SMAD AND BMP EXPRESSION IN RAT FRACTURE HEALING

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Introduction Fracture healing is a complex cascade regulated by many factors. Bone morphogenetic proteins (BMPs) and TGFβ play important roles in promoting fracture healing. They perform their functions by combining with BMP receptors on target cell membranes. Human homologues of Drosophila mother against decapentaplegic proteins (Smads) are recently discovered cytoplasmic mediators that function in downstream signaling of BMPs from cell surface to nucleus. Smads are categorized into at least 3 classes according to their function. Smads 1, 2, 3, 5 and 8 belong to the receptor-regulated class (R-Smad) which are activated by TGFβ receptors, forming heteromers with the common-mediator class (Co-Smad): Smad 4, moving from cell surface to nucleus and positively regulates target gene synthesis. Smads 6 and 7 (I-Smad), however, perform a negative regulatory role in this process. The temporal expression and localization of Smad proteins during fracture healing has not been reported. This study examined the expression of BMPs, TGFβ and Smads during fracture healing in a closed fracture model.

Methods Eighteen 3-month old female CD-COB rats were used after approval from the Animal Care and Ethics Committee. All rats had a closed fracture induced to the right femur using a standard surgical protocol. The left femur of the same animal served as a non-fracture control. The rats were randomly divided into 3 groups with 6 per group and euthanised at day 3, 10 and 28 following fracture. The femurs were harvested, fixed in buffered formalin and decalcified in 10% formic acid – formalin solution. Decalcified tissues were embedded in paraffin and 5 micron serial sections were cut for H&E staining and immunostaining with specific antibodies.

Monoclonal mouse anti-human Smads1 and 4, polyclonal goat anti-human Smads2, 3, 5, 6, 7, BMPs 2, 4, 7 and TGFβ (Santa Cruz, USA) were used as primary antibodies. Non-immunized mouse and goat IgG were applied as negative controls. A Biotin-streptavidin system was applied for amplifying the signals. The positive signals were developed using a peroxidase-DAB system (DAKO, Australia). The intensity and location of the immunostaining was quantified using a colour video image analysis system (Leica, Australia).

Results By day 3 after fracture, the periosteum thickened with undifferentiated mesenchymal cells and osteoprogenitor cells. Intradamembranous ossification occurred with immature bone formation adjacent to the cortex. Undifferentiated mesenchymal cells and polymorphic cells infiltrated the bone marrow near the fracture site. Osteoblast-like cells lined the cortical bone and around woven bone. By day 10, the callus was enlarged and filled with newly formed trabecular bone and cartilage surrounded by mesenchymal cells. By day 28, in 5/6 rats newly formed bone filled the gap and the fracture callus had resolved. The remaining animal without union presented fibrous tissue in the fracture gap.

Immunostaining of Smad 1 to Smad 6 was localized in the cytoplasm and/or the nucleus of the mesenchymal cells, osteoprogenitor cells, osteoblast-like cells and the proliferating chondrocytes in the newly formed bony/cartilaginous areas. The dynamic changes of the Smads expression during fracture healing is illustrated in figure 1. A significant imbalance expression of the R-Smads and the Co-Smad with the I-Smad were noted at day 3 and day 10 post-fracture (p<0.001). Protein expression of BMPs and TGFβ is summarized in table 1. BMP-2 and 7 were peaked at day 3 accompanied by Smads 1 and 5 while BMP-4 elevated at day 3 and peaked at day 10 together with TGFβ and accompanied by Smads 2 and 3. The staining distribution of BMPs and TGFβ was similar to that of Smads, but woven bone and cartilage matrix staining of BMP-4 and TGFβ were noticed.

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Figure 1 Temporal expression of Smad proteins during fracture healing.

Table 1 Quantitative expression of BMPs 2, 4, 7, and TGFβ during fracture healing.

<table>
<thead>
<tr>
<th>Protein</th>
<th>(mean±SEM)</th>
<th>Intact</th>
<th>Day 3</th>
<th>Day 10</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP-2</td>
<td>ND</td>
<td>0.40±0.40</td>
<td>0.38±0.04</td>
<td>ND</td>
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<tr>
<td>BMP-4</td>
<td>2.34±0.56</td>
<td>12.49±0.64</td>
<td>20.64±0.71</td>
<td>2.02±0.08</td>
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<tr>
<td>BMP-7</td>
<td>1.51±0.30</td>
<td>19.41±0.88</td>
<td>0.60±0.08</td>
<td>0.62±0.12</td>
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</tr>
<tr>
<td>TGFβ</td>
<td>3.01±0.40</td>
<td>8.65±0.70</td>
<td>14.62±0.41</td>
<td>3.33±0.32</td>
<td></td>
</tr>
</tbody>
</table>

Discussions Fracture healing is under the control of a number of growth factors, BMPs and cytokines, involved in a complex cascade. The current study reveals the signal transduction molecules, Smads, are expressed in a temporal fashion during fracture healing similar to BMPs. Our data suggest Smad1 and Smad5 may interact with BMP 7 as recently suggested by in-vitro work. Smads2 and 3 work with TGFβ during fracture healing. Smad6 seems to play an inhibitory role in the healing process and is expressed as the tissue moves into a remodelling phase. Control of the Smad expression pathway represents an interesting avenue to explore to control or augment fracture healing.