Effect of Chemical Modification of Tyrosine Residues on Bone Morphogenesis

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Demineralization of compact bone in cold dilute hydrochloric acid produces an organic matrix which is capable of inducing the formation of new bone when implanted into muscle (Urist, 1965; Van de Putte & Urist, 1966; Urist, Dowell, Hay & Strates, 1968). Mineral deposits appear about 10 days after implantation, and calcium content reaches 12-14% of the dry weight of the implant after 32 days (Firschein & Urist, 1971; Strates, Firschein & Urist, 1971). Induction of alkaline phosphatase begins as early as 6 days and the activity of the enzyme is maximal 20 days after implantation (Firschein & Urist, 1971). In rabbits and rats, the yield of new bone expressed as ash content is 400-500mg/g of preimplanted matrix (Urist *et al.* 1968; Urist, Jurist, Dubuc & Strates, 1970). Bone matrices partially digested with trypsin before implantation do not form new bone and do not calcify (Urist, Silverman, Buring, Dubuc & Rosenberg, 1967; Strates & Urist, 1971). The concomitant observation that the tryptic digests contain exceptionally high quantities of tyrosine (Strates & Urist, 1971) raised the question whether chemical modification of the available tyrosine residues of the matrix would influence bone morphogenetic activity.

N-Acetylimidazole has been employed for the acetylation of the phenolic hydroxyl group of tyrosine in polypeptides and proteins (Riordan, Wacker & Vallee, 1965). This reagent acetylates all tyrosine residues of denatured proteins but interacts only with the 'free' tyrosine residues in native proteins. Although thiol groups and to some extent amino groups are also acetylated, there is little or no interaction with aliphatic hydroxyl groups, tryptophan and phenylalanine residues. We found acetylimidazole suitable for the acetylation of bone matrix, because it requires mild conditions for reaction and is more specific than other acetylating agents such as acetic anhydride.

Bone matrix was prepared from femurs and tibias of young adult Sprague-Dawley rats. Fresh bones were demineralized in 0.6M-hydrochloric acid at 4°C for 2 days. At the end of this time bone matrices contained less than 1mg of calcium/g of dry tissue. Mid-diaphyses were cut into cylinders (1cm long), washed with water to remove the acid

and freeze-dried. Samples of the demineralized and freeze-dried matrix, weighing approx. 100mg, were treated with a fresh solution (0.1M) of *N*-acetylimidazole (Sigma Chemical Co., St Louis, Mo., U.S.A.) in 0.01M-tris-HCl buffer, pH 7.5, at a solid/solution ratio 1:100 for 10min, 30min, 1h and 2h. At this pH the reagent has its maximum hydrolytic stability and 'free' tyrosine residues are readily acetylated. At a concentration of 0.01M the effect of tris ions on the acetylation of tyrosine residues is minimal (Riordan *et al.* 1965). Excess of acetylimidazole was removed by dialysis until the diffusate showed no absorption peak at 245nm (Stadtman, 1954). Control matrices were treated in the same manner in buffer without acetylimidazole.

Representative matrices from each reaction time were implanted into the abdominal-wall muscles of animals of the same species as previously described (Urist, 1965). Samples of acetylated and non-acetylated matrix were digested separately with a trypsin (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) solution (2mg/ml of buffer) in 0.1M-potassium phosphate buffer, pH 7.6, for 10min-24h at 15°C. Acetylation of tyrosine was determined from the difference in E_{263} of the tryptic digests. *O*-Acetylation of tyrosine residues would shift the absorption to this wavelength (Riordan *et al.* 1965). All implants were excised 4 weeks after implantation, cleaned of adhering tissues and examined roentgenographically for mineral and histologically for new bone (Urist, 1965). The yield of new bone in implants of non-acetylated and acetylated matrices was assessed by calcium content. Since bone matrix before implantation contains less than 1.5mg of calcium/g of dry tissue and only newly deposited bone calcifies after implantation, the quantity of calcium in the implant is a reliable measurement of bone morphogenetic activity (Firschein & Urist, 1971; Strates *et al.* 1971). Control implants and implants of matrix from each acetylation time were pooled separately, dried at 110°C overnight, weighed, hydrolysed in 6M-hydrochloric acid at 125°C for 18h in an autoclave and analysed for calcium by atomic-absorption spectrophotometry (Willis, 1960).

