Matrix Metalloproteinases and Blastema Formation

Fengyu Song1*, L. Jack Windsor,1, and David Stocum2
1Indiana University School of Dentistry, Indianapolis, Indiana, USA and 2 School of Science, Indiana University-Purdue University Indianapolis, Indianapolis, Indiana, USA. *: Corresponding author

Introduction

The success of the blastema formation determines the limb regeneration occurred in urodele amphibians such as the axolotl (Ambystoma mexicanum). In contrast, mutant type axolotl, short-toe (s/s), never forms blastema after amputation, while African clawed frog Xenopus laevis only forms fibroblastema. Neither s/s nor xenopus froglet is able to regenerate its amputated limb. These differences have been recognized without a persuasive explanation.

The blastema is a mass of mesenchymal stem-like cells that resembles the early embryonic limb bud. Blastema formation has been believed to be achieved by the dedifferentiation and rapid growths of local resident cells, which are released from extracellular matrix (ECM) by enzymatic proteinases, such as matrix metalloproteins (MMPs). MMPs can activate and/or release growth factors from tissue, which play roles during cell differentiation, growth, and possibly cell dedifferentiation to mesenchymal stem-like cells. Recently MMPs have also been suggested the function to maintain the stem cell niche. Studies have shown the alteration of the MMPs expression, such as MMP-2, MMP-9, and MMP-3/-10 (Yang and Bryant, 1994; Park and Kim, 1999; Kato et al, 2003; Vinarsky et al, 2005), during blastema formation in axolotl and newt, but relatively scarce study has been done in the regeneration deficient amphibians such as s/s and xenopus. It is reasonable to hypothesize that the MMPs play a role during blastema formation and therefore their expression patterns will be different in regeneration competent and deficient animals. In this study we used zymography and MMP array to detect the expression of MMPs during the accumulation blastema formation in axolotl, s/s, and xenopus froglet.

Materials and Methods

Wild-type (snout-to-tail length of 10-12 cm), s/s axolotl larvae (12-16 cm) and xenopus stage-60 were amputated through the mid tibia-fibula of the hindlimbs, and the tissues were collected at the stages of wound closure, histolysis / dedifferentiation, and accumulation blastema.

Zymography
Tissue samples were ground and homogenized in 300 µl of cell lysis buffer (RayBiotech MMP Antibody Array, RayBiotech, Inc., Norcross, GA), and centrifuged for 10 minutes at 5000 rpm. The supernatant was collected and stored at -70°C until used. The protein concentration of each sample was determined by the Bradford technique at 595 nm wavelength utilizing bovine serum albumin as a standard and Bio-Rad protein assay dye reagent concentrate (Bio-Rad Laboratories, Inc, Hercules, CA).

Twenty µg of total protein from each samples was resolved at 200 V in 12% SDS-PAGE gels containing 1 mg/ml of gelatin. The gels were washed thoroughly to remove the SDS and then incubated at 37°C overnight with or without the following proteinase inhibitors: 1 mg/ml soybean trypsin inhibitor (STI; Worthington Biochemical Corporation, Lakewood, NJ) or 10 mM 1,10-phenanthroline (Sigma-Aldrich). After staining with Coomassie blue, the proteinases capable of digesting the gelatin were visualized as lytic bands on the blue background of the gels.

MMP Array
RayBio Human Matrix Metalloproteinase Antibody Array (RayBiotech) was used according to the manufacturer’s protocol to identify multiple MMPs and TIMPs expressed in the regenerating and control tissues. The array dots were semi-quantified by NIH SCION image Beta 4.03 software (Scion Cooperation, Frederick, MD). The negative control on the membranes was set as background and the positive control on the membranes was used to normalize the different arrays. The comparison of relative expression levels between the different samples or different animals was performed to determine the change in levels of each MMP and TIMP at the different stages of limb regeneration.

Statistical Analyses
Statistical analyses were performed using One Way Analysis of Variance (ANOVA) and Tukey’s test in the Statistical Package for Social Science 11.5 (SPSS Inc., Chicago, IL). The level of the significance was set at a p<0.05.

