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Synthetic FGF receptor antagonist hampers the nucleic acid synthesis and protein profile in the regenerating tail fin of *Poecilia latipinna*

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ABSTRACT

An earlier study of ours has shown that blocking FGF2 signaling curtails the onset and progression of tail regeneration in fish. The current study was envisaged to understand the mechanistic reasons behind the compromised tail fin regeneration in response to SU5402 treatment in fish Poecilia latipinna. Results showed that the FGF receptor antagonist significantly reduced the DNA, RNA and protein turnover during the wound healing and blastema stages of regeneration. Moreover, striking alterations in the protein profile was also noticed during this period of regeneration in the treated group of fishes. However, upon reaching differentiation stage of epimorphosis the studied parameters by and large were found comparable between the control and treated animals. Therefore, it could be inferred that the FGF2 signalling is inevitable for the successful attainment of initial events of regeneration during fish fin regeneration.

Keywords: Tail fin, regeneration, SU5402, FGFR, Teleost Fish

INTRODUCTION

The teleost fish, *Poecilia latipinna*, has the capacity to completely regenerate their lost part of tail fin within 15 days of amputation through reprogramming and migration of cells that ultimately differentiate to restore the structural integrity of the damaged appendage. The first step of tail regeneration is the closure of the wound. This is a non proliferative event, involving the migration of existing epithelial cells to cover the wound [1]. The epithelial cells need to break through the ECM to reach the site of amputation. The formation of wound epidermis is completed within the first 12 hours post amputation (hpa). Once the amputation surface is covered by a wound epithelium, the next action is characterized by the removal of many existing elements of the extracellular matrix, as the process proceeds towards dedifferentiation and blastema formation, the creation of regeneration cells [1]. Shortly after the wound epidermis is formed, mesenchymal cells immediately beneath this epithelium become disorganized. It has been reported that this tissue remodelling step requires the action of matrix metalloproteinases [3]. Subsequently, a number of cells beneath the amputation plane begin to proliferate and migrate toward the wound epidermis to form blastema [1].

Several signalling pathways have been studied in various regeneration models in order to understand the mechanism of epimorphosis. Among them, FGF signalling is involved in mammalian wound healing and tumor angiogenesis [4-5]. FGF has numerous roles in embryonic development, including induction and/or patterning during organogenesis of the limb, tooth, brain, and heart [6-10]. Evidence obtained from studies of amphibian limb regeneration supports roles for FGFs in regeneration [11-12]. Further, it was also reported that extraneous FGF2 accelerated the blastema formation in *Hemidactylus flaviviridis* [13].

To investigate the role of FGF signalling, some investigators used a pharmacological inhibitor of FGFR1, SU5402, and showed that incubation with SU5402 immediately following amputation prevents blastema formation without affecting wound healing [14].

In our previous study we have noticed that FGF2 signalling unequivocally support the epimorphosis in *P. latipinna* as evident by the compromised regenerative response in animals treated with FGF receptor antagonist SU5402 [15]. As to the problem of the relationship between the level of binding and the mitotic activity of the tissue, Prodi and associates [16] reported a definite increase of binding to DNA in regenerating rat liver. Because RNA is an essential component of protein synthesis, its concentration in tissue often reflects the rate of protein synthesis. The RNA:DNA ratio provides an index of protein synthetic capacity per cell since the amount of DNA per cell is assumed not to vary with condition or with growth rate[17].

Furthermore, the formation of regenerating wound tissue involves not only production of new cells and ECM remoulding, but also synthesis of relatively large amounts of protein [18-21]. Hence, logically it can be inferred that while the new tissue is being formed, nucleic acid metabolism is probably different from that observed in resting state. There has been some indication that this may be the situation for ribonucleic acid during limb regeneration in amphibia [22].

The earliest work on nucleic acids was in connection with exudates from regenerating wound tissue [23]. Subsequent work on such exudates still does not definitely establish whether the nucleic acids originate in the damaged cells, extraneous body tissue, leukocytes, or by synthesis in cells of the regenerating tissue [24-26].

