THE SYSTEM COX-2/PGE2 IN THE KERATINOCYTE INFLAMMATORY RESPONSE AND IN CUTANEOUS NEOPLASIA IN THE DOG

Mar Bardagí i Admetlla

Universitat Autònoma de Barcelona
2012
THE SYSTEM COX-2/PGE2 IN THE KERATINOCYTE INFLAMMATORY RESPONSE AND IN CUTANEOUS NEOPLASIA IN THE DOG

Doctoral Thesis

Mar Bardagí i Admetlla
Facultat de Veterinària
Maig 2012

Universitat Autònoma de Barcelona
El Dr. Lluís Ferrer i Caubet, Catedràric d’Universitat del Departament de Medicina i Cirurgia Animals de la Universitat Autònoma de Barcelona, i la Dra. Dolors Fondevila i Palau, Catedràtica d’Universitat del Departament de Medicina i Cirurgia Animals de la Universitat Autònoma de Barcelona,

FAN CONSTAR:

Que la memòria titulada “The System COX-2/PGE in the keratinocyte inflammatory response and in cutaneous neoplasia in the dog” presentada per la llicenciada Mar Bardagí i Admetlla per optar al títol de Doctora per la Universitat Autònoma de Barcelona, s’ha realitzat sota la nostra direcció, i, en considerar-la conclosa, autoritzem la seva presentació autoritzem la seva presentació per ser jutjada pel Tribunal corresponent.

I per tal que consti als efectes que s’escaigui, signem la present a Bellaterra, el 22 de Març de 2012.

Dr. Lluís Ferrer i Caubet

Dra. Dolors Fondevila i Palau
## CONTENTS

Abbreviations 9

1. **INTRODUCTION** 11
   1.1. General information 13
   1.2. COX-mediated signalling 13
   1.3. COXs in the skin 16
   1.4. Physiologic implications of COX expression in the skin 18
   1.5. COX in acute skin inflammation 19
   1.6. COX in wound healing 22
   1.7. COX in cancer 22

2. **OBJECTIVES** 39

3. **EXPERIMENTAL SECTION** 43
   3.1. Immunohistochemical demonstration of COX-2 expression
       In cutaneous neoplasia and inflammatory dermatoses 45
       3.1.1. Introduction 45
       3.1.2. Materials and Methods 46
       3.1.3. Results 49
   3.2. Study of the COX-2/PGE2 mechanism in the keratinocyte
       inflammatory response 60
       3.2.1. Establishment of a model to investigate the biology
              of the canine keratinocyte as inflammatory cell 60
              3.2.1.1. Introduction 60
              3.2.1.2. Materials and Methods 62
              a. Culture and maintenance of the CPEK cell line 62
              b. Isolation and culture of canine primary keratinocytes 63
              c. Establishment of a method to measure PGE2 in
                 keratinocyte cultures supernatants 65
       3.2.1.3. Results 67
   3.2.2. PGE2 production by stimulated keratinocytes 71
       3.2.2.1. Introduction 71
       3.2.2.2. Materials and Methods 71
       3.2.2.3. Results 75
ABBREVIATIONS

AA: arachidonic acid
Ab: antibody
AK: actinic keratosis
CD-3: cluster of differentiation 3
CETL: cutaneous epitheliotropic T cell lymphoma
CnT-09: keratinocyte culture medium of CELLnTEC Laboratories
COX: cyclooxygenase
COX-1: cyclooxygenase 1
COX-2: cyclooxygenase 2
CPEK: name of the spontaneously immortalized canine keratinocyte cell line developed by CELLnTEC Laboratories (Advanced Cell Systems, Bern, Switzerland)
EDTA: ethylenediaminetetraacetic acid
EGF: epidermal growth factor
ELISA: enzyme linked immunosorbent assay
EP 1 to 4: PGE2 cell surface receptors 1 to 4
IHC: immunohistochemistry
K-1: keratin-1
IL: interleukin
LPS: lipopolysaccharide
NSAID: non-steroidal anti-inflammatory drug
PBS -/+: phosphate buffered saline free of calcium and magnesium
PG: prostaglandins
PGD2: prostaglandin D2
PGE2: prostaglandin E2
PGF2α: prostaglandin F2α
PGI2: prostaglandin I2
PLA2: phospholipases A2
PMN: polymorphonuclear
PMNN: polymorphonuclear neutrophils
PR: pagetoid reticulosis
SCC: squamous cell carcinoma
TNF: tumour necrosis factor
TPA: tetrade canoylphorbol-acetate
UV: ultraviolet
UVB: ultraviolet B
WBC: white blood cells
1. INTRODUCTION
Introduction

1.1. General information

The skin performs critical homeostatic functions including thermoregulation, sensory perception, protection against microbial invasion, and protection against absorption of potentially toxic environmental agents. To perform these functions, the skin must maintain a structural and functional integrity that derives from an array of complex cellular and biochemical interactions (Chu et al. 2003).

The structural homeostasis of the skin is partly the result of regulated cell proliferation and differentiation within the topmost segment of the skin called the epidermis (Fig. 1.1). The epidermis is a cornifying stratified epithelium with the keratinocyte as the major cell type. It has a basal layer of cells (basal keratinocytes) that have the capacity to proliferate. These divide periodically and the daughter cells move upwards to the skin surface. Once suprabasal, cells lose their ability to proliferate. To form the barrier, they pass through different stages of terminal differentiation and finally die and scale off. In the course of terminal differentiation, keratins and other epidermis-specific proteins (i.e., involucrin, loricrin) are cross-linked to form the unique protein envelope of cornified cells. In addition, a complex lipid envelope is deposited between the cornified cells to control trans-epidermal water loss and permeability for exogenous material. Thus, the cornified cell envelope represents a structural and functional interface between the body and the external environment (Candi et al. 2005, Koster and Roop 2007, Feingold 2007)

Alterations in the proliferating and differentiating “compartments” of the epidermis, whether from endogenous and/or exogenous triggers, are associated with a number of skin diseases including skin cancer. It is therefore important to identify and understand the molecular mechanisms that influence and control cell proliferation and differentiation within the epidermis.

1.2. COX-mediated signaling

The prostaglandins (PG), comprising one of the major classes of eicosanoids, are synthesized primarily from arachidonic acid (AA), an essential fatty acid, via activity
that initially was thought to reflect a single enzyme called cyclooxygenase (COX) (Fig.1.2). As a pre-requisite for PG biosynthesis, AA has to be released, a step that is mostly catalyzed by the actions of phospholipases A₂ (PLA₂) (Bonventre et al. 1997, Hirabashi et al. 2004). Since prostaglandin E2 (PGE2) was identified in rat and human skin, (Ziboh and Shia 1971, Jonsson and Anggard 1972), numerous studies have attempted to identify its role in both normal and pathologic skin responses. One of the obstacles to achieving this understanding has been the fact that almost all cell types synthesize some profile of PG and the skin is no exception; consequently identifying the cellular source(s) and the cellular target(s) of PG generation in the context of specific skin responses is paramount. While this process of identification is far from complete, developments in the enzymology of PG generation as well as in the elucidation of PG receptors provide new avenues to explore in the attempt to define how PG modulate mammalian skin function.

Two isoforms of COX, commonly referred to as COX-1 and COX-2 are currently recognized (Simith and DeWitt 1996, Herschman et al. 1997). Early studies on the COX
Figure 1.2: Pathway of prostaglandin E2 (PGE2) synthesis and roles of PGE2 in cancer development. Cyclooxygenase (COX)-1 and COX-2 mediate the conversion of arachidonic acid into PGG2 and PGH2, whereas the enzyme PGE2 synthase converts PGH2 into PGE2. PGE2 contributes to oncogenesis by acting on cell proliferation, tumour angiogenesis, cell invasion and metastasis, and immune modulation.

Isoenzymes focused on inflammatory cells. Investigators found that whereas COX-1 was constitutively expressed, COX-2 required stimulus-based induction (Simith and DeWitt 1996, Herschman et al. 1997, Needleman and Isakson 1997). Consequently, it was postulated that the PG that participate in homeostatic regulation derive from the constitutive COX-1 whereas the PG that participate in inflammation derive from the inducible COX-2. However, research on tissues and organs other than inflammatory cells challenged the concept of COX-2 functioning only as an inducible enzyme under pathophysiological circumstances. In fact, it is currently accepted that the two isoforms of active COX enzymes display similar enzymatic properties but differ in their expression and regulation (Smith et al. 2000, Simmons et al. 2004). Both isoforms are active under physiological and pathophysiological situations. COX-1 as a housekeeping gene is expressed constitutively and ubiquitously and according to numerous genetic and pharmacological studies is thought to generate PG that control normal physiological functions such as maintenance of the gastric mucosa and platelet function, vascular homeostasis, as well as renal blood flow (Simmons et al. 2004, Loftin
et al. 2002). Although not well-understood, COX-1-mediated PG synthesis seems to contribute to tumor progression in various tissues including skin (Loftin et al. 2002, Cha and Dubois 2007). In contrast, COX-2, an immediate-early gene, is below detection levels in many tissues except, for example, brain, pancreatic islets, ovary, uterus, kidney, and skin with anagen hair follicles (see below). As a rule, COX-2 becomes strongly induced by a huge number of diverse stimuli including hormones, environmental stress factors such as physical or chemical carcinogens, and in the course of inflammatory processes and tissue repair (Simmons et al. 2004). Genetic and pharmacological approaches demonstrate a physiological role of COX-2 in renal development, in salt and water regulation by the kidney, in female reproduction, in bone metabolism and in energy homeostasis (Loftin et al. 2002, Simmons et al. 2004, Loftin et al. 2002, Cha and Dubois 2007, Blackwell et al. 2010, Vegiopoulos et al. 2010). Furthermore, studies on the expression of COX-2 in human and mammalian skin suggest that this isozyme may participate in homeostatic function of the epidermis and moreover, overexpression may be a feature of certain types of skin dysplasia and cancer (see below) (Loftin et al. 2002, Cha and Dubois 2007, Wang and Dubois 2010a, Wang and Dubois 2010b).

1.3. COXs in the skin

Early studies of AA deficiency in animals and later in humans receiving hyperalimentation documented profound effects on skin structure and function. The epidermis became hyperkeratotic, hair loss ensued, and barrier function was lost (reviewed by Prendiville and Manfredi 1992). The observation that topical application of PGE2 could reverse epidermal hyperkeratosis in the rat (Ziboh and Hsia 1972), although not restore barrier integrity (Elias et al. 1980), was probably the first indication that PG and, in turn, COX activity, contributed to skin homeostasis. Later on, it was demonstrated that the skin was a major site of PG synthesis (Goldyne 2000). Keratinocytes synthesize PGE2, but also PGF2α and PGD2. While melanocytes also produce PGE2 along with PGD2 (Glehhill et al. 2010), Langerhans cells and mast cells produce PGD2 (Maciejewski-Lenoir et al. 2006, Shimura et al. 2010), fibroblasts, and endothelial cells mainly synthesize PGI2 (Ziboh 1996). The epidermis constitutively expresses COX-1 in individual keratinocytes of the interfollicular epidermis and the

COX-1 and COX-2 isozymes can both be expressed in mouse and human epidermis (Scholz et al. 1995, Goldyne 2000). In neonatal skin, the COX-1 protein was increasingly expressed with differentiation whereas it was equally expressed in all layers in adult skin. In the adult keratinocytes, COX-2 mRNA was barely detectable and no COX-2 protein could be demonstrated. Based on these results, it appears that in mouse skin, COX-2 expression seems to follow the inducible pattern found in inflammatory cells. In fact, expression of COX-2 protein appeared in the basal layer of mouse epidermis following treatment with acetone that disrupts the barrier function of the epidermis (Leong et al. 1996). In human skin, COX-1 immunostaining was observed throughout the epidermis whereas COX-2 immunostaining was more prominent in the more differentiated layers of the epidermis and hair follicle (Leong et al. 1996, Buckman and Gresham 1998, Muller-Decker et al. 1999). Thus, unlike mouse skin, COX-2 protein in human skin may be constitutively expressed in the more differentiated layers of the epidermis. In keeping with this concept, increased expression of COX-2 was present in biopsies of squamous cell carcinomas that derive from more differentiated keratinocytes; in contrast, little if any expression of COX-2 was observed in basal cell carcinomas that derive from the least differentiated basal cells (Leong et al. 1996, Buckman and Gresham 1998, Muller-Decker et al. 1999). One study that also evaluated keratoacanthomas, another type of squamous proliferation that has many features of squamous cell carcinoma, showed increased expression of COX-2 (Muller-Decker et al. 1999). A recent study with rat epidermis, and according with what observed in human skin, the COX-2 protein expression was strongly detected in the suprabasal layers of
the stratified epidermis (Xu et al. 2008).

A part from these studies in human and rodent skin and although constitutive COX-2 expression has been reported in dog, cat and horse kidney (Khan et al. 1998, Newman and Mrkonjich 2006, Thamm et al. 2008, Hamamoto et al. 2009), no studies has been performed in small animal or equine medicine regarding COX-2 and PGE2 expression in non-diseased skin. From the information available on COX-2 expression in canine studies on wound healing or neoplastic disease, it appears that this protein is not expressed in normal canine skin (Hamamoto et al. 2009, Pires et al. 2010). However, in the feline and equine studies on cutaneous neoplasias there is no mention of COX-2 expression in non-diseased epidermis and there are no pictures to allow us to evaluate it (Beam et al. 2003, Thamm et al. 2008).

1.4. Physiologic implications of COX expression in the skin

The extracellular concentration of calcium seems to be critical for induction of keratinocyte differentiation (Yuspa et al. 1989, Pillai et al. 1990). In fact, an extracellular calcium gradient exists in normal human skin with the lowest concentration present in the basal cell region and the highest concentration evident around the more differentiated keratinocytes higher in the epidermis (Menon and Elias 1991). A relationship between COX-2, the epidermal calcium gradient, and/or keratinocyte differentiation is suggested by several studies. Research with human and mouse keratinocyte cultures, leaded to the observation that increasing extracellular calcium resulted in: 1) increased expression of COX-2 mRNA and protein, but not COX-1 protein (Leong et al. 1996), 2) increased PGE2 synthesis (Evans et al. 1993), and 3) induction of cornified envelope or expression of keratin-1 (K1) formation which are recognized markers of keratinocyte differentiation (Sun and Green 1976, Akunda et al. 2003). In the presence of indomethacin or celecoxib (a COX-2 selective inhibitor), however, cornified envelope formation or K1 expression was significantly suppressed, but could be reestablished by adding exogenous PGE2 (Evans et al. 1993, Akunda et al. 2003). This finding of a possible link between PGE2, COX-2 expression, and keratinocyte differentiation is in keeping with another study looking at the effect of cholecalciferol on human keratinocytes (Kanekura et al. 1998). A similar link between
COX-2 and PGE2 synthases expression and cell differentiation and proliferation is also suggested in several other studies (Hume et al. 1991, Hoff et al. 1993, Zhang et al. 1997, Ansari et al. 2008, Hara et al. 2010). However, this link has not been studied in small animal keratinocytes.

