GENERAL CONSIDERATIONS

The relationship among regeneration, life, and death has been concisely summed up by one of the great masters of regenerative biology, Richard J. Goss (1969), in the following words "If there were no regeneration there could be no life. If everything regenerated there would be no death. All organisms exist between these two extremes. Other things being equal, they tend towards the latter end of the spectrum, never quite achieving immortality because this would be incompatible with reproduction". In other words, we are in a constant battle that pits our ability to locally reverse the second law of thermodynamics (regenerate) against inexorable entropic processes, a battle that we ultimately lose as individuals, but win as a species through reproduction. The regeneration of lost body parts and injured organs has captured the human imagination since the time of ancient Greeks. The ambition to induce human tissue regeneration has been reawakened by a number of recent developments.

Regenerative medicine - rebuilding organs and tissues - could conceivably be the 21st century equivalent of antibiotics of the 20th. Before this can happen, researchers must understand the signals that control regeneration. Researchers were puzzled for centuries over how body parts replenish themselves. In the mid 1700s, for instance, Swiss researcher Abraham Trembley noted that when chopped into pieces, hydra could grow back into complete, new organisms. Other scientists of the era examined the salamander's ability to replace a severed tail along with lizards as one of the reptilian model. And a century later, Thomas Hunt Morgan scrutinized planaria, flatworms that can regenerate even when whittled into 279 bits.

Animals exploit three principal strategies to regenerate organs. First, cells that normally do not divide can multiply and grow to replenish the lost tissue, as occurs in injured salamander hearts. Second, specialized cells can undo their training (a process known as dedifferentiation) and assume a more pliable form that can replicate and later respecialize to reconstruct a missing part. Salamanders and newts take this approach to heal and rebuild a severed limb, as do zebrafish to mend clipped fins and lizard to restore its lost tail. Finally, pools of stem cells can step in to perform required renovations. Planarians tap into this

resource when reconstructing themselves. Henceforth, the process of regeneration is broadly divided into two types *viz.* morphallactic and epimorphic regeneration. The process of morphallactic regeneration involves reorganization of the existing cells to form the lost structure, the resulting structure being comparatively smaller in size than the original. However, the epimorphic regeneration involves the generation of new stem cells, either by proliferation of the existing stem cells or by dedifferentiation of adult cells, which redifferentiate to form the lost appendage which is of the similar size as that of the original. The epimorphic regeneration is prevalent in lower vertebrates and can be considered as a substitute for living a normal life for the animal. However, only few classes of lower vertebrates show true epimorphic regeneration like Urodeles and Anurans. More specifically, Urodele amphibians have the impressive ability to perfectly regenerate their tails throughout adulthood (Stocum, 2003). Usually, amputation begins with wound healing, followed by the formation of a blastema of proliferating cells that go on to form a complete array of tissue types. Unravelling the mysteries of regeneration will depend on understanding what separates mammal's wound healing process from that of other animals that are able to regenerate. The difference might be subtle. Researchers have identified one strain of mice that seals up ear holes in weeks, whereas typical strains never do. A relatively modest number of genetic differences seem to underlie the effect. Perhaps altering a handful of genes would be enough to turn us into super-healers, too. But if scientists succeed in initiating the process in humans, new questions will emerge.

In recent years the emphasis in studying vertebrate limb/tail regeneration has shifted from the identification of requirements for regeneration to the characterization of the mechanisms that control the formation of the lost tissue. The requirements of regenerations have been characterized on the basis of inhibition studies. This concept has revealed some of the most important necessities for proper regenerative response *viz.* innervations, cytokines, growth factors, neural and hormonal factors etc. Epimorphic regeneration is outlined by: a wound closure response with the formation of an apical epithelial cap (AEC), with a cellular contribution, via dedifferentiation of the mature tissues and/or stem cells, to form the blastemal mesenchyme. Active cell proliferation response is regulated by multiple tissue interactions, including neurotrophic (NGF, FGF etc.) effect and AEC effect. Lastly, it is the differentiation and growth that takes place for the restoration of lost part (Han *et al.,* 2003).

