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COMPARISON OF BONE INDUCTIVE PROTEINS OF RAT AND PORCINE BONE MATRIX

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SUMMARY: Subcutaneous implantation of demineralized bone matrix in allogenic rats induces a sequence of events resulting in de novo formation of cartilage, bone and bone marrow. In the present study endochondral bone formation by demineralized porcine matrix was studied and compared with the rat bone matrix. Endochondral bone formation was induced by 4M guanidine hydrochloride fraction IV (< 50,000 daltons) of Sepharose CL-6B gel filtration but not by whole extract or by demineralized porcine bone matrix. Sephacryl S-200 gel filtration of the osteoinductive proteins of fraction IV showed the Porcine osteoinductive factor to be associated with protein fraction III (< 20,000 daltons) whereas the rat with fraction II (between 20,000 and 30,000 daltons) of the chromatographic profile indicating an apparent difference in molecular weight of the osteoinductive factors between these two species.

Subcutaneous implantation of demineralized diaphyseal bone matrix results in the local induction of new bone formation (1-3). The sequential endochondral bone induction cascade includes the following steps: chemotaxis and recruitment of mesenchymal cells, proliferation of progenitor cells and differentiation of cartilage and bone (4). Endochondral bone induction is also elicited by dissociative extracts of rat bone after reconstitution with an inactive, collagenous bone matrix residue (5). Further progress in this area requires purification of bone inductive proteins from larger farm animals in order to explore the mechanism of action and possible therapeutic potential. The present communication describes the partial purification of porcine bone inductive protein and a comparison with similar proteins from rat.

MATERIALS AND METHODS

Preparation of demineralized bone: Dehydrated diaphyseal shafts of rat femur and tibia were pulverised in a CRC micromill (Techni Lab Instruments, Vineland, N.J. U.S.A.) and sieved to a discrete particle size of 74-420

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μm(4). Bone shafts were frozen in liquid nitrogen prior to and during pulverisation to avoid possible heat denaturation of matrix components. Mineralized, pulverized porcine matrix was kindly supplied by Helitrex, Princeton, N.J. The bone matrix was demineralized with 0.5 M HCl, followed by further treatment with water, ethanol and ether as previously described and used (4).

Dissociative extraction: Demineralized porcine/rat bone matrix was extracted with 4M Guanidine hydrochloride (4M Gu.HCl) 50mM Tris/HCl buffer (pH 7.4) with 5mM benzamidine hydrochloride/ 0.1M 6-aminohexanoic acid (Sigma)/ 0.5 mM phenylmethyl sulphonyl fluoride (Sigma)/ 5mM N-ethylmaleimide (Sigma) (30ml/g of matrix) for 16h at 4°C according to Sampath and Reddi (5) The extracts were clarified by centrifugation at 20,000 g for 30 min., and the supernatant was concentrated by ultra filtration (Amicon, Diaflo membrane YM-10), dialysed (Spectrapor membrane tubing, ∿ 3.500 molecular weight cut-off) against distilled water and lyophilised (4M Gu.HCl extract). The residue left after the extraction procedure was also washed extensively with distilled water and lyophilised.

Gel filtration on Sepharose CL-6B: The 4M Gu.HCl extract (150 mg of porcine or rat) was subjected to gel filtration through a precalibrated Sepharose CL-6B column (2.6x100cm, two columns in tandem) using 4M Gu.HCl/50mM Tris/HCl (pH 7.0). 10 ml fractions were collected at a flow rate of 15 ml/h. The absorbance of the eluant was continuously monitered at 230 nm. Appropriate fractions were pooled, concentrated by ultrafiltration, dialysed and lyophilised.

Gel filtration on Sephacryl S-200: The bone inducing fraction (IV) of Sepharose CL-6B gel filtrate was subjected to further molecular sieving on a precalibrated Sephacryl S-200 column (1.5 x 100cm, two columns in tandem) using 4M Gu.HCl/50mM Tris/HCl (pH 7.0). The sample (50 mg) was loaded on the column, 4 ml fractions were collected at a flow rate of 16 ml/h. The absorbance of the eluants were monitored at 230nm and appropriate fractions were pooled, concentrated, dialysed and lyophilised as described before.

Reconstitution: The reconstitution of whole Gu.HCl extract, or of the column fractions was carried out according to Sampath and Reddi, (5).

Bioassay: Bioassay of demineralized bone matrix or of the reconstituted fractions was carried out by Subcutaneous implantation in the thoracic region of 28-35 day old (120-150g) male rats of the Long-Evans strain in bilateral sites (2). The day of implantation was designated as day 0. On day 12, the implants were dissected out and cleaned of adherent tissue. The implants were weighed and homogenised in 2 ml of ice-cold 0.15M NaCl/3mM $NaHCO_3$ (pH 9.0). The alkaline phosphatase activity of the supernatant and the calcium content of the acid soluble fraction of the sediment were determined (5). Protein was determined, with crystalline bovine serum albumin (Sigma) as standard (6).

