CHAPTER 3

ROLE OF COX-2 INDUCED PGE_2 IN EPIMORPHOSIS: REGULATION OF STAGE SPECIFIC EXPRESSION OF PROTEINS

INTRODUCTION

It is now well established that prostaglandins participate in a variety of pathological processes such as the induction of fever (Milton and Wendlandt, 1970; DiNarello and Wolff, 1978), hemorrhagic shock (Collier *et al.*, 1973; Feuerstein *et al.*, 1980), haemolysis (Smith *et al.*, 1976), as well as inflammatory responses (Bonney *et al.*, 1979; Kuehl and Egan, 1980; Lord *et al.*, 1980) which might occur with local injury or irradiation. Amputation trauma elicits a localized process similar in some respects to inflammation in the wound healing response of other vertebrates (Mescher, 1996). Prostaglandins are formed by most cells of the body and act as autocrine and paracrine lipid mediators, signalling at or immediately adjacent to their site of synthesis. They are not only the key mediators of inflammation but also involved in apoptosis, cell differentiation and oncogenesis, and play a critical physiological role in tissue homeostasis and function (Cooke, 2005). PGE_2 signalling promotes tumour angiogenesis (Kurie and Dubois, 2001), increases cell proliferation and stimulates oncogenesis (Pai et *al.*, 2002). The inducible isoform of COX accounts for the elevated production of PGs in response to various pro-inflammatory stimuli and other cellular stresses such as in injury and diseases (DeWitt, 1991; Fletcher *et al.*, 1991; Feng *et al.*, 1995).

Inflammatory response coincides with muscle repair, regeneration, and growth. This inflammatory response involves activation and proliferation of satellite cells, followed by their terminal differentiation (Tidball, 2005). In epimorphic regeneration, within an hour of amputation basal epidermal cells at the wound edge mobilize by removing desmosomes and extending pseudopodia (Repesh and Oberpriller, 1978; 1980). Migration of the epithelial sheet from the epidermis across the amputation surface depends on interactions between integrins of the keratinocytes, fibrin and fibronectin of the substrate (Donaldson and Mahan, 1983; Donaldson *et al.*, 1989). Wound closure is generally completed within 24 hours of

amputation. The newly formed epithelium thickens considerably in the next few days due to continued keratinocyte migration and proliferation, forming the wound epithelium (WE) or apical epithelial cap (AEC) which is critically important for limb/tail regeneration. The cells of the WE do not immediately produce a new basal lamina, remaining in direct contact with the fibrin meshwork and with the extracellular matrix (ECM) and cells of the cut tissues (Repesh and Oberpriller, 1980). Ultrastructural evidence indicate role for this epithelium in extruding inert material from the limb/tail stump and in phagocytosis of fibrin and debris. This exocytic and phagocytic function of the epithelium along with the removal of bacteria, is augmented by the action of macrophages and neutrophils in the injured tissues (Singer and Salpeter, 1961; Repesh and Oberpriller, 1980). During this phase the reactive oxygen species (ROS) are generated and this increase in ROS leads to delay in the process of regeneration with a decrease in antioxidant enzymes status of the regenerate (Chapter 2).

Concomitant with the arrival of macrophages is the onset of histolysis and dissociation of injured muscle, connective tissue, nerve sheaths, skeletal elements, and other tissues. It is also known that, constituent cells revert to a mesenchymal, embryonic appearance as collagen; glycosaminoglycans (GAGs) and other extracellular matrix material of these tissues are degraded. Collagenolytic activity in the amputated amphibian limb was assayed in the pioneering work of Goss (reviewed by Stocum, 1995), but characterization of specific extracellular enzymes has only recently been accomplished. Yang and Bryant (1994) demonstrated that activities of five separate gelatinases or collagenases are elevated in axolotl limb tissues during regeneration. The largest and most rapid increase occurs with a 90-kDa gelatinase, an enzyme similar to mammalian matrix metalloproteinase-9 (MMP-9), important for remodelling ECM in many developmental and neoplastic events. Formerly considered "the macrophage gelatinase," MMP- 9 is now known to be secreted by several fibroblastic and epithelial cells (reviewed by Matrisian, 1992), suggesting multiple sources for the enzyme in the limb stump. The increase in urodele MMP-9 activity occurs independently of the WE and was also seen during repair of nonlimb tissues (Yang and Bryant, 1994).

The matrix metalloproteinases (MMPs) especially MMP-2 and MMP-9 are known to play a role in angiogenesis, tumour growth and metastasis (Curran and Murray, 2000; McCawley and Matrisian, 2000; Hoekstra *et al.,* 2001; Coussens *et al.,* 2002; Egeblad and Werb, 2002). MMPs are a family of endopeptidases secreted by a number of cell types, capable of cleaving several macromolecules of the extracellular matrix.

Even though there are sufficient reasons to believe that the regulation of MMPs is being mediated by COX-2 derived PGE_2 (Callejas *et al.,* 2001), there have been no reports regarding the possible role of prostaglandins, prostacyclins, or thromboxanes in the control of extra cellular protein degradation, during injury. Although we were particularly interested in exploring the possible involvement of prostaglandins and related compounds in the protein wasting induced by autotomy, many other physiological factors *viz.* growth factors could contribute to the overall proteolysis in lizard tail. However, the intracellular mediators of these effects are still at large untouched. Therefore, in the current study changes in protein expression and degradation by PGE_2 and affect on MMPs alone were undertaken with the use of selective COX-2 inhibitor etoricoxib. Protein degradation was studied by identifying the activity of MMP-2 and MMP-9 in the regenerate of the animals.

Moreover, several previous studies have reported the release of prostaglandins by skeletal muscles perfused *in situ* (Herbaczynska-Cedro *et al.,* 1974; Young and Sparks, 1979). These experiments give credence to the notion that during caudal regeneration of lizards, the mesenchymal cells of the tail stump might produce PGE_2 and that this autocoid would play an important role in the regulation of protein turnover after caudotomy. Therefore, in the present investigation the role of PGE_2 in protein synthesis and degradation during various stages of reptilian regeneration was explored.

MATERIALS AND METHODS

Animal

A total of sixty lizards of both the sexes were selected and acclimated in the laboratory at 30 $\pm 2^0$ C, for a week prior to experiments (See Materials and Methods for details of dosing and euthanasia). The animals were divided into two groups of thirty animals:

Experimental Design and Drug Dosage

Group I: This group of animals served as control to the experimental groups and injected with vehicle (Tris Buffer of pH 8.8).

Group II: Animals of this group were injected with Etoricoxib (25 mg/kg body weight)

The treatment in each group started four days prior to amputation and was continued till the animals reached differentiation stage. The drug/vehicle was administered every alternate day.

The regenerating animals were sacrificed at three defined stages of regeneration *viz.*, (i) completion of wound healing and appearance of wound epithelium (WE) (ii) in lizards at blastema (BL) stage (iii) in lizards at differentiation (DF) stage with a number of ten animals at each stage in both the groups and the tissues (tail and blood) were collected and processed further for identifying expression of various proteins at each stage using SDS-PAGE technique. Protein content was determined using Bradford assay (Bradford, 1972). Equal amount of total protein was loaded and separated by SDS-PAGE on 10% gels and stained with non-specific coomassie stain and destained with acetic acid -methanol solution.

