mean pack-years 3.1 ± 2.4) and eight matched nonsmokers (controls) were investigated after written informed consent was obtained. Exclusion criteria were chronic skin or systemic diseases. Before examinations, volunteers had to refrain from smoking for at least 3 h.

Instrument

The reflectance CLSM Vivascope1500 (Lucid, Rochester, NY) is a commercially available tool for in vivo investigations of the epidermis and upper dermis. This special kind of confocal microscope characterizes a Gallium Arsenide laser source which emitting long wavelength of 830 nm in the "optical window" of the human skin and thus penetrates deep, up to a controlled depth of 350 μm through the human skin. The laser source generates harmless energy on the skin surface of less than 30 mW. Because of a lateral resolution of 0.4 μm and a vertical resolution of 1.9 μm, it is feasible to visualize tissue on cellular and subcellular levels. The field of vision represents 500 μm × 500 μm. Real time imaging is achievable due to a frame rate of 20 per second. To generate confocal images, a laser beam passes through a light source aperture and then is focused by an objective lens into the skin. The reflected laser light from different structures of the skin (cytoplasm, cell wall, melanin, etc.) is then recollected by the objective lens. A detector aperture separates the reflected light by allowing solely passing a pinhole which is coming from the focal plain. The detected light signal is transforming into an electrical signal that is recorded by a computer. Thus confocal images are acquired point by point and reconstructed with a computer, a process known as optical sectioning.

Parameters

In confocal images the dermal papillae reflect strongly as a bright circular basal layer with the dark focus corresponding to the dermis. The circles are surrounded by the spinous layer. Focusing on the dermal papillae, the lumina of capillary loops were visible as black holes (cf. Fig. 1). The flowing of the brightly reflecting erythrocytes through the dermal capillaries can bee seen clearly in real time imaging. The following parameters were evaluated using in vivo CLSM: Quantitative blood cell flow per minute was counted in the capillary loops of two papillae of interest by off-line analysis of four fields of vision, which were digitally recorded for up to 30 s. Capillary loop diameter was evaluated in digital images of the epidermal-dermal junction using the image analysis program "Image Tool" (cf. data analysis). Two papillae of interest and four fields of vision were measured. In the smoker group, measurements were performed after a resting period of 3 h (baseline), during smoking, as well as 5 and 10 min after smoking of a single cigarette. In the nonsmoker control group, measurements were conducted only once. In a standardized setting, we marked the area to be measured using a special adhesive tissue ring on the inner volar forearm, where the application of the confocal microscope was comfortable. The tissue ring stabilizes the imaging skin site and, because of the adhesive quality, no displacement occurred. Additionally, the corresponding magnetic objective lens head of the microscope captured the fixed tissue ring always at the same position. All measurements were performed

