Research Article

Cutaneous Microdialysis: Cytokine Evidence for Altered Innate Reactivity in the Skin of Psoriasis Patients?

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Abstract. Cutaneous microdialysis demonstrates cytokine production in living human skin. In the present study, microdialysis samples taken from uninvolved and lesional skin in three test subjects with psoriasis over 24 h have been investigated for cytokine content with a bead-based multiplex immunoassay from Luminex. Concentration curves for a set of Th1/Th2 and pro-inflammatory cytokines measured differed from a reference group of ten subjects without psoriasis. The time to return to near baseline values after innate insertion reactivity is between 9 and 16 h. Post-equilibration levels (17–24 h) for the three main cytokines elevated in the reference group were differentially elevated outside the range of the reference group for interleukin-1 β (IL1 β) and IL8 but not so for IL6. Two further cytokines, granulocyte-macrophage colony-stimulating factor and tumor necrosis factor- α not generally elevated in the reference group, showed elevated values in the test subjects. Multivariate time series analysis (chemometry) showed that cytokine patterns for the individual test subjects often fell outside the 99% confidence intervals of a model generated from the reference group. In a clinical research situation, cutaneous microdialysis is feasible, gives generally higher cytokine levels than in the blood and generates interpretable data on an individual's reactivity compared with a reference group. This may well prove useful in delineation of pathogenetic issues, selection of appropriate therapy and monitoring of subsequent response in inflammatory dermatoses such as psoriasis.

KEY WORDS: cytokines; dermis; human; microdialysis; multivariate time series analysis.

INTRODUCTION

Psoriasis is a common, chronic, inflammatory skin disease affecting around 2% of the Caucasian population (1) with significant co-morbidity factors and significant impact on quality of life (2). The cause of psoriasis remains unknown, but it is considered a multi-factorial disease with a complex pathogenesis involving genetic, immunological and environmental factors. Though modern therapies are effective, there is no cure, and challenges in phenotype classification, therapy and management remain at both a group and an individual level (3,4). Numerous “trigger factors” such as infection, physical or psychological stress and medications are known to play a role (5–9). Additionally, it is acknowledged that various psoriasis phenotypes (*e.g.* guttate psoriasis, early-onset psoriasis, *etc.*) exist (10).

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Despite extensive research activity and the general efficacy of newer therapies in psoriasis, there still remain many unanswered questions both in pathogenesis and patient management, *e.g.* on safety, long-term efficacy and the possibility of individualised treatment choices. In clinical research, pathogenetic information from patients has traditionally been gathered through point sampling (biopsies and blood samples). A method which may be considered for use is cutaneous microdialysis (CMD), a minimally invasive technique for the chronological measurement of unbound tissue concentrations of both endogenous and exogenous substances at the site of prime interest—human skin in health and disease (11–19). The present paper investigates use of CMD for the study of cytokines in psoriasis, with reference group data on Th1/Th2 and pro-inflammatory cytokines in normal skin from a previous publication (20).

The insertion of the microdialysis catheter triggers a localised injury response involving increased blood flow and oedema (17,21,22) and release of inflammatory mediators such as histamine and cytokines (23,24). We view the induction of interleukin (IL)1 β as a response to “danger” (20,25), mediated via, *e.g.* hyaluronan (26,27) or ATP (28). Danger signals are known to activate the inflammasome, a cytosol protein complex which can produce IL-1 β in cells such as monocytes/macrophages and Langerhans cells (29,30).

There is wide agreement that, in most situations, 30–135 min is the “equilibration time” required for the skin to

return to “near-normal” in many aspects of function after microdialysis catheter insertion trauma (11,15,16,19,21,31). For cytokines and other biologically active molecules, this period will be longer since local reactive changes meld into repair (20,31). The findings of the pre-equilibration period may be a “bonus” reflecting individual variability/phenotype in regard to innate reactivity to the “non-microbial/sterile” trauma which the catheter insertion constitutes (20,32). We view, however, the cytokine response after microdialysis catheter insertion as a manifestation of innate reactivity similar to the “sterile inflammation” of basic research models (26,33). Such reactivity data may have significance in its own right especially in disease processes such as psoriasis in which innate immune reactivity is known to be involved (34–36).

The aim of this project was to investigate and conceptualise the feasibility and interpretation of CMD for the *in vivo* demonstration of cytokine production in a clinical research situation. Catheters were inserted into uninvolved and lesional forearm skin of three informed volunteers with psoriasis. The cytokines measured over a 24-h period were those for which results from a reference group of subjects not suffering from psoriasis were available. The expected relevance of the findings was the demonstration of a possible role for CMD as a clinical research tool for broad application in the observational and interventional study of psoriasis and other inflammatory dermatoses.