GELANTINASE MMP-2 AND MMP-9 EXPRESSION

Protein degradation following ECM digestion was studied by ***Gelatin Zymography.*** Quantitative zymography was performed. Samples were diluted using zymogrpahic dilution buffer. The samples were loaded into the wells of a 7.5% gelatin gel and electrophoresed. The gel was then incubated for 1 h at room temperature in 100 ml of renaturing buffer (2.5% Triton X-100) on a rotary shaker. The buffer was decanted and replaced with 100 ml of development buffer (50 mM Tris, pH 7.5, 200 mM NaCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 2.5 % triton-X-100). The gel was then incubated at 37 °C for 18 h. Each gel was stained with 100 ml of 0.5% coomassie blue G-250 in 30% methanol and 10% acetic acid for 3 hours and then de-stained with three changes of 30% methanol and 10% acetic acid. The gels were documented using a scanning digitizing system and analyzed using AlphaEase FC software (product of density and band area). Zymography of regenerate tissue homogenate showed the zone of clearance against the dark blue background.

DETERMINATION OF MOLECULAR WEIGHT

Molecular weight of the zymographic bands were determined by using AlphaEase FC software, stand alone for windows, version 4.0.034 (Alpha Innotech, San Learnordo,CA). Briefly the known molecular weights of the protein markers were manually added to the software programme. Based on the values of the known molecular weights the software calibrates a molecular weight curve. Later the molecular weight of a band is calculated based on the graph of the known marker bands. If a query band lay outside of the standard curve, its molecular weight was extrapolated in "Least Squares Fit" mode. The molecular weight data of the reference markers were stored in the software library for further analysis of gels which were run without molecular weight marker but with the similar electrophoresis condition.

SPOT DENSITOMETRY ANALYSIS

Spot densitometry was performed on all the bands using AlphaEase FC software, stand alone for windows, version 4.0.034 (Alpha Innotech, San Learnordo, CA). Densitometry was performed on the scanned images of the gel taken in charged coupled device camera (CCD) and edited in Adobe Photoshop Version 7. Similar areas were analyzed for the densitometry. Auto background subtraction was performed using the AlphaEase FC software. Using densitometric values, quantitative comparison was made in all the bands of interest and the results were expressed in arbitrary units, which was calculated by integration of the intensity of each pixel over the spot area and normalized for the gel background. In case of zymograms, the zone of clearance was quantified on the basis of area enclosed by the region in pixels together with band intensity.

IMMUNOHISTOCHEMISRTY OF MMPs

Fresh frozen cryostat section (5 μm thickness) of regenerate were taken and fixed in ice-cold methanol. Washed in PBS and blocked for 1 hour with blocking solution and then slides were treated and incubated overnight with 1:50 dilution of anti-goat MMP-2 and anti–rabbit MMP-9 ALP conjugated antibodies. After over night incubation of slides in primary antibodies slides were washed in PBS and treated with 1:200 dilutions of secondary antibodies for 2 hours. After washing for 15 minutes in PBS slides were incubated for 30 minutes with NCBI/NBT and washed with PBS and mount with mounting medium and observed under Leica (DMRB) microscope.

RESULTS

All the observations presented here are based on one dimensional gel electrophoresis and these observations points to a possible regulatory role of PGE_2 during the progression of epimorphic regeneration. When the lizard is being subjected to specific COX-2 inhibitor the production of PGE_2 is blocked and this, it was observed, hampers the whole gamut of epimorphosis in lizard (Chapter 1). It was also noticed that PGE_2 can regulate the detoxification of free radicals for proper healing and proliferation mechanism (Chapter 2). Further, it was noticed that biosynthesis of proteins is one of the most important biochemical process during regeneration as reported by Thornton and Bromley (1973). Therefore, it was worth to find the protein alteration occurring during regeneration when the production of autocoid prostaglandin E_2 was inhibited with *in loco* injection of etoricoxib in tail stump of lizard.

The protein fractions of tail were compared with control at all defined stages of reptilian tail regeneration. A large number of polypeptides of identical mobility were found in both experimental and control samples (blood and tissues), although a number of them were absent in experimental samples. There was also a difference in abundance of the proteins with identical mobility, between control and experimental samples as evident by the intensity of these bands (Figure 3.1-3.6).

Alteration of protein expression was observed at wound epithelium (WE) stage, blastema (BL) stage and differentiation (DF) stage of lizard tail regeneration. At WE stage the protein fraction of control animals was enriched with polypeptides having molecular masses of 104.05, 95.08, 76.00, 61.78 and 52.98 kilo Daltons (kDa) (Figure3.1 and Table 3.1). However, these polypeptides which were found in control tail protein fractions remained absent in the treated animals. Moreover, the amount of protein present in the bands was identified by spot densitometry and intensity of the protein expression was measured. It was observed that band of molecular weight 104.05 kDa expressed with the highest intensity of 68 in arbitrary units in control animals and was absent in treated ones (Figure3.1 and Table3.1).

Progression of lizard tail towards blastema stage results in the emergence of new bands with the disappearance of existing bands in experimental animals to that of control animals (Figure3.2 and 3.3, Table 3.2 and 3.3). In experimental animals the absence of three bands of molecular weight 104.36, 89.92 and 84.36 kDa was observed (Figure 3.3, Table 3.3). Moreover, the intensity of the coomassie staining of the bands was much denser in control animals than that of experimental animals. In crude extract of tail homogenate at blastema stage of control animals, the higher molecular mass polypeptides of 137.62 kDa with the high optical density of 41 units was observed in control animals. The predominant, high intensity protein bands of molecular weight 128.19, 95.51, 76.12, 59.60, 50.63, and 37.99 kDa were identified at blastema stage of control animals compared to that of COX-2 inhibited animals (Figure3.3 and Table3.3).

Polypeptide composition of soluble muscle proteins at differentiation stage was determined by SDS-PAGE protocol of Laemmli (1970) and is shown in Figure 3.4 and 3.5. The quantitative and comparative analysis of these soluble muscle proteins was done using AlphaEase FC software. The analysis revealed that the number of polypeptides, which co-

migrate are either present or absent. High molecular weight polypeptides were seen at differentiation stage. Moreover, a total number of seven bands were found to be absent in experimental animals. It was also observed that the low molecular weight proteins have high protein optical density of 145, 148 and 140 for proteins of molecular weight 39.06, 36.71 and 32.55 kDa proteins (Figure 3.4 and 3.5, Table 3.4 and Table 3.5).

The etoricoxib treatment was also found to alter the plasma protein profile. The most striking observation however, was the absence of expression of three bands of polypeptides in the treated animals and later no such difference in expression of the protein was observed. Nevertheless, the intensity and thickness of the bands were less in COX-2 inhibited lizards compared to that of control animals (Figure 3.6, Table 3.6).

Degradation of extracellular matrix plays an important role in cell migration towards the underlying mesenchymal layer to form blastema. Proteolytic activities were detected by clear bands indicating the lysis of the substrate. It was found that in PGE_2 blocked lizards the protease activity was comparatively lower with respect to control animals (Figure 3.7-3.11 and Table 3.7-3.11).

Gel zymography evaluation revealed significantly lower MMP activity in samples obtained from animals treated with specific COX-2 inhibitor when compared to that of control animals. Protease activity in the tissue was identified at all the specific stages. The highest activity was observed at WE stage with highest zone of clearance and optical density of 92 in control animals (Table 3.7). A decrease in the activity of protease was identified with advancement in stages of epimorphic regeneration (Table 3.8). Heightened expression of MMP-2 and MMP-9 were observed in the control animals at blastema and differentiation stage when compared to that of PGE_2 blocked lizards (Table 3.9, 3.10 and 3.11). Further, it was observed that the ratio of active to latent form of MMP-2 increased with the progression in the process of regeneration. The presence of MMP-2 and MMP-9 in reptilian regeneration was further confirmed by immunohistochemical localization (Figure 3.12). The results were similar with zymogram, the activity of these enzymes was found to be less in treated animals to that of control animals (Figure 3.11).

