controls although this was not statistically significant due to the large variation between individual cases (Table 5).

5. Discussion

The present study demonstrated the role of keratins 5 and 10, IVL and FLG in keratinocyte activation and differentiation in CAD. Predominant accumulation of an anuclear keratin in S. corneum layer of CAD reflected orthokeratotic hyperkeratosis. Together with other histopathological findings, the microscopic change of CAD lesional skin in the present study were resemble to the previous reports, showing epidermal hyperplasia, hyperkeratosis, hypergranulosis and spongiosis (Yager and Wilcock 1994; Olivry et al 1997; de Mora et al 2007). The CAD histology was similar to that of human chronic AD (Leung 1999). In addition, similar skin-infiltrating cells contributed of mononuclear cells, neutrophils, mast cells and eosinophils were observed in our study and others (Yager and Wilcock 1994; Olivry et al 1997; Scott et al 2001; de Mora et al 2007).

From the significantly increased expression of Ki-67 in atopic skins, it can be implied that epidermal hyperplasia in lesional CAD skins were also associated with an accumulation of cell number. This hyperproliferation-associated hyperplasia is in agreement with earlier studies in HAD (Kawahira 1999; Sapuntsova et al 2002; Jensen et al 2004; Bovenschen et al 2005). Skin of human AD was shown to be highly proliferated, reflected by *in vitro* incorporation of tritiated thymidine (Van Neste et al 1979). Hyperproliferation was probably to reconstitute normal barrier function and to remove

Table 5 Fold change in gene-specific mRNA expression in CAD lesional, CAD non-lesional and control dog skin

Gene	lesion vs. normal		non-lesion vs. normal		lesion vs. non-lesion	
	Fold change	*P* value*	Fold change	*P* value*	Fold change	*P* value*
K5	*4.947*	0.009	*3.884*	0.011	1.274	0.597
K10	1.271	0.467	0.773	0.520	1.643	0.238
IVL	*4.793*	0.003	1.651	0.289	*2.903*	0.014
FLG	*4.040*	0.001	1.252	0.576	*3.226*	0.009
SPINK5	1.086	0.774	0.986	0.960	1.101	0.705

*Pair wise fixed reallocation randomization test normalizes by reference gene. Results in italics indicate a significant difference ($P < 0.05$).

invaded antigens (Jensen et al 2004). In dog, generally after skin irritation or superficial trauma of epidermis, the mitotic activity of basal epidermal cells are induced to generate new cell population which result in epidermal hyperplasia within 36-48 hr (Yager and Wilcock 1994). The correlations of Ki-67 staining scores with CADESI in the present study suggest an association between hyperproliferation-associated hyperplasia and disease severity.

Hyperproliferation is usually accompanied by disturbed differentiation, probably due to insufficient time for cells to be differentiated, a critical period for the permeability barrier formation (Ekanayake-Mudiyanselage et al 1998; Jensen et al 2004). In the

present study, the reduced and irregular immunostaining of IVL, FLG and K10, marker proteins of keratinocyte differentiation, in lesional and non-lesional CAD samples indicates that hyperkeratosis of CAD epidermis probably resulted from a decelerated desquamation of cornified cells rather than an accelerated cornification (Neufang et al 2001). In addition, since Ki-67 expression showed the hyperproliferation of lesional skin, hence, it could be implied that in CAD, an accumulation of proliferative keratinocytes occurred by obstructing the further postmitotic state which was associated with the reduced expression of the mentioned differentiation-associated proteins.

In our study, K5 immunostaining was found merely in the S. basale in normal controls whereas the staining was extended to suprabasal layers, S. spinosum in lesional or non-lesional skins similar to the HAD study of Jensen et al (2004). Extended K5 immunostaining in suprabasal cells of atopic skins may reflect epitope unmasking in suprabasal cells because K5 protein was reported to be in a masked form in suprabasal normal keratinocytes (Lloyd et al 1995). On the other hand, together with high skin proliferation rate in CAD from Ki-67 immunostaining data and the less K10 staining in the area with high K5 staining in the suprabasal layers, it was suggested that some immature cells could not change to be the mature suprabasal ones. Since the positive correlation between K5 and CADESI was found, K5 protein expression was expected to be increased. However, the aberrantly reduced K5 expression was found. Association of CAD with other proliferation-associated keratins such as K6 and K16, which are highly expressed in the suprabasal levels in lesional AD (Jensen et al 2004), should be further investigated.

In normal control, K10 was expressed in the entire suprabasal compartment similar to the report of Jensen et al (2004). In both lesional and non-lesional CAD skin, K10 was concentrated in S. corneum and the upper part of S. granulosum, higher layers than the granular and upper spinous layers in human AD. In addition, unlike human K10 staining in HAD (Jensen et al 2004), we found that K10 was a sensitive marker for keratinocyte differentiation, as staining in both lesional and non-lesional skin was observed compared with the normal ones. We found the reduction of K10 in lesional skin which is corresponding to K10 expression in HAD (Jensen et al 2004). Reduced K10 was found in dry skin (Engelke et al 1997) and after acute experimental barrier disruption (Ekanayake-Mudiyanselage et al 1998). An impaired permeability barrier was also demonstrated in K10 deficient mice (Jensen et al 2000). Hence, reduced K10 expression may be related to dry skin conditions and to disturbed barrier function.