MATERIALS AND METHODS

Subjects

Three volunteers with a verified diagnosis of psoriasis were given verbal and written information of the procedure. The test subjects comprised a 40-year-old male (PS1), a 59-year-old female (PS2) and a 35-year-old male (PS3). The reference subjects comprised four females between 27 and 56 years of age and six males between 27 and 55 years of age. All subjects were free from systemic medication.

The study design gave subject data solely on age and sex. No other clinical details were available “post hoc” for correlation with CMD data. The study was approved by the Regional Ethics Committee for Human Research at Linköping University (#03250).

Experimental Design

Microdialysis catheters used in this study were commercially manufactured CE marked concentric catheters (CMA71, CMA/Microdialysis AB, Stockholm, Sweden) with a 10 mm polyethersulfone membrane having a molecular weight cut-off of 100 kDa. The point of insertion was anaesthetized with a 0.1-ml intradermal injection of local anaesthetic (Xylocain® 10 mg/ml Astra Läkemedel AB, Södertälje, Sweden). The guide for the microdialysis catheter was an 18-gauge venous catheter (Artsana S.p.A, Italy) tunneled in the deep dermis or subcutaneous tissue for the first 1.5 cm and then intradermally for the last centimetre in order to position the dialysis catheter membrane as superficially as possible under the center of a lesion or in noninvolved skin (Fig. 1). Two portable pumps (CMA 106, CMA/Microdialysis AB, Stockholm Sweden) with a fixed

Fig. 1. Microdialysis catheters inserted into uninvolved and lesional skin in the ventral forearm of a subject with manifest psoriasis. The schematic pictures show how the catheter membrane is positioned in the dermis of the uninvolved skin (to the left) and under a psoriasis plaque (to the right)

flow rate of 0.3 $\mu\text{L}/\text{min}$ was used to pump the perfusate, Ringer Dextran Braun 60 (Apoketsbolaget, Gothenburg Sweden) through the catheters. This flow rate has been previously determined by *in vitro* studies to give the best recovery of cytokines (24). Catheters were inserted in the morning, and the dialysate was collected at 60-min intervals in pre-weighed microcentrifuge tubes (Elkay Products, MA, USA) for the following 8 h. The night sample was composed of dialysate collected in one tube while the individual was sleeping. The following morning, the samples were again collected hourly until termination of the experiment 24 h after catheter insertion.

Microdialysate

The microdialysate from the three individuals was analyzed for cytokines with the Human Cytokine Ten Plex Kit (Biosource, Nivelles, Belgium). Cytokines detected are IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon gamma (INF- γ) and tumor necrosis factor- α (TNF- α). This method is based on multiplex bead technology (37) and modified as earlier described (20,24). The Star Station acquisition program (v2.3 Applied Cytometry Systems, Sheffield, UK) processes the fluorescence intensity of the beads and bound cytokine-antibody complexes. Standard curves are generated by a five-parameter algorithm in the analysis program and plotted on a log-log scale.

Statistical Analysis

The reference group constituted the known base of information (20). The innate reaction to the insertion trauma seen in the ten individuals could be followed in a concentration *versus* time graph over 24 h.

The results for each psoriasis subject were considered individually against the reference group. The median and inter-quartile range is used due to the low number of

individuals that comprised the reference group and large individual variation.

When comparing the reference group (normal skin) and psoriasis individuals (lesional and uninvolved skin) as three groups, a nonparametric test, Kruskal–Wallis and the post-test Dunn's were calculated by GraphPad Prism v5.02 (GraphPad Software Inc., La Jolla, CA, USA).

Multivariate time series analysis was used to compare results for all cytokines. For this the program, SIMCA-P+ 12.0 by Umetrics-MKS was used. The reference data consisted of measurements at 11 local time points for five variables (the cytokines IL1b, IL 6, IL 8, GM-CSF and TNF- α), in the ten reference subjects. The test set consisted of three psoriasis subjects with measurements for the same variables and local time points. The test set measurements were available both for uninvolved and lesional skin. This type of data preferably can be analysed with so-called multivariate batch analysis (subject = batch), an approach developed for process monitoring and early fault detection of new batches. Here, we use the approach suggested by Wold *et al.* 1998, where the reference set measurements for the variables are projected on the settings of a time variable by using the multivariate projection method partial least squares (PLS) regression (38,39) combined with cross-validation (40). The original time series is in the present case reduced from five variables to two PLS components, denoted t1 and t2. By plotting the score values for t1 and t2 for the reference set against the time points, the behavior of the individual subjects can be studied. At each time point, a confidence interval is calculated from the variability of the score values. By fitting a new subject (test set) to the multivariate model, predicted score values at each time point were calculated. Thus, at a particular time point, it is possible to decide if a test set subject falls inside or outside the confidence interval for the reference set subjects. For a general presentation and discussion of approaches to multivariate batch analysis, see Eriksson *et al.* (2006) (41).