Results on the extent of acetylation of the bone

matrix and the calcium content or yield of new bone in 4-week-old implants of matrices acetylated for 10–120 min before implantation are shown in Fig. 1. Incubation of the acetylated matrix in trypsin for 1 h produces digests showing increased E_{263} (Fig. 1a). Trypsin digests of matrices acetylated for only 10 min have E_{263} values significantly higher than those of the non-acetylated matrix. Differences in E_{263} between digests of non-acetylated and acetylated matrices increase rapidly with acetylation time up to 60 min. Beyond this time, acetylation proceeds at a lower rate, which suggests that a large proportion of acetylated residues present in components susceptible to trypsin digestion have been *O*-acetylated. Calcium content, which was in the range 12–13% in implants of non-acetylated matrices, decreased rapidly with acetylation time up to 60 min (Fig. 1b). A further, less abrupt, decrease in calcium content was observed in implants of matrices acetylated for 2 h before implantation.

These results suggest that there is an inverse relationship between the extent of acetylation of the demineralized bone matrix and calcium content of the implants. Under the conditions employed in the present study, acetylation would block tyrosine groups of proteins which might have been denatured during the process of demineralization. Simultaneously, acetylation would block 'free' tyrosine residues present in the undenatured non-collagenous proteins as well as those in the non-helical *N*-terminal portion of the collagen molecules

of the bone matrix. Thiol groups and certain amino groups of various proteins would also be acetylated. However, thiol groups seem not to be associated with bone morphogenetic activity because their chemical modification has no effect on the yield of new bone. Deacetylation of the matrix with hydroxylamine before implantation restores bone morphogenetic activity (Strates & Urist, 1971). Under the deacetylation conditions (Simpson, Riordan & Vallee, 1963) used in the present study, amino groups are not deacetylated (Kronman & Robbins, 1970).

The important observation is that modification of tyrosine residues of the matrix with *N*-acetylimidazole greatly decreases bone morphogenetic activity. Tyrosine residues are often associated with protein conformation and biological activity. 'Free' tyrosine groups are associated with the enzymic activity of pepsin (Herriott, 1935), carboxypeptidase (Simpson *et al.* 1963), fructose diphosphatase (Pontremoli, Grazi & Accorsi, 1966a,b) and aldolase (Pugh & Horecker, 1967). Such tyrosine groups are not essential for activity in trypsin and chymotrypsin (Riordan *et al.* 1965) and in alkaline phosphatase (Christen, Vallee & Simpson, 1971). Exposure of the 'buried' tyrosine groups of trypsin by treatment with 8M-urea results in complete loss of activity, which can be restored by deacetylation (Kronman & Robbins, 1970). In experiments with bone matrix, the observed u.v. shift of tryptic digests of the acetylated samples to wavelengths characteristic of *O*-acetylated tyrosine, and the

Fig. 1. Relationship between the extent of acetylation of matrix and quantity of bone in implants 4 weeks after implantation. (a) E_{263} of 1 h tryptic digests (differences between buffer control and acetylated matrices) for acetylation times up to 2 h; (b) calcium content (as measurement of yield of new bone) in implants of bone matrix acetylated with *N*-acetylimidazole for 10–120 min before implantation. Each point on curve (a) is average of two samples of matrix (approx. 100 mg of matrix per sample); each point on curve (b) is the mean of duplicate determinations on three implants of matrix.

pronounced loss in biological activity with increased acetylation time, implicate tyrosine in bone morphogenesis.

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