DISCUSSION

Regeneration is a complex biological process by which animal restores shape, structure, and function of body parts lost to injury or experimental amputation. In this regard, regeneration of vertebrate appendages has been one of the most extensively studied model systems (Tsonis, 2000; Brockes and Kumar, 2002). Urodeles and zebrafish are among the vertebrate species that retain a significant limb regenerative ability during adulthood. During regeneration, after wound healing, a blastema arises at the surface of the limb stump; the blastema is composed of an epidermal cap, which covers a mass of dedifferentiated mesenchymal cells (Le. blastema cells) that proliferate very actively. After this phase of strong cell proliferation, blastema cell proliferation depends on several different factors which are, as yet not fully understood. For regeneration to occur, this event needs to be preceded by the formation of the apical ectodermal cap (AEC), the multilayered epithelium that covers the wound surface after amputation (Bryant *et al.*, 1987; Poss *et al.*, 2003). During the last decade, and in part with the help of the knowledge gathered during the embryogenesis of the vertebrate limb, some of the molecular and cellular processes involved in AEC and blastema formation have been unveiled (Capdevila and Belmonte, 2001; Poss *et al.* 2003). Members of the *Wnt* and bone morphogenetic protein (BMP) signalling pathways have been shown to be required in vertebrates for the formation of the apical ectodermal ridge (AER). The major signalling pathway such *as Notch, Wnt, Hh* and nuclear receptors play definite role in the maintenance of regenerate (Neumann and Cohen, 1996; 1997; Johnston and Edgar, 1998; Barolo and Posakony, 2002). The regulation of these pathways requires dosage of signal, the strength of the default repression of the target genes, and by the cooperative activation of different transcription factors (Barolo and Posakony, 2002) and also beholden to the particular environment (developmental, functional, etc.) in which the cell may encounter itself. The most important aspect of epimorpic regeneration is dedifferentiation and the products of the genes like *Nrad, radical fringe (rfringe) and Notch.* The expression of all these genes is being associated with its gene products which are proteins.

The differential expression patterns of protein in response to induced autotomy suggest that these proteins have distinct functions during epimorphic regeneration. Sundry amendments, both qualitative and quantitative are being observed. Sophy and Ramachandaran, 2001 noticed the appearance of new proteins in *Hemidactylus flaviviridis* during caudal

regeneration and suggested that these new proteind are formed in response to various developmental needs. In the current study a differential protein expression during various phases of regeneration was observed. Coomasie brilliant blue, stain results in staining of all the proteins present in the tissue at specific stage. Moreover at particular stage, protein bands which are found absent in the experimental animal, but present in control animal, assert the specificity of that protein at specified stages of caudal regeneration. However, the relative electrophoretic mobility of individual bands was very distinct at differentiation stage of tail regeneration. Quantitative variations between co-migrating bands of SDS-PAGE electropherograms of soluble muscle proteins of specific stage may, however, exist (Figure 3.1 and Table 3.1). Such variations in the relative intensities may be attributed to nongenetic reasons such as the physiological state of the animal or due to expression of specific genes during progression of wounded tissue to a restored organ (tail).

Moreover, the present study further unfolds the possible involvement of prostaglandin (PG) and other products in the regulation of protein balance in the regenerate during various stages of regeneration. After induced autotomy there is a state of injury at the amputated tail. Such condition leads to the *de novo* synthesis of prostagalandin from membrane-released arachidonic acid activated by specific cytokine, growth factors and other stimuli (Funk, 2001). The synthesis of PG in the cells is initiated by enzyme cylcooxygenase (COX). There are three different isoforms of COX, *viz.* COX-1, COX-2 and COX-3, of which COX-2 is known to be inducible. It is a product of a gene, which is localized on chromosome 1. The COX-2 gene is about 8 kb long with 10 exons and it is transcribed as 4.6, 4.0, and 2.8 kb mRNAs variants. The apparent molecular mass of COX-2 is 70 kDa. In control animals, the SDS-PAGE gel profile at blastemal stage has expressed a protein of molecular weight 70.15 kDa that stains with high intensity (Figure 3.3 and Table 3.3). A similar band is observed in control animals from differentiation stage that is absent in treated animals. This further lends support to the notion of involvement of COX-2 induced PGE_2 in cell proliferation and differentiation after an injury as worked out in liver regeneration and skeletal muscle regeneration (Casado *et al.*, 2001).

Besides, PGE_2 has been identified by many as an upstream modulator of fibroblast growth factor-2 (Sakai *et al.*, 2001). Ferretti and co-workers in 1997 found that there is an upregulation of FGF-2 in the blastema and ependymal cells of an amphibian regenerating system. Similar observation was made in lizard epimorphic regeneration wherein the growth

of the blastema was observed to increase in control animals (Yadav, 2005). The putative regulators of *fgf* signalling still need to be elucidated.

Sox gene (*SRY-related HMG-box genes*) expression is being regulated in the similar fashion to that of FGF in the regenerating system. SRY (Sex determining region on Y chromosomes) encodes a protein having a DNA-binding motif known as the high-mobility- group (HMG) box (Gubbay *et al.,* 1990; Sinclair *et al.,* 1990). In addition to SRY, genes encoding an SRY-type HMG box (SOX genes/sox). No less than 30 types of SOX gene have been isolated from humans, 19 from mice and rainbow trout. These SOX genes have been considered to be related to the regulation of cell differentiation and to the differentiation pattern (Van de Van de Wetering *et al.,* 1993; Sockanathan *et al.,* 1993; Foster *et al.,* 1994; Wagner *et al.,* 1994; Wright *et al.,* 1993). SDS-PAGE has found one band near the predicted molecular weight of Sox gene (Sox-2 (34kDa) and Sox-11 (41.36kDa)) during all the stages of differentiation was found in gel patterns. This protein band is found to be absent from animals treated with COX-2 inhibitor (Figure 3.1-3.11). It is thus possible that with the blockage of PGE_2 the expression of these gene product is being affected leading to one of the aspect in hampered tail regeneration. It is also seen that Msx-1 gene, which encodes a homebox (Hox) containing transcription repressor might also play role in dedifferentiation. Hox genes *Msx-1* and *Msx-2*, the best studied members of the Msx family, have been shown to be expressed most conspicuously in the areas of epithelial-mesenchymal interactions (Davidson, 1995). High levels of *Msx-1* gene expression have been observed in the developing limb bud (Hill *et al.,* 1989; Robert *et al.,* 1989; Robert *et al.,* 1991; Holland, 1991; Bell *et al.,* 1993; Su and Karin, 1996; Takahashi *et al.,* 1999) in the regenerating limbs (Simon *et al.,* 1995) or fins (Akimenko *et al.,* 1995) and in developing eyes (Monaghan *et al.,* 1991; Levine and Schechter, 1993) Kumar *et al.,* 2004 studied the regenerative plasticity of isolated urodele myofibers and its dependence on *Msx1* . Thus, the change in the expression of the *Msx-1* gene could possibly affect protein expression during the blastema development.

In the current study of the administration of specific COX-2 inhibitor in lizard altered the protein profile not only in the regenerate but also in the plasma. In case of plasma proteins subjected to SDS-PAGE a variation in the number of bands was observed in treated animals compared to that of controls. This change could possibly be due to the blood derived factors controlling regeneration. The results presented here also suggest that SDS-PAGE can be used to identify marker polypeptides (Figure 3.6). Such variations in the electrophoretic behaviour

are most likely to arise due to the absence of PGE_2 in the regenerating system. In SDS-PAGE, constituent polypeptides of the proteins are resolved according to their molecular weights (Weber and Osborn 1969). SDS-PAGE can also be used to study variation of protein expression (as evident by presence or absence of protein band), during amputation and the formation of regenerative tissue (Garling and Tassava, 1984; Stocum, 1984).