Epimorphic regeneration consists of complex processes. Wound healing is one of these critical processes where different multiple cells types, soluble mediators and extracellular matrix (ECM) components are involved. Regulation of all these process in an orchestrated fashion results into appropriate regeneration of the tissues. Dedicated inflammatory cells (macrophages, neutrophils, lymphocytes) play an important role not only in the coordination of the overall inflammatory response to injury/trauma encountered after amputation, but also in directing the subsequent activity of mesenchymal and epithelial cells. Each process may be regulated by many bioactive substances, including growth factors, extracellular matrix components, and eicosanoids.

Eicosanoids such as prostaglandins (PGs), prostacyclins, and thromboxane have been implicated in wound healing in various tissues such as cornea (Joyce and Meklir, 1994), skin (Talwar *et al.*, 1996), gastrointestinal tract (Zushi *et al.*, 1996), and kidney (Cybulsky *et al.*, 1992). Prostaglandin E_2 (PGE_2), which constitutes the major PGs in human and rat skin (Jouvenaz *et al.*, 1970; Jonsson and Änggård, 1972), affects keratinocyte cell proliferation (Lowe and Stoughton, 1977; Pentland and Needleman, 1986), differentiation (Evans *et al.*, 1993), and also promote angiogenesis *in vivo* together with PGE_1 (Ziche *et al.*, 1982; Form and Auerbach, 1983). Talwar and co-workers (1996) have found that synthetic PGE_2 facilitates fibrosis during healing of wounded rat skin. One major class of mediators, prostaglandins, is produced from arachidonic acid by an initial reaction with either of two cycoloxygenase enzymes- COX-1 or COX-2, followed by prostaglandin synthase. In particular, PGE_2, COX-2, and microsomal PGE synthase have been linked with cancer and extensive cell proliferation (Rask *et al.*, 2006). COX-1 is constitutively expressed in most tissues, whereas COX-2 is induced in association with pathological inflammatory sites, including human cancers. For these reasons, it seems appropriate to address the role of prostaglandin E_2 in reptilian caudal regeneration.

The underlying hypothesis driving this line of research is that event, like inflammation that contributes to the release of prostaglandins (PGs), during regeneration largely dictates the final outcome of wound healing. In order to address this hypothesis, attention was focused on issues such as influence of prostaglandin E_2 in initiation and progression of epimorphic regeneration in reptilian system. Here, the hypothesis was tested by blocking the COX induced PGE_2 with the usage of non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs serve as one of the valuable tools for studying the involvement of PGs in physiological

processes by virtue of being the competitive inhibitors of COX activity. There are three known isoforms of COX- COX-1, COX-2 and recently discovered COX-3. Among, all the known isoform it is the COX-2 which is known to be expressed at the time of inflammation. Therefore, the experiment was designed where the non-selective and selective COX-2 inhibiting drugs were used. The mode of administration of the drug was *in loco* to maximize its absorption and effect in the tail stump of lizard. The inhibition of COX-1 and COX-2 was achieved by *in loco* injection of colosprin, a non-selective inhibitor. Cylooxygenase-2 was inhibited by usage of two selective COXibs- celecoxib and etoricoxib.

Hence, the first chapter of the present study dealt in selecting a drug with highest specificity in inhibiting the activity of COX-2 and also to understand the stage specific influence of COX induced PGE_2 in tail regeneration. The experiments were carried out in three stages:

