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Establishment of a 2-week canine skin organ culture model and its pharmacological modulation by epidermal growth factor and dexamethasone

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ABSTRACT

Although canine skin models are already available as either monocellular or organotypic cultures, they only partly recapitulate normal skin morphological features and function. The objective of this study was to establish a canine serum-free skin organ culture model and verify whether dexamethasone could rescue epidermal growth factor-induced changes. The study of morphological changes as a response to pharmacological substances may indeed help to investigate skin physiology and pathology.

Normal skin was obtained from five client-owned dogs subjected to surgical procedures unrelated to dermatological conditions. Two experimental designs were performed: (i) two-week viability of the skin culture; (ii) dexamethasone (DMS) inhibition of epidermal growth factor (EGF)-induced effects. Serum-free submerged organ cultures were established in Williams' E medium supplemented with penicillin-streptomycin, insulin, hydrocortisone and L-glutamine.

General morphological features of skin anatomical structures were well maintained up to day 14, scattered pyknotic nuclei were visible in the epidermis from day 7. Normal keratinocyte differentiation was confirmed by cytokeratin (K) 10, K14 and loricrin immunostaining. Epidermal thickness did not decrease throughout the study. A decrease in keratinocyte proliferation was observed at day 7 and 14. Treatment with EGF induced both keratinocyte proliferation and thickening of the epidermis; both responses were counteracted by DMS. Treatment with EGF increased the length of epithelial tongues at the edge of the skin explants; this effect was further enhanced by DMS supplementation.

Our findings demonstrate the potential use of a full-thickness canine skin organ culture model for the study of skin physiology and pharmacological response to exogenous compounds, especially in the field of re-epithelialisation and keratinization disorders.

1. Introduction

Skin organ culture models in veterinary medicine started with the work of Kondo and colleagues who reported on the maintenance of normal rabbit ear skin explants for up to 12 weeks and its pharmacological modulation (Kondo et al., 1990). More recently, a skin organ culture model from Gottingen minipigs has also been established (Dame et al., 2008). Although canine keratinocyte cultures have been developed (Kimura et al., 2012; Shibata et al., 2010) and organotypic models are available in dogs (Cerrato et al., 2012; Kobayashi et al., 2013; Serra et al., 2007; Yagihara et al., 2011), skin organ culture has not yet been attempted in this species.

Cell cultures are largely used in toxicological studies and cancer research, but still they are insufficient to investigate skin morpho-physiology, epidermal barrier impairment or epithelial-mesenchymal interaction, due to their monocellular composition. Organotypic models overcome this limitation. Unlike the traditional "on-a-plastic" systems, these models reproduce the three-dimensional stratified space within which skin cells normally live and function, nonetheless they only partly recapitulate normal microanatomy and physiology of the skin (Oh et al., 2013).

Skin organ culture methods may thus offer unique opportunities, not only to overcome the concerns surrounding the use of

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animals for biomedical research, but also to study drugs in a biologically relevant environment with the same three-dimensional cell–cell and cell–matrix contacts and communications as are present in intact tissue.

Although in an organ culture model serum was reported to enhance general viability of the epidermis and increase the formation of new stratum corneum (Tammi and Jansen, 1980), its presence was also considered a limitation. This is due to a variable and hardly definable medium composition and the possible interaction of serum molecules deriving both from a different species (i.e. canine skin cultured with bovine serum) and different developmental stage (fetal serum used on adult skin) (Lu et al., 2007).

With this in mind, we developed a serum-free canine model of skin organ culture and investigated the pharmacological modulation thereof. Epidermal growth factor (EGF) and dexamethasone (DMS) were used, this latter agent known to inhibit the effect of the first (Kondo et al., 1990). Since the discovery of EGF (Cohen, 1962) its mitogenic activity has been described in several systems (Zeng and Harris, 2014). Now it is classified as the prototype of the group I EGF family that also includes other growth factors that share the ability to bind the EGF receptor, activate its intrinsic tyrosine kinase activity, and couple the receptor to downstream signaling pathways that are involved in cell proliferation, differentiation, survival, or motility (Carpenter, 1987; Zeineldin and Hudson, 2006; Zeng and Harris, 2014). Keratinocyte proliferation as well as terminal differentiation has been shown to be mediated by the EGF receptor, since its inhibition resulted in growth arrest and terminal differentiation (Peus et al., 1997).

In the study by Kondo and colleagues, EGF was used to stimulate keratinocyte proliferation in a rabbit model of skin organ culture, and DMS was found to inhibit this effect (Kondo et al., 1990). The intracellular mechanisms that regulate such interaction were unknown at that time, while today, they have been more thoroughly described and seem to depend primarily on the extracellular signal-regulated kinase (ERK) pathway (Luo et al., 2007) and glucocorticoid receptors (Kimura et al., 2011).