Further, for successful regeneration several pathways are required to act promptly. Among the early events critical to regeneration are wound healing and dedifferentiation (Stocum, 1995; Tsonis, 1996; Brockes, 1997). One of the earliest events in limb regeneration is the extensive remodelling of the extracellular matrix (ECM). Matrix degrading enzymes are metalloproteinase-2 (MMP-2, a 72 kDa type IV collagenase) and MMP-9 (a 92 kDa type IV collagenase).Yang *et al.* (1999) identified the expression of *Mmp-9* and related MMP genes during axolotl limb regeneration. In order to understand the effect of PGE_2 inhibition on degradative events involved in dedifferentiation, we have examined the involvement of PGE_2 in the activity of matrix MMPs in ECM degradation. Study was performed by using gelatin zymogrpahy to understand the change in the gelatinolytic/collagenolytic factors in regenerating tails of lizards. From the SDS-PAGE gel it was observed that protein bands similar in molecular weights to MMPs, showed a high increase during wound epithelium (Figure 3.7 and 3.8) and blastema stage (Figure 3.9 and Figure 3.10) in control animals to that of animal treated with COX-2 inhibitor. These high molecular weight peptides that were similar to MMPs, were not present in the subsequent stages of regeneration. The gelatinolytic activity of one these peptides (relative molecular weight of 90 kDa) was found to be upregulated after amputation, increased during the dedifferentiation/histolysis stages, peaked at the medium stage, and decreased during redifferentiation stage (Figure 3.11 and Table 3.11). It has been reported in osteoblasts, that growth factor induced PGE_2 production is blocked by indomethacin, a suppressor of gelatinase activity, thus suggesting a role of PGE_2 in MMP induction (Kusano *et al.,* 1998). Moreover, it is well documented that PGE_2 regulates COX-2-dependent, CD44 and MMP-2-mediated invasion in non-small cell lung cancer (NSCLC) in an autocrine/paracrine manner via EP receptor signalling (Dohadwala *et al.,* 2002).

MMP-9 and MMP-2, with its family members are also known to promotes angiogenesis (a critical process required for the survival of dividing cells) by degrading the vascular basement membrane interstitium and also by releasing sequestered vascular endothelial

growth factor (VEGF), which is a well known angiogenic molecule (Afuwape *et al.,* 2002). The process of angiogenesis was found to be negatively affected with the decreased expression of MMP-2 and MMP-9 (Chapter 4) suggesting the role played by PGE_2 regulated MMPs in angiogenesis.

Hence, it can be summarised that prostaglandin assist the cell migration at the site of amputation through regulated expression of MMPs. The PGs are also known to augment cell proliferation (Nishikawa *et al.,* 1997; Rudnick *et al.,* 2001). The rate of proliferation is being examined in the animal by *in vivo* BrdU labelling and acridine orange staining (Chapter 4). Moreover, protein profiling (SDS-PAGE) indicates an altered protein expression in treated animals. The following chapter deals with study describing histological changes and the changes in desmin and myosin heavy chain during regeneration. The study also deals with the alteration in VEGF (angiogenic molecule), macromolecules (nucleic acids) and cell proliferation in the lizards where the inhibition of COX-2 activity and PGE_2 production was achieved by exogenous administration of specific COX-2 inhibitor (etoricoxib).

Table 3.1. Effect of specific COX-2 inhibitor on the protein profile in the tail of control and treated animals at wound epithelium stage.

MOLECULAR WEIGHT	LANE-1 (Optical Density expressed in arbitrary units)	LANE-2 (Optical Density expressed in arbitrary units)	LANE-3 (Optical Density expressed in arbitrary units)	LANE-4 (Optical Density expressed in arbitrary units)
104.05 **kDa**	118	-	-	-
95.08 **kDa**	124	97	71	30
92.26 **kDa**	30	132	80	52
76.00 **kDa**	70	59	49	56
61.78 **kDa**	78	69	49	110
52.98 **kDa**	100	125	128	107
43.06 **kDa**	22	35	43	4
35.48 **kDa**	56	78	69	49
29.82 **kDa**	33	-	-	-
26.44 **kDa**	52	-	-	-

The entire protein profile subjected to densitometric analysis is expressed in arbitrary units. LANE-1 corresponds to control; LANE-2, 3 and 4 corresponds to animals treated with specific COX-2 inhibitor. The blank cells indicate absence of specific protein bands.

Table 3.2. Effect of specific COX-2 inhibitor etoricoxib on the protein profile in the tail of control and treated animals at early blastema stage.

MOLECULAR WEIGHT	LANE-1 (Optical Density expressed in arbitrary units)	LANE-2 (Optical Density expressed in arbitrary units)	LANE-3 (Optical Density expressed in arbitrary units)	LANE-4 (Optical Density expressed in arbitrary units)
67.28 kDa	49	27	-	-
54.42 kDa	41	24	35	55
44.01 kDa	-	-	28	45
38.21 kDa	49	28	32	47
28.57 kDa	45	45	47	43
23.84 kDa	35	48	42	61

The entire protein profile subjected to densitometric analysis is expressed in arbitrary units. LANE-1 and LANE-2 corresponds to control; Lane 3 and Lane 4 correspond to animals treated with specific COX-2 inhibitor. The blank cells indicate absence of specific protein band.

Table 3.3 Effect of specific COX-2 inhibitor on the protein profile in the tail of control and treated animals at late blastema stage.

MOLECULAR WEIGHT	LANE-1 (Optical Density expressed in arbitrary units)	LANE-2 (Optical Density expressed in arbitrary units)
137.62 kDa	41	37
128.19 kDa	68	45
125.05 kDa	25	24
104.36 kDa	22	-
95.51 kDa	47	22
89.92 kDa	17	-
84.36 kDa	22	-
76.12 kDa	45	40
70.15 kDa	12	4
59.60 kDa	60	42
50.63 kDa	78	64
40.92 kDa	45	33
37.99 kDa	56	24
37.58 kDa	25	9
32.96 kDa	25	26
29.74 kDa	27	25
26.83 kDa	15	16

The entire protein profile subjected to densitometric analysis is expressed in arbitrary units. LANE-1 corresponds to control; LANE-2 corresponds to animals treated with specific COX-2 inhibitor. The blank cells indicate absence of specific protein bands.

Table 3.4. Effect of specific COX-2 inhibitor on the protein profile in the tail of control and treated animals at early differentiation stage.

MOLECULAR WEIGHT	LANE-1 (Optical Density expressed in arbitrary units)	LANE-2 (Optical Density expressed in arbitrary units)	LANE-3 (Optical Density expressed in arbitrary units)	LANE-4 (Optical Density expressed in arbitrary units)
168.44 kDa	21	22	-	-
132.20 kDa	10	13	28	28
124.50 kDa	-	-	13	10
112.07 kDa	-	-	12	11
98.91 kDa	10	11	9	14
68.91 kDa	42	24	-	-
56.58 kDa	17	14	-	-
45.85 kDa	27	23	26	30
39.67 kDa	33	45	32	28
29.32 kDa	29	61	52	35
23.92 kDa	29	46	42	47

The entire protein profile subjected to densitometric analysis is expressed in arbitrary units. LANE-1 and LANE-2 corresponds to control; Lane-3 and Lane-4 corresponds to animals treated with specific COX-2 inhibitor. The blank cells indicate absence of specific protein bands.

Table 3.5. Effect of specific COX-2 inhibitor on the protein profile in the tail of control and treated animals at late differentiation stage.