It is important to point out that there are types of cells in which differentiation appears to involve COX-1, rather than COX-2, induction (Simth et al. 1993). Consequently, the physiologic roles of the COX isozymes are most likely cell, tissue and specie specific, and to extrapolate the findings from one type of cell, tissue or specie to other untested needs to be undertaken with caution. While this approach appears self-evident, COX-2 is still often described in the general literature as the inducible form of COX (Doré 2011, Schneider and Pozzi 2011) when, as cited previously, it has been recognized as constitutively expressed in a number of tissues (Lipsky 1999, O’Neill and Ford-Hutchinson 1993, Goldyne 2000, Müller-Decker 2011).

1.5. COX in acute skin inflammation

The skin responds to a spectrum of harmful environmental stimuli with an acute inflammatory reaction characterized by erythema, heat, edema, and pain followed by a reactive epidermal hyperplasia and hyperkeratosis (Hruza and Pentland 1993). Erythema and heat are due to the dilatation of local arterioles and subsequent increased blood flow, while edema and infiltration of the dermis by neutrophils are caused by an increased vascular permeability at the level of the venules. Once thought to be inert, keratinocytes, the predominant cells in the epidermis, have an active participation in the cutaneous immune and inflammatory response. Keratinocytes can mount an immune response through secretion of antimicrobial peptides, eicosanoids, neuropeptides, reactive oxygen species, growth factors and inflammatory cytokines involved in lipid-derived proinflammatory mediator synthesis (Williams and Kupper 1996, Barker et al. 1991, Steinhoff et al. 2001, Kim and Modlin 2003, Albanesi 2010, Nestlé 2010). Keratinocytes constitutively release very low levels of cytokines and independent of the type of stimulus for induction of an acute inflammation and present the predominant responsive cell types of the epidermis (Nestle et al. 2009). Upon injury or stimulation with exogenous factors such as lipopolysaccharides (LPS),
silica, poison ivy cathecols, *Staphylococcus* toxins and UV radiation, keratinocytes secrete high levels of interleukin (IL) -1, IL-6, IL-8, IL-10 and tumour necrosis factor (TNF)-α. These cytokines can induce differentiation and growth of keratinocytes and other resident or migrating cells in the epidermis, dermis and vessels. Furthermore, they are important mediators of both local and systemic inflammatory and immune responses (Williams and Kupper 1996, Barker *et al.* 1991, Steinhoff *et al.* 2001, Kim and Modlin 2003, Nestle *et al.* 2009).

As mentioned previously, a part from cytokines, keratinocytes can also synthetize and secrete PGs. These eicosanoids have been shown to have a modulatory role in acute skin inflammation. Cutaneous irritants, such as ultraviolet (UV) B and tetradecanoylphorbol-acetate (TPA), increase PG levels not only in keratinocytes but also in melanocytes and mesenchymal cells (Ruzicka *et al.* 1983, Pentland and Jacobs 1991, Goldyne *et al.* 1994, Müller-Decker *et al.* 1995, Kuwamoto *et al.* 2000, Gledhill *et al.* 2010). The concentration of PGE2 and PGI2 in the skin increases at the same time that erythema and edema develop (Black *et al.* 1978). And it is been shown that intradermal injection of PGE2, PGD2, and PGI2 induces different aspects of the inflammatory response including erythema and edema (Black *et al.* 1978, Williams 1979). Moreover, individual PGs act synergistically with other eicosanoids and with structurally unrelated pro-inflammatory mediators (Williams 1979, Pentland *et al.* 1990, Rhodes *et al.* 2001). Early PGE2 synthesis as catalyzed by COX-1 depends on both the activation of PLA2 by phosphorylation and increased PLA2 synthesis (Gresham *et al.* 1996). The induction of epidermal hyperproliferation by TPA also depends on immediate COX-1-mediated PGE2 synthesis and, upon progression of the inflammatory response, COX-2 protein is shown to be transiently upregulated in epidermis (Fürstenberg and Marks 1980, Scholz *et al.* 1995 , Buckman *et al.* 1998, Müller-Decker *et al.* 1998, Soriani *et al.* 1999, Athar *et al.* 2001, An *et al.* 2002, Tripp *et al.* 2003, Dazard *et al.* 2003, Akunda *et al.* 2007). In reactive epidermal hiperplasia, an increased number of COX-2-expressing keratinocytes within the basal but not the suprabasal compartment of the interfollicular epidermis is found, coinciding with increased proliferation and apoptosis rates in basal keratinocytes. Simultaneously, the pattern of suprabasal COX-1 expression remains unchanged (Müller-Decker *et al.* 2003, Tripp *et
In inflammatory settings, PGE2 molecules produced via COX-2 and COX-1 have different biological effects. At least in UV B-irradiated skin, COX-2 rather than COX-1 supports keratinocyte proliferation and survival (Tripp et al. 2003, Pentland et al. 2004, Akunda et al. 2007). Like in other cell types, keratinocyte apoptosis seems to be inhibited by COX-2 through the activation of the PGE2 receptors EP2 and EP4. Subsequent activation of downstream effector kinases culminates in increased levels of anti-apoptotic phospho-Bad, an effect that switches the pro-apoptotic Bad into a survival factor (Hoshino et al. 2003, Houchen et al. 2003, Chun et al. 2007). In UV B-irradiated mouse skin, COX-2-selective inhibitors effectively suppress, along with PG synthesis, signs of acute inflammation including proliferation of keratinocytes, edema, infiltration and activation of dermal neutrophils, the formation of acute oxidative damage, and DNA-damaged sunburn cells (Wilgus et al. 2000, Wilgus et al. 2002, Tripp et al. 2003, Wilgus et al. 2003). Moreover, the topical application of non-steroidal anti-inflammatory drugs (NSAIDs) is also potent in inhibiting the UV light-induced acute sunburn reactions such as redness (Snyder and Eaglestein 1974, Black et al. 1980). And the COX-2-inhibition also leads to a reduction of the epidermal proliferation index and increases apoptosis in basal cells (Tripp et al. 2003, Rodriguez-Buford et al. 2005).

The impact of PGE2 present during an inflammatory response on both immune effector cells and surrounding stromal cells is determined by the array of receptors the cells express and the intracellular pathways to which they are coupled. Activation of these receptors, even when coupled to similar pathways, might evoke different responses because of different levels of expression (both constitutive and induced), different patterns of desensitization, and differential affinity to metabolites of the primary ligand (Rundaugh et al. 2011). Thus, determining the role of prostanoids in a given inflammatory response requires not only knowledge of the lipid mediators present in the lesion, but also the receptor profile on surrounding cells and the biochemical signaling of these receptors under specific ligand concentrations. The potential pro- and anti-inflammatory roles of PGE2 highlights these complexities. A part from the pro-inflammatory effects of PGE2 explained above, this PG has also been shown to possess anti-inflammatory properties (Tilley et al. 2001, Tizard 2004). In particular,
PGE2 has been shown to inhibit a wide range of T and B cell functions (Chouaib et al. 1985, Pène et al. 1988, Roper et al. 1994). PGE2 has also been reported to attenuate inflammatory responses initiated by mast cell degranulation (Hitchcock 1978, Raud et al. 1988) and in models of inflammatory colitis, hepatitis and allergic airway disease, an overall anti-inflammatory role for PGE2 and COX-2 has been suggested (Morteau et al. 2000, Yin et al. 2007).

1.6. COX in wound healing

Similar to the transient upregulation of COX-2 in UV B or TPA-induced acute inflammation, it is COX-2 expression that is predominantly upregulated in a transient manner in basal epidermal keratinocytes as well as fibroblasts, macrophages, and endothelial cells at the wound site, independent of whether wounding has been superficial, incisional, or excisional in mouse, human and canine skin (Scholz et al. 1995, Abd-El-Aleem et al. 2001, Fugatami et al. 2002, Müller-Decker et al. 2002, Blomme et al. 2003, Kämper et al. 2003, Schäfer and Werner 2008, Hamamoto et al. 2009).

1.7. COX in cancer

One of the more intensively studied fields of COX function is that relating to carcinogenesis (Fig. 1.2.). A large body of evidence suggests that COX-derived PG contribute to tumorigenesis in humans (Greenhough et al. 2009). Colorectal cancer represents the first cancer where the role of COX-2 and PG was suspected after epidemiological studies had revealed that the regular intake of low doses of aspirin reduced the risk of developing this neoplasia (Thun and Heath 1995, Cuzick et al. 2009). This initial indication of a role for COX-2 in colorectal cancer was then followed by a series of studies demonstrating that COX-2 is overexpressed in these cancers (Eberhart et al. 1994, Kargman et al. 1995, Sano et al. 1995). Immunohistochemical studies revealed that COX-2 was present in colonic epithelial neoplastic cells (Sano et al. 1995). Moreover, the levels of PGE2 are elevated in cancerous colonic tissues compared to normal tissues (Rigas et al. 1993, Pugh and Thomas 1994).

PGE2 exerts its actions via binding to 4 specific cell surface G protein-coupled
receptors (designated EP1 to EP4) and can influence many cellular events that contribute to cancer development. Characterization of the precise role of each EP subtype in carcinogenesis is another field of investigation. Different EP receptors appear to be involved at various stages of carcinogenesis and in different types of cancer (Fulton et al. 2006, Wang and DuBois 2006, Rundhaug et al. 2011). For example, experimental evidence indicates that EP1 and EP4 play a role in colon cancer, whereas EP2 is involved in mouse skin tumor development (Sung et al. 2005, Wang and DuBois 2006, Brouxhon et al. 2007). The presence of EP1 has also been demonstrated in human breast cancer cells, and EP4 antagonists have been shown to inhibit breast cancer metastasis (Ma et al. 2006, Thorat et al. 2008). Via its receptors, PGE2 is able to promote cell proliferation and survival (through different mechanisms, including suppression of apoptosis), promote tumor angiogenesis (through the production of proangiogenic growth factors such as vascular endothelial growth factor and basic fibroblast growth factor), increase cell invasion and metastasis, and suppress the immune response in order for the tumor cells to escape immunosurveillance (Fig.1.2) (Tsuji et al. 1998, Harris et al. 2002, Gupta et al. 2007, Greenhough et al. 2009).

In addition to colorectal cancer, numerous studies document the aberrant overexpression of COX-2 as a consistent feature of a significant number of human premalignant and malignant tumors of epithelial and non-epithelial origin such as such as breast, pulmonary, head and neck, pancreatic, prostatic, and gastric cancers including various tumor entities of the skin, while in general, COX-1 expression is unaltered as compared to healthy tissue (Hida et al. 1998, Gupta et al. 2000, Soslow et al. 2000, Half et al. 2002, Gallo et al. 2002, Denkert et al. 2003, Forones et al. 2008, Hermanova et al. 2009, Müller-Decker 2011). Regarding the skin, COX-2 overexpression has been detected in human Merkell cell carcinoma, melanoma, basal cell carcinomas and and has been found strongly overexpressed in a high percentage of squamous cell carcinomas (SCC) and in cutaneous epitheliotropic T cell lymphoma (CETL) (revised in Müller-Decker 2011, Kopp et al. 2010). In most of these neoplasias, a direct relationship between COX-2 overexpression and neoplasia malignancy and progression has been postulated. Most importantly, COX-2 overexpression is observed
in precursor lesions of SCC such as actinic keratosis (AK) and Bowens’s disease clearly indicating that COX-2 upregulation is an early premalignant event in the development of SCC (Müller-Decker et al. 1999, An et al. 2002, Akita et al. 2004, O’Grady et al. 2004, Nijsten et al. 2004). UV B-induced COX-2 seems to be causally related to the development of AK, since UV B exposure leads to a strong upregulation of COX-2 expression in a significant portion of these benign tumors and AK are primarily found on chronically sun-exposed skin sites (Black et al. 1978, Buckmann et al. 1998, Athar et al. 2001, Chun et al. 2007, Fischer et al. 2007, Müller-Decker 2011).

Regarding canine cancers, COX-2 elevated expression has been detected in several neoplasias (revised in Doré 2011). COX-2 is overexpressed with different intensities in several canine epithelial neoplasias, including mammary tumors, prostatic carcinomas, transitional cell carcinomas, intestinal adenocarcinomas, nasal tumours, renal cell carcinomas, ovarian carcinomas and SCC. Other neoplasias in which COX-2 overexpression has been detected are oral and cutaneous melanomas, osteosarcomas and meningiomas (Mohammed et al. 2004, Mullins et al. 2004, Rossmeisl et al. 2009, Martínez et al. 2011). As in humans, COX-2 expression in SCC has also been shown to be present in a large proportion (56–100%) of canine neoplastic growths originating from various locations (oral, cutaneous, or digital SCCs) (Pestili de Almeida et al. 2001, Mohammed et al. 2004). One clinical study has evaluated the effect of a nonspecific COX-2 inhibitor (piroxicam) with a reported remission (including complete or partial) rate of 18% and stable disease in 29% of dogs suffering from oral SCCs (Schmidt et al. 2001). A higher remission rate (56%) was obtained when dogs suffering from oral SCCs were treated with piroxicam in combination with cisplatin (Boria et al. 2004). Oral fibrosarcomas, lymphomas, hemangiosarcomas, histiocytic sarcomas, and mast cell tumors of dogs do not express COX-2 (Mohammed et al. 2004, Heller et al. 2005). Definitive intraneoplastic COX-2 expression was found only in 3.5% of canine uveal melanocytic neoplasms, but the presence of COX-2 in many normal ocular structures made the exact determination of COX-2 positivity in neoplastic cells difficult (Paglia et al. 2009).

Studies of COX-2 expression in feline neoplasias are less numerous and have included mostly epithelial tumours (revised in Doré 2011). Mammary carcinoma, oral SCC and
transitional cell carcinoma showed different degrees of positivity to COX-2 immunostaining. On the contrary, other carcinomas including cutaneous SCC, pulmonary and intestinal adenocarcinomas, lymphomas and vaccine-associated sarcomas were reported COX-2 negative on IHC (Beam et al. 2003). A study of 8 feline pancreatic adenocarcinomas reported that only 2 were COX-2 positive, with less than 10% of the cells expressing an intense staining (Newman and Mrkonjich 2006).

