

# DESMOGLEIN 1 IN OVINE MUZZLE SKIN DETECTED BY IMMUNOHISTOCHEMISTRY AND IMMUNOBLOTTING

## DESMOGLEINA 1 NEL MUSELLO DI PECORA: INDAGINE DI IMMUNOISTOCHEMICA E DI IMMUNOBLOTTING

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### SUMMARY

Desmosomes are epithelial adhesion structures that assure the mechanical cell-cell adhesion, by linking the intermediate filaments of two adjacent cells. Several proteins have been characterised as component of these structures and, among them, desmogleins are known to be the autoantigens in some autoimmune diseases that go by the name of pemphigus complex diseases. In particular Desmoglein 1 is the targeted antigen in Pemphigus foliaceus. This study aims at revealing the presence of Desmoglein 1 in the ovine muzzle skin using a commercial monoclonal antibody, raised against human desmoglein-1, on samples obtained from sheep muzzle. Immunohistochemical analyses (streptavidin-peroxidase method) and immunoblotting analyses (chemiluminescence method) have been performed. Immunohistochemical evaluation allowed for detection of a pericellular staining of keratinocyte cell membranes in the spinous and the basal layers of epidermis. A positive immunoreaction was obtained when the anti-Dsg-1 antibody was reacted with human cheek epidermis used as positive control. Specificity of the immune reaction was confirmed by the lack of staining in samples incubated with the anti-vimentin antibody used as negative control. Densitometric analyses of the chemiluminescence film showed three bands. One of them could be referred to Desmoglein 1, the second one to Desmoglein 3 and the last one to a Desmoglein-Plakoglobin complex.

Key words: muzzle; sheep; desmoglein; immunohistochemistry; immunoblotting.

### RIASSUNTO

I desmosomi sono strutture di adesione tipiche dei tessuti epiteliali che connettono i filamenti intermedi del citoscheletro di due cellule adiacenti, assicurando la loro adesione meccanica. Tra le proteine che compongono queste strutture, le desmogleine sono note come antigene target in alcune malattie autoimmuni riunite sotto il nome di malattie del complesso del pemfigo. La desmogleina 1, in particolare, si comporta da auto antigene nel pemfigo foliaceo. Lo scopo della presente ricerca è stato quello di mettere in evidenza questa proteina su campioni di cute prelevate da musello di pecora con l'utilizzo di un anticorpo monoclonale indotto contro la desmogleina 1 dell'uomo. Su di essi sono state condotte indagini immunohistochemiche (metodo streptavidina-perossidasi) e di immunoblotting (metodo della chemiluminescenza). L'indagine immunohistochemica ha rilevato una distribuzione della proteina uni-

forme e pericellulare negli strati basale e spinoso dell'epidermide. La reazione positiva si è ottenuta anche su cute di guancia di uomo usata come controllo positivo. La specificità del test è stata confermata dall'assenza di positività in campioni incubati con un anticorpo anti-umentina, utilizzato come controllo negativo. L'indagine di immunoblotting ha evidenziato tre bande di cui una potrebbe corrispondere alla desmogleina 1, la seconda alla desmogleina 3 e la terza ad un complesso Desmogleina-Placoglobina.

Parole chiave: musello; pecora; desmogleina; immunistochemica; immunoblotting.

## INTRODUCTION

Desmosomes are adhesion structures mainly found in epithelial tissues as well as in myocardium, meninges and Purkinje cells (Suter et al., 1997). They assure a mechanical adhesion by linking intermediate filaments of adjacent cells (Scott et al., 2001). Several desmosomal plaque proteins (desmoplakin I and II, plakoglobin, and plakophilin) and desmosomal core glycoproteins (desmoglein [Dsg] and desmocollin) have been characterised as structural components of desmosomes in humans and other animals (Suter et al., 1997). The desmosomal core glycoproteins belong to the cadherin's family and, via an homophilic link, they work as a cell-cell bridge in the intercellular space. Among the desmosomal cadherins, Dsgs are known to play a role in some autoimmune blistering diseases known as pemphigus complex, whose prototypal are Pemphigus Foliaceus (PF) and Pemphigus Vulgaris (PV) (Payne et al., 2004; Yancey, 2005). PF has been documented in the human (Bystryn & Rudolph, 2005), canine (Shinya et al., 1996), feline (Preziosi et al., 2003), equine (Zabel et al., 1995) and caprine (Pappalardo et al., 2003) species and Dsg-1 has been characterised as the targeted antigen in humans and dogs (Mahoney et al., 1999; Suter et al., 1993; Iwasaki et al., 1997; Steeves et al. 2002;) although, recently, it has been shown that Dsg-1 might be only a minor antigen in the canine species (Olivry et al., 2005).