MOLECULAR WEIGHT	LANE-1 (Optical Density expressed in arbitrary units)	LANE-2 (Optical Density expressed in arbitrary units)
156.14 kDa	-	40
142.52 kDa	14	46
135.60 kDa	20	38
126.85 kDa	-	54
113.92 kDa	-	47
104.41 kDa	-	39
95.70 kDa	27	42
89.18 kDa	-	76
77.78 kDa	63	81
65.61 kDa	45	68
56.74 kDa	84	75
48.67 kDa	81	99
42.97 kDa	-	99
39.06 kDa	104	145
36.71 kDa	109	148
32.55 kDa	114	140
29.83 kDa	-	63

The entire protein profile subjected to densitometric analysis is expressed in arbitrary units. LANE-1 corresponds to animals treated with specific COX-2 inhibitor; LANE-2 corresponds to control at differentiation stage. The blank cells indicate absence of specific protein bands.

Table 3.6. Effect of specific COX-2 inhibitor on the protein profile in the blood of control animals.

MOLECULAR WEIGHT	LANE-1 (Optical Density expressed in arbitrary units)	LANE-2 (Optical Density expressed in arbitrary units)
167.57 kDa	92	76
124.60 kDa	69	76
96.04 kDa	49	32
76.03 kDa	10	12
56.44 kDa	14	18
46.83 kDa	4	19
37.41 kDa	65	76
30.43 kDa	46	48

The entire protein profile subjected to densitometric analysis is expressed in arbitrary units. LANE-1 corresponds to control and LANE-2 corresponds to animals treated with specific COX-2 inhibitor. The blank cells indicate absence of specific protein bands.

Table 3.7 Densitometric analysis of zymograms at wound epithelium stage (WE) in control animals.

GELATINOLYTIC ZONE	LANE-1 (Optical Density expressed in arbitrary units)	LANE-2 (Optical Density expressed in arbitrary units)	LANE-3 (Optical Density expressed in arbitrary units)	LANE-4 (Optical Density expressed in arbitrary units)
MMP-2	67	62	53	68
MMP-9	57	73	92	77

The zymogram subjected to densitometric analysis is expressed in arbitrary units. LANE-1 to LANE-4 corresponds to samples with different concentration of protein. The blank cells indicate absence of specific gelatinolytic zone. In order to minimize background error a total of 5 randomly selected background was normalised.

Table 3.8. Densitometric analysis of zymograms showing the effect of specific COX-2 inhibitor etoricoxib in the tail of treated animals at wound epithelium (WE) stage.

GELATINOLYTIC ZONE	LANE-1 (Optical Density expressed in arbitrary units)	LANE-2 (Optical Density expressed in arbitrary units)
MMP-2	42	48
MMP-9	28	33

The zymogram subjected to densitometric analysis is expressed in arbitrary units. LANE-1 to LANE-4 corresponds to different concentration of protein. In order to minimize background error a total of 5 randomly selected background was linked to normalization.

Table 3.9. Densitometric analysis of zymograms at blastema (BL) stage in control animals.

GELATINOLYTIC ZONE	LANE-1	LANE-2	LANE-3
MMP-2	-	35	-
MMP-9	85	32	11

The zymogram subjected to densitometric analysis is expressed in arbitrary units. LANE-1 to LANE-4 corresponds to samples with different concentration of protein. The blank cells indicate absence of specific gelatinolytic zone. In order to minimize background error a total of 5 randomly selected background was normalised.

Table 3.10. Densitometric analysis of zymograms showing the effect of specific COX-2 inhibitor in the experimental animals at blastema (BL) stage.

GELATINOLYTIC ZONE	LANE-1 (Optical Density expressed in arbitrary units)	LANE-2 (Optical Density expressed in arbitrary units)
MMP-2	8	15
MMP-9	19	28

The zymogram subjected to densitometric analysis is expressed in arbitrary units. LANE-1 to LANE-2 corresponds to samples with different concentration of protein. The blank cells indicate absence of specific gelatinolytic zone. In order to minimize background error a total of 5 randomly selected background was normalised.

Table 3.11. Densitometric analysis of zymograms at differentiation stage.

GELATINOLYTIC ZONE	CONTROL (Optical Density expressed in arbitrary units)		DIFFERENTIATION (Optical Density expressed in arbitrary units)	
	(LANE-2) WITHOUT APMA	(LANE-3) WITH APMA	(LANE-5) WITHOUT APMA	(LANE-6) WITH APMA
MMP-2	43	53	57	64
MMP-9	-	51	-	54

The zymogram subjected to densitometric analysis is expressed in arbitrary units. The blank cells indicate absence of specific gelatinolytic zone. In order to minimize background error a total of 5 randomly selected background was linked to normalization. APMA = Alpha phenyl mercuric acetate. (Lane-2= Control without APMA activation, Lane-3= APMA activated sample, MW = Molecular weight marker, Lane 5 = Differentiation stage without APMA activation, Lane 6 = APMA activated at differentiation stage). Lane 1 (unmarked, Figure: 3.11) also contains APMA activated samples but densitometry analysis has been ignored due to smearing of gel.

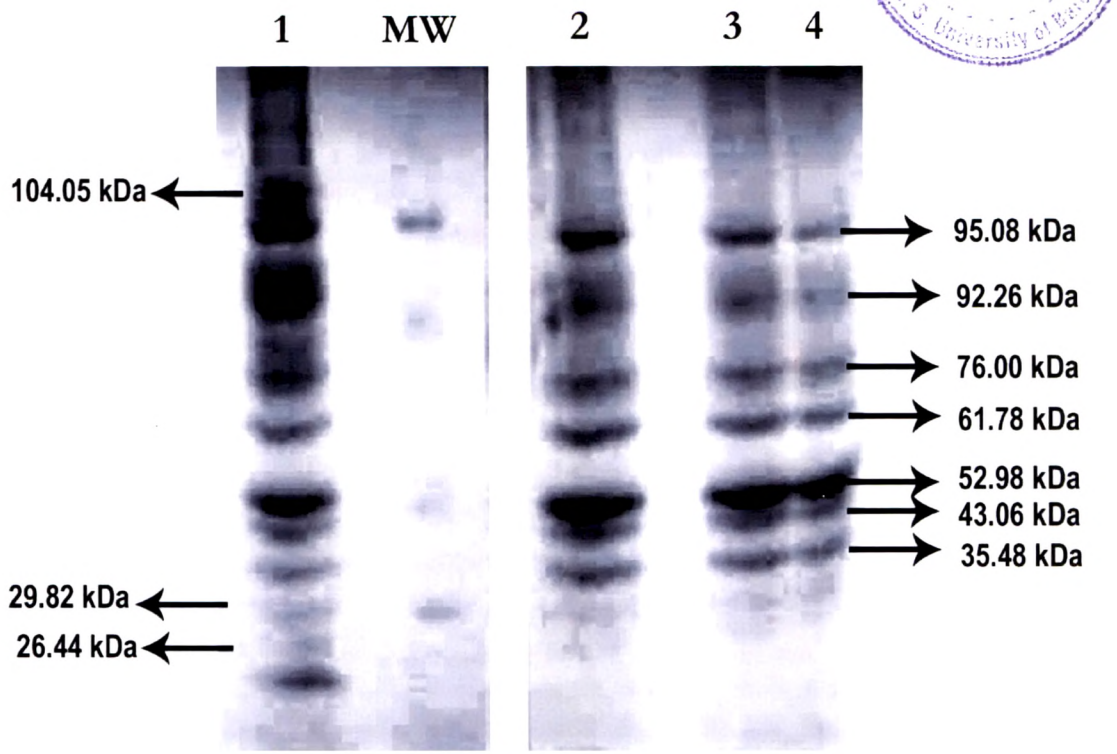

Figure 3.1: Effect of specific COX-2 inhibitor on the protein profile in the tail of control and treated animals at wound epithelium stage. LANE-1 corresponds to control; LANE-2, 3 and 4 corresponds to animals treated with specific COX-2 inhibitor. MW corresponds to molecular weight marker.