Equine SCC positivity to COX-2 IHC has reported conflicting results, from negative to strong staining neoplasias depending on the study (revised in Doré 2011). Only one study has examined COX-2 expression in other equine tumors finding that melanomas were positive for COX-2 (28% with a moderate to strong staining) and that COX-2 was weakly expressed in very few equine sarcoids (14%) (Thamm et al. 2008).
References:


Albanesi C. Keratinocytes in allergic skin diseases (2010). Current Opinion in Allergy and Clinical Immunology, 10: 452-6.


Black AK, Greaves MW, Hensby CN (1980). The anti-inflammatory and pharmacological
effects of topically applied flurbiprofen on human skin 24 hours after ultraviolet B irradiation. Prostaglandins and Medicine, 5: 405–413.


Fürstenberger G, Marks F (1978). Indomethacin inhibition of cell proliferation induced by the phorbolester TPA is reversed by prostaglandin E2 in mouse epidermis in vivo.


Harris RE, Chlebowski RT, Jackson RD, Frid DJ, Ascenseo JL et al. (2003). Breast cancer


Pène J, Rouset F, Brière F, Chrétien I, Bonnefoy JY et al. (1988). IgE production by


Smith CJ, Morrow JD, Roberts LJ, Marmett LJ (1993). Differentiation of monocytoid


Ziboh VA, Shia SL (1971). Prostaglandin E2: biosynthesis and effects on glucose and


2. OBJECTIVES
Objectives

As explained above, there are multiple evidences linking the system COX-2/PGE2 with physiologic, inflammatory and neoplastic processes. With this background, we planned to investigate the route COX2/PGE2 in epidermal involving neoplasia and epidermal inflammation.

The two objectives of the thesis were:

1. To investigate COX-2 expression in cutaneous neoplasia with epidermal involvement in dogs, cats and horses.
2. To investigate the route COX-2/PGE2 in the canine keratinocyte inflammatory response.

In order to reach these objectives, two different studies were designed and carried on (see scheme in the following page):

1. Experiment 1:
   To investigate COX-2 expression in cutaneous pre-neoplastic (actinic keratosis) and neoplastic processes (squamous cell carcinoma, melanoma and cutaneous epitheliotropic T cell lymphoma) and in inflammatory dermatoses an immunohistochemical study was carried on. The pattern of COX-2 expression and its association to inflammation was studied.

2. Experiment 2:
   To investigate the participation of keratinocytes in the inflammatory cutaneous response an in vitro model of keratinocytes culture was implemented. To establish the role of keratinocytes in the inflammatory response through the COX-2/PGE2 route, this in vitro model was used to evaluate PGE2 production by keratinocytes in response to different stimuli (lipopolysaccharide and inflammatory cells).
Overall structure of the thesis (objectives and experiments to carry on):

**OBJECTIVES**

**OBJECTIVE 1**
To investigate COX-2 expression in cutaneous neoplasia with epidermal involvement in dogs, cats and horses

**OBJECTIVE 2**
To investigate the role of the COX-2/PGE2 route in the epidermal inflammatory response

**EXPERIMENT 1**
COX-2 immunohistochemical study of pre-malignant, malignant and inflammatory disorders with epidermal involvement

**EXPERIMENT 2**
Set up an in vitro model to study the keratinocyte inflammatory response.

Evaluate in vitro production of PGE2 in response to different stimuli
3. EXPERIMENTAL SECTION
3.1. Immunohistochemical demonstration of COX-2 expression in cutaneous neoplasia and inflammatory dermatoses*

3.1.1. Introduction

In this first experiment we aimed to investigate COX-2 expression in cutaneous pre-neoplastic, neoplastic and inflammatory dermatoses involving, all of them, the epidermis.

First of all, pre-neoplastic (AK) and neoplastic (SCC) disorders in which the keratinocytes are primarily involved were studied. As explained previously, in human and rodent beings, it has been demonstrated that COX-2 is overexpressed in AK and SCC, and COX-2 has been postulated as being causally related to its development (Buckman et al. 1998, An et al. 2002, Müller-Decker 2011). However, no studies on COX-2 expression in companion animals AK have been performed. Moreover, immunohistochemical studies regarding SCC and COX-2 expression in dogs and cats have yielded divergent results. COX-2 has been shown to be present in a large proportion of canine SCCs but cutaneous feline SCC are reported to be COX-2 negative (Pestili de Almeida et al. 2001, Mohamed et al. 2004, Beam et al. 2003).


We included this neoplasia in the experiment because it is a common neoplasia in dogs.

* Part of the results have been published in Journal of Comparative Pathology 2012; 146 (1): 11-7. See annex.
and horses that affect a resident cell in the epidermis and because the conflicting results reported in previous veterinary and human studies.

The last neoplasia studied was the lymphocytic proliferative disorder CETL. The neoplastic lymphocyte is not an epidermal resident cell, but due to its epitheliotropism, the epidermis becomes primarily involved in its pathogenesis. Moreover, a recent study has reported COX-2 overexpression in human CETL (Kopp et al. 2010). This neoplasia seemed interesting to us because its epidermal involvement, and because invasion of the epidermis by neoplastic cells often causes erosion and/or ulceration. This could allow us to study, a part of COX-2 expression in neoplastic lymphocytes, the relationship between COX-2 expression and the presence of inflammation in eroded or ulcerated neoplastic lesions as we have seen in the previous experiment.

Finally, we aimed to study COX-2 expression in inflammatory dermatoses of dogs and cats. We wanted to study them because even though there is evidence that the COX-2/PGE2 route has important roles in the cutaneous inflammatory response (Goldyne 2000, Müller-Decker 2011), no studies in either humans or animals have been reported regarding different cutaneous inflammatory diseases. This study would allow us to broaden the understanding of the role of COX-2/PGE2 in the inflammatory epidermal response.

3.1.2. Materials and methods:

Tissue samples

All samples were selected from the Veterinary Pathology Diagnostic Service of the Universitat Autònoma de Barcelona. A careful file history review was carried out to exclude from the study those samples from animals for which the history stated or was indicative of steroid or NSAID treatment previous to biopsy retrieval.
Canine and feline actinic keratosis and squamous cell carcinoma: 18 were AK (9 feline and 9 canine) and 36 SCC (27 feline and 9 canine).

Canine and equine melanocytic neoplasms: 22 samples were selected. Twelve (6 melanocytomas and 6 melanomas) were canine samples and 10 (8 melanocytomas and 2 melanomas) were equine.

Canine cutaneous epitheliotrophic T cell lymphoma: 20 samples were selected. Two of them were classified as Pagetoid reticulosis (PR) (neoplastic infiltrate confined to the epidermis and adnexal structures) and the rest (18) as “classical” mycosis fungoides (with neoplastic infiltrates present in the epidermis, adnexa and underlying dermis) (Gross et al. 2005).

Canine and feline inflammatory dermatoses: 24 samples were selected. These included pemphigus foliaceous (2 feline and 3 canine), pustular staphylococcal infection (1 feline and 5 canine), superficial hyperplastic perivascular dermatitis without pustules (2 feline and 3 canine) and dermatophytosis (3 feline and 5 canine).

Immunohistochemistry:

The standard streptavidin biotin technique was used for detecting COX-2 protein in all samples.

Five-micron-thick paraffin wax embedded tissue sections were cut and placed on positively charged glass slides. Sections were deparaffinised and endogenous peroxidase activity was quenched by exposure to 3% hydrogen peroxide for 5 minutes. Bleaching with 0.25% potassium permanganate for 30 minutes followed by 1% oxalic acid for 5 minutes was performed. Heat-induced epitope retrieval was carried out at 90 to 100ºC in citrate buffer solution for 10 minutes with 30 minutes of cooling at ambient temperature. The sections were then blocked with 2% bovine serum albumin (Sigma-Aldrich, Germany). The primary antibody (polyclonal murine COX-2 (Cayman Chemical, Ann Arbor, MI)) was applied, and slides were incubated overnight at 4ºC.
Slides were then incubated with a biotinylated polyclonal goat anti-rabbit secondary antibody (Dako, Denmark) for one hour and then with an avidin-biotin-peroxidase complex (ImmunoPure, Thermo Fisher Scientific, Rockford, IL) for another hour. Slides were developed with 3,3-diamino-benzidine tetrahydrochloride (Sigma-Aldrich, Germany) and counterstained with haematoxylin.

The rabbit anti-COX-2 polyclonal antibody used in this study had been previously shown to detect COX-2 in feline and canine cells of the macula densa and validated in the horse specie (Newman and Mrkonjich 2006, McInnis et al. 2007, Hammamoto et al. 2009).

In the CETL study, for each sample included, two consecutive five-micron-thick paraffin wax embedded tissue sections were cut and placed on silane-coated glass slides. COX-2 IHC was performed in one section, and for those samples positive to this IHC, a CD-3 IHC was performed on the other consecutive section. As in COX-2 IHC, a standard streptavidin biotin technique was used for detecting CD-3 molecules. Sections were deparaffinised and endogenous peroxidase activity was quenched by exposure to 3% hydrogen peroxide for 30 minutes. Bleaching with 0.25% potassium permanganate for 30 minutes followed by 1% oxalic acid for 5 minutes was performed. The sections were treated with protease 0.1% in Tris buffer for 8 minutes at 37ºC and incubated with a rabbit polyclonal anti-CD-3 antibody (Dako, Copenhagen, Denmark) overnight at 4ºC. The sections were then incubated with a biotinylated polyclonal goat anti-rabbit secondary antibody (Dako, Denmark) for one hour and then with an avidin-biotin-peroxidase complex (ImmunoPure®, ABC Peroxidase Staining Kit Standard, Pierce, Thermo Fisher Scientific, Rockford, IL) for another hour. The immunologic reaction was developed with 3,3-diamino-benzidine tetrahydrochloride (Sigma-Aldrich, Germany) and counterstained with haematoxylin. The positive controls were paraffin-embedded sections of a canine lymph node. The negative controls were sections incubated in normal rabbit serum in place of specific CD-3 antiserum.
All samples were closely assessed in an attempt to define whether neoplastic and epidermal cells were specifically labelled with COX-2 antibody and, in the CETL samples, with CD-3 antibody.

### 3.1.3. Results:

Immunoreactivity to COX-2 was observed in pre-neoplastic and neoplastic keratinocytes (AK and SCC samples) and melanocytes (melanocytic neoplasm samples) but not in neoplastic lymphocytes (CETL samples). However, positive neoplastic cells where those found in association with a granulocytic infiltrate. On the contrary, positive pre-neoplastic keratinocytes of the AK samples were not associated to inflammatory infiltrate. In the inflammatory dermatoses samples, all keratinocytes that were positive to COX-2 had a granulocytic inflammatory infiltrate around them.

The cellular pattern of COX-2 immunoreactivity was in all cases granular cytoplasmatic and located mainly in the perinuclear zone.

**Actinic keratosis and squamous cell carcinoma:**

Regarding AK, 4 out of 9 feline and 3 out of the 9 canine samples were positive to COX-2 IHC (Table 3.1). Immunoreactivity was observed in basal epidermal cells, but scattered suprabasal positive cells were also detected (Fig 3.1). The positive cells were in the proximity of an inflammatory infiltrate only in one canine sample.

All feline and canine SCC showed COX-2 positive neoplastic cells (Table 3.1). Staining was heterogeneous throughout the tumour sections. Basal cells and more differentiated cells were found to be positive, mainly in and below areas were there was superficial erosion or ulceration of tumours, but positive tumour cells were also observed to be scattered deeper in all samples (Fig 3.2). In most cases, the positive IHC reaction in keratinocytes was associated with the presence of polymorphonuclear (PMN) cells (Fig 3.2). No COX-2 positive reaction was observed in the non-
eroded/ulcerated margins of the tumours. In 6/27 feline cases and 1/9 canine cases, positive neoplastic keratinocytes were observed without the presence of an associated inflammatory response.

<table>
<thead>
<tr>
<th>SAMPLES</th>
<th>COX-2 IHC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Disease</td>
</tr>
<tr>
<td>AK</td>
<td>F</td>
</tr>
<tr>
<td>AK</td>
<td>C</td>
</tr>
<tr>
<td>SCC</td>
<td>F</td>
</tr>
<tr>
<td>SCC</td>
<td>C</td>
</tr>
</tbody>
</table>

**Table 3.1:** Summarize of results of COX-2 immunohistochemistry (IHC) on canine and feline actinic keratosis (AK) and squamous cell carcinoma (SCC) samples. (C, canine; F, feline; n, number of samples; PMN, polymorphonuclear)

**Figure 3.1.** Representative photomicrographs of COX-2 immunohistochemistry of actinic keratoses samples. A and B: feline and canine samples respectively. COX-2 immunoreactivity is observed mainly in the basal (arrows) and in few suprabasal keratinocytes (arrowheads).
Figure 3.2. Representative photomicrographs of COX-2 immunohistochemistry of squamous cell carcinoma (SCC) samples. A and B: feline SCC. C: canine SCC. Immunoreactivity of neoplastic keratinocytes to COX-2 antibody is observed mainly at the eroded surface of the tumours (arrows) but also scattered deeper positive cells are detected (arrowheads). Positive immunoreactivity of keratinocytes is associated mainly with the presence of polymorphonuclear cells (asterix).
Melanocytic neoplasms:

Expression of COX-2 protein in neoplastic melanocytes was found in only 36% (8 of 22) of the samples examined and 64% (14 of 22) showed negative staining results (table 3.2).

In these positive sections, immunoreactivity was detected in scattered neoplastic melanocytes (5 to 15 per section). These positive neoplastic cells were detected in areas were ulceration and polymorphonuclear (PMN) infiltrate was present (Fig. 3.3). However, not all ulcerated areas had COX-2 positive cells. No positive cells were detected in the centre of any neoplasia. If the neoplasia was not ulcerated, there were no COX-2 positive neoplastic melanocytes or keratinocytes.

In the canine neoplasias, the five samples that were positive corresponded to melanomas (3 oral, 1 mucocutaneous and 2 cutaneous) and none of the melanocytomas (which were not ulcerated) showed immunoreactivity to COX-2. Only one melanoma, which was not ulcerated, was negative.

In the equine samples, the 3 positive specimens were ulcerated melanomas. As in the canine samples, one not ulcerated melanoma and all melanocytomas (which all of them were not ulcerated), were negative to COX-2 IHC.