Four isoforms of Dsgs have been described in the human (Whitlock, 2003), one in the bovine (Koch et al., 1990; Puttagunta et al., 1994), two in the canine (Muller et al., 2000; Aoki et al., 2002), six in the murine (Whitlock, 2003) and, recently, one in the swine species (Nishifuji et al., 2005). Cellular distribution of Dsg-1 in epidermis has already been documented in the human (Mahoney et al., 1999), canine, feline (Miragliotta et al., 2005) and equine species (unpublished data).

This study aims at revealing the presence of Dsg-1 in the ovine muzzle skin and assessing the suitability of a commercial monoclonal antibody raised against human desmoglein, in evaluating the desmosomal pattern distribution in this species by immunohistochemistry and immunoblotting.

## MATERIALS AND METHODS

### *Samples*

Skin biopsy samples were collected at the slaughterhouse either with an 8 mm punch and with a scalpel blade from 4 sheep muzzles that did not show any dermatologic lesions. Samples were promptly frozen in liquid nitrogen. Punch samples were cryo-embedded (Microm Int., Walldorf, Germany) and cryostat sections of about 6  $\mu\text{m}$  were obtained and either stained with hematoxylin-eosin for histological evaluation or used for immunohistochemical analyses. Scalpel blade samples were used for immunoblotting analyses. Skin from human cheek was used as control.

### *Immunohistochemistry*

A mouse anti-Dsg-1 monoclonal IgG (Progen, Heidelberg, Germany) and a mouse anti-vimentin monoclonal IgG (Progen, Heidelberg, Germany) were used in the experiments. Immunohistochemistry was performed with the biotin-streptavidin peroxidase method as previously reported (Miragliotta et al., 2005).

### *Protein extraction*

Epidermis was isolated from the dermis using a scalpel blade. The obtained fragments were powdered in liquid nitrogen and powder aliquots stored at  $-80\text{ }^{\circ}\text{C}$ . Aliquots were suspended in 10 ml/g of powder of 20 mM Tris-HCl, pH 8, for 30 minutes; a protease inhibitor cocktail was added (1,5 ml/g of powder) (Protease Inhibitor Cocktail, Sigma-Aldrich, St. Louis, MO, USA). A pellet was obtained, by centrifugating the crude extract at 14000 g for 20 minutes, and resuspended for 1 hour mixing in 20mM Tris-HCl pH 8, 2% SDS, 2M Urea; the same protease inhibitor cocktail was added (1,5 ml/g of powder). The supernatant obtained after a 14000 g centrifugation for 20 minutes was used for electrophoresis and immunoblotting analyses.

### *Western Blot*

The total protein concentration was estimated by Lowry's method (Lowry et al., 1951). Thirty  $\mu\text{g}$  of proteins were separated on 6% T sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and electrophoretically transferred on a PVDF membrane (Hybond-P PVDF, Amersham Bioscience, Italy), previously activated in methanol, using the Multiphor II (Amersham Biosciences Corp, San Francisco, USA) 1 hour at  $0,8\text{ mA}/\text{cm}^2$ , according to the method described by Towbin and colleagues (Towbin et al., 1979). SigmaMarker™ High Molecular Weight Range (Sigma-Aldrich Corporation, St. Louis, Missouri, USA) have been used as molecular weight marker.

Strips of membrane were blocked with 3% skim milk in Tween 20-Phosphate Buffered Saline (PBS-Tween 20-M) for 1 h at room temperature and incubated with an anti-Dsg-1 monoclonal antibody (Progen Biotechnik GmbH, Heidelberg, UK) diluted 1:100 with PBS-Tween 20-M at  $4\text{ }^{\circ}\text{C}$  overnight. Strips were then incubated

with a goat anti-mouse IgG-HRP (Santa Cruz Biotechnology) diluted 1:2000 with PBS-Tween 20-M. Between each incubation, strips were washed with PBS-Tween 20-M four times for 10 minutes. Signal were then detected by chemiluminescence using the Luminol reagent (ECL Western Blotting Detection Amersham Bioscience, UK).

