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COX-2 activity and expression pattern during regenerative wound healing of tail in lizard *Hemidactylus flaviviridis*

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ABSTRACT

Cyclooxygenase-2 (COX-2) is an important mediator of the immune response. It is found upregulated after pathogen invasion or tissue injury and also in many cancers. Of the lesser known functions of this enzyme is its role in effecting epimorphic regeneration. We have previously shown that COX-2 activity is essential for proper regeneration of tail in lizard *Hemidactylus flaviviridis*; however, the pattern of its activity and expression during the early stages of regeneration was unknown. The present work provides the first report of the trend in COX-2 activity and expression during the wound healing in epimorphic regeneration. It was found in *H. flaviviridis* that COX-2 gene was induced on the first day after amputation of the tail and expression and activity remained high through the course of wound healing. Further it was revealed that the COX-2 signal was mediated through the PKA/cAMP pathway via binding with the prostaglandin E_2 receptor 2 (EP2). In order to delineate the mechanism of epimorphic regeneration, we must understand the regulation of the major regulatory molecules therein. Therefore, the current study on the role of COX-2 during the regenerative wound healing is of paramount significance. Optimistically, such a mechanistic insight will help us achieve large scale tissue regeneration in humans in the future.

1. Introduction

The inducible isoform of Cyclooxygenase (COX) − COX-2–has been recognised for various roles in cell proliferation and expansion, over and above its well-known immunomodulatory effects [1,2]. COX-2 derived Prostaglandin E_2 (PGE_2) is reported to be a crucial mediator of cell-proliferation in various cancers. PGE_2 promotes the proliferation of head and neck squamous carcinoma (HNSC) cells by signalling through PGE_2 receptors expressed on most of these cells [3]. Also, COX-2 expression is found upregulated in pancreatic and intestinal tumors [4,5]. Studies involving the inhibition of COX-2 in cancer systems further validate the importance of this enzyme in the process [6,7]. While reports on the role of this enzyme in regeneration are few, studies on mammals, which have fairly modest regenerative ability, have implicated COX-2 and its major product PGE_2 as essential promoters of healing, repair and proliferation of various tissue types. Stimulation of the PGE_2-EP2 pathway in rabbit enhanced the regeneration of cartilage tissues [8]. The activity of this inducible enzyme was also discovered to be necessary for the healing of fractures in rats [9]. Studies in our lab have revealed that COX-2 activity is crucial for successful regeneration of the amputated tail in lizard *Hemidactylus flaviviridis* [10].

Epimorphic regeneration of the lizard tail involves formation of a regeneration blastema, a collection of progenitor cells, which orchestrates further outgrowth [11]. Essential for and prior to blastema formation is the covering of the amputation site with a wound epithelium, which marks the first major milestone after amputation and is crucial to the success of regeneration. It has been demonstrated previously in our lab that COX-2 activity promotes cellular and molecular processes of regeneration such as angiogenesis, myogenesis, proliferation of the wound epithelial cells and normal functioning of signalling pathways in the lizard [12,13]. It was however not known thus far when COX-2 is induced in the course of regeneration and how its signal is relayed. The current study was taken up to understand the activity and expression pattern of COX-2 immediately following amputation of the tail leading to formation of a wound epithelium in *H. flaviviridis*, since the fate of an amputated appendage heavily relies on the process of wound healing. Further, to gain a mechanistic insight, the receptors of PGE_2 were screened for their expression in the tail tissue and the expression of possible downstream signalling intermediates was also assessed.

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Table 1
Details of primers used for gene expression studies.

Gene	Primer Sequence		NLM accession number
	Forward	Reverse	
COX-2	ACGTCTTGACATCACGATCCC	GGAGAAGGCTTCCCAGCTTTT	NM_001167718.1
EP1	AGTTCGAACGTTGGTCACGA	AAGACCCAGGGGTCGATGAT	NM_013641.3
EP2	AGTTCAGCCAGAGCGAGAAC	AAGACCCAGGGGTCGATGAT	NM_001083365.1
EP3	GACGATGGCGTGCAACCT	CATCTTCAGCATCGTTACCAGCA	NM_001040468.1
EP4	CATTCCTCTGGTGGTCCGTG	GCTTGCAGGTCAGGGTTTTG	NM_001081503.1
18S rRNA	GGCCGTTCTTAGTTGGTGGA	TCAATCTCGGGTGGCTGAAC	NR_003278.3

2. Material and methods

2.1. Animal procurement and maintenance

Adult *Hemidactylus flaviviridis* were procured from urban habitat, without any preference of sex. The lizards were housed in wooden cages and allowed to acclimatise to the animal house conditions for one week before commencement of experiments. Ambient conditions of 36 ± 2 °C were maintained with a 12:12 h light-dark cycle daily. The lizards were fed with cockroach nymphs once daily and water was provided *ad libitum*. Experimental procedures were approved by the Institutional Animal Ethics Committee vide protocol no. 984/07/2014-2.