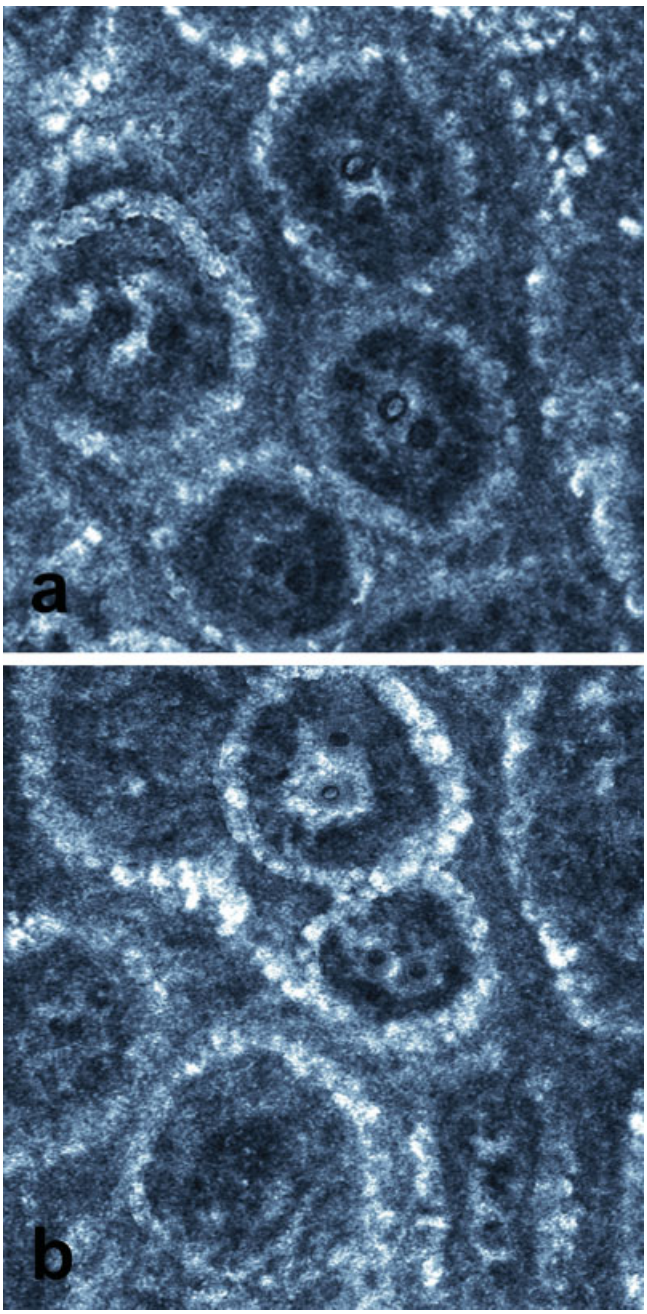

Fig. 1. Confocal images depict the epidermal-dermal junction as a bright circular basal-layer with the dark center corresponding to the dermal papillae. The circles are surrounded by the spinous layer. Focusing on the dermal papillae, the lumina of capillary loops were visible as black holes. The bright structures in the focus of the black holes represent blood cells. During smoking (b) the size of the capillary loops has a slighter aspect compared with the control area (a). The field of view measures 200 μm × 200 μm.

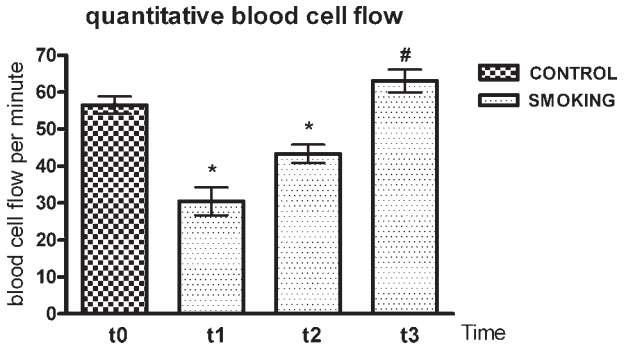

Fig. 2. The quantitative blood cell flow decreased during smoking (t1) and increased to a level below the baseline value 5 min (t2) after smoking, compared with the control (baseline value before smoking, t0). By contrast, 10 min after smoking (t3), a blood cell flow exceeding the baseline value was measured. A total of ~800 values in 24 volunteers were evaluated. The bars represent standard deviation. $^{*}P < 0.05$; $^{\#}P > 0.05$ analyzed by one-way ANOVA test and in comparison with the control (t0).

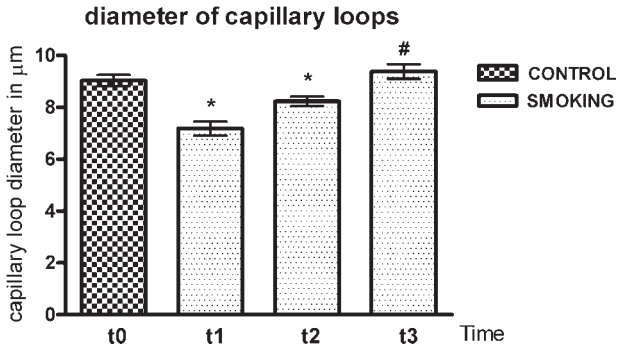

Fig. 3. Capillary loop diameter (dcl) decreased during (t1), respectively, 5 min after (t2) smoking of a single cigarette, compared with the baseline value (t0). Ten minutes after smoking (t3), the size of the capillaries increased, however, this was not statistically significant. A total of roughly 800 values in 24 volunteers were evaluated. $^{*}P < 0.05$; $^{\#}P > 0.05$ analyzed by one-way ANOVA test and in comparison with the control (t0). Values are mean standard deviation.

at 22 ± 4°C with 50% humidity in a standardized manner at the same position on the corners of an imaginary quadrate of 500 µm × 500 µm.

Data and Statistical Analysis

The sizes of capillary loops were evaluated by offline-analysis of confocal images using the image analysis software "Image Tool version 3.0" (UTHSCA, San Antonio, TX). This provides several image analysis tools, such as area or distance measurements. Moreover, the results are presented in table format and assist the export to statistical software. Statistical analyses were performed using SPSS version 13.0. Analysis of distribution was performed by Kolmogorov-Smirnov-test. Data were statistically analyzed using a paired student's *t*-test among results obtained within each time-point and one-way ANOVA test for independent observations between the corresponding time-values and an unpaired *t*-test to compare the control and smoker group. *P* values of less than 0.05 were considered significant.

