Salivary leptin induces increased expression of growth factors in oral keratinocytes

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Abstract

We investigated the influence of salivary leptin, purified by affinity chromatography, on the proliferation of human oral keratinocytes. Furthermore we determined the time- and dose-dependency of the incubation with salivary leptin on the production of epidermal growth factor (EGF) and keratinocyte growth factor (KGF), which are growth factors relevant to keratinocyte proliferation. The analysis was performed both intra- and extracellularly. The relationship between the three cytokines in cell proliferation was studied by successive blocking with specific antibodies. The incubation of oral keratinocytes with recombinant and native leptin led to a significantly increased, dose-dependent cell proliferation (P<0·001). A further significant increase in proliferation was observed after incubating the cells with sterile filtered saliva (P<0·001). The increase in proliferation could not be observed by simultaneous incubation with salivary leptin and specific antibodies against either leptin or KGF (P<0·001). We found a significant dose-dependency between leptin incubation and production of KGF and EGF at the RNA and protein level. Both cytokines were expressed intracellularly and released into the culture medium, where they could be quantified by ELISA. Furthermore, there was a dose- and time-dependent increase in the phosphorylation of STAT-1 and STAT-3, indicating that Ob-Rb (the long form of the leptin receptor) expressed by the keratinocytes is functional. It is conceivable that the leptin-induced proliferation in keratinocytes is mediated by this signalling pathway. This is the first study to show a physiological role of salivary leptin as a growth factor for keratinocyte proliferation in the oral cavity. We could demonstrate its influence on the production of other growth factors essential for this proliferative effect. Based on the findings of our study we assume an important role for salivary leptin in wound healing within the vulnerable oral cavity.

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Introduction

Leptin was first described as an adipocyte-derived hormone (Zhang et al. 2000) influencing food intake and energy expenditure by interaction with receptors in the feeding centres of the hypothalamus (Elmqquist et al. 1997). However, increasing numbers of publications demonstrate that this cytokine is also produced by other tissues, such as the placenta (Zhao et al. 2003), gastric mucosa (Sobhani et al. 2002) and neuronal tissues (Morash et al. 1999). In addition to the well-described influence of leptin on nutrition, multiple peripheral metabolic effects of this hormone have been discussed (Muio & Dohm 2002).

Previous investigations of leptin in human saliva (Gröschl et al. 2001, 2002, Randeva et al. 2003) and its influence on the growth and weight of the salivary glands in rodents (Higa et al. 2002) indicate that, in addition to the circulating cytokine, local leptin production might play an important role in digestion or wound healing in the gastrointestinal tract. The idea of an autonomous glandular leptin production and active release into saliva is supported by the localisation of leptin within salivary microglobules, whereas seromucous material was negative (De Matteis et al. 2002).

The hypothesis of the current study was that leptin found in human saliva is biologically active and affects the proliferation of oral mucosal cells in vitro. This hypothesis is supported by previous publications describing the beneficial influence of leptin on wound healing in the gastrointestinal tract (Brzozowski et al. 1999, Konturek et al. 2001) and proliferation of various other tissues and cell lines (Wolf et al. 1999, Martin Romero et al. 2000, Hu et al. 2002, Wu et al. 2002). Previous studies demonstrating the beneficial effect of leptin on the proliferation of cutaneous keratinocytes in rodents were of special interest for our current investigation (Frank et al. 2000). Interestingly, in murine leukaemic cells this pro-proliferative effect can be inhibited by blocking the receptor with specific antibodies (Iversen et al. 2002).