There are several studies which have reported that there is a series of proteomic changes that occur during the process of fin regeneration [27-29]. Thus, obtaining differential protein expressions and the association of the various proteins during the process of regeneration might lead to a new understanding of the regeneration mechanism.

In the present study attempts were made to evaluate nucleic acids as well as protein levels in the regenerating fins of the control and SU5402 treated fishes to understand the alterations caused due to block of FGF2 signalling and performed SDSPAGE analysis to study the regulation of stage specific expression of proteins in the normal as well as the treated fish fin regenerates.

MATERIALS AND METHODS

Maintenance of animals

Sailfin Molly, *Poecilia latipinna* (Lesueur, 1821), of both the sexes of same age (size 4-5cm), weighing about 4-5g were purchased from a commercial supplier and maintained in glass aquariums containing sterile dechlorinated water with constant aeration. The daily photoperiod was 12h (hours) of light and 12h of darkness, and the water temperature was kept in the range of 26 ± 2°C. Animals were daily fed with daily readymade fish food (whiterose fish food, Mumbai, India) *ad labium*. Handling and processing of fish were carried out according to the ethical principles (Drugs and Cosmetics Rules, 2005) approved by the Institutional Animal Ethics Committee [No. ZL/IAEC/15-2010] constituted as per the guidelines of CPCSEA, India.

Drug dosage and experimental set up

40 animals were divided into two groups. 20 animals of control group were injected with 1%DMSO and treated group were dosed with 2µM/g body wt. of freshly prepared SU5402 in 1% DMSO. The fishes were dosed a day prior to amputation and continued every day till the control animals reach the differentiation stage. The fins were amputated from both the groups at three defined stages of regeneration *viz.* wound healing (1dpa), blastema (4dpa), differentiation stage (7dpa).

Nucleic acids and Protein estimation

The fins from each group were pooled, homogenized for 10% and then further processed for estimating the nucleic acids as well as the protein contents in the tissue sample. Extraction of nucleic acids was done by the method described by Schneider [30] and the DNA and RNA levels were estimated by the DPA and Orcinol methods respectively [31]. The protein estimation was done according to BCA (Bicinchoninic acid) assay kit (Genei Products, Merck, USA) as described by Smith *et al*. [32].

SDS-PAGE

Expression of various proteins at each stage was identified using SDS-PAGE technique. Protein content was determined using BCA assay. Equal amount of total protein was loaded and separated by SDS-PAGE on 12.5% gels; stained with silver staining method

Determination of molecular weight and spot densitometry analysis of gel

Molecular weight and spot densitometry of the protein bands were determined by using Doc-ItLs software (GeNei, Merck, USA). Analysis was performed on the scanned images of the gel taken in charged coupled device (CCD) camera and edited in Adobe Photoshop. Auto background subtraction was performed using the same 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.

RESULTS

Transcriptional and translational profiling of regenerating caudal fin

The amount of DNA contained in regenerating tissue of caudal fin at three specific stages is shown in Tables 1. The amount of DNA (µg/100mg tissue) in the regenerating tissue appeared to increase at blastemal stage (about 4dpa) as compared to wound healing stage (1dpa) and thereafter gradually decreased by the time it reached the differentiation stage (7dpa).

The amount of RNA (µg/100mg tissue) also reached its maximum at the blastemal stage. In all the cases, however, SU5402 treated group always showed a lower concentration of DNA and RNA as compared to the controls. Similar results were obtained for protein content also during all three stages (Table 1).

Accordingly, the DNA:RNA as well as RNA:Protein ratios were also found to be lowered during the early regeneration followed by an intense increase at the blastemal stage and then a gradual decline as the cells begin to redifferentiate to compensate the lost structure. However, the SU5402 treatment reduced the ratios significantly at all the three stages of regeneration, (Table 2).