RESULTS

The baseline value of quantitative blood cell flow was 55.50 ± 2.33 per minute in habitual smokers and did not differ significantly ($P > 0.05$) compared with the nonsmoker group (53.70 ± 3.24 per minute). Compared with the baseline, blood cell flow decreased during smoking by over 45% to 30.43 ± 3.76 per minute ($P = 0.02$). It remained nearly 22% lower at 43.33 ± 2.45 per minute 5 minutes after smoking ($P = 0.01$). By contrast, 10 min after smoking, quantitative blood cell flow tended to increase by more than 13% to 63.00 ± 3.10 per minute ($P > 0.05$) compared with the baseline value (cf. Fig 2). In smokers, capillary loops diameter was at 9.03 ± 0.22 µm before smoking (baseline). It did not differ significantly ($P > 0.05$) compared with the control nonsmoker group (9.28 ± 0.42 µm). In comparison with the baseline, the size of the capillaries decreased during smoking by ~21% to 7.18 ± 0.28 µm ($P = 0.03$) and by about 9% to 8.23 ± 0.18 µm ($P = 0.02$) 5 min after smoking. By contrast, capillary loop diameter tended to increase 10 min after smoking by roughly 4% to 9.38 ± 0.28 µm ($P > 0.05$) in comparison with the baseline value (cf. Figs. 1 and 3).

DISCUSSION

Using reflectance CLSM, we observed in accordance with previous studies a prompt and dramatic microcirculatory reactivity in young healthy smokers with relatively limited exposure in terms of pack years, both during and immediately after smoking. Hitherto, CLSM has been used to investigate the histomorphology in both the burn care arena and dermatology (Aghassi et al., 2000; Altintas et al., 2008a,b in press; Gonzalez et al., 1999a; Rajadhyaksha et al., 1999; Sauermann et al., 2002). CLSM results were demonstrated to correlate well to histological examination (Aghassi et al., 2000; Gonzalez et al., 1999a; Rajadhyaksha et al., 1999; Sauermann et al., 2002) with both a high sensitivity (88.15%) and specificity (97.60%) (Gerger et al., 2004). The present study is the first to our knowledge to analyze the dynamic alterations of the cutaneous microcirculation using in vivo CLSM. We believe that this methodology is particularly suited for microcirculation studies, since reflectance CLSM opens a window onto living tissue and provides real time images of the microvascular network up to a depth of 350 µm. The influence of smoking on microcirculatory disturbances was previously described and controversly discussed (Cunningham et al., 2005; Parsa et al., 2006). Regardless of the fact that neither study designs, nor the volunteers investigated, nor the methods used were ever standardized and therefore allowed only limited comparison all studies concluded that smoking decreases the cutaneous blood flow. Accordingly, Sarabi and Lind (2000) observed that cigarette smoking induced vasoconstriction in the forearm of young habitual smokers as soon as 5 min and persisting for up to 30–50 min after smoking of one cigarette; Using reflectance CLSM, we observed, as an acute effect of cigarette smoking, a similar reaction with reduced blood flow of over 45% during smoking. Although not as severely, these reduced blood flow values persisted for up to 5 min afterwards. Pellaton et al.

(2002) examined young smokers and did not find any impairment in reactive hyperemia 5 min after smoking a single cigarette. Based on the data evaluated, we are able to confirm these findings in part only, as we too could not determine a hyperemic phase during the first 5 min after smoking; however, we observed a hyperemic phase 10 min after smoking in a similar group of young smokers. Although the vasodilatatory and vasoconstrictive effects of nicotine has been discussed controversial (Black et al., 2001), the pathophysiological mechanism of blood flow reduction was explained by the vasoconstrictive effects of nicotine and the associated decrease of the arterial oxygen pressure (Jensen et al., 1991; Ludbrook et al., 1974). Accordingly, Dalla Vecchia et al. (2004) reported a dysfunctional vasodilatation because of irritated regulation of the peripheral cutaneous vascular activity in young patients after cigarette smoking. Although we did not quantify the oxygen pressure, we observed similar smoking-induced microvascular reactivity, as we demonstrated a vasoconstriction of over 20% during smoking. These findings may explain the previously described, smoking-induced dramatically reduced blood flow. However, the capillaries are without smooth muscle and the question remains open, how they have this reduced diameter. Despite, confocal microscopy evaluates significant changes in capillary sizes and confirm previously studies on smoking induced alterations on microvascular network. However, these reports are based mainly on indirect blood flow measurements of relative large vessels (e.g., by flowmetry) for want of a modality that allowed observation of the smallest-calibre vessels. Using CLSM and the direct visualization of the microcirculation on a cellular level, we have demonstrated that even the smallest cutaneous vessels, the dermal capillaries, are certainly influenced by smoking, as it has already been demonstrated for large vessels (Dalla Vecchia et al., 2004; Ijzerman et al., 2003). To establish the effects of smoking on microvascular diseases, additional clinical trials are required. Although confocal microscopy provides a new option to visualize microcirculatory alterations, however, it is not without limitations, as this technique allows the acquisition of high resolution images up to a depth of 350 μm. Beyond this depth, however, image resolution is limited. Hence, only superficial vessels of the skin can be investigated using this technique. In spite of this, confocal microscopy is currently the only method that allows observation of the cutaneous micromorphology, including the microcirculation in vivo and in real time on cellular and subcellular levels. In conclusion, in vivo reflectance CLSM enables qualitative and quantitative observation of dynamic processes of cutaneous microcirculation by direct visualization of microvascular reactivity and associated morphological patterns on cellular and subcellular levels.

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