Leptin receptors (Ob-Rs) are located ubiquitously (Halaas & Friedman 1997) and are present in various...
receptor isoforms (Sweeney 2002) inducing different modes of signal transduction. Research has focused on the long receptor isoform Ob-Rb, which causes phosphorylation of a receptor-bound janus kinase (JAK) using STAT proteins as second messengers (Rosenblum et al. 1996, Wang et al. 1996, Hakansson & Meister 1998, Banks et al. 2000). In the cutaneous keratinocytes of humans and rodents, JAK/STAT signalling has been described as the main second messenger pathway. In humans and rodents, JAK/STAT signalling has been described as the main second messenger pathway (Stallmeyer et al. 2001, Goren et al. 2003). Other truncated isoforms, in particular the soluble receptor, seem to have a leptin-binding function rather than a signal-transducing function (Lammert et al. 2001). Recently, functional Ob-R were identified in the gastric mucosa (Breidert et al. 1999) and in other parts of the gastrointestinal tract (Morton et al. 1999). The role of salivary leptin in the biology of the oral and gastrointestinal mucosa remains to be fully elucidated.

It was the objective of the present study to answer this question by investigating the effect of salivary leptin on the proliferation of primary keratinocytes derived from the oral cavity of healthy humans. Moreover, we sought to demonstrate the interaction between salivary leptin with other cytokines. Our interest focused on the analysis of the epidermal growth factor (EGF) and the keratinocyte growth factor (KGF; FGF-7), both well known for being involved in the proliferation of keratinocytes and wound healing (Alison & Sarraf 1994, Slonina et al. 2001).

Materials and methods

Cell culture

Primary human oral non-gingival keratinocytes obtained from healthy subjects (Lauer et al. 2001) were cultured in a 5% CO2 humidified atmosphere at 37°C in Keratinocyte-SFM medium (Invitrogen) supplemented with bovine pituitary gland extract and EGF, containing 100 U/ml penicillin, and 100 mg/ml streptomycin (Sigma). During the experiments the culture medium was not supplemented with EGF or KGF.

Isolation and purification of salivary leptin

Saliva from healthy volunteers was collected (~500 ml) using a Salivette with polyester swabs (Sarstedt, Nümbrecht, Germany). Saliva was pooled, filtered and centrifuged to remove cellular and mucous components (4000 g; 1 h). For the purification of salivary leptin, we extracted the clear solution using N-hydroxysuccinimide (NHS)-activated columns (HiTrap, 1 ml; Amersham Pharmacia) coated with leptin-specific antibodies. Ligand coupling was performed using a standard coupling buffer (0·2 M NaHCO3, 0·5 M NaCl; pH 8·3). After equilibration of the column with ten volumes of PBS buffer, pH 7·4, saliva was applied in 1 ml portions to the column (flow rate 0·2–1 ml/min). During this step of the procedure leptin binds to the antibodies. After repeated washing steps (five column volumes PBS; pH 7·4) salivary leptin was eluted by alteration of the pH value (three column volumes 100 mM glycine/0·5 M HCl; pH 2–7). The elute was buffered with Tris buffer (pH 9). The leptin content of the elution buffer was determined by RIA (Gröschl et al. 2000). The material was lyophilised and stored at −80°C until use.

Expression of cytokines

The influence of salivary leptin on the expression of EGF and KGF was examined by culturing the cells in six-well plates (Falcon, Heidelberg, Germany). Cells were sown in plates at a density of 2 × 104/well. After 48 h the medium was changed to contain either 0, 1·2, 2·5, 5, 10 or 20 ng/ml salivary leptin in the six different wells of the plates. After 24, 48 and 72 h two of each of the plates were harvested. The medium was collected for the analysis of extracellular proteins, the cells were harvested for Western blot analysis in SDS/mercaptoethanol lysis buffer and Trizol reagent for PCR determination.

Cell proliferation experiments

The direct determination of cell proliferation by counting cells was inconvenient, due to the non-homogeneous shape of our adherent epithelial cells. Therefore we decided to use an indirect method by determination of the DNA synthesis during mitosis.

In the first experiment, keratinocytes were transferred to sterile 96-well microtitre plates (MTPs) (Falcon) at a density of 2 × 103 cells/well. After adhesion of the cells (24 h), the culture medium was changed, containing either 0, 1·2, 2·5, 5, 10 or 20 ng/ml recombinant human leptin (Sigma). The different leptin concentrations were placed in ascending order within the MTPs.

The second experiment was carried out using salivary leptin purified as described above and adjusted to the same concentrations as in the first experiment.

