THE EFFECTS OF PAPAIN, VITAMIN A, AND CORTISONE ON CARTILAGE MATRIX *in VIVO*

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ABSTRACT The extreme and, apparently, selective vulnerability of chondromucoprotein in cartilage matrix to the action of proteolytic enzymes *in vivo* provides a useful model for studying factors involved in the transport, inhibition, and activation of a protease, papain, in the blood and tissues. The lysis of cartilage matrix which occurs in hypervitaminosis A is the result of release, probably from chondrocytes, of cathepsins normally contained within lysosomes. Cortisone possesses two properties which are not only of importance for this experimental model but also may have more general bearing on the physiological functions of this hormone with respect to connective tissue. One property is to prevent the resynthesis or deposition of chondroitin sulfate in cartilage matrix, after depletion of the latter. The other, which may be relevant to the "anti-inflammatory" actions of cortisone, is to increase the stability of lysosomes and prevent release of the acid hydrolytic enzymes contained in these organelles.

This paper is concerned with several techniques which can be used to bring about rapid depletion of chondroitin sulfate from cartilage matrix *in vivo*. These techniques have provided opportunities to manipulate and examine some of the physiological mechanisms governing the regulation of this constituent of connective tissue.

In 1956, it was reported that an intravenous injection of crude papain in rabbits caused, within a few hours, loss of the normal rigidity of cartilage to such an extent that the ears of the animals collapsed (1). Cartilaginous tissues in all parts of the body were found to have lost the normal degree of basophilia and metachromasia, and, in the ear cartilage, much of the actual substance of the matrix disappeared, leaving a shrunken cartilage plate with normal appearing chondrocytes packed side by side. Within 2 or 3 days, the matrix was restored, and the ears were again upright.

Apart from the change in cartilage matrix, no other lesions were encountered in these animals (although Kellner (2), using larger doses, had previously described necrotizing changes involving the myocardium following intravenous papain). The only evidence of inconvenience to the animals was respiratory difficulty in some, attributable to partial collapse of the softened trachea and bronchial tubes. No his-

tological evidence of damage or loss of metachromasia in connective tissue other than cartilage matrix was found.

The disappearance of metachromatic matrix became evident in the cartilage plates of the ear and trachea within 3 or 4 hours after papain, but the epiphyseal plates, which seemed much more vulnerable to the effect, were found by Westerborn (3) to show depletion as early as 10 minutes after injection.

Biochemical evidence for the release of chondroitin sulfate from cartilage into the circulating blood was obtained by Bryant *et al.* (4), Tsaltas (5), and Potter *et al.* (6), employing direct assays for mucopolysaccharide in the blood and urine, or measuring the release of S^{35} from previously labeled cartilage. Radioautographs of ear cartilage showed depletion of S^{35} within 4 hours after papain.

The active principle in papain was found to be the crystallizable papain proteinase originally isolated by Kimmel and Smith (7). Initial experiments with this material failed to produce the phenomenon, but it was then showed by McCluskey and Thomas (8) to be highly active when injected in its oxidized, inactive state. When activated by cysteine, intravenous injection of the enzyme caused no gross or microscopic change in cartilage matrix, but when *inactivated* by dialysis, or by the addition of antithiols such as parachloromercuribenzoate, iodoacetate, or iodoacetamide, the material was rapidly effective in a dose of 2 mg per kilo.

This paradoxical behavior of the enzyme *in vivo* might suggest that its biological properties with respect to cartilage in the living animal are the precise opposite of its known proteolytic action *in vitro*. However, it was then shown that the ear cartilage plates removed from animals injected with papain by vein 15 minutes earlier displayed the same wilting and depletion of chondroitin sulfate, and at about the same rate as *in vivo,* when held at 37°C (6, 8). This provided a useful method for approaching the problem more closely. Cartilage plates from animals injected with oxidized papain underwent wilting *in vitro,* but not those from animals injected with reduced papain, indicating that the enzyme had not reached the cartilage in the latter cases. Moreover, when the cartilage containing papain was placed in buffer containing antithiols, wilting was prevented, while in cysteine the process was much accelerated, indicating that the conditions for its activation are the same *in vivo* as *in vitro,* once it had succeeded in entering the cartilage.

The experiments with papain inactivated by iodoacetamide posed a special problem, since it is generally recognized that the acetamide inactivation is not reversible. It was at first thought that cartilage tissue must have its own technique for accomplishing this feat, but Potter *et al.* (6) showed that the reactivation was really only apparent, and was attributable to a portion of the enzyme present in the disulfide form before the addition of iodoacetamide, and therefore not susceptible to alkylation. When pains were taken to increase the proportion of enzyme in reduced form, by repeated exposure to cysteine, iodoacetamide lost its capacity to restore the activity of papain *in vivo.*

Apart from these observations with iodoacetamide, it is worth emphasizing that the crystalline papain inactivated by other means, *e.g.* parachloromercuribenzoate, and rendered completely inert *in vitro,* was *fully* effective when injected by vein. The findings suggested that not only is activated papain prevented from reaching cartilage, but also there must exist a reducing system of some sort in cartilage to restore its activity once it has gained access to this tissue.

The mechanism by which reduced, activated papain is prevented from getting into cartilage is of some interest. The likeliest explanation is that papain in this form becomes rapidly bound to a protein or proteins in the blood, perhaps as substrate, and thus fails to leave the circulation. It was shown that papain becomes bound to a protein migrating between α_2-globulin and β-globulin soon after injection (6). Furthermore, utilizing the *in vitro* method with whole cartilage plates, it was found that oxidized papain mixed with fresh serum caused lysis of the matrix when added to normal plates, while reduced, fully activated papain was prevented by some factor in serum from affecting the plate.