A decreased amount of DNA and RNA in the tissue during the wound healing juncture are to be expected because, during healing of the wound, the cells do not undergo cell division and so can be considered as being in the lag phase; and the mitotic activity and DNA synthesis begins after this phase. After this period, a vigorous DNA synthesis starts as it is evident from the higher amounts of DNA in the regenerating fins at the blastemal stage. The values then gradually decrease as the fin proceeds to the end of regeneration course (Table 1).

Table 1: Nucleic acid levels in the fin regenerates of control and SU5402 treated fish

Experimental Groups	WH STAGE (1dpa)		BL STAGE (4dpa)		DF STAGE (7dpa)	
	DNA (µg/100mg tissue)	RNA (µg/100mg issue)	DNA (µg/100mg tissue)	RNA (µg/100mg tissue)	DNA (µg/100mg issue)	RNA (µg/100mg tissue)
Control	18.233±0.145	3.510 ± 0.006	28.167±0.167	5.033 ± 0.033	22.333 ± 0.167	4.590 ± 0.038
Treated	11.867 ±0.186*	2.637 ± 0.020*	18.667 ±0.441*	4.100 ± 0.058*	15.333 ± 0.333*	3.830 ± 0.012*

Table 2: Cellular proliferative and synthetic activities in the fin regenerates of control and SU5402 treated fish

Experimental Group	WH STAGE		BL STAGE		DF STAGE	
	DNA: RNA	RNA: Protein	DNA: RNA	RNA: Protein	DNA: RNA	RNA: Protein
Control	5.337 ± 0.114	4.399 ± 0.156	5.597 ± 0.061	5.593 ± 0.108	4.865 ± 0.015	5.399 ± 0.104
Treated	4.503 ± 0.046*	3.769 ± 0.081*	4.557 ± 0.172*	5.083 ± 0.108*	4.003 ± 0.088*	4.996 ± 0.046*

*@Values are expressed as Mean ±SEM; n=5; Control: 1% DMSO; Treated: 2µM/gm body Wt. SU5402; *p<0.001*

Protein profiling by SDS-PAGE

Alterations in the protein content were found at all the three stages, *viz.*, Wound healing, Blastema formation and Differentiation stages during regeneration (Table 3). Some of the protein bands which were observed in the control group remained absent in the SU5402 treated groups. Also, the intensity of protein bands was found to be low in the treated samples as compared to the control ones.

Table 3: Protein content in the fin regenerates of control and SU5402 treated fish

Experimental Groups	Protein Content (mg/100mg tissue) in WH(1dpa) stage	Protein Content (mg/100mg tissue) in BL(4dpa) stage	Protein Content (mg/100mg tissue) in DF(7dpa) stage
Control	0.801±0.02[@]	0.900±0.012[@]	0.860±0.012[@]
Treated	0.712±0.012*	0.807±0.018*	0.767±0.007*

*[@]Values are expressed as Mean ±SEM; n=5; Control: 1% DMSO; Treated: 2µM/gm body Wt. SU5402; *p<0.001*

At wound healing stage (1dpa), the protein fraction of control animals were enriched with polypeptides having molecular masses of 73.42, 59.71, 54.36, 28.61, 18.09, 13.78 and 10.71 kilo Daltons (kDa). The intensity of these bands was much less in the SU5402 treated groups as compared to the control with the exception of the band 59.71 kDa that showed the intensity somewhat greater in the treated as compared to the control ones. However, it was noticed that the difference in the intensity was not very striking (Table 4).

The blastemal stage showed absence of many prominent polypeptides that were observed with great intensity in the control groups. The band of the molecular weights 116.54, 67.32 and 54.36 remained absent in the treated groups whereas they were observed with high intensity in the control groups. Some other bands with similar molecular masses were also observed (107.04, 93.24, 45.92, 13.78 kDa); nevertheless, the intensity in treated always remained low as compared to the control group (Table 5).

At differentiation stage, the protein fraction of control animals were enriched with polypeptides having molecular masses of 116.54, 107.04, 93.24, 73.42, 67.32, 59.71, 54.35, 45.92, 28.61, 23.71, 18.09, 13.78, 10.71 kDa. The intensity of these bands was much less in the SU5402 treated groups as compared to the control with the exception of the band 67.32 kDa (Table 6).