RESULTS

Results from the three test subjects demonstrate that cytokines can be measured in uninvolved and lesional skin in subjects with psoriasis, over a 24-h period. The procedure is well-tolerated and instructed subjects were able to perform the vial changes necessary during periods in which they were not in the laboratory. The individuality of vial changing was not expected to affect the cytokine concentrations at a detectable level due to the slow flow rate of the perfusate and the chosen analysis method. Temperature variations caused by different transport times to the lab the following morning might affect the standard error in the final readouts. Many of the cytokines measured here are, however, quite robust to temperature changes as has been shown in *in vitro* testing.

Results shown are absolute microdialysate levels without modelling for blood flow or recovery. The reactivity over the whole experimental period (both post-equilibration and pre-equilibration) for the five cytokines showing elevated levels in the three test subjects as well as the reference group ($n=10$) is shown in Electronic supplementary file a and b.

In the usual analysis of microdialysis data, an equilibration time of 60–90 min is adequate to account for axon reflex-mediated blood flow changes after insertion trauma (11,16,17). The equilibration time required with regard to cytokines induced by catheter insertion is longer (20,31). Results suggest that it may be as long as 17 h before the tissue around the catheter can be presumed to have returned to a state, which can reflect the actual inflammatory state of the subject's tissue—a “post-equilibration” state.

Figure 2 shows the “post-equilibration” values for the five cytokines which were elicited in the three test subjects and the reference group. Apart from IL-6, values in test subjects from both uninvolved and involved skin were often outside the range of the reference group. This was seen most consistently with GM-CSF and IL-8. Other cytokines were either within the range of the reference group or had disparate (higher or lower) values in the individual subject, indicating that the three subjects were, in regard to cytokines, not reacting in the same way and were thus either not homogeneous as to their phenotypes or were in different disease activity phases. Plasma levels taken at the cessation of the experiment are also shown in Fig. 2. With the exception of one test subject all plasma levels were at the extremely low levels previously seen in our reference group and reported by the kit manufacturer for “normal blood donors” (Biosource Nevelles, Belgium).

The “Pre-equilibration” Period

Analysis of “pre-equilibration” results is not usually performed, the findings dismissed as being an artefact with consequent difficulty in interpretation of data.

In Fig. 3, we suggest that the time interval for protein production during the pre-equilibration period can be analysed in three phases. In the first 2 h, presence of cytokines in the microdialysate should imply presence of cytokines in the tissue, prior to induction of cytokines caused by the catheter insertion itself. The second period (3–8 h) can be interpreted as illustrating the response of the tissue (release of cytokines) to the insertion manoeuvre, which we view as a manifestation of innate skin reactivity to the minimal wound which the catheter insertion constitutes (20,32,42). The third period in the pre-equilibration phase (9 to 17 h period) is a recovery phase in which values move towards baseline. This in our material involved, for practical considerations in the overnight experimental situation, pooled rather than hourly sampling. The data presently available does not permit a more exact conclusion.

Summary of Findings for Individual Cytokines

GM-CSF

The findings for GM-CSF were striking. In the post-equilibration phase (Fig. 2), two of three subjects with psoriasis had elevated values in lesional skin, and the post-test Dunn multiple comparison test showed statistical significance ($p<0.05$) for GM-CSF when the grouped lesional and non-involved skin were compared with the controls.

Values for both uninvolved and lesional skin (in the pre-equilibration phase) were markedly elevated for all subjects

◀ **Fig. 2.** The figure shows the median concentration of five proinflammatory cytokines in cutaneous microdialysis samples of three subjects with psoriasis (ps1 blue; ps2 red; ps3 light green) collected between 18 and 24 h (the “post-equilibration” period) after catheter insertion from uninvolved and lesional skin. Plasma concentrations for each test subject collected at 24 h are represented as a color-coded star. The median cytokine concentrations from uninvolved skin of a reference group (black, $n=10$) for the same time period are shown with the median and inter-quartile range

but not in the first 2 h. The post-test Dunn multiple comparison test showed statistical significance ($p<0.05$) for GM-CSF at 3–8 h when the lesional and non-involved skin groups were compared with the control group. We conclude that GM-CSF is not constitutively present in the skin of subjects with psoriasis because of negative findings in the first 2 h. We propose that it is induced by the insertion trauma, in marked contrast to the skin of non-psoriasis subjects in the reference group.