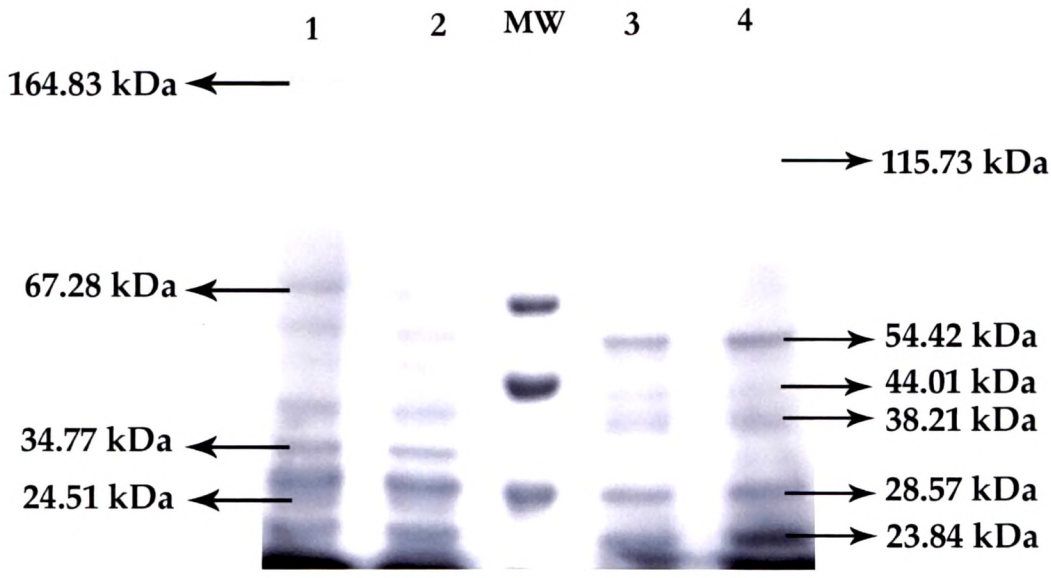

Figure 3.2: Effect of specific COX-2 inhibitor etoricoxib on the protein profile in the tail of control and treated animals at early blastema stage. LANE-1 and LANE-2 corresponds to control; Lane 3 and Lane 4 correspond to animals treated with specific COX-2 inhibitor.

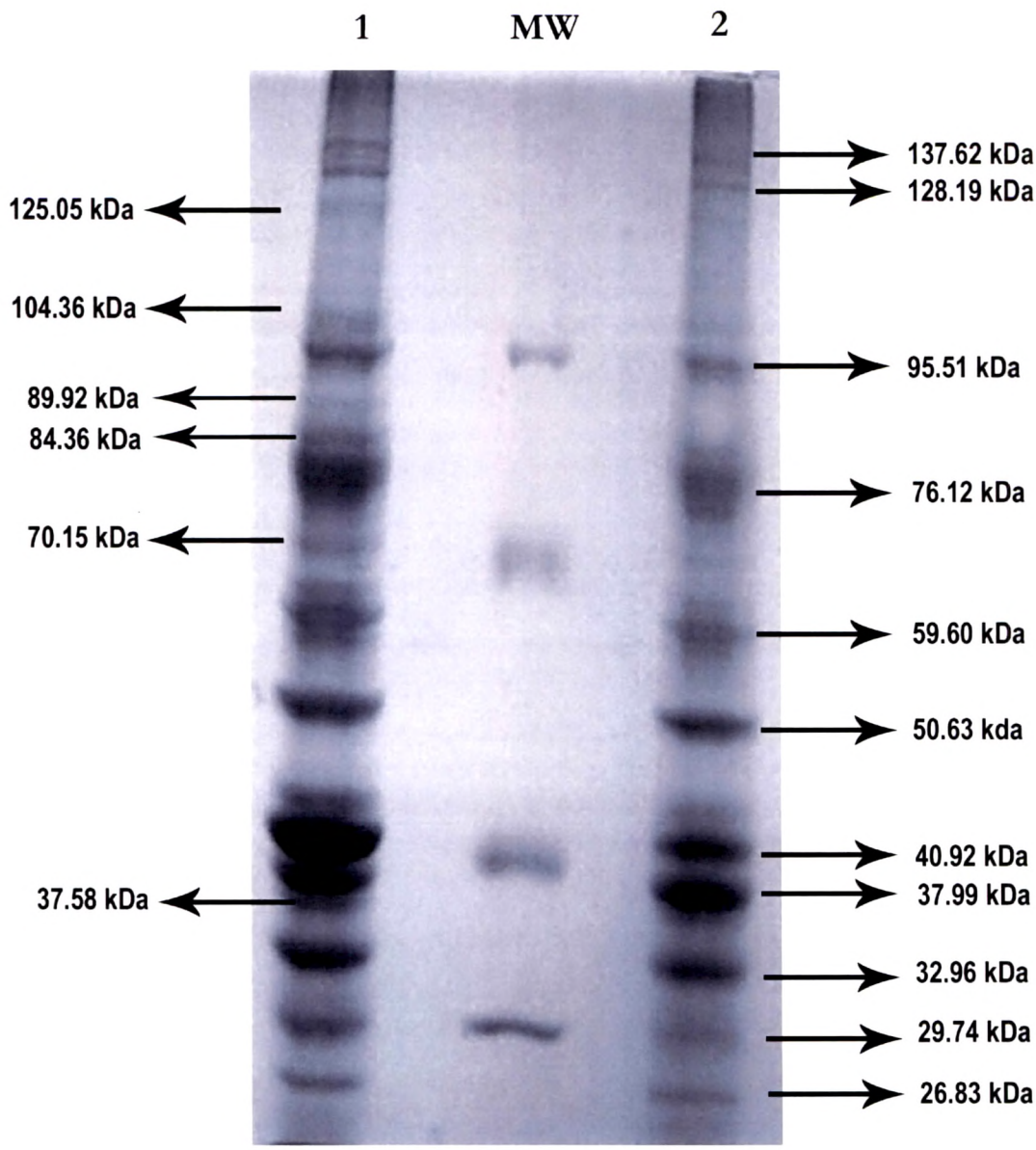

Figure 3.3: Effect of specific COX-2 inhibitor on the protein profile in the tail of control and treated animals at late blastema stage. LANE-1 corresponds to control; LANE-2 corresponds to animals treated with specific COX-2 inhibitor. MW corresponds to molecular weight marker.

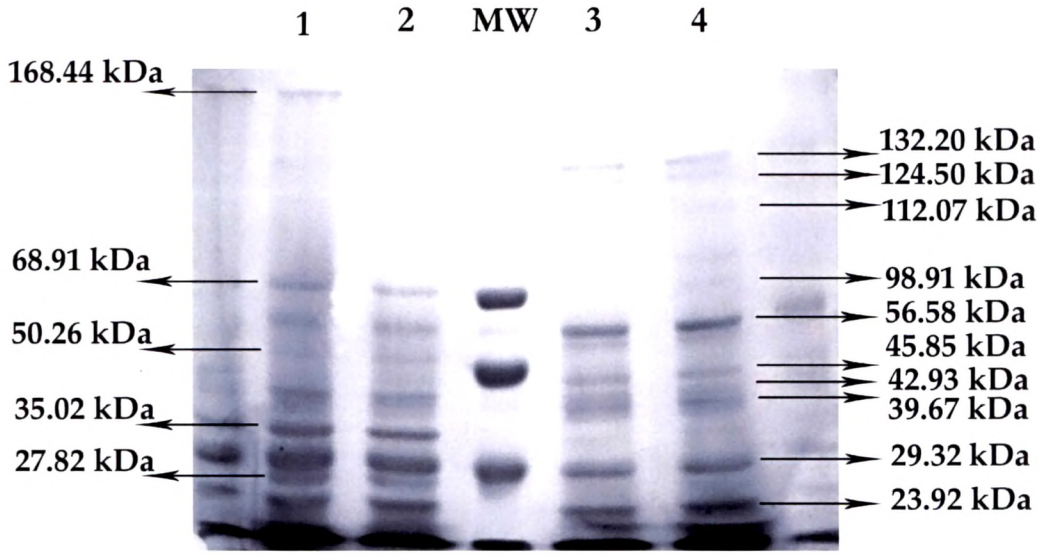

Figure 3.4: Effect of specific COX-2 inhibitor on the protein profile in the tail of control and treated animals at early differentiation stage. LANE-1 and LANE-2 corresponds to control; Lane-3 and Lane-4 corresponds to animals treated with specific COX-2 inhibitor at early differentiation stage. MW corresponds to molecular weight marker.