2.2. Experimental design

Lizards were divided into four experimental groups (n = 6), each representing a different time-point in regeneration. Tails from all groups were amputated using the blunt edge of a scalpel at a pre-determined segment, after which the regenerating (distal-most) segment was collected at 1, 2, 3 and 4 days post-amputation (dpa). 4 dpa corresponds to attainment of wound epithelium stage under conditions of our housing facility. Tissue for 0 dpa (resting stage tail) was collected from the above groups during the amputations. Collected tissue from all groups was used further for enzyme activity assay, gene and protein expression analyses and immunohistochemistry experiments. For the study of signalling intermediates, COX-2 activity was inhibited *in vivo* by oral administration of etoricoxib, a pharmacological inhibitor of COX-2. For this, lizards were divided into two groups (n = 6), *viz.*, control and etoricoxib. Drug administration and dosage was as described by Buch et al. [13]. After validation of COX-2 inhibition by etoricoxib *in vivo* [10], tissues were collected at wound epithelium stage and processed for western blot.

2.3. COX-2 activity assay

10% w/v homogenates were prepared in cold Tris-EDTA buffer from regenerating tail tissue collected at 0, 1, 2, 3 and 4 dpa. Activity of COX-2 in these homogenates was assayed using a microplate assay kit (Cayman Chemical Co., USA; cat no. 760151), wherein formation of N,N,N',N'-tetramethyl-*p*-phenylenediamine (TMPD) is measured at 590 nm as an indicator of the enzyme's peroxidase activity.

2.4. Protein expression analysis

Western blot was used to determine relative protein expression in various experimental groups. 30 µg total protein, isolated from tissue regenerates and quantified by the Bradford assay [14], was electrophoresed on a 12% polyacrylamide gel and transferred on to a PVDF membrane. Membranes were incubated in 0.1 µg/ml of either Anti-COX-2 IgG Mouse (Sigma Aldrich, USA), Anti-phospho-PI3 K IgG Rabbit (SantaCruz Biotech, USA), Anti-phospho-CREB IgG rabbit (Cell Signaling Technology, USA), Anti-phospho-p38 MAPK IgG Mouse (Thermo Fisher, USA) or Anti-β-Actin (SantaCruz Biotech, USA) overnight and probed using the ALP-BCIP/NBT system.

2.5. Gene expression analysis

Relative quantification of gene expression was carried out using reverse transcription PCR. Total RNA was isolated from tissue samples using TRIzol reagent (Life Technologies, USA) and was quantified using the Qubit assay on a Qubit 3.0 fluorimeter (Life Technologies, USA). 1 µg RNA was reverse transcribed to cDNA using High Capacity RT kit (Life Technologies, USA). Amplification from the cDNA samples was performed on a T100 thermal cycler (BioRad, USA) using ReadyMix (Sigma, USA) as per the following program: 3 min at 95 °C, 40 cycles (each cycle consisted of 10 s each at 95 °C, 60 °C and 72 °C). This was followed by electrophoresis on a 2% agarose gel and imaging on gel documentation system (GeNei, India). Primers were designed using the online primer blast tool of NCBI. Details are outlined in Table 1.

2.6. Immunohistochemistry

Tissue regenerates at wound epithelium stage were collected and embedded in cryoembedding medium (Tissue-Tek, Sakura Finetech, Japan). Fresh frozen sections of 12 µm thickness were cut on a cryostat microtome (Cryocut E, Reichurt-Jung, USA) and blocked in BSA-blocking buffer for 1 h at room temperature, followed by incubation in Anti-COX-2 IgG mouse (Sigma Aldrich, USA) at 0.3 µg/ml. The ALP-BCIP/NBT system was used for colour development. Observation and documentation was made on a DM2500 microscope (Leica, Germany) with Leica EZ digital camera.

2.7. Statistical analysis

A one-way ANOVA, followed by Bonferroni post-hoc analysis was carried out in SPSS v17.0 (IBM, USA) for comparison of the means.

3. Results

3.1. COX-2 activity during early stages of regeneration

COX-2 activity was measured at 0, 1, 2, 3 and 4 days post-amputation (dpa). Wound epithelium formation was achieved at 4 dpa in the ambient conditions of our set-up. Activity was seen to increase significantly ($p < .001$) at the first measured time-point 1 dpa from the resting (uncut) state (Fig. 1). Activity remained high for rest of the tested time-points. This reflects an early induction of the enzyme after amputation.

3.2. COX-2 protein expression

An immunoblot analysis was carried out at the said time-points. Results showed COX-2 expression increasing by over 1.7 folds early on in regeneration from the first measured time-point 1 dpa (Table 2, Fig. 2), as seen from densitometric analysis of the blot. Protein