Regarding epidermal keratinocytes, their positivity paralleled that of neoplastic melanocytes. All those samples that had positive neoplastic melanocytes, had positive epidermal keratinocytes in the same areas. Like the positive neoplastic cells, positive keratinocytes were found in inflamed areas with ulcers and PMN infiltrate.
<table>
<thead>
<tr>
<th>Sample number</th>
<th>Sp</th>
<th>M/ Mc</th>
<th>Neoplasia location</th>
<th>Ulcers</th>
<th>COX-2</th>
<th>COX-2 positive cells</th>
<th>COX-2 positivity associated to PMN infiltrate, ulcers, both or neither</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C</td>
<td>M</td>
<td>Oral</td>
<td>Yes</td>
<td>+</td>
<td>Kcs &amp; mel</td>
<td>both</td>
</tr>
<tr>
<td>2</td>
<td>C</td>
<td>Mc</td>
<td>M-cut</td>
<td>No</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>3</td>
<td>C</td>
<td>Mc</td>
<td>Cut</td>
<td>No</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>4</td>
<td>C</td>
<td>M</td>
<td>Cut</td>
<td>Yes</td>
<td>+</td>
<td>Kcs &amp; mel</td>
<td>both</td>
</tr>
<tr>
<td>5</td>
<td>C</td>
<td>Mc</td>
<td>Cut</td>
<td>No</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>6</td>
<td>C</td>
<td>M</td>
<td>M-cut</td>
<td>Yes</td>
<td>+</td>
<td>Kcs &amp; mel</td>
<td>both</td>
</tr>
<tr>
<td>7</td>
<td>C</td>
<td>M</td>
<td>Oral</td>
<td>Yes</td>
<td>+</td>
<td>Kcs &amp; mel</td>
<td>both</td>
</tr>
<tr>
<td>8</td>
<td>C</td>
<td>Mc</td>
<td>Cut</td>
<td>No</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>9</td>
<td>C</td>
<td>M</td>
<td>Cut</td>
<td>Yes</td>
<td>+</td>
<td>Kcs &amp; mel</td>
<td>both</td>
</tr>
<tr>
<td>10</td>
<td>C</td>
<td>Mc</td>
<td>Cut</td>
<td>No</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>11</td>
<td>C</td>
<td>Mc</td>
<td>Cut</td>
<td>No</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>12</td>
<td>C</td>
<td>M</td>
<td>M-cut</td>
<td>No</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>13</td>
<td>E</td>
<td>Mc</td>
<td>Cut</td>
<td>No</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>14</td>
<td>E</td>
<td>M</td>
<td>Cut</td>
<td>Yes</td>
<td>+</td>
<td>Kcs &amp; mel</td>
<td>both</td>
</tr>
<tr>
<td>15</td>
<td>E</td>
<td>M</td>
<td>Cut</td>
<td>Yes</td>
<td>+</td>
<td>Kcs &amp; mel</td>
<td>both</td>
</tr>
<tr>
<td>16</td>
<td>E</td>
<td>M</td>
<td>Cut</td>
<td>Yes</td>
<td>+</td>
<td>Kcs &amp; mel</td>
<td>both</td>
</tr>
<tr>
<td>17</td>
<td>E</td>
<td>Mc</td>
<td>Cut</td>
<td>No</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>18</td>
<td>E</td>
<td>Mc</td>
<td>Cut</td>
<td>No</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>19</td>
<td>E</td>
<td>Mc</td>
<td>Cut</td>
<td>No</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>20</td>
<td>E</td>
<td>Mc</td>
<td>Cut</td>
<td>No</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>21</td>
<td>E</td>
<td>Mc</td>
<td>Cut</td>
<td>No</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>22</td>
<td>E</td>
<td>M</td>
<td>Sclera</td>
<td>No</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

**Table 3.2:** Summarize of results of COX-2 immunohistochemistry (IHC) on canine and equine melanocytic neoplasms samples. (-, negative; +, positive; C, canine; Cut: cutaneous; E: equine; kcs: keratinocytes; n/a, not applicable; Sp: specie; M: melanoma; Mc: melanocytoma; M-cut: mucocutaneous; mel: melanocytes; PMN, polymorphonuclears)
Figure 3.3. Representative photomicrographs of COX-2 immunohistochemistry of canine (A, C, E) and equine (B, D, F) melanomas. Immunoreactivity of neoplastic melanocytes (arrows) and of keratinocytes (arrowheads) to COX-2 antibody is observed mainly at the eroded surface of the tumours. Images C and E are closer views of keratinocytes (C) and melanocytes (E) of sample A. Images D and F are closer views of keratinocytes (D) and melanocytes (F) of sample B.
**Cutaneous epitheliotropic T cell lymphoma:**

COX-2 immunoreactivity was absent in the neoplastic cell population in 19/20 cases. The only case in which immunoreactivity was detected in the neoplastic infiltrate was a very mild case with only 2 cells being positive. No microscopic signs of inflammation were detected around these positive cells (table 3.3).

Few keratinocytes (5-15 cells/section) in 10/20 samples expressed COX-2 (Fig. 3.4). It was confirmed that they were keratinocytes because they were CD-3 negative and phenotypically compatible with epidermal cells.

COX-2 expression in keratinocytes was associated with ulcers or polymorphonuclear (PMN) infiltration in 9/10 of cases (Fig. 2.4). From them, 6 were associated to the presence of ulcers and in 2 there were no ulcers but PMN were present around the positive COX-2 cells.
### Table 3.3: Summarize of results of COX-2 immunohistochemistry (IHC) on canine cutaneous epitheliotropic T cell lymphoma samples. (-, negative; +, positive; CMF, classical mycosis fungoides; E, epidermis; D, dermis; n/a, not applicable; n/k, not known; PMN, polymorphonuclear; PR, Pagetoid reticulosis)

<table>
<thead>
<tr>
<th>Sample number</th>
<th>PR or CMF</th>
<th>COX-2 + (E or D)</th>
<th>COX-2 positive cells</th>
<th>COX-2 positivity associated to PMN infiltrate, ulcers or neither</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CMF</td>
<td>+ (E)</td>
<td>keratinocytes</td>
<td>ulcer</td>
</tr>
<tr>
<td>2</td>
<td>CMF</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>3</td>
<td>CMF</td>
<td>+ (E)</td>
<td>keratinocytes</td>
<td>ulcer</td>
</tr>
<tr>
<td>4</td>
<td>PR</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>5</td>
<td>CMF</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>6</td>
<td>CMF</td>
<td>+ (E)</td>
<td>keratinocytes</td>
<td>ulcer</td>
</tr>
<tr>
<td>7</td>
<td>CMF</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>8</td>
<td>CMF</td>
<td>+ (E)</td>
<td>keratinocytes</td>
<td>ulcer</td>
</tr>
<tr>
<td>9</td>
<td>CMF</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>10</td>
<td>CMF</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>11</td>
<td>CMF</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>12</td>
<td>CMF</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>13</td>
<td>PR</td>
<td>+ (E)</td>
<td>keratinocytes</td>
<td>PMN</td>
</tr>
<tr>
<td>14</td>
<td>CMF</td>
<td>+ (E)</td>
<td>keratinocytes</td>
<td>neither</td>
</tr>
<tr>
<td>15</td>
<td>CMF</td>
<td>+ (D)</td>
<td>lymphocytes</td>
<td>neither</td>
</tr>
<tr>
<td>16</td>
<td>CMF</td>
<td>+ (E)</td>
<td>keratinocytes</td>
<td>Ulcer</td>
</tr>
<tr>
<td>17</td>
<td>CMF</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>18</td>
<td>CMF</td>
<td>+ (E)</td>
<td>keratinocytes</td>
<td>PMN</td>
</tr>
<tr>
<td>19</td>
<td>CMF</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>20</td>
<td>CMF</td>
<td>+ (E)</td>
<td>keratinocytes</td>
<td>Ulcer</td>
</tr>
</tbody>
</table>
Figure 3.4. Representative photomicrographs of COX-2 immunohistochemistry (A and C) and CD3 immunohistochemistry (B and D) of a cutaneous epitheliotropic T cell lymphoma samples. C and D are high-power magnifications of the indicated areas of A and B, respectively. COX-2 is expressed by keratinocytes at the eroded surface (arrows). COX-2 positive cells are CD3 negative (arrowheads).
**Inflammatory dermatoses:**

Of the 24 samples of inflammatory dermatoses tested, IHC positive keratinocytes were observed in 4 feline (2 pemphigus foliaceus and 2 dermatophytoses) and 6 canine (3 pemphigus foliaceus, 2 pustular staphylococcal infections and 1 dermatophytosis) cases (Table 3.4). In all positive samples, basal and suprabasal positive keratinocytes were always associated with the exocytosis of PMN neutrophils or eosinphils in the epidermis or follicular epithelium (Fig. 3.5). In the absence of PMN exocytosis, the epithelium was invariably negative to IHC. In 3 cases (1 feline and 2 canine dermatophytoses) there was focal and mild exocytosis of neutrophils to the follicular epithelium but no positive keratinocytes were observed. Lymphocytic mural folliculitis in dermatophytosis was not associated with immunoreaction in the epithelium in any of the samples.

<table>
<thead>
<tr>
<th>SAMPLES</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease</td>
<td>Specie</td>
<td>n</td>
<td>Positive samples (%)</td>
<td>Positivity associated to PMN infiltrate (% of positive samples)</td>
</tr>
<tr>
<td>---------</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Pemphigus foliaceus</td>
<td>F</td>
<td>2</td>
<td>2/2 (100)</td>
<td>2/2 (100)</td>
</tr>
<tr>
<td>Pemphigus foliaceus</td>
<td>C</td>
<td>3</td>
<td>3/3 (100)</td>
<td>3/3 (100)</td>
</tr>
<tr>
<td>Pustular staphylococcal infection</td>
<td>F</td>
<td>1</td>
<td>0/1 (0)</td>
<td>n/a</td>
</tr>
<tr>
<td>Pustular staphylococcal infection</td>
<td>C</td>
<td>5</td>
<td>2/5 (40)</td>
<td>2/2 (100)</td>
</tr>
<tr>
<td>SHPVD without pustules</td>
<td>F</td>
<td>2</td>
<td>0/2 (0)</td>
<td>n/a</td>
</tr>
<tr>
<td>SHPVD without pustules</td>
<td>C</td>
<td>3</td>
<td>0/3 (0)</td>
<td>n/a</td>
</tr>
<tr>
<td>Dermatophytosis</td>
<td>F</td>
<td>3</td>
<td>2/3 (66)</td>
<td>2/2 (100)</td>
</tr>
<tr>
<td>Dermatophytosis</td>
<td>C</td>
<td>5</td>
<td>1/5(20)</td>
<td>1/1 (100)</td>
</tr>
</tbody>
</table>

**Table 3.4:** Summarize of results of COX-2 immunohistochemistry (IHC) on canine and feline inflammatory dermatoses. (C, canine; F, feline; n, number of samples; n/a, not applicable; PMN, polymorphonuclear; SPVD, superficial hyperplastic perivascular dermatitis)
**Figure 3.5.** Representative photomicrographs of COX-2 immunohistochemistry of Inflammatory dermatoses. A: feline pemphigus foliaceous. B: feline dermatophytosis. C: canine pemphigus foliaceous. COX-2 immunoreactivity of keratinocytes is associated with the exocytosis of polymorphonuclear cells in the epidermis and follicular epithelium (arrows).
3.2. Study of the COX-2/PGE2 mechanism in the keratinocyte inflammatory response

3.2.1. Establishment of a model to investigate the biology of the canine keratinocyte as inflammatory cell

3.2.1.1. Introduction

As mentioned in chapter 1, keratinocytes are more than barrier cells that protect the body from the external environment and dehidratation. Keratinocytes have numerous immunologic functions and produce numerous inflammatory and immunologic mediators. Like the immune systems cells, keratinocytes are also considered immune sentinels and have a very important role in the cutaneous inflammatory immune response (Nestle et al. 2009). Keratinocytes are pro-inflammatory effector cells that are strategically positioned at the outermost layer of the body to react in a timely fashion to harmful insults by the coordinated production of antimicrobial peptides, pro-inflammatory cytokines (including type I interferons, interleukins (IL)-1β, IL-6, IL-10, IL-18, tumour necrosis factor), eicosandois (PGE2 being the more important) and chemokines that selectively attract T cells, neutrophils or Langerhan cells. Moreover, keratinocytes display features of antigen presenting cells with the potential for both antigen-specific tolerization and activation. In fact, keratinocytes can function as instigators of cutaneous inflammation (Barker et al. 1991, Williams and Kupper 1996, Steinhoff et al. 2001, Nestle et al. 2009). As mentioned in chapter 1, PGE2 has both physiologic and pathologic roles in the skin. PGE2 and its precursor COX-2 are implicated in numerous cutaneous inflammatory conditions, including solar induced dermatitis and irritant contact dermatitis (reviewed in Müller-Decker 2011).

Identification of keratinocytes as instigators and modulators of cutaneous inflammation has lead to investigate on different drugs and nutrients that modulate their inflammatory response (like cyclosporine A, cortisol, vitamin D, polysaccharides and grape extract as examples) (Tomaino et al. 2006, Cirillo and Prime 2011, Dai et al. 2011, Hibino et al. 2011, Miodovnik et al. 2012).
Within this mark, appears the need to have a simple, well standardized and characterized in vitro model to allow assessing the effects of different agents on the inflammatory response of canine keratinocytes. In the present model we elected to assess PGE2 production by keratinocytes. PGE2 is a well known mediator of cutaneous inflammatory and neoplastic processes (Müller-Decker 2011). PGE2 inhibit keratinocyte apoptosis in inflammatory and neoplastic settings and produces dermal edema and keratinocyte proliferation among other actions. A part of the proinflammatory properties, PGE2 has also anti-inflammatory activites in other tissues including lung, liver or gut (reviewed in Tilley et al. 2001). However, the anti-inflammatory properties of PGE2 in the skin, if present, remain to be clarified and explained.

Against this background, we decided to develop a simple model of canine keratinocyte culture and its PGE2 production.

In the late 1990s the CPEK cell line was developed by CELLnTEC laboratories (Advanced Cell Systems, Bern, Switzerland). CPEK is a spontaneously immortalised keratinocyte cell line derived from the epidermis of an adult Beagle dog. To date, this keratinocyte cell line is the only one available for veterinary research. It has been phenotypically characterized and used in three different in vitro studies (Shibata et al. 2008, Maeda et al. 2009, Shibata et al. 2011a, Shibata et al. 2011b). We decided to use this cell line and PGE2 concentration in the supernatant as a marker of activation and production of inflammatory mediators.

However, the use of this cell line presented two limitations: 1) Being an immortalised cell line, may present some differences with primary keratinocytes and 2) It would not allow to perform interventions and in vivo/in vitro studies in living dogs. For example, working with the cell line would not allow performing studies to assess the response of keratinocytes after a dietary or pharmacologic interventions to the living animal. In consequence, we decided to develop also a more complex model to work also with primary cultures of canine keratinocytes.
3.2.1.2. Materials and Methods

a. Culture and maintenance of the CPEK cell line.