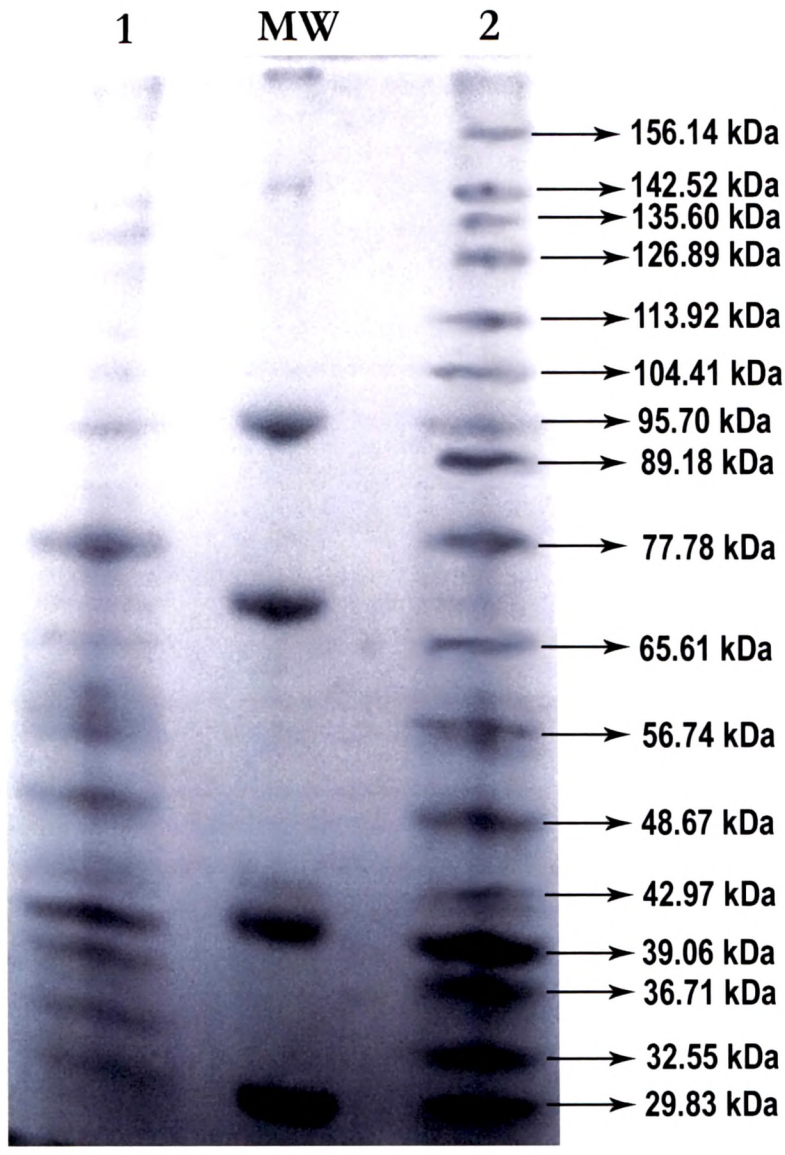

Figure 3.5: Effect of specific COX-2 inhibitor on the protein profile in the tail of control and treated animals at late differentiation stage. LANE-1 corresponds to animals treated with specific COX-2 inhibitor; LANE-2 corresponds to control. MW corresponds to molecular weight marker.

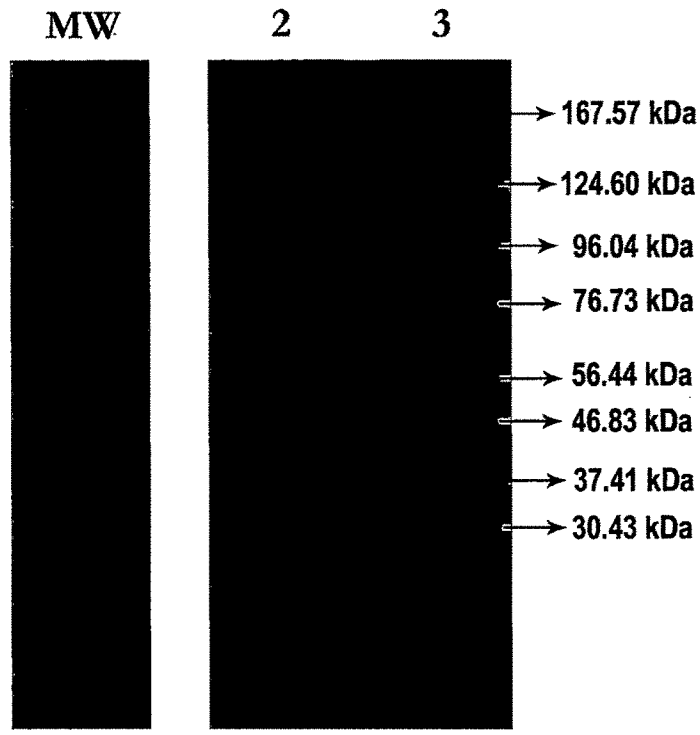

Figure 3.6: Effect of specific COX-2 inhibitor on the protein profile in the blood of control and treated animals. LANE-2 corresponds to control and LANE-3 corresponds to animals treated with specific COX-2 inhibitor. MW corresponds to molecular weight marker.

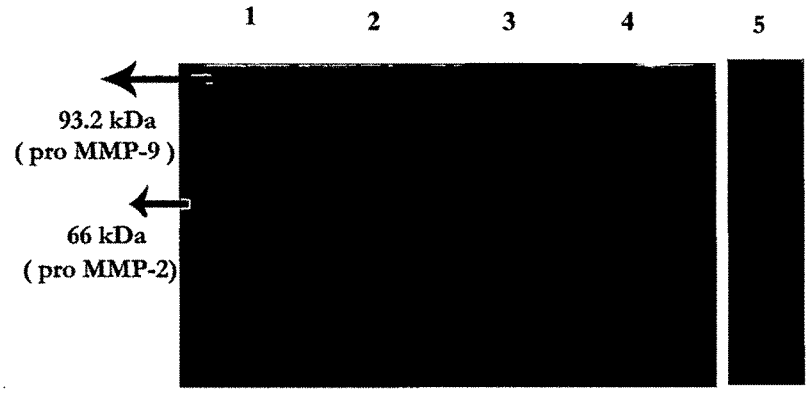

Figure 3.7: Expression of MMP-2 and MMP-9 in the wound epithelium (WE) stage in control animals. LANE-1 to LANE-4 corresponds to samples with different concentration of protein. Lane-5 corresponds to molecular weight marker.