During the first stage, selective and non-selective COX inhibitors were administered before autotomy was induced in the lizards. Data presented (Chapter 1) on the progression of the tail regeneration in lizards revealed that the animals administered with etoricoxib delayed the process of wound healing to the maximum as compared to other inhibitors and also to the control animals. Thus, it appeared that the extraneously administered etoricoxib (a selective COX-2 inhibitor), that causes blockage of PGE_2, might have retarded the healing process in the experimental animals. Moreover, the formation of wound epithelium (WE) is a very critical event and is controlled by many factors which possibly include PGE_2. Once a functional WE is formed it releases the necessary signals for further events in the process of tail regeneration. Hence, in the present study the administration of drug which blocks the synthesis of PGE_2, when administered before amputation, delayed the formation of WE and this delayed formation of WE further hampered the subsequent stages of caudal regeneration (blastema stage and differentiation stage). Thus, the late healing of the wound could be attributed to the absence of local inflammatory mediator PGE_2. Previous studies have demonstrated the presence of PGE_2 during the healing of the wound (Buntrock *et al.,* 1982; Joyce and Meklir, 1994; Futagami *et al.,* 2002; Savla *et al.,* 2001) which corroborate the current notion.

The formation of WE is followed by rapid cycles of cell division to accumulate a mass of pleuripotent cells called blastema. PGE_2 might be involved in the increased proliferation of blastemal cells in the regenerating tail of lizard, since it is known to regulate cell

proliferation. In the current study, a significant delay in formation of blastema had occurred in the animals administered with an exogenous blocker for PGE_2. However, on measurement of the rate of growth of the regenerates from 2-12 mm and 12-24 mm of growth of tail from different groups of lizards, it was apparent that etoricoxib retarded the rate of growth of regenerate the maximum as compared to that of colosprin and celecoxib treated animals. Delayed onset of differentiation in the treated animals could be due to less accumulation of blastemal cells. Moreover, the role of PGE_2 in cell proliferation has been observed in various cell cultures as well as in different animal models (Zalin, 1987; Joyce and Meklir, 1994; Kaneko *et al.,* 1995; Moreno, 1997; Otis *et al.,* 2005; Bondesen *et al.,* 2007). Thus, it was evident from this experiment that extraneous administration of different PGE_2 blockers before amputation has a profound influence on the process of regeneration in lizards with etoricoxib, exerting the highest detrimental impairment in regeneration.

In the second set of experiments, the animals were injected with the drugs just after amputation, then at the attainment of WE stage and at BL stage. The results of these set were very much similar to the above set, wherein the inhibition in rate of growth of regenerate was reflected at all the stages of regeneration. Therefore, it might be possible that the presence of PGE_2 has a positive influence on regeneration and its absence leads to a negative effect which was evident from the (reduced) rate of growth of regenerate. Moreover, when the animals were injected with high dose of COX inhibitors, it resulted in growth arrest of the inflamed tail (Chapter 1). When comparing the rate of regeneration by measuring the length of the regenerate of animals treated with etoricoxib demonstrated least growth of the regenerate among other COX inhibitors. Thus, in the later experiments etoricoxib was used for blocking the endogenous production of PGE_2 and the injections were given before induced autotomy. Moreover, Fourier Transform Infrared Spectroscopy (FTIR) was done in order to confirm the absorption of the drug in the tail stump and the regenerate of the animal. The peaks obtained were very much similar to that of standard etoricoxib. Therefore, from this morphometric study it could be concluded that PGE_2 might be playing a definite role in caudal regeneration of lizards.

The cellular events that occur during regeneration are orchestrated by a number of growth factors and cytokines. These cellular events are also known to generate reactive oxygen species (ROS) which serve as normal signalling molecules (Suzuki and Griendling, 2003). In addition to this, it is also known that prostaglandin may increase intracellular reactive

oxygen species production (Nencioni *et al.*, 2003). Cellular processes such as cell division are accompanied by the formation of some by-products that might be harmful for progression into further events of the regeneration. One such noxious by-product is the formation of reactive oxygen species that are formed during normal cellular processes (Halliwell and Gutteridge, 1985). However, the cells possess antioxidant mechanisms to buffer the harmful effects of these oxidants or ROS and maintain the homeostasis. The factors which are required for the successful resolution of inflammation are the cyclooxygenase pathways and release of ROS for destroying contaminating bacteria. Hence, a study was envisaged to understand the changes if any on the antioxidant status of the animal when PGE_2 synthesis is blocked (Chapter 2).