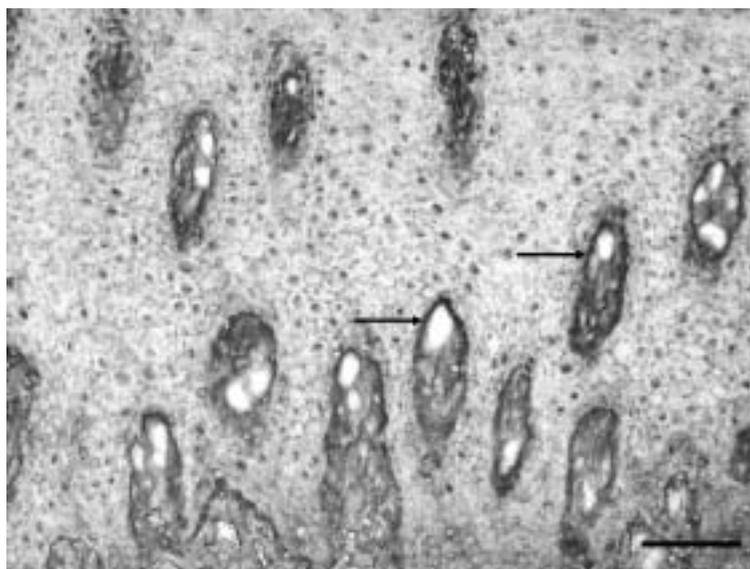
#### *Densitometric analyses*

Gels and membranes have been analysed using Quantity One (Bio-Rad Hercules, CA, USA).

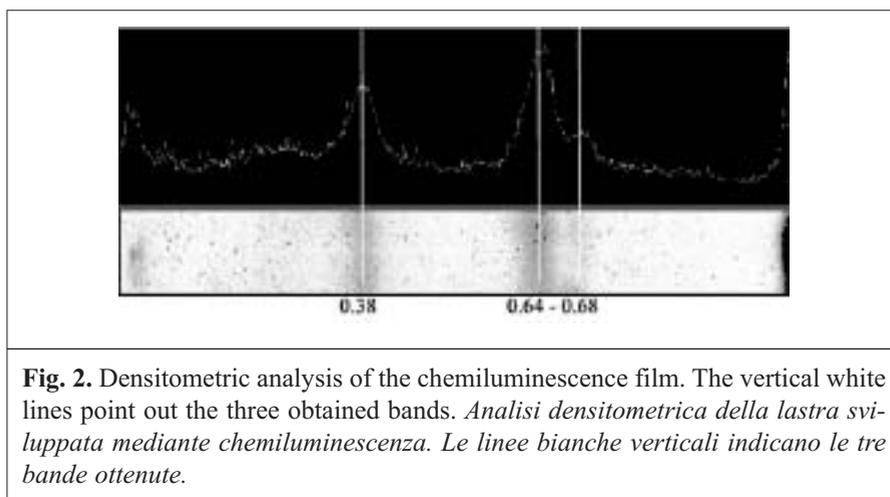
## RESULTS

#### *Immunohistochemistry*

In the ovine muzzle epidermis the use of anti-Dsg-1 antibody allowed for detection of a pericellular staining of keratinocyte cell membranes in the spinous and the basal layers (Fig. 1). A positive immunoreaction was obtained when the anti-Dsg-1



**Fig. 1.** Photomicrograph of normal sheep muzzle epidermis. Positive reaction appears as pericellular dark tiny spots in both basal (arrows) and spinous layers. Streptavidin-biotin peroxidase method. Bar = 70  $\mu$ m. *Epidermide normale del musello di pecora. La positività della reazione risulta sottoforma di punti scuri pericellulari sia nello strato basale (frece) che nello strato spinoso. Metodo streptavidina-perossidasi. Barra = 70  $\mu$ m.*



**Fig. 2.** Densitometric analysis of the chemiluminescence film. The vertical white lines point out the three obtained bands. *Analisi densitometrica della lastra sviluppata mediante chemiluminescenza. Le linee bianche verticali indicano le tre bande ottenute.*

antibody was reacted with human cheek epidermis used as positive control. Specificity of the immune reaction was confirmed by the lack of staining in samples incubated with the anti-vimentin antibody used as negative control.

