The Carboxy Terminus of Secreted Phosphoprotein 24 kD (spp24) Contains a Second BMP/TGF-β Binding Site and Can Independently Affect BMP-2 Activity

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Introduction: Spp24 is a bone matrix protein isolated during attempts to identify osteogenic proteins1. It is not osteogenic but performs other important roles in the regulation of bone metabolism, at least in part, by binding to and affecting the activity of members of the BMP/TGF-β family of cytokines2. Spp24 exists in a number of forms that preserve the N-terminus and are truncated at the C-terminus. The hypothesized cytokine binding domain is present within the cystatin domain which is preserved in all of the N-terminal products3. In order to clarify the biological function of the protein and to define properties that can be exploited to engineer biotherapeutics, we have undertaken a series of studies on the binding properties and biological actions of the various N-terminal size forms and the corresponding released C-terminal products.

Methods: Two C-terminal proteins C-term c/t 18.1 and C-term c/t 14.5 were generated, corresponds to the fragment of the full-length protein that would remain after the production of spp18.1 and spp14.5 respectively. Surface plasmon resonance analyses of protein interactions between the C-terminal proteins and BMP-2 or TGF-β were performed. Mouse hindquarter ectopic bone formation assay was used to detect the bone formation ability of C-terminal proteins only or combined with BMP-2. DEXA, µCT were performed to quantify the ectopic bone formation, and histology and immunohistology were used to observe the ectopic bone morphology. The activity of Smad signal was also tested by immunostaining.
**Results:** C-term c/t 14.5 shows high binding affinity to BMP-2/TGF-β, while there is barely none binding between C-term c/t 18.1 and BMP-2/TGF-β. In the mouse hindquarter ectopic bone formation test for osteogenic activity, C-term c/t 14.5 did not demonstrate any such activity when implanted alone and effectively inhibited BMP-2 induced bone formation when the materials were co-implanted (Fig. 1A). Histological examination found trabecular bone only in the BMP-2 only group but not in the sponge only, C-term c/t 14.5 only, and BMP-2 + C-term c/t 14.5 groups. Positive Alcian Blue and osteocalcin staining were also seen in the BMP-2 only group indicating active bone formation, and C-term c/t 14.5 effectively inhibited this effect, although slight Alcian Blue and osteocalcin staining could still be identified in association with fibrotic inflammation and metaplasia. Immunohistochemical staining for phospho-Smad 1/5/8 was done for each group and positive staining, in the nuclei of some cells associated with trabecular bone, was seen only in the BMP-2 alone group indicating an inhibition of that pathway by the addition of C-term c/t 14.5 to BMP-2. C-term c/t 18.1 produced a dramatically different effect from that of C-term c/t 14.5. Rather than inhibiting BMP-2 induced bone formation, it appeared to increase the amount of calcified bone induced by BMP-2. The amount of calcium deposited when C-term c/t 18.1 was co-implanted with BMP-2 was about half again the amount deposited in BMP-2 alone samples. Interestingly, C-term c/t 18.1 alone samples also contained about two thirds as much calcium deposition as did the BMP-2 alone samples (Fig 1B). Histologically, the BMP-2 group showed bone shelling with abundant internal adipocytes while more trabecular bone and fewer adipocytes were seen in the BMP-2 + C-term c/t 18.1 group. In the C-term c/t 18.1 alone group, remnants of the carrier sponge are apparent and there is a moderately fibrotic inflammatory response with foreign body multinucleated giant cells, macrophages, and some lymphocytes present. Furthermore, the BMP-2 + C-term c/t 18.1 group appeared to exhibit greater proteoglycan deposition, as manifested by Alcian Blue staining, than the BMP-2 alone group, indicating more active bone formation. Positive staining of Smad4 was seen in the nuclei of some cells around trabecular bone in the BMP-2 only group, and in accordance with the Alcian Blue staining and osteocalcin staining, more and stronger nuclear staining was seen in the BMP-2 + C-term c/t 18.1 group. No positive staining was found in the sponge only group. Interestingly, positive staining was also seen in the nuclei and cytoplasm of cells surrounding the “bone-like structure” in samples from the C-term c/t 18.1 group, indicating activation of the Smad signal pathway.

**Discussion:** In this report we describe two C-terminal fragments of spp24 that exhibit totally different effects. C-term c/t 14.5 bound to BMP-2 and inhibited the osteogenic effect of BMP-2 and Smad signal activation caused by BMP-2 in a manner similar to that of full length spp24. In contrast, the shorter fragment, C-term c/t 18.1 did not bind BMP-2 with a high affinity and, furthermore, when delivered together, C-term c/t 18.1 and BMP-2 showed a synergetic effect. C-term c/t 18.1 improved the new bone quality induced by BMP-2 that more trabecular bone and fewer adipocytes were seen in the samples from the BMP-2 + C-term c/t 18.1 group when compared to BMP-2 only. C-term c/t 18.1 also promote Smad signal activation cause by BMP-2.

**Significance:** Several potential biotherapeutics have been engineered from spp24 and the further definition of the properties of the various fragments increases the repertoire of materials available for testing in pre-clinical models. These results provide more information on the function of spp24 and provide other materials that can be exploited for clinical interventions.
Fig 1. Radiological examination of implants. A: Radiograph of hindquarters of mice implanted with different materials. Note the ectopic bone induced by BMP-2 and that C-term c/t 14.5 inhibited the osteogenic effect of BMP-2. Note especially the calcium deposition in the C-term 18.1 alone group. B: Mineral contents as assessed by DEXA. The mineral content induced by BMP-2 + C-term c/t 14.5 was significantly lower than that of BMP-2 only group. The mineral content of BMP-2 + C-term c/t 18.1 was significantly higher than BMP-2 only group (p<0.01), whereas the mineral content of BMP-2 + scrambled peptide showed no significant difference with BMP-2 group (p>0.05). **: p<0.01, ***: p<0.001.
ORS 2015 Annual Meeting
Poster No: 0550