The current study has demonstrated that the administration of COX-2 inhibitor in the animal resulted in the decrease of major antioxidant enzymes in tail tissue which is in a state of inflammation after induced caudotomy. A disparate change was observed due to the differences in the amount, nature and activities of these enzymes in particular tissue. The status of the enzymes was examined in all the stages of regeneration wherein the tissue undergoes several amendments from lost structure to a replaced organ. Under these prerequisite the production of ROS and its removal from the system plays an important role (Steilieng *et al.*, 1999; Thannickal and Fanburg, 2000; Finkel, 2003; Gordillo and Sen, 2003; Diegelmann and Evans, 2004; Johar *et al.*, 2004). The amount of ROS production was estimated by the indirect evidence from available pool of antioxidants.

Current findings suggest that the presence of PGE_2 at wound site is important and is essential for wound repair and anti-oxidative effects. Similar observations made by Shen *et al.* (2006) give credence to the present findings. Some of the key antioxidant enzymes which may contribute to the oxidative stress were quantified. Superoxide dismutase (SOD) is an important antioxidant. Data gathered in the current study vouch for oxidative stress in the regenerating system with decreased activity of SOD, indicating decreased ability of the tissues to handle O^{-}_2 radicals. Similar findings on SOD have been reported in the tissues of mice exposed to high fluoride intake (Patel and Chinoy, 1998; Sharma and Chinoy, 1998; Vani and Reddy, 2000). This decrease was consistent till the WE and BL stages was attained. The SOD activity reaches to the basal level at DF stage of the regenerating tail. Moreover, similar trend was apparent even for other antioxidant enzymes (GSH, GPx, CAT, LPO, GST, Total-SH, and LPO), which were biochemically estimated at DF stage.

Further, in order to understand whether similar oxidative burden exists in other organs during regenerative phase, antioxidant status was also estimated in other organs of the body like liver, kidney, intestine and blood. The observed discrepancy in the antioxidant enzymes in respective tissues might be due to several reasons. First pool of reasons could be-differences in the route, time, duration, and dose of etoricoxib (COX-2 inhibitor) administered. Second group of reasons could be the state of the tissue and type of the cells present in it and the stimulus to which they have to respond, etc. A decrease in MDA level was observed in the tail at WE and BL stage. This decrease could support the minimum process of lipid peroxidation occurring in the tissue due to the inhibition of COX-2 activity. Studies conducted by Moseley *et al.* (2004) highlighted the roles of ROS/antioxidants in skin wound healing, their possible involvement in chronic wounds and the potential value of ROS-induced biomarkers in wound healing prognosis.

Considering the experimental data (Chapter 2), it is necessary to discuss other factors involved in the protective mechanism of animal. ROS if remained untreated would eventually lead to oxidative damage of DNA, proteins, carbohydrates and lipids (Thannickal and Fanburg, 2000). A higher susceptibility of tissue towards an oxidative stress depends upon both antioxidant capacity and the level of free radicals produced by the species (Prior, 2004). These amendments in the levels of the antioxidant enzymes could be due to the non-existence of PGE_2 in the lizards after the injury caused by induced autotomy. Further, in order to scrutinize the effect of increased ROS and blockage of PGE_2 on proteins and DNA damage, protein expression was studied with SDS-PAGE (Chapter 3) and the rate of cell proliferation was quantified with the administration of DNA marker dye (Chapter 4).