Polyacrylamide gelelectrophoresis: The column fractions and the whole Gu.HCl extract were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (7) with 10-20% acrylamide gradient gels. The spacer gel contained 3% acrylamide and 2M urea. Samples were heated at 100°C for 5 min in 1% SDS in the presense of 0.5% 2-mercaptoethanol. The gels were stained with coomassie brilliant blue.

RESULTS AND DISCUSSION

Implantation of demineralized porcine matrix in rats does not induce endochondral bone differentiation as evident from alkaline phosphatase

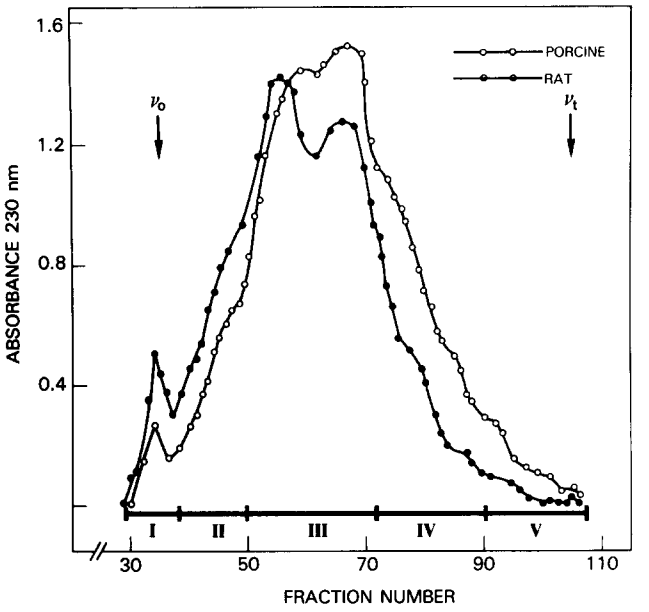


Fig. 1 Sepharose CL-6B profile of 4M Gu.HCl extract of porcine and rat.

activity and calcium content of day 12 plaques (Fig.2). In order to compare the osteoinductive potential of porcine bone with that of rat, the 4M Gu.HCl extract of porcine and rat bone were subjected to gel filtration through Sepharose CL-6B. Fig.1 shows the chromatographic profile. The osteoinductive potential of each of the fractions in terms of alkaline phosphatase activity and calcium content of day 12 plaques as shown in Fig. 2 imply that porcine fractions IV of Sepharose CL-6B profile is comparable with that of rat. The SDS-PAGE pattern of the osteoinductive fraction of porcine and rat matrix are shown in Fig. 5 lane C and F.

When the osteoinductive fraction IV of Sepharose CL-6B chromatography was subjected to gel filtration on Sephacryl S-200, the gel filtration pattern as shown in Fig. 3 was obtained. From Fig. 4 it is evident that Fraction III of porcine is capable of inducing endochondral bone formation. In the case of rat, unlike that of porcine, the osteoinductive potential was found to be associated with fraction II of Sephacryl S-200 profile which indicates that the chondro-osseous factor of porcine and rat are different in molecular weight. Fig. 5 lane D and G indicates the SDS-PAGE