IVL, regarded as a marker of keratinocyte differentiation, is a major component of the cornified cell envelope (CE) (Steinert and Marekov 1997; Candi et al 2005; Proksch et al 2008). IVL binds with ester linkages to ceramide, one of the component lipids that make up sphingomyelin in lipid bilayer of cell membrane. (Wertz and Downing 1986; Marekov and Steinert 1998). In our study, IVL staining was observed in the entire nucleated epidermal layers to lower horny layer in normal dog skins. Human IVL was described to be normally expressed in upper spinous layers and the S. granulosum. We found that it was difficult to differentiate immunostained cell layers in normal dog skins because dog's skin (10-30 μm) is thinner than that of human (50-100 μm) (Lloyd and Garthwaite 1982; Kanistakis 2002). Hence, dog's skin is more sensitive. However, similar to HAD,

IVL protein expression was significantly decreased in CAD skins ($P< 0.001$) although the immunostaining band of atopic skins was broadened. Reduced IVL staining in HAD due to less S. spinosum formation in AD caused the reduced number of ceramides (Seguchi et al 1996; Jensen et al 2004; Jarzab et al 2010). The band was expanded to the lower spinous layer in non-lesional and lesional skin (Jensen et al 2004). An inverse correlation between IVL staining scores and disease severity scores CADESI-03 strongly supported the role of IVL in CAD. However, the aberrant increased IVL expression in CAD was demonstrated (Chervet et al 2010).

During epidermal differentiation, FLG and IVL play a crucial role in the formation of the CE, an insoluble protein structure underneath the plasma membrane (Sandilands et al 2007; Sandilands et al 2009) and establishment of epidermal barrier function. In dogs, several studies of FLG immunostaining were demonstrated. Bardagi et al (2007) showed the expression of filaggrin in cytoplasmic and keratohyalin granules in the epidermal granular layer in normal dog skin. In our study, FLG staining in normal skins was found in all epidermal layers, owing to very thin layers of dog skin as above-mentioned. We also showed that the FLG staining in atopic dogs was significantly reduced compared to the controls. This result is corresponding to reduced FLG expression in 39% (7 of 18) of dogs with AD compared with that of controls when using the N- and C-terminal FLG antibodies (Chervet et al 2010). Immunostaining observed in most epidermal layers probably indicated the cross-reactivity with other epidermal proteins as suggested by Marsella et al (2009). However, the significant reduction of the FLG protein expression in both lesional and non-lesional skins of CAD supported its role as a CE protein in skin

maintenance. In addition, decreased FLG immunostaining was remarkably found in both lesional and non-lesional skins of HAD (Seguchi et al 1996; Jensen et al 2004).

Increased IVL and FLG expression together with the decreased keratinocyte proliferation were used as protein makers, showing recovery of treated keratinocyte as well as improvement of skin barrier permeability e.g. after AD therapy with suberythemal UV B (Hong et al 2008a), after treatment of primary keratinocyte with rose absolute oil (Kim et al 2010), after treatment of mite antigen-stimulated human skin keratinocyte, HaCat cells, with a phytosphingosine-like substance from starfish (Choi et al 2010), after treatment of HaCat cells with K6PC-5, a direct activator of sphingosine kinase 1 (Hong et al 2008b), and after treatment of primary keratinocyte with silica and mud from Blue Lagoon (Grether-Beck et al 2008).

A serine protease inhibitor LEKTI protein expression was found in the present study to be significantly increased in CAD lesional samples. This is consistent with the lower expression of FLG since LEKTI protein has been demonstrated to inhibit proteases matriptase which has an important function in proteolytically cleavage of profilaggrin, leading to the loss of proteolytically processed filaggrin (List et al 2003; O'Regan et al 2009). LEKTI staining scores was also significantly correlated with the clinical severity scores. This is corresponding to the trend of inversed correlation of FLG staining scores and the clinical severity scores although not reaching statistical significance (data not shown).

To understand biological events or pathogenesis of CAD at the mRNA level, diagnostic techniques such as quantitative RT-PCR were performed. The up-regulation of

IVL, FLG and *K5* mRNA expression in lesional skin compared to the control was observed. This was not associated with a decrease in their protein expression. A significant upregulation of *IVL* RNA has been described in lesional HAD skin (Jarzab et al 2010), on a contrary, a reduced IVL protein expression was demonstrated (Seguchi et al 1996; Jensen et al 2004). For FLG, a non-associated mRNA-protein expression of FLG was shown. Whereas *FLG* mRNA did not significantly change, the protein was decreased in HAD compared to the normal ones (Jarzab et al 2010).

In accordance with molecular central dogma, mRNAs is referred to as the transcriptome, then serves as the template for protein synthesis (Crick 1970); however, it is yet unclear how strongly specific mRNA levels correlate with the abundance of corresponding protein since several researches demonstrated different results between mRNA and protein expressions (Chen et al 2002; Strömberg et al 2007; Fournier et al 2010). The absent correlation between mRNA and protein levels may be due to i.) variable and complicated post-transcriptional steps, which are not well defined, including protein translation, post-translational modification, the number of isoforms for each gene (Szallasi 1999; Baldi and Long 2001; Chen et al 2002; Strömberg et al 2007), ii.) different protein turnover rate or different *in vivo* half lives due to varied protein synthesis and degradation (Glickman and Ciechanover 2002; Pratt et al 2002) and iii.) the noise and error in mRNA and protein expression experiments including the specificity of the antibody used (Qian et al 2003; Strömberg et al 2007). Moreover, the mutation of the gene may lead to reduced protein expression without any effect on the transcription level (e.g. FLG gene). Lack of C-terminal FLG was observed in some AD dogs suggesting the