CPEK frozen cells and the CnT-09 keratinocyte culture medium were purchased at CELLnTEC Advanced Cell Systems (Bern, Switzerland). The CnT-09 medium is a keratinocyte growth medium developed by CELLnTEC Advanced Cell Systems. It is composed by medium with bovine fetal calf serum, growth factors and has low calcium content.

Immediately upon reception the frozen 1mL vial was transferred to liquid nitrogen storage tank until thawed. Before thawing the cells, 30 mL of CnT-09 medium divided into two culture flasks of 75 cm² (T-75) were equilibrated for 30 minutes in a 5% CO₂ and 37ºC incubator. This process pre-warms the medium and adjusts its physiological pH. Frozen cells were thawed with gently swirling in water bath at 37ºC during 90 seconds until just melted while still a small amount of ice was left. Cells were resuspended in the cryovial by pipetting up and down three times and 500 µL transferred to each T-75 cell culture flask with the equilibrated CnT-09 medium. After 24 hours the medium was changed to remove the residual DMSO of the freezing medium. Seventy-two hours after thawing, the cells were 90% confluency.

At this stage the first passaging was carried on. CnT-09 medium was aspirated from the T-75 culture flasks. The cells were washed with 8 mL of PBS (without calcium and magnesium) at 37ºC. Five mL of detachment enzyme (TrypLE Express, Gibco, Rockville, MD, USA) were added to the cells and incubated at 37ºC until cells were rounded and started to detach (10 minutes). The culture flask was tapped and under the microscope it was checked that cells had been detached. In order to inhibit the detachment enzyme, 8 mL of CnT-09 medium was added to the cell suspension. Cell suspensions of the two T-75 culture flasks were transferred to a centrifugation tube and centrifuged 5 minutes at 1200 rpm at room temperature.
The supernatant was aspirated and the pellet resuspended in 2 mL of CnT-09. The cells were placed on ice and a 20 μL sample taken to perform cell count with tripan blue dye. Cell counting yielded 10*10^6 cells/mL approximately. For passaging, cell concentration was adjusted to 0.8*10^6 cells/mL and 1mL/culture flask was seeded into four T-75 culture flasks. For freezing, cell concentration was adjusted to 2 x 10^6 cells/mL with cold (4ºC) CnT-09 medium. The same amount of freezing medium (CnT-CRYO-50, CELLnTEC Advanced Cell Systems, Bern, Switzerland) was drop-wisely added while gently swirling the tube. One mL of the suspension was added to labelled, sterile and 4ºC pre-cooled cryotubes. Cryotubes were immediately transferred to a Nalgene® Cryo 1ºC-freezing container “Mr.Frosty” pre-cooled at 4ºC and the “Mr.Frosty” placed at -80ºC overnight. Afterwards, the cryotubes were transferred from -80ºC to liquid nitrogen for long-term storage.

These procedures (passaging and freezing) were repeated eight times during the following two months. This allowed us to create a bank of frozen CPEK cells stored in a liquid nitrogen tank.

b. Isolation and culture of canine primary keratinocytes:

Excision of cutaneous tissue:

Cutaneous tissue was obtained from canine patients undergoing surgery under general anesthesia or from canine patients recently euthanasied for humane reasons. In all cases a previous signed consent form of their owners was obtained. The hair was clipped and the surgical site prepared aseptically. A skin square of 6x3 cm² was excised according to standard surgical methods. Immediately after excision, the cutaneous tissue was placed in DMEM culture medium (Gibco, Rockville, MD, USA) containing 1% Penicillin-streptomycin (Pen Strep, Gibco, Rockville, MD, USA) and transferred to the cell culture laboratory. Time from excision to further processing of the cells was from 15 to 60 minutes.
Isolation of primary canine keratinocytes:

Under aseptic conditions, skin samples were cleaned with 70% etanol bath during 8 minutes followed with a povidone-iodine bath during 8 minutes before starting with cell isolation. Fat tissue and blood vessels were removed from the skin using curved scissors and then samples were washed with PBS in Petri dishes (3 washes of 10 minutes each one) and then cut into small fragments (0.5 cm²). Fragments were placed in a sterile recipient with a magnet inside and 25 mL of 0.05% trypsin-0.02% EDTA (Gibco, Rockville, MD, USA). After 45 minutes of incubation at 37ºC under agitation 25 mL of DMEM (Gibco, Rockville, MD, USA) were added to the tissue suspension. The supernatant was collected and spun at 1200 rpm during 10 minutes. The supernatant was discharged and the pellet was re-suspended with 5 mL of cell culture medium. Culture medium was composed of DMEM/F12 (3:1) with 10% fetal calf serum (FCS) (Gibco, Rockville, MD, USA), 10⁻⁶ M hydrocortisone, 10⁻⁶ M isoproterenol and 10⁻⁷ M insulin (Sigma, St. Louis, MO, USA). The 5 mL suspension of cell culture medium and cells was then transferred to a 25 cm² (T-25) cell culture flask.

Fresh medium was added to cells after discharging the previous one every 48-72 hours. Twenty-four hours after the seeding of the cells few cells were observed attached to the base of the culture flask. Seventy-two hours later, islands of keratinocytes and fibroblasts were observed. At this point, epidermal growth factor (EGF) at 10 ng/mL was added to the medium to stimulate the growth of keratinocytes in front of fibroblasts.

Seventy-two hours later, the keratinocytes were the predominant cell and few islands of fibroblasts could be observed. At this point rapid trypsinization of cells was performed in order to detach fibroblast (which are more weakly attach to the flask base than keratinocytes) and keep the keratinocytes attached to the flask base. Two washes of the cells with PBS at 37ºC was performed before adding 4 mL of 0.05% trypsin-0.02% EDTA (Gibco, Rockville, MD, USA) followed by 3 minutes of incubation at 37ºC. Trypsin-EDTA was inactivated by adding culture medium to the cell culture flask. Detached cells and medium were aspirated and discharged. New culture medium with EGF (at 10 ng/mL) or without EGF depending on the need of rapid growth of
keratinocytes was added to the flask. In 24 hours in case EGF presence or after 48-72 hours if EGF was not added to the medium, confluent cultures of keratinocytes were obtained (figure 2). Cell density in a 25 cm² cell culture flask at this stage was around 4*10⁶ cells/flask. Depending on each different isolation protocol, cells could be passaged between 4 or 7 times without losing their proliferation potential and keeping their normal keratinocyte phenotypic characteristics.

This method proved to be a good, easy and cheap method to obtain primary canine keratinocytes for *in vitro* studies. Other less invasive methods were tried (like trying to obtain hair follicle keratinocytes from epilated hairs or superficial skin scrapings) without success. Basal keratinocytes are the ones with greater proliferative potential within the epidermis and therefore, are the cells that have to be isolated for culture purposes. To obtain basal keratinocytes full thickness skin samples with epidermis and dermis are needed. The reason why the less invasive methods did not prove to be a good source of keratinocytes for *in vitro* culture was that they yielded superficial keratinocytes instead of basal keratinocytes.

c. Establishment of a method to measure PGE2 in keratinocyte cultures supernatants:

*Seeding of cells in 24 wells plaques*

For primary keratinocytes, the cell seeding density for 24 wells plaques was 60,000 cells/well and needed between 5 and 10 days to be 80% confluent. On the contrary, due to its greater proliferation potential, the amount of CPEK cells needed was 35,000 cells/well and in 48 hours were 80-90% confluent.

*Measurement of PGE2 concentration in cell culture supernatants*

Two commercial enzyme linked immunosorben assay (ELISA) kits were tried with similar results: one from R&D Systems (PGE2 Assay –item number KGE004-,
Minneapolis, MN, USA) and one from Cayman (PGE2 EIA Kit-Monoclonal –item number 514010-, Cayman Chemical Company, Ann Arbor, MI, USA).

The final protocol to measure PGE2 in keratinocytes supernatants was the following:

When cells in wells were at 80% confluency, the medium was replaced by 500 µL identical medium supplemented with 10 µM AA (Sigma-Aldrich, St Louis, MO, USA). Cells with the supplemented medium were incubated 15 minutes at 37ºC and 5% CO₂. Afterwards, the supernatants were harvested and centrifuged at 1300 rpm during 10 minutes. Samples were stored at -80ºC until processed with one of the two ELISA kits following the manufacturer instructions.

*Cellular kinetics-PGE2 production study with CPEK cells:*

PGE2 has physiological roles on keratinocytes, mainly on its proliferation and maturation. For this reason, a kinetic study on cell proliferation and PGE2 production was performed using the CPEK cells. We did not use primary keratinocytes for this study because working with primary cells was difficult. The isolation of primary keratinocytes was well standardized, but their proliferation was very different among isolates, making kinetics studies very difficult. This is why we kept working using the only the CPEK keratinocytes. Working with these cells was easier since their proliferation was consistent. PGE2 production was measured (as explained above) by triplicate on CPEK cells supernatants at 50, 60, 74 and 97 hours post-seeding times. After collecting the supernatants for PGE2 concentration dermatination, cells were washed with PBS */- pre-warmed at 37ºC and 500 µL of TrypLE Express (Gibco, Rockville, MD, USA) added. The cells were incubated during 10 minutes at 37ºC until detached and TrypLE Express was inactivated by adding 700 µL of CnT-09 medium. Detached cells and medium were aspirated and placed into an sterile 1.8 mL tube and 20 µL sample taken to perform cell count with the tripan blue exclusion dye test. This process was repeated at 50, 60, 74 and 97 hours post-seeding time.
3.2.1.3. Results:

*PGE2 production by primary keratinocytes and CPEK cells at 80% confluency*

The median production of PGE2 production by primary keratinocytes at 80% confluency was 6600 pg/mL per well. The results were similar that the production of CPEK cell line, which was 7100 pg/mL.

*CPEK cellular kinetics*

As explained before, cell confluency to 80% was reached at 48-50 hours post seeding time. The number of cells between 50 and 74 hours was between 105,000 and 450,000 cells, showing an exponential growth that reached around 1*10^6 cells at 97 hours post-seeding time (Fig. 3.6). Between 50 and 74 hours, there was no mortality but at 97 hours, when cell confluency was 100% with focuses of cornification (Fig. 3.7), cells began to die (table 3.5).

*PGE2 production by CPEK cells in relation to cell number:*

The concentration of PGE2 in the cell culture medium remained constant over time with cell confluency between 80 and 100% corresponding to 48 to 72 hours period after seeding; however, at 96 hours, when cell confluency was over 100% with focuses of cornification, PGE2 concentration in the cell culture medium decreased abruptly (table 3.5 and figure 3.8). Accordingly, when the concentration of PGE2 was calculated per viable cells or total cells, the concentration of PGE2 per cell declined as the number of cells raised (figures 3.6 and 3.9). Because cell mortality was very low and there was almost no difference between total cells and viable cells, the values of PGE2 concentration per cell or per viable cell were very similar (table 3.5).
Figure 3.6. Number of viable CPEK cells at different post-seeding times (48 to 96 hours)

Figure 3.7. CPEK cells at 97 hours post-seeding time. One hundred per cent confluency is observed with focuses of cornification. Optical microscopy (40x).
Table 3.5: CPEK keratinocytes production of PGE2 depending on post-cell-seeding time, cell confluency and cell density.
* 100+: 100% confluency with focuses of cornification

<table>
<thead>
<tr>
<th>Sample #</th>
<th>Post seeding time (hours)</th>
<th>Cell confluency (%)</th>
<th>Cells/mL</th>
<th>Viable cells/mL</th>
<th>PGE2 (pg/mL)</th>
<th>PGE2/cell (pg/mL)</th>
<th>PGE2/viable cell (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>80</td>
<td>140000</td>
<td>140000</td>
<td>7770</td>
<td>0,05550</td>
<td>0,05550</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>80</td>
<td>105000</td>
<td>105000</td>
<td>12900</td>
<td>0,12285</td>
<td>0,12285</td>
</tr>
<tr>
<td>4</td>
<td>60</td>
<td>90</td>
<td>240000</td>
<td>240000</td>
<td>12690</td>
<td>0,05287</td>
<td>0,05287</td>
</tr>
<tr>
<td>5</td>
<td>60</td>
<td>90</td>
<td>170000</td>
<td>170000</td>
<td>8280</td>
<td>0,04870</td>
<td>0,04870</td>
</tr>
<tr>
<td>6</td>
<td>60</td>
<td>90</td>
<td>295000</td>
<td>295000</td>
<td>12120</td>
<td>0,04108</td>
<td>0,04108</td>
</tr>
<tr>
<td>7</td>
<td>74</td>
<td>100</td>
<td>360000</td>
<td>360000</td>
<td>10980</td>
<td>0,03050</td>
<td>0,03050</td>
</tr>
<tr>
<td>8</td>
<td>74</td>
<td>100</td>
<td>435000</td>
<td>430000</td>
<td>16560</td>
<td>0,03807</td>
<td>0,03851</td>
</tr>
<tr>
<td>9</td>
<td>74</td>
<td>100</td>
<td>450000</td>
<td>430000</td>
<td>9510</td>
<td>0,02113</td>
<td>0,02211</td>
</tr>
<tr>
<td>10</td>
<td>97</td>
<td>100+</td>
<td>1075000</td>
<td>1035000</td>
<td>540</td>
<td>0,00050</td>
<td>0,00052</td>
</tr>
<tr>
<td>11</td>
<td>97</td>
<td>100+</td>
<td>970000</td>
<td>955000</td>
<td>480</td>
<td>0,00049</td>
<td>0,00050</td>
</tr>
<tr>
<td>12</td>
<td>97</td>
<td>100+</td>
<td>950000</td>
<td>905000</td>
<td>450</td>
<td>0,00047</td>
<td>0,00049</td>
</tr>
</tbody>
</table>
Figure 3.8. PGE2 concentration in the cell supernatant from 48 to 96 hours post-seeding time.

Figure 3.9. PGE2 production per viable cell at different post-seeding times (48 to 96 hours)
3.2.2. PGE2 production by stimulated keratinocytes

3.2.2.1. Introduction

Once established the method to measure PGE2 in canine keratinocytes cultures and after knowing the kinetics of PGE2 production in non-stimulated cells, we aimed to study the production of PGE2 by keratinocytes stimulated with different concentrations of lipopolysaccharide (LPS) (first part of the experiment) and white blood cells (WBC) (second part of the experiment). Being LPS a potent stimulator of innate immune response and because keratinocytes have demonstrated to respond to it (Chadebech et al. 2003, Pivarsi et al. 2003, Kobayashi et al. 2005, Köllisch et al. 2005, Baümer and Kietamann 2007, Hirasawa et al. 2009), we wanted to know if canine keratinocytes responded to LPS by secreting PGE2 as had preliminary been shown by Baümer and Kietamann (2007). Moreover, PGE2 secretion in response to LPS stimulation in keratinocytes, would allow us to compare it with stimulation with canine WBC containing more than 90% of neutrophils. Results of experiment 1 had shown that keratinocytes expressed COX-2 when a granulocytic infiltrate was present. Because PGE2 is the main COX-2 metabolite in the skin, our hypothesis was that incubation of keratinocytes with neutrophils would stimulate the expression of COX-2 and secretion of PGE2 in the culture medium.