In the third experiment, geometrically diluted (1/1 to 1/8; in sterile PBS) and sterile-filtered (Minisart 0·45 µm; Sartorius, Göttingen, Germany) saliva was added to the culture medium.

A fourth experiment was designed in order to determine the relationship between salivary leptin and EGF and KGF in keratinocyte proliferation. Cells in these MTPs were incubated with salivary leptin (10 ng/ml). Additionally, antibodies against either leptin, EGF or KGF were added in ascending order to the rows of the MTPs. As controls, we added rows including either no antibodies or rabbit IgG (RIA grade;
Sigma) to exclude any apoptotic effects of the rabbit immunoglobulin on human cells.

In a final experiment we sought to investigate how far salivary leptin is responsible for oral mucosal proliferation in native saliva. For this reason we prepared saliva free of leptin, EGF or KGF by immunoprecipitation of the cytokines with specific rabbit antibodies and goat anti-rabbit secondary antibodies. Keratinocytes were stimulated with saliva (1:1 in medium), and each of the cytokine-free aliquots. PBS replaced saliva in the controls.

After 24 h incubation, 5-bromo-2′-deoxyuridine (BrdU), a pyrimidine analogue, was added to the wells. The incorporation of BrdU during mitosis is an accepted measure of cell proliferation via assessment of DNA synthesis (Tsuchiya et al. 1999, Yoshizawa et al. 2000). The cell proliferation assay was purchased from Roche (Mannheim, Germany). After BrdU incorporation and fixation of the cells, a BrdU-specific antibody was added and, after further incubation, the turnover of the substrate tetramethylbenzidine was measured at 370 nm. In order to avoid edging effects, only the ten inner samples of each row of the MTPs were used.

Molecular biology

RNA extraction and reverse transcription

Total RNA was extracted from the cells using guanidine–thiocyanate acid phenol (Trizol; WAK Chemie, Medical GmbH, Bad Homburg, Germany). RNA concentrations were determined spectrophotometrically. One microgram of RNA was reverse transcribed in a volume of 20 µl at 39 °C for 60 min (chemicals from Boehringer Mannheim, Germany).

TaqMan real-time PCR

The method has been validated in our laboratory and successfully used before. This method is based upon the 5′ exonuclease activity of the Taq polymerase. Briefly, within the amplicon defined by a gene-specific oligonucleotide primer pair a oligonucleotide probe labelled with two fluorescent dyes is designed. As long as the probe is intact, the emission of a reporter dye (i.e. 6-carboxy-fluorescein, FAM) at the 5′-end is quenched by the second fluorescence dye (6-carboxy-tetramethylrhodamine, TAMRA) at the 3′-end. During the extension phase of the PCR, the Taq polymerase cleaves the probe releasing the reporter dye. An automated photometric detector combined with a special software (ABI Prism 7700 Sequence Detection System; Perkin-Elmer Applied Biosystems, Foster City, CA, USA) monitors the increasing reporter dye emission. The algorithm normalises the signal to an internal reference (ΔRn) and calculates the threshold cycle number (Ct), when the ΔRn reaches 10 times the standard deviation of the baseline. The Ct values of the probes are interpolated to an external reference curve constructed by plotting the relative or absolute amounts of a serial dilution of a known template vs the corresponding Ct values.

Commercial reagents (TaqMan PCR Reagent Kit; Perkin-Elmer) and conditions according to the manufacturer’s protocol were employed. cDNA (2·3 µl) (reverse transcription mixture) and oligonucleotides with a final concentration of 300 nM of primers and 200 nM of TaqMan hybridisation probe were added to 25 µl reaction mix. The oligonucleotides of each target of interest were designed using the Primer Express software (Perkin-Elmer) using uniform selection parameters that allowed the application of the same cycle conditions confirmed by primer optimisation. All of the primers and probes were purchased from Perkin-Elmer and Eurogentec (Liège, Belgium). The thermocycler parameters were 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 94 °C for 15 s and 60 °C for 1 min. Serial dilutions of one of the samples served as a reference providing relative quantification of the unknown samples.