Results
Figure 1: Gelatin Zymography. 20 µg of total protein from all the time points of wild-type axolotl, s/s axolotl, and xenopus stage 60 were mixed with loading dye (without β-mercaptoethanol (Panel A, B and C) and resolved in a 12% SDS-PAGE with 1 mg/ml gelatin at 200V. The gels were washed thoroughly to remove SDS and incubated for 24hrs at 37°C with or without inhibitors, 10 uM 1, 10-phenanthroline (1, 10-P) or 1 mg/ml soybean trypsin inhibitor (data not shown) and then stained with Coomassie blue to visualize lytic bands. 1: Wound closure stage; 2: histolysis/dedifferentiation; and 3: accumulation blastema.

Figure 2: MMP Antibody Array. 20 µg of total protein from individual stages from wild-type axolotl, s/s axolotl, and xenopus stage 60 were incubated with the MMP membrane following the manufacture’s instruction. The signals of the different MMPs and TIMPs were detected by exposing the membranes to X-ray film for 30 seconds and the films were developed. The density of each dot was measured by NIH SCION image Beta 4.03 software. Positive control was used as an internal control to normalize the blots, while the negative control was measured as background.

Table 1: Matrix Metalloproteinase Antibody Arrays

<table>
<thead>
<tr>
<th>Stage</th>
<th>MMP-1</th>
<th>MMP-2</th>
<th>TIMP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>1.01 ± 0.07</td>
<td>1.06 ± 0.04 *</td>
<td>1.03 ± 0.11</td>
</tr>
<tr>
<td>s/s axolotl</td>
<td>1.17 ± 0.12</td>
<td>1.25 ± 0.14</td>
<td>1.10 ± 0.13</td>
</tr>
<tr>
<td>Xenopus</td>
<td>1.04 ± 0.02</td>
<td>1.17 ± 0.07 *</td>
<td>1.10 ± 0.04</td>
</tr>
</tbody>
</table>

Table 2: The expression of MMP and TIMP expression detected by MMP array from wildtype axolotl, s/s axolotl, Xenopus stage 34 and Xenopus stage 60.
Discussion and Conclusions

The data suggest that four of MMPs might play major roles in ECM degradation in the amputated wild-type axolotl limb, which is the standard for regenerative competence. These were MMP -8, -9, and -10, all of which were significantly up regulated throughout blastema formation, and MMP-13, which was significantly up regulated at histolysis/dedifferentiation. Within this group, MMP-9 and-10 were the most highly up regulated, as has been found by others using transcript analysis (Yang and Bryant, 1994; Park and Kim, 1999; Kato et al, 2003; Vinarsky et al, 2005). MMP-1 and -3 were significantly up regulated only at histolysis/dedifferentiation stage, a period of intense histolysis. These MMPs would thus also appear to play a significant role in histolysis of the regenerating wild-type axolotl limb. Histological studies have shown visible evidence of histolysis within 2-3 days post-amputation in larval urodeles and within 4-5 days in adults.

In contrast, the s/s axolotl and Xenopus froglet limbs failed to up regulate the MMPs in a pattern comparable to the wild-type axolotl limbs, suggesting that subnormal histolysis is at least in part responsible for the poor blastema formation characteristic of both the s/s and froglet limbs. The patterns of TIMP expression followed the same trends as the MMP expression patterns in each group of animals (Data not shown).

The role played by ECM degradation in dedifferentiation is not well understood. Recently ECM has been recognized for its critical roles to maintain a special local environment for adult stem cells, stem cell niche, and ECM degradation involves in the stem cell differentiation, activation and/or release. Degradation of the ECM by proteases would break contacts between ECM molecules and integrin receptors, leading to changes in cell shape and reorganization of the actin cytoskeleton (Juliano and Haskill, 1993). In conjunction with Na⁺ influx and H⁺ efflux (Adams et al, 2007), secondary messenger signals such as inositol trisphosphate (IP₃) (Rao et al, 2009), and growth factors provided by the AEC (Christensen, 2001 and 2002; Xu, 2007), this reorganization might activate signal transduction pathways that down regulate phenotype-specific transcription programs and up regulate programs characteristic of a less specialized state that allow blastema cell migration and response to proliferation and patterning signals. The molecular characterization of this reorganization, including blastema cell surface antigens, transcription factors, and microRNAs, and studies of changes in epigenetic marks via chromatin-modifying enzymes will be crucial for understanding the mechanisms of dedifferentiation in regenerating amphibian limbs.

In conclusion, the different patterns of MMP and TIMP expression after limb amputation among the three amphibian animal models result in the differences in the histolysis. This information suggested that MMPs might play a crucial role during the blastema formation and possibly affect animal’s capability of the regeneration.

References


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Disclosures

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