In epimorphic regeneration the process of wound healing is known to be accompanied by matrix reorganization, angiogenesis and the formation of a functional wound epithelium (Cohn *et al.*, 2002). To ascertain the influence of COX-2 induced PGE_2 alteration on protein expression and matrix degrading enzymes, in the regenerate of lizards, the experimental animals were subjected to the exogenous injection of etoricoxib, a specific COX-2 inhibitor. This agent is known to inhibit the activity of COX-2 enzyme and hence, the synthesis of PGE_2. It was observed that the biosynthesis of proteins is one of the most important biochemical processes during regeneration (Thornton and Bromley, 1973). Therefore, it was considered worth to investigate the change in protein expression occurring during

regeneration, when the production of autocoid prostaglandin E_2 is inhibited with *in loco* injection of etoricoxib in tail stump of lizard. All the observations presented in Chapter 3 were based on one dimensional gel electrophoresis. These observations points to a possible regulatory role of PGE_2 during the progression of epimorphic regeneration. A difference in abundance and intensity of the protein bands were found in all the specific stages of regenerate asserting the specificity of that protein at specified stages of caudal regeneration.

The most important aspect of epimorphic regeneration is dedifferentiation. The genes that are known to play a role in bringing this into effect are *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 were observed. Sophy and Ramachandran, (2001) noticed the appearance of new proteins in *Hemidactylus flaviviridis* during caudal regeneration and opined that these are formed in response to various developmental needs. The expression of protein band of 70.15 kDa in control animal is comparable to the molecular weight of COX-2 and absence of this band in treated animals support 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; Shen, 2005). SDS-PAGE has revealed one band near the predicted molecular weight of Sox gene product SOX-2 (34kDa) and SOX-11 (41.36kDa) during differentiation stage. This protein band is found to be absent from animals treated with COX-2 inhibitor. It is thus possible that with the blockage of PGE_2, the expression of these gene products is being affected and hence, contributing its share to hamper tail regeneration in treated animals.

Further, for successful regeneration, several modulators are required to act in unison. 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 matrix metalloproteinase 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 matrix metalloproteinases (MMPs) known to be upregulated by PGE_2 in

ECM degradation. Study was performed by using gelatin zymogrpahy, to understand the change in the gelatinolytic/collagenolytic factors in regenerating tails of lizards. An appreciable reduction in the activity of MMP-2 and MMP-9 was observed in the regenerate of treated animals. Suppression of gelatinase activity was reported when PGE_2 is blocked in osteoblast cells (Kusano *et al.*, 1998). All these amendments were investigated and reported in the last chapter of the thesis via experiments identifying the histoarchitectural alteration, localization of muscle marker proteins, VEGF (angiogenic molecule), rate of cell proliferation and nucleic acid localization.

Chapter four focussed on some of the finer aspects of epimorphic regeneration. Histological studies confirmed the results of Chapter 1, where a delay in rate of growth of regenerate was seen. The presence of number of promuscle was less in the regenerate of etoricoxib treated animals. Also, the thickness of apical epithelial cap (AEC) was less in sections of experimental animals resulting in a delay of restoration of lost tail in lizards. Muscle alterations were confirmed by localizing the muscle specific marker protein (desmin and myosin). Intensity and localization of desmin and myosin was less in comparison to control animals. Yazawa *et al.* (2004) reported that selective inhibition of COX-2 inhibits endothelial cell proliferation by induction of cell cycle arrest. Present study revealed that the VEGF localization, which is important for endothelial cell proliferation and angiogenesis, was adversely affected by the usage of COX-2 inhibitor. Nevertheless, it is known that COX-2 derived prostaglandin E_2 regulates the angiogenic switch (Wang and DuBois, 2004). In addition to influencing the cellular processes, PGE_2 also influences the levels of macromolecules including DNA, RNA and protein. The influence on protein is discussed in chapter 3 of the thesis. Effect on nucleic acids at different stages of regeneration were studied by acridine orange (AO) staining. Castaño *et al.* (2000) analysed that two selective inhibitors of COX-2 NS-398-[N-(2-cyclohexyloxy-4-nitrophenyl) methanesulfonamide] and SC-236-(4-[5-(4-chlorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzenesulfonamide) inhibit the DNA synthesis induced by platelet-derived growth factor (PDGF) in Swiss 3T3 fibroblasts and that this effect is mediated through the inhibition of COX-2 activity and hence, decrease in PGE_2 production. Marked decrease in DNA synthesis was observed after administration of etoricoxib (COX-2 inhibitor). O'Keefe *et al.* (1992) found that when growth plate of chondrocyte was exposed to prostaglandins of the E series (E1 and E2), a 13 fold increase in DNA synthesis was observed at an exposure of 24 hours. Histofluorescence of nucleic acid (DNA and RNA) showed that the intensity of green fluorescence (DNA) was