EGF and KGF gene expression was related to the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin. For primers and TaqMan probes see Table 1.

Protein analysis

ELISA

Specific immunoassays for the analysis of EGF and KGF in the culture medium were purchased from R&D Systems (Wiesbaden, Germany; #DEG00 and DKG00). Sample volumes required per assay were 200 µl for EGF and 100 µl for KGF. Neither assay had obvious cross-reactivates to other cytokines. The linear range for measurement was 3·9–250 pg/ml for EGF and 31·2–2000 pg/ml for KGF. Limits of detection were listed at 0·7 pg/ml (EGF) and 15 pg/ml (KGF). Detailed information on the assays are provided in the product information.

Immunoblot

The proteins of the keratinocyte lysates were separated on 10–20% gradient SDS-polyacrylamide gels and transferred to PVDF membranes (Roth, Karlsruhe, Germany). Analysis was performed using polyclonal rabbit antisera raised against the soluble Ob-R. Detailed specification is provided in the original publication (Lammert et al. 2001). Monoclonal antibodies raised against human EGF and KGF were purchased from R&D Systems (#MAB236 and #AF-251-NA), poly-
clonal antibodies raised against the specific cytokine receptors EGF-R and KGF-R were purchased from New England Biolabs (Frankfurt, Germany; #2232) and Acris Antibodies (Hiddhausen, Germany; #AB5476). Detailed information on these antibodies are provided in the product information. All antibodies were used in a dilution of 1:1000 in blocking buffer (Roth). Protein markers were obtained either from New England Biolabs (biotin-labelled protein ladder) or from BioRad (colour-labelled protein markers).

Analysis of leptin signalling

The course in phosphorylation of STAT proteins over time was investigated in keratinocytes after incubation with 20 ng/ml recombinant leptin for 5, 10, 15 and 40 min. In an additional experiment keratinocytes were incubated with logarithmically increasing leptin concentrations between 0·01 and 100 ng/ml for 15 min. Untreated keratinocytes served as controls. Additionally, interferon (IFN)-α-treated HeLa cells served as positive and untreated HeLa cells served as negative controls (New England Biolabs).

After washing with PBS, 0·5 ml of lysis buffer (SDS/mercaptoethanol) was added to the cells. After 5 min of lysis, the cell suspension was heated to 90 °C for 10 min. After cooling on ice, samples were centrifuged and transferred to 12% SDS gels. The sample material was adjusted to a standardised protein content.

Proteins were blotted to nitrocellulose membranes (Roth). After blocking the membranes with 5% fat-free milk in PBS, the membranes were incubated overnight (4 °C) with monoclonal antibodies against phosphorylated and non-phosphorylated STAT-3 and STAT-1 (New England Biolabs; #9176; # 9132).

Data calculation and statistics

ANOVA (Kruskal–Wallis with Dunn’s multiple comparison test) was used to calculate significance of differences in cytokine production and BrdU incorporation. A $P$ value below 0·05 was considered significant. Values of cytokine release into the culture medium are provided as concentrations. Furthermore the values where normalised to the cytokine content of the daily non-stimulated control sample, set as baseline level.
Results

Expression of cytokine receptors

Using Western blot analysis of non-stimulated human oral keratinocytes, we found that the receptors for leptin, KGF and EGF were being expressed by the cells. The receptors displayed molecular masses of approximately 100 and 140 (Ob-R; Fig. 1a), 92 and 115 (KGF-R; Fig. 1b) and 175 (EGF-R, data not shown) kDa. The molecular masses are in accord with the data provided by the manufacturers of the antibodies and the literature (Marchese et al. 1995, Hackel et al. 1999, Shintani et al. 1999, Zwick et al. 1999, Lammert et al. 2001, Visco et al. 2004) and indicate that oral keratinocytes are potential targets for the three cytokines which are the focus of our investigations. The heavier Ob-R fragment with the expected size of 140 kDa was very weak in the keratinocytes, whereas the lighter band was well expressed in the cells and glandular tissues. The KGF-R was also weak but present in the keratinocytes and in line with the bands of the positive controls from gastric and gut mucosa. Interestingly, the salivary glands did not significantly express either band of the KGF-R.