Here we present a 14-day setup of a serum-free model of canine skin organ culture and its pharmacological modulation with EGF and DMS.

2. Materials and methods

2.1. Animals and sample collection

Normal skin was obtained from five client-owned dogs subjected to surgical procedures unrelated to dermatological conditions (removal of mammary neoplastic lesions). A written informed consent was obtained from each dog's owner prior to surgery. Before anesthesia with propofol 4 mg/kg IV and isoflurane 2% in oxygen, hair was clipped and the surgical site aseptically prepared. Skin to be cultured was collected at the periphery of the surgical site in order to avoid atrophic changes due to the presence of either the tumor or lymph node enlargement.

2.2. Set-up of the dog skin organ culture for 14 days

During the surgical procedure, skin strips were collected and promptly immersed in the isolation medium (99% Williams' E Medium-L-glutamine free supplemented with 1% antibiotic antimycotic solution containing 10,000 IU penicillin, 10 mg streptomycin and 25 μg amphotericin B per mL). The sample was kept at 4 °C until the establishment of the organ culture (within 2 h of sampling).

To perform skin organ cultures, full-thickness 4 mm skin biopsy punches were collected from the isolated skin after mechanical removal of excess of subcutaneous fat by using a surgical scalpel. Three skin biopsies from each dog were immersed in 10% buffered formalin solution (pH 7.4) and regarded as Day 0 samples. Cultures were established from each dog in triplicate (three biopsies per time-point); skin biopsies were placed into 6-well plate Petri dish containing Williams' E medium supplemented with 1% 10000 IU/ml penicillin-10 mg/ml streptomycin, 0.1% 10 μg/ml insulin, 0.02% 10 ng/ml hydrocortisone and 1% 200 mM L-glutamine. All reagents were commercially obtained (Williams' E Medium Cat. N° W4128. Antibiotic antimycotic solution Cat. N° A5955. Penicillin-streptomycin solution cat N° P4333; Insulin cat N° I9278; Hydrocortisone cat N°H0135; L-glutamine cat N° G7513; Sigma Aldrich S.R.L. Milan, IT).

Medium was replaced after 24 h and then at 2-day intervals. Skin samples were kept at 37 °C in a humidified incubator with 5% CO_2 for 14 days. Three cultured biopsies per each selected time-point (day 1, 4, 7, 14) were collected and fixed in 10% buffered formalin solution (pH 7.4) and routinely processed for paraffin embedding, thus allowing a time-course for the evolution of epidermal, dermal and adnexal conditions during culture. The following histological parameters were then assessed: morphological features, epidermal thickness, keratinocyte proliferation and epidermal differentiation.

2.3. Epidermal growth factor and dexamethasone effect on canine cultured skin

The effect of EGF supplementation was evaluated on skin obtained from three different dogs. DMS was used to inhibit EGF effects. Biopsy triplicates were treated for three days – from day 2 to day 5 – (Kondo et al., 1990) with either vehicle (culture medium), or 10 or 20 ng/mL EGF (epidermal growth factor human; cat N° E9644 Sigma Aldrich S.R.L. Milan, IT). A second set of triplicates was treated with either DMS 10 μg/mL alone, or EGF 10 ng/mL-DMS 10 μg/mL or EGF 20 ng/mL-DMS 10 μg/mL. The following parameters were assessed according to this experimental set up: morphological features, epidermal thickness, keratinocyte proliferation, length of epithelial tongues forming at the periphery of the cultured biopsy and epidermal differentiation.

2.4. Morphological features

Morphological features were evaluated on H&E stained 5 μm sections. The presence of culture-induced changes was qualitatively evaluated on epidermis, dermis and adnexal structures.

2.5. Epidermal thickness

Epidermal thickness was measured on 20, 400× captured fields per time point, by manually tracing 16 segments at regular intervals per captured field with NIS-Elements Br Microscope Imaging Software (NIS-Elements Br Microscope Imaging Software. Nikon Instruments, Calenzano, Italy). Segments were perpendicular to the basement membrane and extended from the germinative layer to the beginning of the stratum corneum.

2.6. Keratinocyte proliferation

Ki67 immunostaining was performed to evaluate keratinocyte proliferation. Briefly, superfrost-Plus mounted sections were deparaffinized and rehydrated. Epitope retrieval was carried out at 120 °C in a pressure cooker for 3 min with a Tris/EDTA buffer pH 9.0. Quenching of endogenous peroxidases was carried out by incubation with 1% H_2O_2 in phosphate buffer 0.1 M pH 7.4 (PBS), 5 min, at room temperature (RT). Non-specific binding was prevented by incubation of slides with 5% normal goat serum (Normal

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