#### *Western blot*

The chemiluminescence film showed three bands. Densitometric analyses compared to the molecular weight markers revealed a 130 kDa (0.68 Rm), a 160 kDa (0.64 Rm) and a 250 kDa (0.38 Rm) bands (Fig. 2).

## DISCUSSION

The immunohistochemical investigation documents, for the first time, the presence of Dsg-1 in the ovine muzzle epidermis and indicates the suitability of the used antibody to define the distribution pattern of desmosomes on sheep cryo-sectioned skin samples. As reported in humans and dogs, the spinous layer of epidermis showed a positive immune reaction also in sheep.

Moreover, the anti-Dsg-1 antibody strongly outlined also the keratinocyte cell membranes of the basal layer although it is known from the literature this latter normally expresses only Dsg-3 (Mahoney et al., 1999).

Despite the specificity of the monoclonal antibody in humans, the immunoblotting results pointed out three bands in the blotted sheep muzzle proteins.

The 0.64 Rm band which has an estimated molecular weight of 160 kDa could be imputable to Dsg-1, in agreement to that reported by Aoki et al. (2002).

The 0.68 Rm band was instead detectable at the molecular weight of 130 kDa. This finding might be addressed to a cross-reactivity of the antibody with Dsg-3 since this latter is known to have the same molecular weight (Aoki et al., 2002).

Therefore, on the basis of the present data, it is not possible to establish whether the presence of the 130 kDa band is due to a cross-reactivity of the used antibody against Dsg-3 or another protein.

Moreover, the 0.38Rm band, located in a 250 kDa area could be referred to Desmoglein-Plakoglobin complex as reported by Calvanico et al. (1991).

Experiments are in progress to define the isoform distribution of Dsgs in sheep epidermis and to verify if the unknown cross-reactive protein could be Dsg-3.

#### REFERENCES

- AOKI M., NISHIFUJI K., AMAGAI M., NISHIKAWA T., PARK S., IWASAKI T. (2002). Distribution and expression of desmosomal proteins, desmoglein-1 and -3, in normal canine skin and mucous membrane. In: *Advances in veterinary dermatology*. Vol 4. Ed: K.L. Thoday, C.S. Foil, R. Bond, Blackwell Publishing, Oxford. pp. 30-36.
- BYSTRYN J.C., RUDOLPH J.L. (2005). Pemphigus. *Lancet*, 366: 61-73.
- CALVANICO N.J., MARTINS C.R., DIAZ L.A. (1991). Characterization of pemphigus foliaceus antigen from human epidermis. *J. Invest. Dermatol.*, 96: 815-821.
- IWASAKI T., SHIMIZU M., OBATA H., ISAJI M., YANAI T., KITAGAWA H., SASAKI Y. (1997). Detection of canine pemphigus foliaceus autoantigen by immunoblotting. *Vet. Immunol. Immunopathol.*, 59: 1-10.
- KOCH P.J., WALSH M.J., SCHMELZ M., GOLDSCHMIDT M.D., ZIMBELMANN R., FRANKE W.W. (1990). Identification of desmoglein, a constitutive desmosomal glycoprotein, as a member of the cadherin family of cell adhesion molecules. *Eur. J. Cell Biol.*, 53: 1-12.
- LAEMMLI U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227: 680-685.
- LOWRY O.H., ROSEBROUGH N.J., FARR A.L., RANDALL R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, 193: 265-275.
- MAHONEY M.G., WANG Z., ROTHENBERGER K., KOCH P.J., AMAGAI M., STANLEY J.R. (1999). Explanations for the clinical and microscopic localization of lesions in pemphigus foliaceus and vulgaris. *J. Clin. Invest.*, 103: 461-468.
- MIRAGLIOTTA V., COLI A., RICCIARDI M.P., PODESTÀ A., ABRAMO F. (2005). Immunohistochemical analysis of the distribution of desmoglein 1 and 2 in the skin of dogs and cats. *Am. J. Vet. Res.*, 66: 1931-1935.
- MULLER E., CALDELARI R., LEVINE R., KAPLAN S., BARON A., ROHRBACH B., WYDER M., BALMER V., SUTER M.M. (2000). Cloning of canine Dsg1 and evidence for alternative polyadenylation. *J. Invest. Dermatol.*, 114: 1211-1213.
- NISHIFUJI K., FUDABA Y., YAMAGUCHI T., IWASAKI T., SUGAI M., AMAGAI M. (2005). Cloning of swine desmoglein 1 and its direct proteolysis by *Staphylococcus hyicus* exfoliative toxins isolated from pigs with exudative epidermitis. *Vet. Dermatol.*, 16: 315-323.
- OLIVRY T., LAVOY A., DUNSTON S.M., BROWN R.S., LENNON E.M., WARREN S.J., PRISAYANH P., MULLER E.J., SUTER M.M., DEAN G.A. (2005). Desmoglein-1 is a minor autoantigen in dogs with pemphigus foliaceus. *Vet. Immunol. Immunopathol.*, in press.
- PAPPALARDO E., ABRAMO F., NOLI C. (2002). Pemphigus foliaceus in a goat. *Vet. Dermatol.*, 13: 331-336.
- PAYNE A.S., HANAKAWA Y., AMAGAI M., STANLEY J.R. (2004). Desmosomes and disease: pemphigus and bullous impetigo. *Curr. Opin. Cell. Biol.*, 16: 536-543.