Signal transduction

It was our aim to prove the functionality of the Ob-Rb receptor isoform being expressed by oral keratinocytes. Potential transducers of this proliferative effect after incubation with leptin are STAT proteins. After incubation of oral keratinocytes with 20 ng/ml human leptin, phosphorylation of STAT-1 and STAT-3 was obviously upregulated in a time- and dose-dependent manner. In our experiments the most intense bands for STAT-1P (STAT-1 α 91 kDa; STAT-1 β 84 kDa) and STAT-3P (STAT-3 α 79 kDa; STAT-3 β 86 kDa) were observed after 30 and 15 min of incubation respectively (Fig. 2a and b). Interestingly, even very small concentrations of leptin (0·1 ng/ml) led to a detectable phosphorylation of STAT-1 (Fig. 2a). The much higher intensity of the non-phosphorylated signal peptides is presented as a loading control in both figures. This finding proves the functionality of the Ob-Rb isoform expressed in the oral mucosa (Bohlender et al. 2003). It remains to be determined whether this way of signal transduction is responsible for the increased cell proliferation.

Expression of cytokines

The levels of specific mRNA for both EGF and KGF increased dose-dependently on day 1 and day 2 after the leptin stimulus (P<0·001) with lower expression of EGF. On day 3, however, the specific mRNA expression dropped down to a level as low as the initial value of non-stimulated cells. Since data of day 1 and 2 looked very similar, we decided to present the mean of the 2 days for both cytokines in Fig. 3. All values were normalised to the housekeeping genes β-actin and GAPDH.

We found EGF and KGF expressed intracellularly in leptin-stimulated cells. In addition to the specific banding for EGF (6 kDa; Fig. 4a) and KGF ( ~ 20 kDa; expected size 21 kDa; Fig. 4b), additional bands were found for both cytokines. However, we could not find significant differences in the intensity of the bands in
Figure 2 (a) Time- and dose-dependency of STAT-1 and STAT-1P in human oral keratinocytes after incubation with 20 ng/ml leptin for 0, 5, 10, 15 and 30 min, or with logarithmically ascending doses (0·01, 0·1, 1, 10, 100 ng/ml) of leptin for 15 min. IFN-α-treated HeLa cells served as positive (K+), untreated keratinocytes (0 min) and HeLa cells (K−) as negative controls. M: biotinylated protein marker. STAT-1α: 91 kDa; STAT-1β: 84 kDa. (b) Western Blot analysis of STAT-3 and STAT-3P after incubation of human oral keratinocytes with 20 ng/ml leptin for up to 30 min (0, 5, 10, 15 and 30 min; 20 µg protein/lane). IFN-α-treated HeLa cells served as positive (K+), untreated keratinocytes (0 min) and HeLa cells (K−) as negative controls. M: biotinylated protein marker. STAT-3α: 79 kDa; STAT-3β: 86 kDa.
relation to the concentrations of salivary leptin used or the duration of stimulation.

In the culture medium collected from the identical assay, a significant dose- and time-dependency was observed for both EGF and KGF expression after incubation with salivary leptin.

EGF values remained low in culture supernatants without stimulation with leptin (day 1: 7.1 ± 1.8 pg/ml; day 2: 5.7 ± 1.8 pg/ml; day 3: 7.2 ± 2.0 pg/ml; n.s.). In contrast, incubation with 20 ng/ml leptin led to significantly increased EGF values after 48 and 72 h (day 1: 7.2 ± 0.4 pg/ml; day 2: 22.1 ± 1.3 pg/ml; day 3: 28.9 ± 4.6 pg/ml; P < 0.001). The dose- and time-dependency of leptin on EGF release is presented in Fig. 5a. When normalised to the daily control value without leptin incubation, it represents an increase of 206% at 48 h and 300% at 72 h (P < 0.001).