TNF- α

TNF- α , with a prominent place in the pathogenesis and therapy of psoriasis, did show elevated values in the post-equilibration phase for two of the subjects. The same two subjects had elevated values during the pre-equilibration period whereas the third did not. Thus, the three patients showed differentiated responses, suggesting that they were of different phenotypes or in different phases of their disease. Group analysis failed to show statistical significance.

IL-8

IL-8 (CXCL8) was elevated in both uninvolved and lesional skin in two of three subjects with psoriasis in the post-equilibration period. The same two subjects showed marked elevation in the pre-equilibration period though not convincingly in the first 2 h. The subject with low IL-8 values was consistently low throughout the experiment and showed thus marked difference compared with the other two patients. In the group analysis, despite this dichotomy, statistical significance of $p<0.05$ was shown at the 0–2 h time period in lesional *versus* reference group.

IL-1 β and IL-6

Neither IL-1 β nor IL-6 showed systematically elevated values compared with controls in the pre-equilibration period. In the post-equilibration periods, IL-6 was not elevated in any subject whereas IL-1 β was elevated in two subjects in lesional skin.

The remaining measured cytokines IL-2, IL-4, IL-5, IL-10 and INF- γ were only above detection thresholds in isolated samples.

Can Patterns of Cytokine Production be Analysed?

Analysis of the “cytokine fingerprint” for a range of cytokines or other mediators in the total output of a microdialysis examination over 24 h is a potential basis for the consideration of an individual subject’s possible difference

from a reference group. The multivariate batch analysis gave two PLS components (t1 and t2) according to cross-validation (39,40). The score values for the t1 component at the different time points are plotted in Fig. 4 together with their 99% confidence interval at each time point. One of the test persons, PS1, behaved rather similarly to the reference group, with score values just outside the confidence interval for a few time points (time 7–9 h for t1 and although not shown, time 9–16 for t2) for uninvolved skin and minor deviations for t1 at 3 and 4 h for lesion skin. This is in contrast to the results for the other two subjects (PS2 and PS3), both for uninvolved and lesional skin. For PS2 and PS3, a strong deviation from the reference group are found for both t1 and t2 from 3 h except for PS2 lesional skin where the strong deviation appeared from 6 and continued to 24 h. The deviations are less pronounced at the last time point 17–24 for PS1. The proposed phases of the pre- and post-equilibration periods are also shown in the plots.

DISCUSSION

Although this study, of only three test subjects, was designed primarily to demonstrate feasibility and concepts of use and interpretation of data generated by CMD in a clinical research setting, the findings for GM-CSF were noteworthy because of their occurrence in both uninvolved and lesional skin in the psoriasis subjects.

GM-CSF, a growth factor working mainly in a paracrine and an autocrine fashion, is produced by several different cell types found in the skin. Non-inflammatory cells such as keratinocytes, endothelial cells, mast cells and fibroblasts can produce GM-CSF when activated by inflammatory stimuli (43–45). GM-CSF stimulates dendritic cells and monocytes/macrophages, also normally present in the skin, to produce IL-6 and IL-23 (46). These two key cytokines are, together with GM-CSF, essential in psoriasis for the differentiation of memory T cells into Th17 lymphocytes and their continued survival and production of IL-17 (47,48). The study’s findings of increased GM-CSF in all test subjects in the 3–8 and 9–16 h phases of the pre-equilibration period and in two of the three subjects with psoriasis in the post-equilibration period must be interpreted as significant (outside the inter-quartile range of the reference group) despite the small number of subjects. The fact that findings were seen in both nonlesional and lesional skin gives strength to the hypothesis that the skin of patients with psoriasis has, in its entirety, differing function despite a “normal” appearance of nonlesional skin. Observations of abnormalities in nonlesional skin in psoriasis patients have been noted in previous studies for, *e.g.* GM-CSF (49) nerve growth factor (50) and serotonin (51). With regard to the cellular origin of the GM-CSF in nonlesional skin, keratinocytes (no epidermal activation in the model (20)) and blood-derived inflammatory cells (very sparse infiltrate in normal dermis and epidermis) are not likely causes. Resident (stromal) cells of the dermis such as dendritic cells, endothelial cells, macrophages, mast cells and fibroblasts are candidates (5,26). Future studies combining CMD and “end-point biopsy” (32) may shed light on the matter.

