Calcineurin Activity and Inhibition in Skin and (Epi)Dermal Cell Cultures

NPM Smit[1], HH Van Rossum[1], FP Romijn[1], KJ Sellar[1], M Breetveld[2], S Gibbs[2] and J Van Pelt[1]

Calcineurin (Cn) is the target of the immunosuppressive drugs cyclosporine A (CsA), tacrolimus (Trl), and pimecrolimus (Prl). Trl and Prl are often used topically for treatment of various skin diseases. The Cn inhibitors CsA and Trl are mostly used for maintenance therapy of transplant patients. Their long-term use, however, causes a dramatic increase in skin cancer risk. By using a newly developed assay for Cn measurement in blood, we were able to demonstrate Cn activity in total skin homogenates. A significantly higher activity was found in epidermis compared to dermis. In skin cell cultures, fibroblasts showed the highest activity as compared to keratinocytes and melanocytes. Of the Cn inhibitors, Trl showed stronger inhibition than CsA and Prl (57 and 55% in fibroblast and keratinocyte cultures, respectively). Also, the lowest IC_{50} (the half maximal inhibitory concentration) values were found for Trl (0.5 and 1.3 nM in two different fibroblast cultures). Cn activity and its inhibition can thus be studied in dermatological samples. The effects of Cn inhibition in fibroblasts and keratinocytes may be of influence on the overall functioning of the skin immune system.

Journal of Investigative Dermatology (2008) **128,** 1686–1690; doi:10.1038/sj.jid.5701244; published online 17 January 2008

INTRODUCTION

The enzyme calcineurin (Cn) plays a key role in the immune response. In T cells, it is responsible for the dephosphorylation of the NFAT (nuclear factor of activated T cells), and its subsequent nuclear translocation ultimately results in T-cell activation (Winslow *et al.*, 2003). In organ transplantation patients, Cn is the main target for immunosuppression by the Cn inhibitors cyclosporine A (CsA) and tacrolimus (Trl).

Besides its role in T cells, Cn plays a role in various tissues and also in the skin (Reynolds and Al Daraji, 2002; Aramburu *et al.*, 2004). Al Daraji *et al.* (2002) have shown that in keratinocytes, the nuclear translocation of NFAT is regulated by Cn and influenced by Cn inhibitors. Furthermore, a role for Cn in the differentiation of keratinocytes has been described (Santini *et al.*, 2001). Cn activity has been demonstrated before in cultured lung fibroblasts with an assay using ^{32}P-radiolabeled substrate and the activity could be inhibited by CsA (Chen *et al.*, 2003). A role for Cn in melanocytes is suggested, as CsA was shown to decrease pigmentation in cultured human melanocytes, and Cn inhibitors are used topically for treatment of vitiligo (Lee and Kang, 2003; Taieb, 2005). The Cn inhibitors CsA, Trl, and pimecrolimus (Prl) are also used for the treatment of other dermatological diseases, such as psoriasis and atopic eczema (Griffiths, 2001; Reynolds and Al Daraji, 2002).

For a long time, the treatment of organ transplant patients with Cn inhibitors has been known to result in an increased incidence of non-melanoma skin cancer (Hartevelt *et al.*, 1990), and nowadays the occurrence of various other types of post-transplant malignancies are becoming an important cause of mortality (Buell *et al.*, 2005).

The studies mentioned above indicate a role for Cn in skin. However, so far no direct measurement of Cn activity in skin tissue or skin cells has been described. Recently, we developed a new sensitive assay for spectrophotometric measurement of Cn in leukocytes (Sellar *et al.*, 2006). The increased use and topical application of Cn inhibitors in dermatological practice and the strong elevation of non-melanoma skin cancer in transplant patients treated with Cn inhibitors was the rationale to use this assay for our study on Cn activity in the skin and the cells, which are the main constituents of the dermis and epidermis.

RESULTS

In this study, the Cn assay was used to study Cn activity in skin tissue and cultured cells derived from the skin. Our first measurements using a total skin tissue homogenate prepared in lysis buffer showed that Cn could be measured in the sample with the activity of 1.9 ± 0.1 nmol per min per mg protein ($n = 4$; 95% confidence interval (CI) 1.82–1.98 nmol min^{-1} mg^{-1}). After 14 days of culture (in Fei medium), the keratinocytes derived from the epidermis of the same skin showed a Cn activity of 11.1 ± 0.9 nmol $min^{-1}mg^{-1}$. After the separation of the dermis and the epidermis of the skin from four other donors, an 8.0 × higher Cn activity was measured in the epidermis

[1]*Department of Clinical Chemistry, Leiden University Medical Center, Leiden, The Netherlands and* [2]*Department of Dermatology, VU University Medical Center, Amsterdam, The Netherlands*

Correspondence: Dr NPM Smit, Department of Clinical Chemistry, L01-036, Leiden University Medical Center, Albinusdreef 2, Leiden 2333 ZA, The Netherlands. E-mail: N.Smit@lumc.nl

Abbreviations: Cn, calcineurin; CsA, cyclosporine A; Prl, pimecrolimus; Trl, tacrolimus

Received 22 May 2007; revised 10 October 2007; accepted 27 November 2007; published online 17 January 2008

(1.7 ± 0.4 and 14.0 ± 3.2 nmol min^{-1} mg^{-1} in dermis and epidermis, respectively) (Figure 1).