The other growth factor KGF was found in considerably higher concentrations in the keratinocyte supernatants. Without prior incubation with leptin, values of 83.7 ± 39.7 pg/ml (day 1), 198 ± 22.1 pg/ml (day 2) and 221.8 ± 29.3 pg/ml (day 3) were found. The stimulation with the maximal leptin concentration (20 ng/ml) also led to significantly increased values after 48 and 72 h (day 1: 87.4 ± 2.7 pg/ml; day 2: 310.2 ± 9.4 pg/ml; day 3: 540.9 ± 63.7 pg/ml). The continuous rise of KGF concentrations in relation to the incubation with leptin is shown in Fig. 5b. After normalisation to the daily control value without leptin stimulation we calculated an increase of 355% after 48 h and 619% after 72 h (P < 0.001).

For both growth factors we found significant increases in release at lower, physiological doses of salivary leptin. The findings of this experiment indicate that salivary leptin is a potent stimulus for the expression of the two cytokines relevant to keratinocyte proliferation. Interestingly, KGF expression rises in non-stimulated cells during the 3 days, whereas EGF concentrations remained unchanged in the supernatants of cells without incubation with leptin.

**Cell proliferation assay**

Near physiological amounts of salivary leptin enhanced the proliferation of keratinocytes. A significant increase in DNA synthesis was demonstrated in concentrations as low as 2.5 ng/ml (P < 0.05; 5 ng/ml: P < 0.01; 10 and 20 ng/ml: P < 0.001). Using the same concentrations of recombinant human leptin, an almost identical effect could be observed (Fig. 6a). Moreover, sterile dilutions of whole saliva with the native content of salivary leptin also led to a dose-dependent increase in BrdU incorporation (Fig. 6b) compared with the cells in saliva-free culture medium.

The findings so far indicated that salivary leptin, in addition to other salivary factors, promotes oral keratinocyte proliferation. Consequently, we wished to determine to what extent oral keratinocyte proliferation is based on an interrelationship between salivary leptin and the two other cytokines EGF and KGF, which were found to be upregulated by incubation with salivary leptin. The simultaneous incubation of the cells with a constant concentration of salivary leptin (10 ng/ml) and antibodies against either leptin or EGF or KGF was designed to capture one of the three cytokines and investigate their individual significance for the proliferation of the keratinocytes.

We found that the addition of antibodies raised against human leptin neutralises the positive effect of salivary leptin on keratinocyte proliferation (Fig. 7a). The proliferation remained on a significantly lower level compared with the leptin-stimulated control cells (P < 0.001). Moreover, the addition of antibodies raised against human KGF led to comparable effects with the proliferation remaining at levels much lower in comparison with leptin-stimulated cells without simultaneous inhibition by antibodies (P < 0.001). In contrast, the addition of antibodies raised against human EGF did not significantly reduce the mitogenic effect of salivary leptin.

It was essential to prepare a control assay using non-specific rabbit IgG in order to guarantee that the inhibition of cell proliferation was based on the specific capturing of the cytokines and not on a general apoptotic effect of rabbit antibodies on human cells.
However, the addition of RIA grade rabbit IgG to the salivary leptin-stimulated cells did not hamper the proliferation. In fact, the stimulatory effect of salivary leptin on the cells was as significant as in the assay without addition of IgG.

Finally our experiment using immunprecipitated human saliva showed that both salivary leptin and EGF equally contribute to the pro-proliferative effect of saliva \( (P<0.001) \), whereas saliva after removal of KGF was as stimulatory as under native conditions (Fig 7b). This was not surprising, since our analysis showed that native saliva does not contain KGF in detected concentrations (below the limit of quantification of our ELISA).

**Discussion**

The neuroendocrine activity of leptin in the metabolism of the adipose tissue as a trigger to the CNS has been intensively studied with special focus on the regulation of food intake (Seeley et al. 1996, Elmquist et al. 1998, Loftus 1999, Thomas et al. 2001). The discovery of leptin and Ob-R in the stomach (Bado et al. 1998, Breidert et al. 1999, Cinti et al. 2000) and the presence of leptin in saliva (Gröschl et al. 2001) have led to the suggestion that salivary leptin in the gastrointestinal tract and the oral cavity influences food uptake, digestion, or protection of the epithelia in an, as yet, unknown manner.