In complex diseases, analysis of interrelations between cytokines and cytokine patterns may increase the value of data. Multivariate time series analysis in so-called batch mode

Fig. 3. The median concentrations of five pro-inflammatory cytokines in cutaneous microdialysis samples collected hourly from three subjects with psoriasis (ps1 blue; ps2 red; ps3 light green) in uninvolved and lesional skin and a reference group of ten individuals (black, $n=10$) are shown. The individual samples for the first 16 h (the "pre-equilibration" period) have been shown in sub-groups: 0-2, 3-8 and 9-16 h (the sample collected during the night). The median and inter-quartile range of the concentrations are also shown for the reference group

Fig. 4. Batch multivariate analysis of five cytokines (IL1- β , IL-6 IL-8, GM-CSF and TNF- α) from ten individuals in the reference group is shown for the t1 PLS component (see “Materials and Methods”) over 24 h. The reference set measurements for the variables are projected on the settings of time variables by using the multivariate projection method PLS combined with cross-validation. At each time point, a 99% confidence interval is calculated from the variability in the individual score values ($n=10$). The mean and ± 3 SD are shown. Background shading indicates the three “pre-equilibration” periods used in Fig. 4 (blue, yellow and white) and the “post equilibration” period in pink. PS1, PS2 and PS3 show the plotted results for each test subject

clearly demonstrates an interesting approach to model results for a number of variables (cytokines) from a normal group of subjects over time. The model is then used to monitor the “performance” of new subjects over the same time period and to determine whether they follow the levels and the time dependence of the reference group. In the present study, only five variables were studied, and the model is based on rather few normal persons, but for this type of modelling, there is no restrictions to the number of variables, number of subjects and how often the sampling is done. A multivariate approach also makes it possible to detect deviations that cannot be detected by univariate analysis due to type 2 errors. Another possibility is to interpret in more detail which variables cause deviations. A more comprehensive description of the analysis

of the present multivariate will be given in a separate publication. The approach needs a consistently measured and representative group of normal persons, and it is important also that the test persons are measured with the same procedure. The obvious virtue of the statistical technique is the capacity to analyse the outcome for an individual test subject against a reference group (38–41,52), which represents a significant advance in the interpretation of CMD data, an analysis now often used. This opens the possibility of study design and hypothesis generation built “case by case” rather than on groups of subjects. Whilst this methodology may appear complex, it is by no means as complex as other methodologies currently required for optimal interpretation of data (53,54).

A general feature of the findings was that cytokine levels in the tissue were generally higher than in the blood. Additionally, cytokine concentrations in lesional skin were not generally higher than that in uninvolved skin. Normalisation (modelling) for membrane performance (recovery) and blood flow (high blood flow “leaches” molecules) has not been performed, since there is, at present, no fully accepted method for this modelling. *In vivo* recovery estimations are difficult to apply for biologically active molecules such as cytokines. Given this situation, our view and that of other authors (31) is that display of raw data is most appropriate at this point in time. If normalisation for increased blood flow in lesional skin was to be performed, values may well be two- to ten- (highly likely) or 100-fold (less likely) higher.

The ubiquitous response observed to the actual catheter introduction trauma reflects the reactive capability of the skin in the individual studied. The mechanism may involve induction of the inflammasome through ATP or hyaluronic acid fragments (26–28). Other forms of reactivity are more sporadic, *e.g.* the Koebner phenomenon can occur in psoriasis and some other dermatoses (*e.g.* lichen ruber, lupus erythematosus). Pathergy is another example of aberrant reactivity in, *e.g.* Behcet’s disease (42), and anergy describes reduced reactivity in sarcoidosis, severe systemic disease and trauma. We propose that CMD is a method well suited to the study of the chronology of skin reactivity in health and disease and possibly in the mapping of response to therapeutic intervention.

In Summary

Although there have, over the last three decades, been many clinical publications on psoriasis, the day to day management of psoriasis patients in the clinical workplace remains a potentially rich but as yet poorly tapped source of clinical research data. With newer methods of data generation, better use could be made of opportunities for data collection at the clinical frontline, where patients regularly present in exacerbation, respond to treatment or resolve spontaneously. CMD using suspension arrays and other recent advances in analytical technique, in a protocol such as the one used here over 24 h, is a technique which could well be applied in the clinical workplace as an additional research tool to currently used patient registries, phenotypic and severity assessments, blood test and skin biopsy. There are numerous, as yet under-investigated, inflammatory dermatoses other than psoriasis for which a similar approach could be applied to gain knowledge at an individual and group level, of relevance to diagnosis, therapy and prevention.

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Conflict of Interests The authors identify no conflicts of interest.

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