Table 4: Effect of FGF receptor inhibitor SU5402 on the protein profile on the fin regenerates of control and treated fish at Wound Epithelium stage (1dpa)

LANE 1 (Control)		LANE 2 (Treated)	
Molecular Weight (kDa)	Optical Density (Arbitrary Unit)	Molecular Weight (kDa)	Optical Density (Arbitrary Unit)
116.54	487.13	116.54	608.13
107.04	455.68	107.04	26.36
93.24	473.85	93.24	376.24
73.42	394.58	73.42	251.46
67.32	611.33	67.32	111.24
59.71	839.22	59.71	921.1
54.36	485.89	54.36	347.93
45.92	479.72	45.92	301.79
28.61	374.97	28.61	167.46
23.71	490.53	23.71	295.05
18.09	561.24	18.09	337.03
13.78	629.03	13.78	415.98
10.71	660.99	10.71	446.42

Table 5: Effect of FGF receptor inhibitor SU5402 on the protein profile on the fin regenerates of control and treated fish at Blastema stage (4dpa): blank cells indicate absence of specific protein band

LANE 1 (Control)		LANE 2 (Treated)	
Molecular Weight (kDa)	Optical Density (Arbitrary Unit)	Molecular Weight (kDa)	Optical Density (Arbitrary Unit)
116.54	289.48	116.54	-
107.04	142.83	107.04	114.51
93.24	241.12	93.24	112.2
73.42	1.5	73.42	249.45
67.32	110.43	67.32	-
59.71	236.84	59.71	457.57
54.36	104.26	54.36	-
45.92	160.13	45.92	104.55
28.61	214.68	28.61	212.22
23.71	436.32	23.71	4.12
18.09	0	18.09	268.66
13.78	331.15	13.78	326.49
10.71	4.36	10.71	421.66

Table 6: Effect of FGF receptor inhibitor SU5402 on the protein profile of the fin regenerates of control and treated fish at Differentiation stage (7dpa)

LANE 1 (Control)		LANE 2 (Treated)	
Molecular Weight (kDa)	Optical Density (Arbitrary Unit)	Molecular Weight (kDa)	Optical Density (Arbitrary Unit)
116.54	495.3	116.54	384.8
107.04	366.39	107.04	205.73
93.24	399.62	93.24	292.15
73.42	347.48	73.42	237.33
67.32	277.95	67.32	278.01
59.71	374.75	59.71	296.64
54.36	366.08	54.36	278.69
45.92	363.83	45.92	297.44
28.61	386.78	28.61	316.98
23.71	397.36	23.71	345.81
18.09	521.69	18.09	472.32
13.78	593	13.78	523.2
10.71	563	10.71	504.05

DISCUSSION

The fins of teleosts are appendices capable of regenerating by an epimorphic process that completely restores the original shape and size in a few days post amputation. The process of regeneration is a multifaceted one which begins with amputation and results in the complete replacement of the structures and tissues removed. It is well documented that two key signals namely soluble growth factors and extracellular matrix (ECM) directly influence the initial stages of regeneration [33-36].

After a partial amputation, the process that follows include, wound healing and blastema formation to form a particular cell population responsible for the building of the different tissue elements. This extensive process comprises, essentially, dedifferentiation and cell proliferation. It is known that the interaction between the cells and the extracellular matrix is, in part, responsible for the control of both processes [2]. This interaction has been widely studied in several *in vitro* and *in vivo* models [37], and earlier studies have proved that the teleost fin is a good *in vivo* model for the study of the regenerative processes [38-40].

Inhibition of fin regeneration by SU5402, a specific FGF receptor inhibitor, was already established by our previous morphometric study of fish fin regeneration [15]. However, we wanted to determine whether SU5402, played any cardinal role in affecting the nucleic acids and protein levels of the regenerating caudal fins whereby hampering the progression of epimorphosis. Thus, the present study deals with the alterations of the DNA-RNA-Protein levels in the pharmacological inhibitor treated group as well as observation of the alterations in proteins by SDS-PAGE in the SU5402 treated animals compared to control group of animals.