3.2.2.2. Materials and Methods

*CPEK keratinocyte culture:*

The canine keratinocyte cell line CPEK was used in this experiment (CELLnTEC Advanced Cell Sytems CA, Bern, Switzerland). CPEK cells were seeded into 24 well plates (35,000 cell/well) and cultured in CNT-09 medium (CELLnTEC) until 80% confluence (48 hours) as described in the previous chapter (section 3.2).
Stimulation of cultured CPEK keratinocytes with 3 different concentrations of LPS:

Keratinocytes were incubated only with CNT-09 medium (controls) and with CNT-09 with 100, 10 or 1 µg/mL of lipopolysaccharide (LPS, *Escherichia coli*; 0111; B4, Sigma) and incubated during 1, 6, 24 and 48 hours. After each incubation time the cell supernatant was discharged and fresh medium supplemented with AA (10 µM) was added to all keratinocytes cultures. After an additional 15 minutes at 37ºC, cells supernatants were collected and stored at -80ºC until PGE2 measurement was carried on.

Isolation of neutrophils - white blood cells (WBC) -:

Canine blood from a healthy donor was collected by yugular venipuncture into an EDTA treated collecting tube, maintained in ice and processed within 30 minutes. Red blood cell lysing buffer (ACK Lysing Buffer, Lonza Group Ltd., Basel, Switzerland) was added at a ratio of 1:2 (blood: buffer lysis) and incubated 5 minutes at room temperature. Afterwards, the lysed blood sample was centrifuged at 1200 rpm during 8 minutes and the supernantant discharged. The buffy coat was carefully resuspended in CNT-09. The cell suspension differential count yielded 94% of neutrophils, 3% of monocytes, 1% of lymphocytes and 1% of eosinophils. Viability of the cells, which were in a concentration of 2.435 x 10⁶ cells/mL, was greater than 99% as determined by tripan blue dye exclusion. The whole cell isolation procedure was carried out steriley at room temperature.

Stimulation of CPEK keratinocytes with LPS and neutrophils:

Results of the previous experiment stimulating keratinocytes with different concentrations of LPS showed that the higher concentration of LPS used to stimulate keratinocytes was necessary to obtain a slight increase in PGE2 concentration in the cells supernatant and this concentration of LPS did not cause mortality among keratinocytes (see results in section 3.2.2.3). Because that, we elected to use 100 µg/mL of LPS in this experiment.
CPEK keratinocytes were incubated with CnT-09 medium alone (controls), with CnT-09 medium with 100 µg/mL of LPS (LPS, Escherichia coli; 0111; B4, Sigma-Adrich) or with CnT-09 medium and WBC suspension (with 94% of neutrophils). WBC was added at a concentration of 1.2175 x 10^6 cells/well. Incubation times were 2, 24 and 48 hours. After these times, the keratinocytes incubated with WBC were washed twice with phosphate buffered saline free of magnesium and calcium (PBS−/−) in order to eliminate all WBC. Microscopic inspection of the wells confirmed that the vast majority of WBC had been eliminated after the second wash with PBS−/− (see figure 3.10). CNT-09 and CNT-09 with LPS was discharged from control and LPS stimulated keratinocytes. Fresh CNT-09 medium supplemented with arachidoic acid (10 µM) was added to all keratinocytes cultures. After an additional 15 minutes at 37ºC, cells supernatants were collected and stored at -80ºC until PGE2 measurement was carried on.

Measurement of PGE2:

PGE2 concentration was determined in culture supernatants using a non-radioactive enzyme-linked immuno-assay (EIA) from Cayman Chemical (Ann Arbor, MI, USA). The supernatants were diluted 1:50 in EIA Buffer, PGE2 determined according to the manufacturer’s protocol and results were expressed in pg/mL PGE2. Cross-reactivities were <0.04% for 6-keto PGF1, and <0.01% for LTB4, TXB2, and AA. Three determinations for each variable was done and each one was read twice with the EIA.

The differences in PGE2 concentration in the cell supernatants, cell counts and cell mortality were evaluated using non-parametric tests (Mann-Whitney U test), with P < 0.05 regarded as statistically significant.
Figure 3.10: A: control CPEK keratinocytes incubated with CNT-09. B, C and D: CPEK keratinocytes incubated 2 hours with white blood cells (94% neutrophils). B numerous round cells are observed on the keratinocyte underlying monolayer before PBS washes. C: after the first PBS wash, much less WBC are observed on the keratinocyte monolayer. D: keratinocyte monolayer after the second PBS wash: no WBC are detected. Optical microscopy (40x).
3.2.2.3. Results

*PGE2 production by keratinocytes (CPEK) incubated with LPS at different concentrations:*

There were no differences among the concentrations of PGE2 in the cell culture medium of keratinocytes incubated with LPS at 100, 10 and 1 µg/mL or without LPS (control cells) (Fig. 3.11 and table 3.6). The concentration of PGE2 remained the same for the 4 groups of cells until 24 hours post-stimulation (72 hours post seeding). After that time, at 48 hours post-stimulation (96 hours post seeding), some of the cells incubated with LPS at 100 µg/mL produced more PGE2 (Fig. 3.11 and table 3.5). The curve of PGE2 production per viable cell was the same as for the previous experiment with a general proportional reduction of PGE2 as the cell density increased. However, at 48 hours post-stimulation (96 hours post seeding), two samples (one of keratinocytes incubated with 100 µg/mL and one of control cells) produced more PGE2 (Fig. 3.12). The cellular proliferation remained with the same exponential growth as with keratinocytes (Fig. 3.13) and stabilized at 24 hours post-stimulation (72 hours post-seeding); however, the cell incubated with different concentrations of LPS proliferated more than control cells (keratinocytes without LPS) (Fig. 3.13). The proliferation curves of total cells and viable cells were very similar; indicating that cells incubated with LPS had no more mortality than control cells (Fig. 3.13, Fig. 3.14 and table 3.6).
Figure 3.11: PGE2 production by CPEK keratinocytes incubated at different concentrations of LPS (LPS 100, LPS 10 and LPS 1 corresponding to LPS at 100, 10 and 1 µg/mL) or without LPS (LPS 0).

Figure 3.12: Production of PGE2 per viable CPEK keratinocyte incubated at different concentrations of LPS (LPS 100, LPS 10 and LPS 1 corresponding to LPS at 100, 10 and 1 µg/mL) or without LPS (LPS 0).
Table 3.6: CPEK keratinocyte production of PGE2 depending on LPS incubation at different concentrations

<table>
<thead>
<tr>
<th>Sample #</th>
<th>LPS (µg/mL)</th>
<th>Post seeding time (hours)</th>
<th>Post stimulation time (hours)</th>
<th>Cell Confluency (%)</th>
<th>Cells/mL</th>
<th>Viable cells/mL</th>
<th>PGE2 (pg/mL)</th>
<th>PGE2/viable cell (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>49</td>
<td>1</td>
<td>80</td>
<td>280000</td>
<td>230.000</td>
<td>9270</td>
<td>0.04030</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>49</td>
<td>1</td>
<td>80</td>
<td>255000</td>
<td>235000</td>
<td>10860</td>
<td>0.04621</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>49</td>
<td>1</td>
<td>80</td>
<td>185000</td>
<td>180000</td>
<td>11760</td>
<td>0.06533</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>49</td>
<td>1</td>
<td>80</td>
<td>170000</td>
<td>165000</td>
<td>10110</td>
<td>0.06127</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>49</td>
<td>1</td>
<td>80</td>
<td>185000</td>
<td>185000</td>
<td>9390</td>
<td>0.05075</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>49</td>
<td>1</td>
<td>80</td>
<td>185000</td>
<td>180000</td>
<td>9390</td>
<td>0.05216</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>49</td>
<td>1</td>
<td>80</td>
<td>155000</td>
<td>145000</td>
<td>9510</td>
<td>0.06558</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>49</td>
<td>1</td>
<td>80</td>
<td>120000</td>
<td>120000</td>
<td>8730</td>
<td>0.07275</td>
</tr>
<tr>
<td>9</td>
<td>100</td>
<td>54</td>
<td>1</td>
<td>80</td>
<td>250000</td>
<td>230000</td>
<td>9210</td>
<td>0.03684</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>54</td>
<td>6</td>
<td>80-90</td>
<td>215000</td>
<td>215000</td>
<td>6840</td>
<td>0.03181</td>
</tr>
<tr>
<td>11</td>
<td>10</td>
<td>54</td>
<td>6</td>
<td>80-90</td>
<td>210000</td>
<td>205000</td>
<td>10950</td>
<td>0.05341</td>
</tr>
<tr>
<td>12</td>
<td>10</td>
<td>54</td>
<td>6</td>
<td>80-90</td>
<td>335000</td>
<td>310000</td>
<td>8880</td>
<td>0.02864</td>
</tr>
<tr>
<td>13</td>
<td>1</td>
<td>54</td>
<td>6</td>
<td>80-90</td>
<td>245000</td>
<td>230000</td>
<td>7710</td>
<td>0.03352</td>
</tr>
<tr>
<td>14</td>
<td>1</td>
<td>54</td>
<td>6</td>
<td>80-90</td>
<td>190000</td>
<td>170000</td>
<td>6600</td>
<td>0.03882</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>54</td>
<td>6</td>
<td>80-90</td>
<td>165000</td>
<td>155000</td>
<td>8190</td>
<td>0.05283</td>
</tr>
<tr>
<td>16</td>
<td>0</td>
<td>54</td>
<td>6</td>
<td>80-90</td>
<td>215000</td>
<td>200000</td>
<td>8520</td>
<td>0.04260</td>
</tr>
<tr>
<td>17</td>
<td>100</td>
<td>72</td>
<td>24</td>
<td>100</td>
<td>730000</td>
<td>715000</td>
<td>8820</td>
<td>0.01233</td>
</tr>
<tr>
<td>18</td>
<td>100</td>
<td>72</td>
<td>24</td>
<td>100</td>
<td>725000</td>
<td>720000</td>
<td>16920</td>
<td>0.02350</td>
</tr>
<tr>
<td>19</td>
<td>10</td>
<td>72</td>
<td>24</td>
<td>100</td>
<td>580000</td>
<td>565000</td>
<td>13560</td>
<td>0.02400</td>
</tr>
<tr>
<td>20</td>
<td>10</td>
<td>72</td>
<td>24</td>
<td>100</td>
<td>620000</td>
<td>530000</td>
<td>12600</td>
<td>0.02377</td>
</tr>
<tr>
<td>21</td>
<td>1</td>
<td>72</td>
<td>24</td>
<td>100</td>
<td>640000</td>
<td>605000</td>
<td>9510</td>
<td>0.01571</td>
</tr>
<tr>
<td>22</td>
<td>1</td>
<td>72</td>
<td>24</td>
<td>100</td>
<td>610000</td>
<td>540000</td>
<td>8580</td>
<td>0.01588</td>
</tr>
<tr>
<td>23</td>
<td>0</td>
<td>72</td>
<td>24</td>
<td>100</td>
<td>535000</td>
<td>335000</td>
<td>7560</td>
<td>0.02256</td>
</tr>
<tr>
<td>24</td>
<td>0</td>
<td>72</td>
<td>24</td>
<td>100</td>
<td>450000</td>
<td>360000</td>
<td>13200</td>
<td>0.03666</td>
</tr>
<tr>
<td>25</td>
<td>100</td>
<td>96</td>
<td>48</td>
<td>100</td>
<td>910000</td>
<td>675000</td>
<td>17370</td>
<td>0.02573</td>
</tr>
<tr>
<td>26</td>
<td>100</td>
<td>96</td>
<td>48</td>
<td>100</td>
<td>900000</td>
<td>780000</td>
<td>67230</td>
<td>0.08619</td>
</tr>
<tr>
<td>27</td>
<td>10</td>
<td>96</td>
<td>48</td>
<td>100</td>
<td>645000</td>
<td>590000</td>
<td>9720</td>
<td>0.01647</td>
</tr>
<tr>
<td>28</td>
<td>10</td>
<td>96</td>
<td>48</td>
<td>100</td>
<td>765000</td>
<td>660000</td>
<td>14280</td>
<td>0.02163</td>
</tr>
<tr>
<td>29</td>
<td>1</td>
<td>96</td>
<td>48</td>
<td>100</td>
<td>765000</td>
<td>645000</td>
<td>16320</td>
<td>0.02530</td>
</tr>
<tr>
<td>30</td>
<td>1</td>
<td>96</td>
<td>48</td>
<td>100</td>
<td>710000</td>
<td>605000</td>
<td>25140</td>
<td>0.04155</td>
</tr>
<tr>
<td>31</td>
<td>0</td>
<td>96</td>
<td>48</td>
<td>100</td>
<td>440000</td>
<td>390000</td>
<td>15060</td>
<td>0.03861</td>
</tr>
<tr>
<td>32</td>
<td>0</td>
<td>96</td>
<td>48</td>
<td>100</td>
<td>415000</td>
<td>405000</td>
<td>36810</td>
<td>0.09089</td>
</tr>
</tbody>
</table>
**Figure 3.13:** Number of total CPEK cells at different post-stimulation times depending on LPS concentration in the cell medium. LPS 100, LPS 10 and LPS 1 corresponding to LPS at 100, 10 and 1 μg/mL and LPS 0 as cells incubated without LPS.

**Figure 3.14:** Number of viable CPEK cells at different post-stimulation times depending on LPS concentration in the cell medium. LPS 100, LPS 10 and LPS 1 corresponding to LPS at 100, 10 and 1 μg/mL) and LPS 0 as cells incubated without LPS.
4.3.2. PGE2 production by keratinocytes (CPEK) incubated with LPS and neutrophils (WBC):

PGE2 concentration in the keratinocyte (CPEK) culture medium after incubation with WBC (in which neutrophils (PMNN) represented 94% of the cells) during 2, 24 and 48 hours was significantly more elevated than PGE2 concentration in the keratinocyte culture medium after incubation with LPS at a 100µg/mL or in keratinocytes alone (control cells) (Fig. 3.15, Fig. 3.16 and table 3.7). Two hours of incubation with WBC was enough to produce an almost 3 fold production of PGE2 by keratinocytes compared to control and LPS-stimulated keratinocytes. This difference in PGE2 production raised to the maximum (7.5 fold) at 24h and, even not that high, the production of PGE2 by WBC stimulated keratinocytes kept 5 fold higher than control and LPS-stimulated cells at 48 hours incubation time. Keratinocytes incubated with LPS produced more PGE2 than control cells but this difference was not significant (Figl. 3.16 and table 3.7). These results confirmed the findings of the previous experiment that found no significant difference in PGE2 production between LPS stimulated and non-stimulated keratinocytes, even at high LPS concentration (100 µg/mL) The same difference was noted when PGE2 per viable cell was calculated (table 3.7).