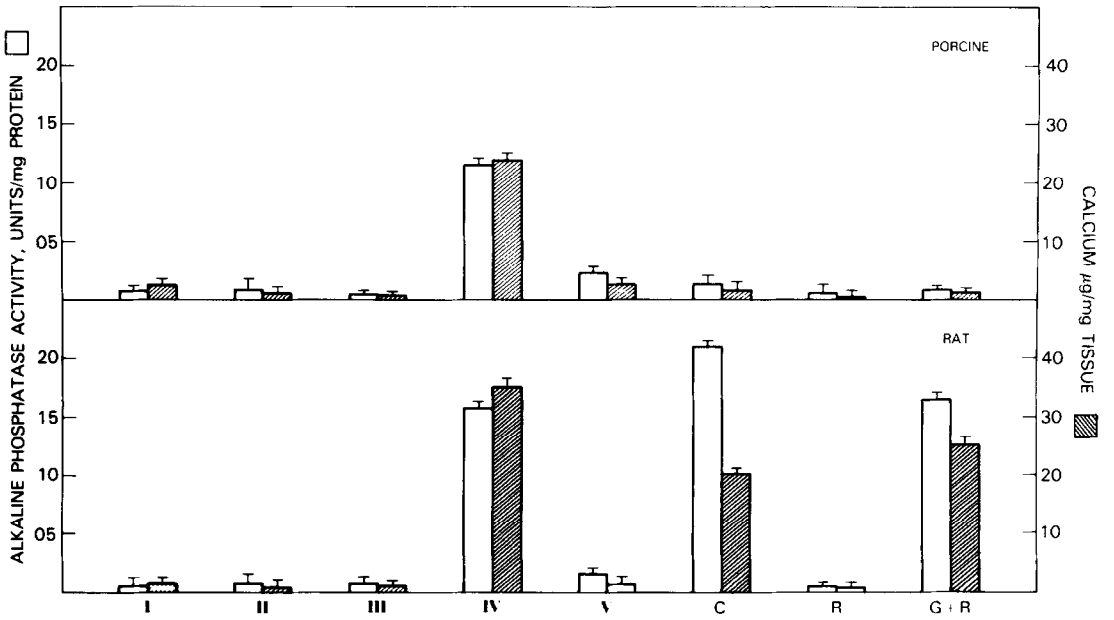
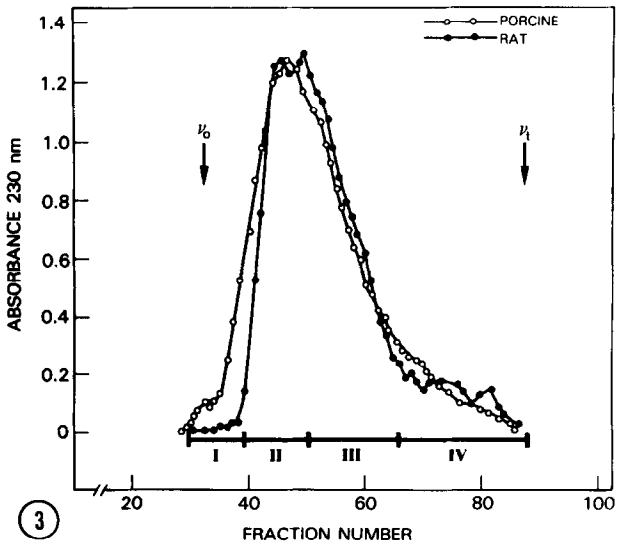


Fig. 2 Alkaline phosphatase activity and calcium content of day 12 plaques of reconstituted Sepharose CL-6B fractions. Demineralized bone matrix (C); Residue, 4M Gu.HCl extraction (R); 4M Gu.HCl extract reconstituted with rat residue (G+R).

of the biologically active fraction derived from Sephacryl S-200 chromatography.



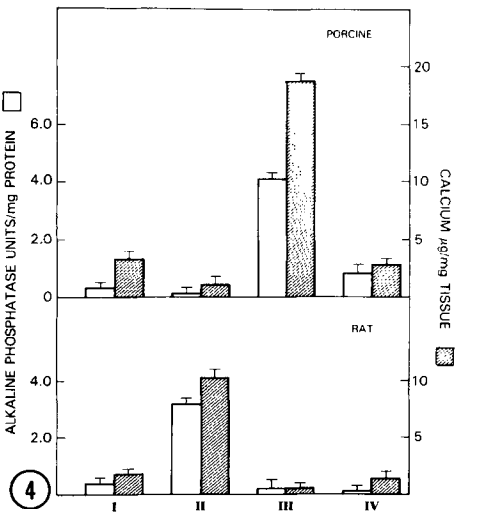


Fig. 3 Sephacryl S-200 profile of osteoinductive fraction (IV) of Sepharose CL-6B chromatography.

Fig. 4 Alkaline phosphatase activity and calcium content of Sephacryl S-200 fractions.

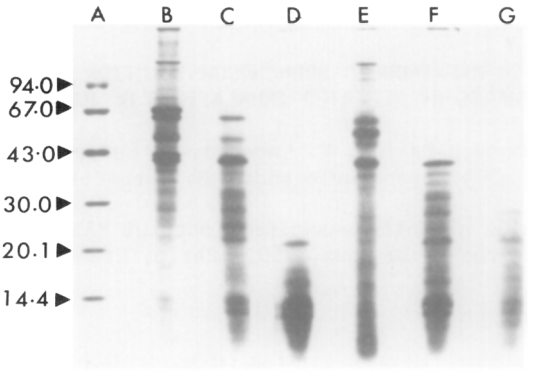


Fig. 5 SDS-PAGE - pattern of different fractions: Protein standard mixture including phosphorylase b (92,500 daltons) BSA (60,000 daltons) Ovalbumin (45,000 daltons) Carbonic anhydrase (30,000 daltons) Soybean Trypsin inhibitor (21,500 daltons) and α Lactalbumin (14,500 daltons) (Lane A); 4M Gu.HCl extract of porcine (Lane B) and rat (Lane E). Fraction IV of Sepharose CL-6B gel filtration porcine (Lane C) and rat (Lane F). Sephacryl S-200 fraction III of porcine (Lane D) and fraction II of rat (Lane G).

The presence of a bone inductive fraction with high molecular weight in rat compared to porcine may be due to proteolytic processing of precursor proteins to biologically active forms. Alternatively, there may be multiple molecular forms of homologous proteins. In conclusion this study indicates that porcine osteoinductive factors are homologous to rat in terms of biological response and also that there is an apparant difference in molecular size between the two species.

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