In the present study, we investigated the physiological function of leptin produced by the salivary glands on oral keratinocytes with a focus on a potential epithelial-protecting action. Keratinocytes play an active part in local immune defence in oral mucosa and skin. They synthesise and release inflammatory cytokines and chemokines in response to a variety of environmental stimuli, such as bacterial products, ultraviolet light and other inflammatory cytokines (Kondo 1999). The impact of leptin as a mitogenic factor in skin repair has been intensively studied (Stallmeyer et al. 2001).
It was therefore of special interest to investigate the impact of salivary leptin on EGF and KGF, the latter known to be expressed by oral keratinocytes (Formanek et al. 1999) and their contribution to proliferation of these particular cells.

It was important to confirm the presence of the specific cytokine receptors in the cells used for our experiments. In addition to previous findings demonstrating the presence of Ob-R in the salivary glands (Bohlender et al. 2003), we confirmed by Western blotting that oral keratinocytes are potential target cells for the three cytokines of interest, which has been described previously for KGF (Partridge et al. 1996, Drugan et al. 1997) and EGF (Jurukovski & Simon 2000). Furthermore we could demonstrate the functionality of the Ob-Rb expressed by the keratinocytes. The incubation with salivary leptin led to the phosphorylation of STAT-1 and STAT-3.

**Figure 5**
(a) Release of EGF in supernatants of salivary leptin-stimulated keratinocytes. A significant dose- and time-dependency was observed after incubation with both low and higher doses of salivary leptin ($P<0.001$). In comparison with the daily control value (0 ng/ml), the highest leptin concentration (20 ng/ml) led to a 206% increase after 48 h and to a 300% increase after 72 h ($P<0.001$). (b) Release of KGF in supernatants of salivary leptin-stimulated keratinocytes. A significant dose- and time-dependency was observed after incubation with both low and higher doses of salivary leptin ($P<0.001$). In comparison with the daily control value (0 ng/ml), the highest leptin concentration (20 ng/ml) led to a 355% increase after 48 h and to a 619% increase after 72 h ($P<0.001$). Data are expressed as means±S.D. ($n=4$).

**Figure 6**
(a) Proliferation of human oral keratinocytes after incubation with leptin compared with medium controls (2.5 ng/ml: $P<0.05$; 5 ng/ml: $P<0.01$; 10 and 20 ng/ml: $P<0.001$). Cells incubated with recombinant human leptin. • Cells incubated with salivary leptin obtained from human saliva by affinity chromatography and lyophilisation. Index for proliferation of the cells is the incorporation of BrdU into the DNA. Data are expressed as means±S.D. ($n=10$). (b) BrdU incorporation in cells incubated with sterile filtered human saliva, serially diluted in PBS and mixed with an equal volume of medium compared with the medium controls (*$P<0.05$; ***$P<0.001$). Index for proliferation of the cells is the incorporation of BrdU into the DNA. Data are expressed as means±S.D. ($n=10$).
Figure 7 (a) BrdU incorporation in oral keratinocytes incubated simultaneously with 10 ng/ml salivary leptin and antibodies against either leptin, or EGF or KGF. Leptin antibodies neutralised the enhanced proliferation following salivary leptin incubation (**P<0.001). Antibodies against KGF also reduced proliferation significantly (***P<0.001) whereas antibodies against EGF had no significant effect. The control assay using non-specific rabbit IgG did not hamper the proliferation. (b) BrdU incorporation in cells incubated with sterile filtered human saliva (1:1 in culture medium) with its native cytokine composition, and after immunoprecipitation of leptin, EGF or KGF, compared with the controls with PBS replacing saliva (**P<0.01; ***P<0.001). Index for proliferation of the cells is the incorporation of BrdU into the DNA.
We therefore agree with recent findings describing the leptin signalling pathways in human and murine keratinocytes (Stallmeyer et al. 2001, Goren et al. 2003). However, it remains to be fully determined whether the signal transduction via the JAK/STAT pathway is responsible for the increased keratinocyte proliferation.