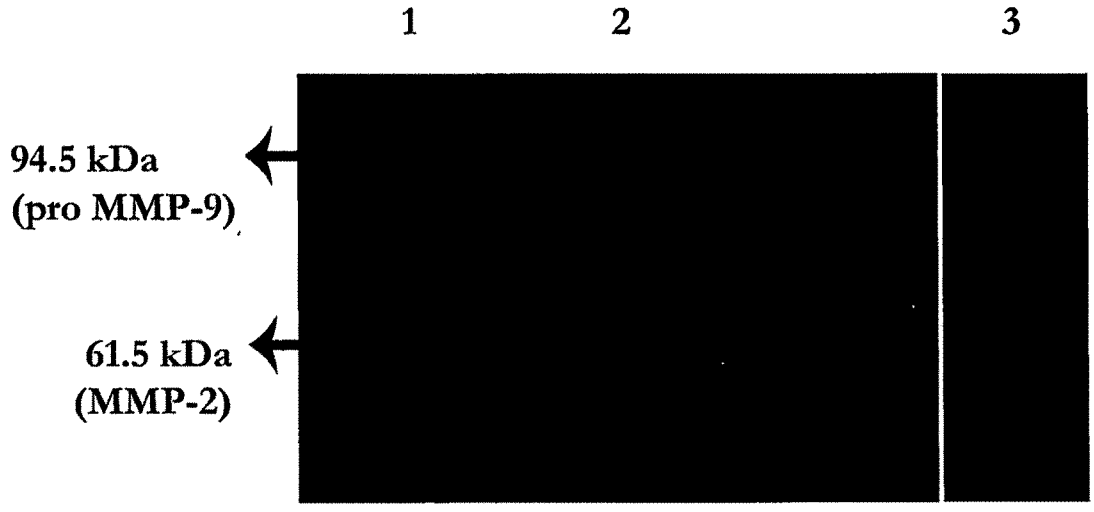

Figure 3.8: Expression of MMP-2 and MMP-9 in the wound epithelium (WE) stage in experimental animals. LANE-1 to LANE-2 corresponds to samples with different concentration of protein. Lane-3 corresponds to molecular weight marker.

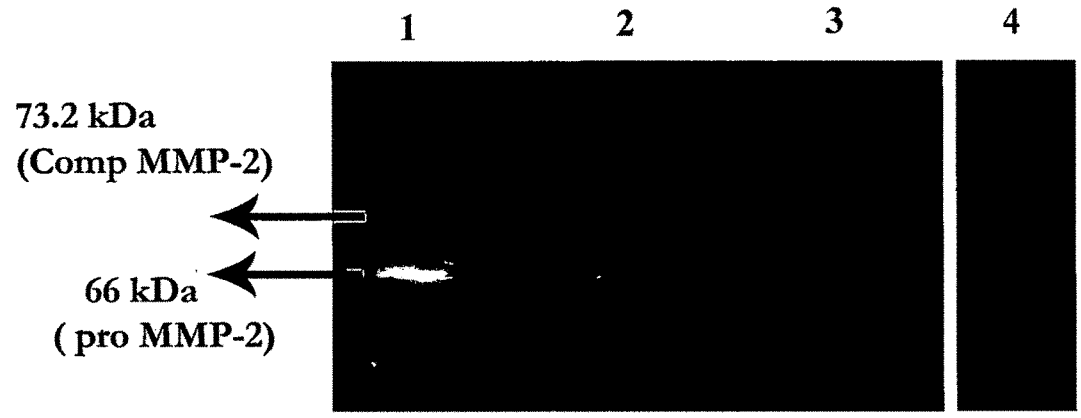

Figure 3.9: Expression of MMP-2 and MMP-9 in the blastema (BL) stage in control animals. LANE-1 to LANE-3 corresponds to samples with different concentration of protein. Lane- 4 corresponds to molecular weight marker.

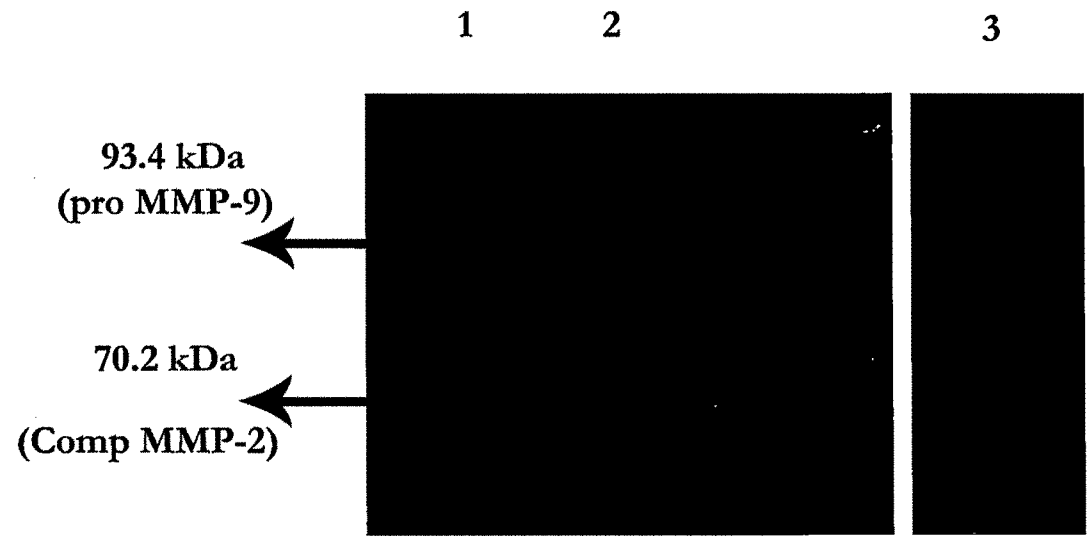

Figure 3.10: Expression of MMP-2 and MMP-9 in the blastema (BL) stage in experimental animals. Lane-1 and Lane-2 corresponds to samples with different protein concentration. Lane-3 corresponds to molecular weight marker.

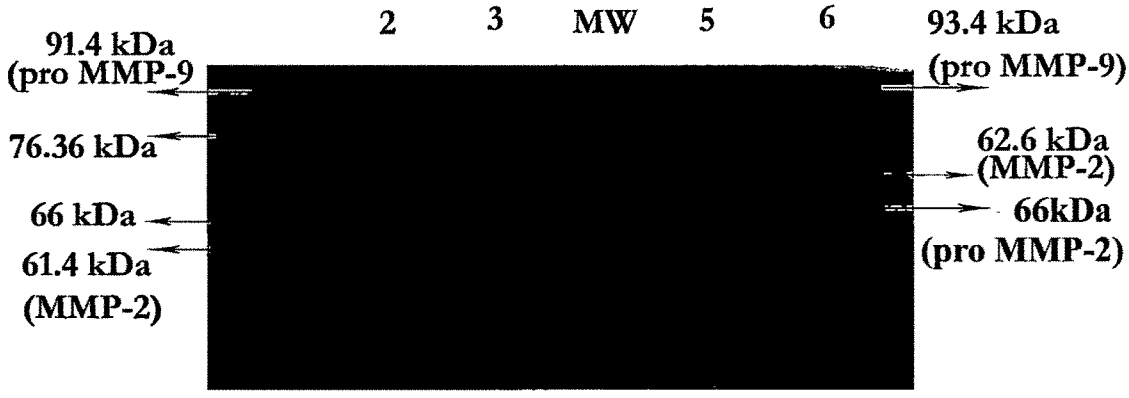

Figure 3.11: Expression of MMP-2 and MMP-9 in the differentiation stage in control and treated animals. (Lane-2 = Control without APMA activation, Lane-3= APMA activated sample, MW = Molecular weight marker, Lane 5 = Differentiation stage without APMA activation, Lane 6 = APMA activated at differentiation stage). Lane 1 (unmarked) also contains APMA activated samples but densitometry analysis has been ignored due to smearing of gel.

Figure 3.12 Histochemical Localization of MMP-2 and MMP-9