In cultures of fibroblasts derived from the dermis and keratinocytes from the epidermis of the same donor, a 40% lower Cn activity was found in the keratinocytes. Inhibitions of Cn activity with 124 nM Trl were 74 and 76% in the fibroblasts and keratinocytes, respectively (see Table 1). For another donor, a 55% lower activity was found for the melanocytes compared to the corresponding keratinocytes. Inhibition with 124 nM Trl was 89 and 75% in the keratinocyte and melanocyte cultures, respectively (Table 1). With 831 nM CsA, this was 25 and 49%, respectively. Mean Cn activity was higher in fibroblasts (13.6 ± 7.0 nmol per min per mg protein) than it was in keratinocytes (7.2 ± 3.0 nmol per min per mg protein) ($P = 0.018$) (Figure 2). A considerable spread was observed for the Cn activities in the fibroblast and keratinocyte cultures (95% CI 9.5–17.7 and 4.9–9.5 nmol per min per mg protein, respectively). Activities in melanocytes were the lowest (2.7 ± 0.9 nmol min^{-1} mg^{-1}). Low Cn activities were also found for two melanoma cell cultures (1.7 ± 1.0 and 1.4 ± 0.5 nmol min^{-1} mg^{-1} for An ($n = 8$) and M14 ($n = 5$), respectively).

Figure 3 shows the inhibition curves for the inhibition of Cn after a 24-hour incubation with CsA in two different fibroblast cultures (F0502, F0503). Maximal inhibition of 63% was found in the culture F0503, and for both cultures similar IC_{50} (the half maximal inhibitory concentration) values were found (Table 2). Inhibition curves for Trl and Prl were measured using the same two cultures. As can be seen in Table 2, maximum inhibition of Cn was stronger for Trl than for CsA and Prl, and IC_{50} values calculated for Trl are lower than those for CsA and Prl. All three Cn inhibitors were more effective in the culture F0503 than in F0502.

As can be concluded from these experiments and our earlier work with CsA (Sellar *et al.*, 2006), maximal inhibitions are achieved at concentrations of $\geqslant$831 nM CsA, $\geqslant$124 nM Trl, and 123 nM Prl. Numerous inhibition experiments were subsequently performed using the inhibitors at these higher concentrations. In Figure 4, it can be seen that maximal reduction of Cn activity is found for Trl in both fibroblast and keratinocyte cultures. Somewhat milder inhibition is obtained with both CsA and Prl (CsA, 36 and 33%; Trl, 57 and 55%; and Prl, 43 and 35% inhibition in fibroblast and keratinocytes, respectively). As described above, inhibition of Cn activity in melanocytes with 831 nM CsA and 124 nM Trl was only measured once. Both melanoma cell cultures showed relatively low Cn activity compared to that of the melanocytes. After 24 hours treatment of the melanoma cells, reductions in Cn activity were similar for CsA and Trl (both $n = 6$) (71 and 69%, respectively).

Table 1. Activity and inhibition of Cn in cell cultures from two different donors

	Cn activity[1] (nmol min^{-1} mg^{-1})		
	Control	+ Trl	
Donor 1			%[2]
Fibroblasts	12.7	3.3	26
Keratinocytes	7.7	1.9	24
Donor 2			
Keratinocytes	8.5	1.0	11
Melanocytes	3.8	1.0	25

Cn, calcineurin; Trl, Tacrolimus.
[1]Fibroblasts and keratinocytes originating from one donor and keratinocytes and melanocytes originating from a second donor. Measurements were performed in duplicate cultures for donor 1 and single cultures for donor 2. Incubations were performed for 24 h with 124 nM Trl. Keratinocytes were grown in Fei medium.
[2]The percentage (%) activity compared to untreated control.

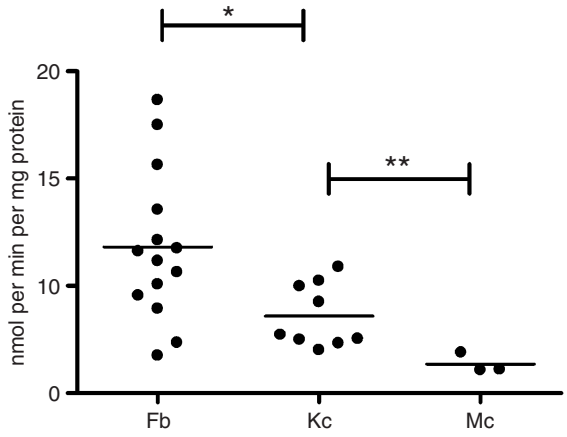

Figure 2. Calcineurin activity measurements in different fibroblast, keratinocyte, and melanocyte cultures. Cn activity measurements were performed in lysates of fibroblasts (Fb, $n = 14$), keratinocytes (Kc, $n = 9$), and melanocytes (Mc, $n = 3$). Significant differences in Cn activity were observed between fibroblasts and keratinocytes (*$P = 0.018$) and between keratinocytes and melanocytes (**$P = 0.030$). All keratinocyte cultures were maintained in Fei medium.

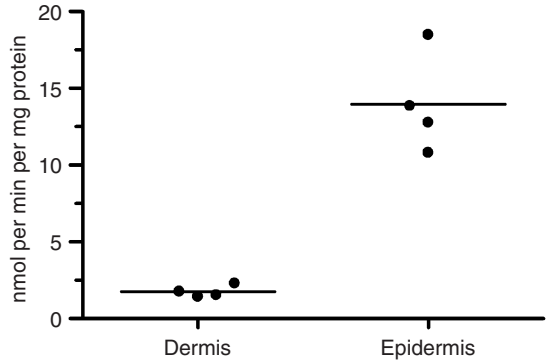

Figure 1. Measurement of calcineurin activity in dermis and epidermis. Dermis and epidermis were separated by dispase treatment, homogenized, and lysed, and calcineurin activity was measured. Material was used from four different donors.

DISCUSSION

Using a Cn enzyme assay, we were able to investigate Cn activity in skin. In four different skin samples, the highest