The first step was to evaluate how much the inhibitor affects nucleic acid and protein levels in the animals of control (1% DMSO) and treated (SU5402) groups. We examined the variation in total DNA, total RNA, total Protein as well as the DNA: RNA and RNA: Protein ratios during different stages of fin regeneration. Experiments dealing with mitotic counts [41] and with cytophotometric analysis of total DNA content [42] or measurements of DNA, RNA and Proteins by ^{32}P [43] conducted on regenerating planarians have already showed the significance of DNA-RNA-Protein ratio in epimorphic regeneration. In our results, overall, the DNA: RNA: Protein content was minimal during the early stages of regeneration as compared to the later stage and then slowly lowered by the time it reached differentiation stage. We found a radical decrease in the total DNA, RNA and protein contents as well as the DNA: RNA and the RNA: Protein ratios in the fin regenerates of SU5402 treated fishes compared to that of controls

Decreased DNA content in the regenerating fins of SU5402 treated fishes is suggestive that these cells could not enter the new cycles, main reason probably being the insufficient availability of FGF signals. Thus, there is probability of a defect in cell cycle regulation, following retardation in the rate of replication of the dividing cells, that consequently results in the low DNA content in the SU5402 treated fins. Apart from the synthesis of DNA, the proliferating blastemal cells also transcribe RNA and synthesize new proteins to meet the demands of the rapidly dividing cells. Synthesis of RNA is followed by the translation of mRNA into proteins in the regenerates. The concentration of RNA and protein as well were lesser in the receptor inhibitor treated groups as compared to the control ones. These low rates of DNA, RNA and Protein in the treated animals, to some extent, reflect the unavailability of growth factors to the injured tissue, thereby lowering their transcriptional and translational levels. Thus, the demonstration of such changes in the DNA-RNA concentration and protein metabolism of amputated animals as compared to normal animals, points to the possibility that there also may be some change in the nucleic

acid metabolism of the wounded animals. It seems quite probable that some further clue to the metabolism of the nucleic acids may be obtained from consideration of the nucleotide and protein content of the regenerating tissue. Hence, the present study considers an alteration in the metabolism of nucleic acids and thereby protein synthesis in regenerating fins when treated with FGF receptor inhibitor SU5402.

Significance of FGF2 was already noticed by observing the delayed wound epithelium in the tyrosine kinase inhibited (SU5402) group [15]. Thus, it was apparent to find out its role in the protein turnovers that occur during ECM remodelling and how the receptor inhibitor affects former. Therefore, the expression of proteins was evaluated in the control and SU5402 treated groups. Results depicted a high significance of FGF2 during each of the stages of regeneration.

Also, a regulation of differential expression of proteins is required for a successful fin regrowth. The proteins expressions in the regenerating fins of the control and treated were evaluated by SDS-PAGE and the intensity of the band was measured using spot densitometry. Lower content of protein in the treated fins as compared to the control ones is showed by the absence of few of the band in the treated fins. These may be the proteins that are required for regeneration, and could not be expressed as a result of the signal inhibitor treatment; thus proving the importance of FGF2 in the expression of various essential proteins required for regeneration. Therefore, it is valid to assume that FGF2 down regulated many of the essential proteins that may have significance during caudal fin regeneration. No significant difference in the protein bands at differentiation stage between both the groups leads to suppose that the later stage of regeneration is by and large independent of FGF2 signalling. However, minor alterations in the expression of bands in both the groups cannot be neglected.

CONCLUSION

Study provides evidence that inhibition of FGF2-FGFR1 signalling pathways inhibits caudal fin regeneration. This inhibition may be mediated by unsuccessful interaction between the FGF2 and its receptor, thereby resulting in lower nucleic acids and protein contents and lowered protein expression in the fins. Interactions between the ECM and growth factors via receptors are an important affair that helps in the further responses of the fin regrowth. Thus, FGF2 signalling is unavoidable for the fin regeneration.

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