Regarding total cell count, there were no differences in viable cells in different mediums in any incubation time, there was difference in total cell count at 48 hours incubation time between control cells and keratinocytes incubated with PMNN and there were almost differences between control cells and keratinocytes incubated with PMNN and controls cells and keratinocytes incubated with LPS. The proliferation of cells depending on the cell medium (alone, with LPS or with neutrophils) was similar for the 3 groups of cells at 2 and 24h incubation times, and the statistically significant difference noted was with total cell count at 48 hours incubation period between keratinocytes incubated with neutrophils (717,500 cells) and control cells (450,000) in which the p value was p=0.021 (table 3.8). The presence of LPS or neutrophils did not increase the rate of mortality of keratinocytes statistically significant in any incubation time, despite it was higher (but not statiscally significant with p values of 0.057) in these groups compared to keratinocytes at 48 hours (table 3.8).
Figure 3.15: PGE2 production by non-stimulated CPEK keratinocytes (kcs) and keratinocytes incubated with white blood cells (containing 96% of neutrophils (PMNN)) or with 100 µg/mL of lipopolysaccharide (LPS).
Figure 3.16: PGE2 concentration in wells supernatants of non-stimulated CPEK keratocytes (kcs) and keratocytes incubated with white blood cells (containing 96% of neutrophils (N)) or with 100 µg/mL of lipopolysaccharide (LPS). A: 2 hours incubation time; B: 24 hours incubation time; C: 48 hours incubation time. * p <0.05
<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Stimulus</th>
<th>PGE2 (pg/mL) (median [range])</th>
<th>PGE2/viable cell (pg/mL) (median [range])</th>
</tr>
</thead>
<tbody>
<tr>
<td>2h</td>
<td>None</td>
<td>2660 (1060-32059)</td>
<td>0.0079 (0.002944-0.009712)</td>
</tr>
<tr>
<td></td>
<td>LPS 100</td>
<td>2970 (2490-5990)</td>
<td>* 0.0094 (0.00673-0.019967)</td>
</tr>
<tr>
<td></td>
<td>PMNN</td>
<td>7760 (6900-8530)</td>
<td>* 0.0253 (0.022056-0.03)</td>
</tr>
<tr>
<td>24h</td>
<td>None</td>
<td>2407.5 (2180-29859)</td>
<td>0.0029 (0.002224-0.006708)</td>
</tr>
<tr>
<td></td>
<td>LPS 100</td>
<td>2875 (2250-3050)</td>
<td>* 0.0031 (0.002564-0.007349)</td>
</tr>
<tr>
<td></td>
<td>PMNN</td>
<td>19745 (16065-22875)</td>
<td>* 0.0365 (0.03-0.4867)</td>
</tr>
<tr>
<td>48h</td>
<td>None</td>
<td>3022 (2675-3115)</td>
<td>0.0069 (0.005877-0.008231)</td>
</tr>
<tr>
<td></td>
<td>LPS 100</td>
<td>3830 (2480-4340)</td>
<td>* 0.0081 (0.004593-0.009136)</td>
</tr>
<tr>
<td></td>
<td>PMNN</td>
<td>17632.5 (15180-21450)</td>
<td>* 0.0261 (0.023833-0.047185)</td>
</tr>
</tbody>
</table>

**Table 3.7**: Production of PGE2 by non-stimulated CPEK keratinocytes (kcs) and keratinocytes incubated with LPS at 100 µg/mL (LPS 100) or neutrophils (PMNN). *p < 0.05
Table 3.8: Cellular proliferation of non-stimulated CPEK keratinocytes (kcs) and keratinocytes incubated with LPS at 100 µg/mL (LPS 100) or neutrophils (PMNN). *: p < 0.05

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Stimulus</th>
<th>Total cells/mL (median (range))</th>
<th>Viable cells/mL (median (range))</th>
<th>Mortality (%) (median (range))</th>
</tr>
</thead>
<tbody>
<tr>
<td>2h</td>
<td>None</td>
<td>352500</td>
<td>345000</td>
<td>1.35 (0.00-2.94)</td>
</tr>
<tr>
<td></td>
<td>LPS 100</td>
<td>320000</td>
<td>315000</td>
<td>3.22 (0.00-3.90)</td>
</tr>
<tr>
<td></td>
<td>PMNN</td>
<td>325000</td>
<td>320000</td>
<td>1.06 (0.00-3.23)</td>
</tr>
<tr>
<td>24h</td>
<td>None</td>
<td>870000</td>
<td>850000</td>
<td>2.33 (1.01-10.10)</td>
</tr>
<tr>
<td></td>
<td>LPS 100</td>
<td>845000</td>
<td>822500</td>
<td>2.69 (0.45-8.79)</td>
</tr>
<tr>
<td></td>
<td>PMNN</td>
<td>530000</td>
<td>502500</td>
<td>6.15 (0.93-9.62)</td>
</tr>
<tr>
<td>48h</td>
<td>None</td>
<td>450000</td>
<td>442500</td>
<td>1.73 (0.93-8.45)</td>
</tr>
<tr>
<td></td>
<td>LPS 100</td>
<td>590000</td>
<td>490000</td>
<td>11.03 (4.35-25.4)</td>
</tr>
<tr>
<td></td>
<td>PMNN</td>
<td>717500</td>
<td>600000</td>
<td>16.34 (5.76-28.32)</td>
</tr>
</tbody>
</table>
3.3. References


Mohammed SI, Khan KNM, Sellers RS et al. (2004). Expression of cyclooxygenase-1 and


4. DISCUSSION
5. CONCLUSIONS
6. SUMMARY / RESUM
Summary

Cyclooxygenase (COX)-2 and its main metabolite, prostaglandin E2 (PGE2), are important players in the skin physiology (maturation and proliferation of keratinocytes) and pathophysiology (wound healing, skin inflammation and carcinogenesis). Despite the evidence that suggests that COX-2 derived prostaglandins contribute to carcinogenesis, conflicting results have been reported from numerous human and mammalian studies. Moreover, the involvement of the COX-2/PGE2 route in cutaneous inflammatory processes in companion animals have not been investigated. This has left a desert field regarding therapeutic strategies involving the pharmacologic manipulation of the COX-2/PGE2 system in these species.

In this thesis COX-2 expression on cutaneous pre-neoplastic (actinic keratosis (AK)), neoplastic (squamous cell carcinoma (SCC), melanocytic neoplasias and cutaneous epitheliotropic T cell lymphoma (CETL)) and inflammatory disorders with an epidermal involvement was studied.

We detected COX-2 expression in non-neoplastic canine, feline and equine keratinocytes present in neoplastic or inflammatory dermatoses. COX-2 expression was also detected in pre-neoplastic and neoplastic canine and feline keratinocytes of the AK and SCC samples. A low number of canine and equine melanocytes in the melanocytic neoplasias also expressed COX-2. However, and except for the AK samples, in all these cases COX-2 expression was observed mainly at the surface of the samples and was highly associated to the presence of infiltrating granulocytes. On the contrary, we did not detect COX-2 expression in neoplastic lymphocytes of CETL or in neoplastic and non-neoplastic keratinocytes and neoplastic melanocytes in the absence of a polymorphonuclear infiltrate.

We set up an in vitro model to study the keratinocyte inflammatory response and evaluated the in vitro production of PGE2 in response to different stimuli. Keratinocytes cultures were stimulated with different concentrations of LPS and with canine white blood cells (WBC) (containing more than 90% of neutrophils). We could
observe that PGE2 production by kerationcytes was highly associated to the presence of WBC (neutrophils) in the culture media.

Overall, our results suggest that the COX-2/PGE2 pathway may be involved in the AK pathogenesis as in its human counterpart. However, it is not an essential mechanism in the carcinogenesis process in SCC, melanocytic neoplasias and CETL in the canine, feline and equine species. It is suggested that COX-2 expression or PGE2 production is an epiphenomenon associated to the inflammatory response in these neoplasias and in neutrophilic inflammatory dermatoses.
Resum

La ciclooxigenasa (COX)-2 i els seu metabòlit principal, la prostaglandina E2 (PGE2), són importants en la fisiologia cutània (maduració i proliferació dels queratinòcits) i la seva patofisiologia (processos de cicatrització, inflamació cutània i carcinogènesi). Malgrat l’evidència que suggereix que les prostaglandines derivades de la COX-2 contribueixen a la carcinogènesi, elsresultats de múltiples estudis en humans i mamífers són sovint contradictoris. Endemés, el paper de la ruta COX-2/PGE2 en els processos inflamatoris cutanis en animals de companyia no s’ha investigat, deixant desert el camp de les estratègies terapèutiques que involucren la manipulació farmacològica d’aquest sistema en aquestes espècies.

En aquesta tesi, hem estuddiat l’expressió de la COX-2 en lesions cutànies pre-neoplàstiques (queratosis actíniques (QA)), neoplàstiques (carcinoma de cèl·lules escatoses (CCE), neoplàstiques melanocitàques, limfoma cutani epiteliotòpic de cèl·lules T (LCECT)) i en dermatosis inflamatories amb afectació epidèrmica.

Els resultats han revelat expressió de COX-2 en queratinòcits no neoplàstics canins, felins i equins de les dermatosis inflamatories i neoplàstiques estudiades. L’expressió de COX-2 es va detectar també en queratinòcits pre-neoplàstics i neoplàstics canins i felins de les mostres de QA i CEE. I un baix nombre de melanòcits neoplàstics dels processos cancerosos melanocitàtics també expressaren COX-2. Tot i així, i amb l’excepció de les mostres de QA, en tots aquests casos l’expressió de COX-2 es va observar sempre a la superfície de les mostres i majoritàriament en aquelles cèl·lules que tenien un infiltrat inflamatori granulocitàtic al seu voltant. En canvi, no vàrem detectar l’expressió de COX-2 en els limfòcits neoplàstics del LCECT. Tampoc no vàrem detectar expressió de COX-2 en queratinòcits (neoplàstics i no neoplàstics) o melanòcits neoplàstics en absència d’un infiltrat inflamatori de cèl·lules polimorfonuclears.

Es va posar a punt un model in vitro per estudiar la resposta inflamatòria dels queratinòcits. Amb aquest model es va estudiar la producció de PGE2 per part
d’aquestes cèl·lules davant de diferents estímuls. Aquestes cèl·lules es varen estimular amb diferents concentracions de lipopolisacàrid i amb leucòcits sanguinis canins (que contenien més d’un 90% de neutròfils). Amb aquests estudis vàrem poder observar que la producció de PGE2 per part dels queratinòcits era molt elevada quan aquests s’havien cultivat en presència de leucòcits circulants (principalment neutròfils).

Tots aquests resultats suggereixen que la via COX-2/PGE2 podria estar involucrada en la patogènia de les QA tal i com ho està en la QA humana. En canvi, indiquen que aquest no és un mecanisme essencial en el procés carcinogènic del CCE, neoplàsies melanocitiques i LECT en les espècies canina, felina i equina. Els nostres resultats suggereixen, en canvi, que l’expressió de COX-2 o producció de PGE2 és un epifenomen associat a la resposta inflamatòria d’aquestes neoplàsies i en les dermatosis inflamatòries neutrofíliques.
7. ANNEX
NEOPLASTIC DISEASE

Immunohistochemical Detection of COX-2 in Feline and Canine Actinic Keratoses and Cutaneous Squamous Cell Carcinoma

M. Bardagí, D. Fondevila and L. Ferrer

Department of Animal Medicine and Surgery, Universitat Autònoma de Barcelona, Spain

Summary

Cyclooxygenase-2 (COX-2) overexpression and its causal role in epidermal carcinogenesis have been demonstrated in human actinic keratoses (AK) and cutaneous squamous cell carcinoma (SCC). The aim of this study was to determine immunohistochemically the level of expression of COX-2 in feline and canine AK \( (n = 18) \), SCC \( (n = 36) \) and inflammatory dermatoses \( (n = 24) \). COX-2 immunoreactivity was detected in all feline and canine SCC. In all specimens, labelled basal and suprabasal neoplastic keratinocytes were localized within and below areas of superficial erosion or ulceration and only scattered deeper tumour cells were positively labelled. In most cases, positive immunoreactivity of keratinocytes was associated with the presence of granulocytes. COX-2 expression was detected in 3/9 canine and 4/9 feline cases of AK and in only one case was associated with inflammation. Inflammatory dermatoses were characterized by positively labelled epidermal and follicular basal and suprabasal keratinocytes that were always associated with granulocyte exocytosis. These results indicate that further study of the effect of using COX-2 inhibitors in the management and prevention of feline and canine cutaneous SCC is warranted. The association between inflammatory cells and COX-2 expressing epidermal cells opens a new line of research regarding the role of COX-2 in SCC oncogenesis. Moreover, further studies should investigate the role of COX-2 in the pathogenesis and management of AK in animals.

Correspondence to: M. Bardagí. (e-mail: Mar.Bardagi@uab.cat).

Introduction

Cyclooxygenase (COX) isoenzymes COX-1 and COX-2 catalyze the initial step in the biosynthesis of prostaglandins (PGs) from arachidonic acid (Dubois et al., 1998). While there are notable exceptions, a simplified view is that COX-1 is constitutively expressed throughout the body and catalyzes the synthesis of PG involved in homeostasis, whereas COX-2, generally regarded as the inducible isoenzyme, is found primarily at sites of inflammation and its main role is to aid in producing PGE\(_2\) (Jones and Budsberg, 2000; Rouzer and Marnett, 2009). A multitude of inflammatory signals, mitogens and carcinogens can induce COX-2 activity in numerous cell types (Jones et al., 1993).