lower in the regenerates of the animals treated with COX-2 inhibitor at WE stage, unlike control which showed high intensity of histofluorescence for DNA. This observation indicated that the process of DNA synthesis was inhibited in experimental animals. However, vehicle treated controls showed a normal replication process. Otto *et al.* (1982) indicated that PGE_1 and PGE_2 probably act through a common mechanism with an event inovled in regulating the rate of initiation of DNA synthesis. Thus, the labelling and detection of nucleic acid was done by processing the fresh frozen section with fluorescent dye acridine orange, which interacts with both DNA and RNA (fluorescence green (Em~525 nm) when bound to DNA, and fluorescence red (Em~650 nm) when bound to RNA). The intensity of fluorescence for DNA and RNA showed a marked decrease at BL stage and DF stage. Therefore, it could be deduced that the application of COX-2 inhibitor in the lizards resulted in a decrease in the synthetic activities of the cells along with a marked decrease in nucleic acid contents which in turn affected the rate of cell proliferation and caused less number of cells to renter the cell cycle. To supplement these observation cell proliferation studies were undertaken.

Further, dedifferentiation of cells is the most important process of epimorphic regeneration (Holly *et al.,* 2003). Increase in cell proliferation in the regenerate is essential so that regenerate can step into the next stages without any hindrance. A recent study performed by Otto *et al.* (1982) revealed that untransformed fibroblasts in tissue culture may exhibit two extreme physiological growth states: quiescence where the majority of the cells are in the G_0-G_1 phase of the cell cycle, or active proliferation. Transition from the quiescent to the rapidly proliferating state can be regulated by the concentration of essential nutrients in serum or other growth promoting factors, such as insulin, present in the medium. Most of these factors seem to act on the cell surface, and hence, the continuation of the stimuli which trigger growth reinitiation within the cell. This probably requires the generation of intracellular signals in the vicinity of the plasma membrane. Changes in the intracellular concentrations of cyclic AMP, cyclic GMP, Ca^{2+} ions, and the influx of essential nutrients into the cell have been suggested as such regulatory signals. Furthermore, transformed fibroblasts and cancer cells as well as untransformed fibroblasts, before the mitotic period, show marked increase in synthesis of some prostaglandins, and this raises the possibility that such substances can act as both negative and as positive extracellular factors to regulate cell proliferation.

Mitotic index of the regenerate was identified by labelling the cells with BrdU when cells are at S-phase of cell cycle. BrdU labelling of the cells in the regenerate was performed by administering an intra peritoneal injection of BrdU at the blastema and differentiation stage, where maximum cell proliferation takes place. Current study has demonstrated that PGE_2 is one of the extracellular factors to exhibit positive regulation on cell proliferation. The blockage of COX-2 induced PGE_2 by a specific COX-2 inhibitor resulted in less number of cells in S-phase of the cell cycle and more number of cells in the quiescent state when compared to control animals. Therefore, it could be possible that the absence of PGE_2 leads to a decrease in frequency of cells re-entering the cell cycle and the induction of G_0/G_1 cell cycle arrest.

Finally, it could be inferred from the present study that COX-2 induced PGE_2 is a general and mandatory factor for proper regenerative response in lizards and is essential for migration, proliferation and differentiation of cells in response to induced autotomy in lizards. Moreover, endogenous production of PGE_2 is also found to act as an inducer for the matrix digestion and angiogenesis. Nevertheless, the finer mechanisms by which the this autocoid (PGE_2) influences tail regeneration in *Hemidactylus flaviviridis* need to be further validated in a suitable mammalian model (e.g. MRL mice) for its subsequent exploitation as one of the tools in regenerative medicines.