This study shows that native leptin isolated from human saliva exhibits biological activity comparable to recombinant leptin on oral keratinocytes in culture. The pro-proliferative effect has been shown before for non-oral keratinocytes, but only using recombinant leptin (Frank et al. 2000, Goren et al. 2003). We were able to elucidate the importance of salivary leptin on oral cell proliferation by investigating the effect of salivary leptin on the expression and production of further pro-proliferative cytokines. Both EGF and KGF were significantly upregulated by salivary leptin on mRNA and protein level. It was extremely interesting to find that mRNA for both cytokines was dose-dependently upregulated over 2 days followed by a sharp decline on day 3, showing that the cellular answer to the leptin stimulation on the RNA level does not parallel the protein synthesis. However, we consider this finding understandable, since protein synthesis is known to be a longer-lasting process than RNA transcription.

We were surprised to find EGF release into the culture medium remaining unchanged over 3 days without prior stimulation with salivary leptin. The slight decrease in the EGF values in the supernatant on day 3 in some leptin concentrations (5 and 10 ng/ml) may be explained by the degradation of the peptide, or adhesion to the plate surface.

In contrast, KGF release was gradually increased by the non-stimulated keratinocytes proliferating on a basal level. Knowing that EGF is present in human saliva (Olsen et al. 1984, Tebar et al. 2000, Thulesen et al. 2002) but not KGF, we interpret these data to indicate that EGF, supported by leptin, is predominantly an exogenous, saliva-derived stimulus for growing oral keratinocytes, whereas KGF is the main endogenous growth factor in keratinocyte proliferation (Slonina et al. 2001, Dörr et al. 2002, Sanaie et al. 2002). This hypothesis is underlined by our finding that the inhibition of KGF action by adding specific antibodies suppresses keratinocyte proliferation to a basal level. In contrast, the addition of antibodies against EGF did not reduce proliferation significantly. Moreover, this hypothesis is supported by recent publications demonstrating the importance of salivary EGF on cutaneous wound healing (Jahovic et al. 2004).

It was also interesting to observe that the inhibition of salivary leptin by simultaneous addition of antibodies against leptin reduced the proliferation to a basal level. We therefore postulate a hierarchical system of cytokine action in keratinocyte proliferation, which we have summarised in Fig. 8. In this model we represent figuratively the effect of salivary leptin binding to Ob-R molecules, leading to an increased expression of EGF and KGF possibly via STAT phosphorylation. The release of these cytokines and their autocrine...
or paracrine binding to specific receptors, finally accelerates the cellular proliferation.

So far we have demonstrated that salivary leptin shows significant physiological effects in oral keratinocytes. This is important for wound healing in the extremely vulnerable oral mucosa. The wound-healing effect of saliva is well known in animals (Bodner 1991, Bodner et al. 1992, 1993) and salivary cytokines such as salivary leptin, together with salivary EGF, may be responsible for this in addition to the cooling and antiseptic effects of saliva (Hart et al. 1987, Hart & Powell 1990). As shown in our study, sterile filtered native saliva containing salivary leptin and many other salivary components such as EGF and also the recently discovered ghrelin (Gröschl et al. 2005) accelerates keratinocyte proliferation, clearly demonstrating the importance of saliva for oral health.

It fits in very well with the findings of our study, that leptin has recently emerged as an important regulator of mucosal inflammatory response to bacterial infection. It has been shown that recombinant leptin prevents the reduction in salivary mucin synthesis by oral bacterial lipopolysaccharides (Slomiany & Slomiany 2003). Furthermore, a recent study of Ducroc et al. (2005) using Ussing chamber technology, proves that there are significant differences between leptin action in the circulation and in the gastrointestinal lumen. The authors demonstrated the inhibition of glucose uptake in rat jejunum being significantly decreased when leptin was supplied from the luminal in comparison with the serosal compartment.

Based on these findings and the data provided in our study, it becomes clear that salivary leptin may be a multifunctional factor in the maintenance of oral health.

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