Human epidemiological, pharmacological, clinical and experimental studies document the importance of PG signalling in carcinogenesis and suggest that the long-term use of non-steroidal anti-inflammatory drugs (NSAIDs), particularly the highly selective COX-2 inhibitors, is linked to a lower incidence of several types of epithelial neoplasms, including premalignant actinic keratoses (AK) and squamous cell carcinoma (SCC) (Fischer et al., 1999; Dempke et al., 2001; Shaheen et al., 2002; Thun et al., 2002; Butler et al., 2005; Greenhough et al., 2009). As reviewed by Rundhaug et al. (2007), human and murine studies have demonstrated that an increase in COX-2 expression, leading to increased levels of PGE\(_2\), is one of the early events in ultraviolet (UV) light-induced skin carcinogenesis and COX-2 overexpression and its causal role in epidermal carcinogenesis has been demonstrated in AK and cutaneous SCC (Buckman et al., 1998; An et al., 2002; Müller-Decker and Fürstenberger, 2007). Moreover, studies in vitro have shown that skin cancer cell growth is suppressed by
inhibition of COX-2 expression, and several trials have reported significant regression of AK lesions after topical treatment with diclofenac, a potent inhibitor of COX-2 (Rivers and McLean, 1997; Higashi et al., 2000; Wolf et al., 2001; Rivers et al., 2002; Gebauer et al., 2003; Nelson et al., 2004). As reviewed by Greenhough et al. (2009), the carcinogenic potential of the COX-2/PGE2 pathway is mediated by its ability to induce cell proliferation, evasion of apoptosis and insensitivity to anti-growth signals, promotion of angiogenesis and tissue invasion and metastasis and evasion of the anti-tumour immune response.

COX-2 expression has been shown in canine and equine cutaneous SCC (Pestili de Almeida et al., 2001; Elce et al., 2007; Thamm et al., 2008), but not in feline cutaneous SCC or normal canine skin (Beam et al., 2003; Hamamoto et al., 2009). COX-2 expression in AK in animals has not been investigated.

The initial aim of the present study was to investigate immunohistochemically COX-2 expression in feline and canine AK. However, since preliminary results had also shown COX-2 expression in feline SCC and a possible correlation between COX-2 expression and inflammation, we also aimed to investigate the expression of COX-2 in feline and canine SCC and in inflammatory dermatoses and its possible correlation with inflammatory infiltration.

**Materials and Methods**

**Tissue Samples**

Seventy-eight samples were selected from the archive of the Veterinary Pathology Diagnostic Service of the Universitat Autònoma de Barcelona. These included 18 cases of AK (nine each feline and canine), 36 cases of SCC (27 feline and nine canine) and 24 inflammatory dermatoses. Inflammatory dermatoses included pemphigus foliaceus (two feline and three canine cases), pustular staphylococcal infection (one feline and five canine cases), superficial hyperplastic perivascular dermatitis (SHPVD) without pustules (two feline and three canine cases) and dermatophytosis (three feline and five canine cases). The clinical history of each case was reviewed in order to exclude samples from animals that had received glucocorticoid or NSAID treatment prior to biopsy sampling.

**Immunohistochemistry**

The standard streptavidin—biotin technique was used for the detection of COX-2 protein by immunohistochemistry (IHC). Sections (5 μm) from formalin-fixed and paraffin wax-embedded tissues were dewaxed and endogenous peroxidase activity was quenched by exposure to H2O2 3% in distilled water for 5 min. Bleaching with 0.25% potassium permanganate for 30 min followed by 1% oxalic acid for 5 min was performed subsequently. Heat-induced epitope retrieval was carried out at 90–100°C in citrate buffer (pH 6.0) for 10 min with 30 min of cooling at ambient temperature. The sections were then blocked with 2% bovine serum albumin (Sigma--Aldrich, St Louis, Missouri, USA). The primary antibody (polyclonal rabbit anti-murine COX-2; Cayman Chemical, Ann Arbor, Michigan, USA) was applied, and slides were incubated with biotinylated polyclonal goat anti-rabbit secondary antibody (Dako, Glostrup, Denmark) for 1 h and then with an avidin–biotin–peroxidase complex (Immunopure; Thermo Fisher Scientific, Rockford, Illinois, USA) was applied, and slides were incubated overnight at 4°C. Slides were then incubated with biotinylated polyclonal goat anti-rabbit secondary antibody (Dako, Glostrup, Denmark) for 1 h and then with an avidin–biotin–peroxidase complex (Immunopure; Thermo Fisher Scientific, Rockford, Illinois, USA) for 1 h. Labelling was ‘visualized’ with 3,3′diaminobenzidine tetrahydrochloride (Sigma–Aldrich) and counterstained with haematoxylin. Negative control sections were included in each

<table>
<thead>
<tr>
<th>Disease</th>
<th>Species</th>
<th>n</th>
<th>Positively labelled samples (%)</th>
<th>COX-2 expression associated with granulocytic inflammation (%) of positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCC</td>
<td>Feline</td>
<td>27</td>
<td>27/27 (100)</td>
<td>21/27 (77)</td>
</tr>
<tr>
<td>SCC</td>
<td>Canine</td>
<td>9</td>
<td>9/9 (100)</td>
<td>8/9 (88)</td>
</tr>
<tr>
<td>AK</td>
<td>Feline</td>
<td>9</td>
<td>4/9 (44)</td>
<td>0/4 (0)</td>
</tr>
<tr>
<td>AK</td>
<td>Canine</td>
<td>9</td>
<td>3/9 (33)</td>
<td>1/3 (33)</td>
</tr>
<tr>
<td>Pemphigus foliaceus</td>
<td>Feline</td>
<td>2</td>
<td>2/2 (100)</td>
<td>2/2 (100)</td>
</tr>
<tr>
<td>Pemphigus foliaceus</td>
<td>Canine</td>
<td>3</td>
<td>3/3 (100)</td>
<td>3/3 (100)</td>
</tr>
<tr>
<td>Pustular staphylococcal infection</td>
<td>Feline</td>
<td>1</td>
<td>0/1 (0)</td>
<td>n/a</td>
</tr>
<tr>
<td>Pustular staphylococcal infection</td>
<td>Canine</td>
<td>5</td>
<td>2/5 (40)</td>
<td>2/2 (100)</td>
</tr>
<tr>
<td>SHPVD without pustules</td>
<td>Feline</td>
<td>2</td>
<td>0/2 (0)</td>
<td>n/a</td>
</tr>
<tr>
<td>SHPVD without pustules</td>
<td>Canine</td>
<td>3</td>
<td>0/3 (0)</td>
<td>n/a</td>
</tr>
<tr>
<td>Dermatophytosis</td>
<td>Feline</td>
<td>3</td>
<td>2/3 (66)</td>
<td>2/2 (100)</td>
</tr>
<tr>
<td>Dermatophytosis</td>
<td>Canine</td>
<td>5</td>
<td>1/5 (20)</td>
<td>1/1 (100)</td>
</tr>
</tbody>
</table>

n/a, not applicable.
experiment and comprised sections incubated with normal rabbit serum in place of the primary antiserum. The anti-COX-2 reagent used in this study has been shown previously to detect COX-2 in feline macula densa (Newman and Mrkonjich, 2006).

**Results**

Expression of COX-2 was observed in samples of AK, SCC and inflammatory dermatoses. The pattern of immunoreactivity in keratinocytes was granular cytoplasmic and located mainly in the perinuclear zone.

All feline and canine SCC contained positively labelled neoplastic cells (Table 1). Labelling was heterogeneous throughout the tumour sections. Basal cells and more differentiated cells were labelled, mainly in and below areas of superficial erosion or ulceration, but positively labelled tumour cells were also observed to be scattered deeper in all samples (Fig 1). In most cases, the positive labelling of keratinocytes was associated with the presence of granulocytes (Fig. 1). There was no COX-2 expression at the non-eroded/ulcerated margins of the tumours. In 6/27 feline cases and 1/9 canine cases, positively labelled neoplastic keratinocytes were observed without the presence of an associated inflammatory response.

Four out of nine feline and three out of the nine canine samples of AK expressed COX-2 (Table 1). Immunoreactivity was observed in basal epidermal cells,

---

Fig. 1. (A–D) Sections of feline SCC. (E–F) Section of canine SCC. (B), (D) and (F) are high-power magnifications of the indicated areas of (A), (C) and (E), respectively. Expression of COX-2 by neoplastic keratinocytes is observed mainly at the eroded surface of the tumours (arrows), but scattered deeper positive cells are also present (arrowheads). Labelling of keratinocytes is associated with the presence of granulocytes (asterix). IHC.
but scattered suprabasal cells were also labelled (Fig. 2). The positively labelled cells were in the proximity of an inflammatory infiltrate only in one canine sample.

Of the 24 samples of inflammatory dermatoses tested, labelled keratinocytes were observed in four feline (two pemphigus foliaceus and two dermatophytoses) and six canine (three pemphigus foliaceus, two pustular staphylococcal infections and one dermatophytosis) cases (Table 1). In all samples, basal and suprabasal labelled keratinocytes were always associated with exocytosis of granulocytes into the epidermis or follicular epithelium (Fig. 3). In the absence of granulocyte exocytosis the epithelium invariably failed to express COX-2. In three cases (one feline and two canine cases of dermatophytoses) there was focal and mild exocytosis of neutrophils into the follicular epithelium, but no labelled keratinocytes were observed. Lymphocytic mural folliculitis in dermatophytosis was not associated with immunoreactivity of the epithelium in any of the samples.

**Discussion**

The results of the present study indicate that COX-2 occurs in feline cutaneous SCC as shown for other animal species (Pestili de Almeida et al., 2001; Elce et al., 2007; Thamm et al., 2008). Additionally, COX-2 expression was shown for the first time in feline and canine AK and inflammatory dermatoses.

The expression of COX-2 by feline SCC is in sharp contrast to the absence of COX-2 expression in feline SCC reported previously (Beam et al., 2003). The lack of detectable COX-2 expression in the previous study of feline cutaneous SCC may have been due to the small sample size \((n = 4)\) or to differences in the methodology, such as the antigen retrieval methods, the antibody used or prolonged fixation, as already suggested by the authors of that study (Beam et al., 2003).

COX-2 immunoreactivity in neoplastic keratinocytes was generally associated with the presence of granulocytic inflammation and only a minority of tumour cells showed COX-2 expression in the absence of inflammation. COX-2 expression was also more prevalent among cells close to the eroded or ulcerated surface of the tumours. Because inflammatory cells are normally present at the surface of ulcerated tumours such as SCCs, and most of the labelled neoplastic keratinocytes were associated with granulocytes, this association may help to explain the distribution of the observed immunolabelling. A similar pattern of expression has been described in equine ocular and adnexal SCC, in which COX-2 labelling was confined to relatively

![Fig. 2](image-url)
superficial cells (Smith et al., 2008). The authors of that study suggested that a plausible explanation for this superficial distribution of COX-2 expression is that it could be related to inflammation; however, there was no mention of the presence of inflammatory cells in that report. Moreover, the finding in the present study that some keratinocytes expressed COX-2 in some of the inflammatory dermatoses only when there was granulocyte exocytosis into the epithelium, further supports this relationship. At this point, we do not know if it is the presence of inflammatory cells that stimulates COX-2 expression by neoplastic keratinocytes or if it is COX-2 expression by the tumour that facilitates the infiltration of inflammatory cells.

It has been shown that inflammatory mediators, such as interleukin (IL)-17, IL-1β, tumour necrosis factor (TNF)-α and platelet-activating factor, stimulate the production of COX-2 by keratinocytes and other cells (Pei et al., 1998; Biswas et al., 2005; Farley et al., 2005; Kanda et al., 2005) and IL-1β and TNF-α have been shown to induce COX-2 expression in cancer cells (Chen et al., 2000; Cui et al., 2006). This could help to explain the expression of COX-2 by feline and canine SCC and inflammatory dermatoses. However, COX-2 plays an important role in granulocyte recruitment and activation via production of prostanoids (Biswas et al., 2005). Accordingly, COX-2 could be produced by the tumour and facilitate the infiltration of inflammatory cells. In human medicine, a close

Fig. 3. (A–F) Sections from cases of inflammatory dermatosis. (A) and (B) Feline pemphigus foliaceus. (C) and (D) Feline dermatophytosis. (E) and (F) Canine pemphigus foliaceus. (B), (D) and (F) are high-power magnifications of the indicated areas of (A), (C) and (E), respectively. COX-2 expression by keratinocytes is associated with the exocytosis of granulocytes into the epidermis and follicular epithelium (arrows). IHC.
relationship between COX-2 expression and infiltration of inflammatory cells has been observed in colorectal cancer and hepatocellular carcinoma (Cervello et al., 2005; Naghshvar et al., 2009). Studies in vitro may help to distinguish between these two possibilities; however, it is also possible that the two phenomena may be present at the same time or that other unknown factors may be implicated in this interrelationship. In fact, some of the inflammatory dermatoses did not have COX-2 expression and some neoplastic cells showed strong labelling without a clear association with the presence of inflammatory cells.

Another objective of the study was to investigate the expression of COX-2 by feline and canine AK. Numerous studies have shown that UVB irradiation significantly increases COX-2 gene expression at both mRNA and protein levels in human skin as well as in cultured keratinocytes (Buckman et al., 1998; An et al., 2002). Moreover, studies in mice and human patients have shown that following UV light exposure, COX-2 expression is induced in the basal and suprabasal keratinocytes of the interfollicular epidermis and precedes keratinocyte proliferation (Buckman et al., 1998; Tripp et al., 2003). In the present study, COX-2 expression was detected in the basal and suprabasal keratinocytes in some cases of feline and canine AK. Unlike the results for SCCs, in 95% of these samples the labelled keratinocytes were not associated with the presence of inflammatory cells.

These findings support the possible role of COX-2 in AK and the possible implication of an arachidonic acid/PG pathway in photocarcinogenesis in canine and feline patients as has been suggested in man and mice (Rundhaug et al., 2007). It could be argued that finding COX-2 expression unassociated with inflammation in pre-neoplastic lesions (AK) could facilitate infiltration of inflammatory cells in more advanced lesions (SCC), but this would not explain why labelling of neoplastic cells in SCC was observed mainly in areas of surface erosion or ulceration with the presence of inflammatory cells.

In conclusion, the strong labelling observed in feline and canine skin SCC indicates the need for further studies of the effect of using COX-2 inhibitors for the management and prevention of cutaneous SCC. The association between inflammatory cells and COX-2 expression by epidermal cells opens a new line of research regarding the role of COX-2 in SCC oncogenesis. Moreover, further in-vitro and in-vivo clinical studies may be indicated in order to investigate the role of COX-2 and COX-2 inhibitors in animal AK. This may open the door to new therapeutic approaches for management of this disease in animals, such as the use of topical diclofenac, which has been demonstrated to be effective and well tolerated for the treatment of human AK (Rivers and McLean, 1997; Wolf et al., 2001; Rivers et al., 2002; Gebauer et al., 2003; Nelson et al., 2004).

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

The authors are grateful to T. Rivero, who performed the IHC, and to M. Palau for helping with the review of cases. This work was carried out at the Universitat Autònoma de Barcelona, Spain, and the study was self-funded. The results of this study were presented at the North American Veterinary Dermatology Forum, Savannah, April 2009.

References


[Received, August 3rd, 2010]

[Accepted, March 23rd, 2011]