- PREZIOSI D.E., GOLDSCHMIDT M.H., GREEK J.S., JEFFERS J.G., SHANLEY K.S., DROBATZ K., MAULDIN E.A. (2003). Feline pemphigus foliaceus: a retrospective analysis of 57 cases. *Vet. Dermatol.*, 14: 313-321.
- PUTTAGUNTA S., MATHUR M., COWIN P. (1994). Structure of DSG1, the bovine desmosomal cadherin gene encoding the pemphigus foliaceus antigen. Evidence of polymorphism. *J. Biol. Chem.*, 269: 1949-1955.
- SCOTT D.W., MILLER W.H., GRIFFIN C.E. (2001). Structure and function of the skin. *Muller & Kirk's Small Animal Dermatology*, Ed. WB Saunders Co, 2001; pp. 1-70.
- SHINYA K., NOMURA K., WADA S., MORIOKA H., UMEMURA T. (1996). Pemphigus foliaceus with typical histological and immunohistological findings in a dog. *J. Vet. Med. Sci.*, 58: 815-817.
- STEEVES E.B., CHELACK B.J., CLARK E.G., HAINES D.M. (2002). Altered immunohistochemical staining for desmoglein in skin biopsies in canine pemphigus foliaceus. *J. Vet. Diagn. Invest.*, 14: 53-56.
- SUTER M.M., CRAMERI F.M., OLIVRY T., MUELLER E., VON TSCHARNER C., JENSEN P.J. (1997). Keratinocyte biology and pathology. *Vet. Dermatol.*, 8: 67-100.
- SUTER M.M., ZIEGRA C.J., CAYATTE S.M., SMITH C.A., CRAMERI F.M. (1993). Identification of canine pemphigus antigens. In: *Advances in veterinary dermatology* Vol 1 Ed: Ihrke P.J., Mason I.S., White S.D. Pergamon Press, Oxford, UK, pp. 367-380.
- TOWBIN H., STAHELIN T., GORDON J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci., USA.* 76: 4350-4354.
- WHITTOCK N.V. (2003). Genomic sequence analysis of the mouse desmoglein cluster reveals evidence for six distinct genes: characterization of mouse DSG4, DSG5, and DSG6. *J. Invest. Dermatol.*, 120: 970-980.
- YANCEY K.B. (2005). The pathophysiology of autoimmune blistering diseases. *J. Clin. Invest.*, 115: 825-828.
- ZABEL S., MUELLER R.S., FIESELER K.V., BETTENAY S.V., LITTLEWOOD J.D., WAGNER R. (2005). Review of 15 cases of pemphigus foliaceus in horses and a survey of the literature. *Vet. Rec.*, 157: 505-509.