Thus, there is available an experimental model in which a potent protease can circulate in the blood in its activated form and cause no tissue damage, while the inactivated (and therefore enzymatically undetectable) form of the same enzyme can produce, selectively, widespread disintegration of a particular tissue. The implications of this are of considerable interest in speculating about ways in which other potentially harmful enzymes may play a role in tissue damage.

A similar state of affairs was found to hold for two other—SH activatable plant proteases, bromelin and ficin. When injected by vein in reduced state, they displayed severe systemic toxicity, sometimes causing acute shock and death, but had no effect on cartilage. When oxidized before injection, on the other hand, they were non-toxic, but produced the same lytic action on cartilage matrix as papain (8).

It is surprising that the removal of so much of the bulk of cartilage matrix, and the sharp decrease in the normal rigidity of this tissue, produce so little evidence of disablement or secondary damage at weight-bearing surfaces. Westerborn (3) noted some evidence of degenerative changes in chondrocytes of the epiphyses following papain, and in some animals transverse cracks appeared at the junction between epiphysis and metaphysis, but these changes were rapidly reversible. Even in rabbits maintained on repeated injections of papain for many weeks, in which cessation of skeletal growth and dwarfism occurred (1, 8), normal growth was restored soon after discontinuing papain. Whatever the physiological functions of chondroitin sulfate in cartilage may be, they are not such as to entail obvious degrees of dysfunction when this component is removed.

The Effects of Cortisone on Replacement of Matrix. The inhibiting action of cortisone on the synthesis of chondroitin sulfate in connective tissue has been studied by several investigators, mainly by measurement of effects on the incorporation of S^{35}. Layton (9) found that cortisone reduced the fixation of S^{35} in the skin of

rats; this was confirmed by Boström and Odeblad, who found the same effect in costal cartilage (10). The findings were interpreted to mean interference by cortisone with the exchange of the ester sulfate group of chondroitin sulfate. Schiller and Dorfman (11), however, have presented evidence indicating that inhibition of sulfate incorporation is based on impaired synthesis of the entire mucopolysaccharide molecule.

The papain effect proved to be a useful and convenient method for studying this action of cortisone in the intact animal. It was found that the daily administration of cortisone following a single administration of papain completely prevented the reconstitution of cartilage matrix, and maintained the tissue in its depleted state for as long as 4 weeks (1, 12). The inhibition was demonstrated to be due to a direct action of cortisone on cartilage; intraarticular injections of small doses of prednisolone prevented recovery of the articular cartilage in that joint, and also inhibited the local uptake of S^{35}, without affecting the cartilage in other areas (12).

Similarities between the Effects of Papain and Vitamin A on Cartilage

In 1952, Fell and Mellanby (13) described the effects of hypervitaminosis A on embryonic bone explants in organ cultures. Within 4 or 5 days after growth in media containing excess vitamin A, fetal chick and mouse bones exhibited a marked degree of loss of metachromatic material from cartilage matrix, resulting in small, deformed bones with greatly impaired growth. When removed from excess vitamin A, redeposition of metachromatic matrix occurred, and normal growth was resumed.

The histological resemblances between the effects of papain in the rabbit and those of vitamin A on embryonic bones were noted by Fell and Thomas in 1958, and a collaborative study of the relation between the events was undertaken. It was found that the administration of vitamin A alcohol to rabbits resulted in collapse of their ears within 2 or 3 days, and the cartilaginous tissues of these animals showed changes indistinguishable from those following an injection of papain (14). Basophilia and metachromasia disappeared from the matrix during the period of treatment, and reappeared after discontinuation. The epiphyseal cartilage, as with papain, was the most sensitive of all tissues to the lytic effect of vitamin A.

The two agents were found by Fell and Thomas (15) to exert similar effects in embryonic bone cultures. The addition of crystalline papain protease to organ cultures of mouse and chicken bone rudiments caused the rapid disappearance of metachromatic material from cartilage matrix, and impairment of growth. It was of special interest that oxidized papain was as effective in cultures as reduced papain, suggesting, as in the rabbit experiments, some mechanism in cartilage matrix for activation of the enzyme. No other elements of the bone rudiments were damaged by papain, nor did lysis of the plasma clots occur.

In the light of these experiments, it was suggested, as a working hypothesis, that "the changes in cartilage seen in experimental hypervitaminosis A may be the result

of activation of a proteolytic enzyme or enzymes with properties similar to papain" (15).

Lucy, Dingle, and Fell (16) demonstrated the presence of a proteolytic enzyme in embryonic cartilage by incubating samples in hypotonic buffer; at pH 3-5 there was rapid loss of metachromatic material from the matrix. Aqueous extracts of the tissue contained an active protease with a pH optimum against hemoglobin of about 3.0. When cartilage was homogenized in isotonic sucrose, there was little or no release of enzyme, but fractional centrifugation of the sucrose homogenates, followed by hypotonic treatment of the fractions, revealed that about 85 per cent of the enzyme was contained in an inactive, particulate form. Hypotonicity and acidification brought about full activity in the sediments of sucrose homogenates. Thus, it appeared that the cathepsins of cartilage might be contained in lysosomes, as de Duve (17) had previously demonstrated in various tissues, and Dingle (18) undertook a study of the action of vitamin A on lysosomes. Rat liver was chosen as the most convenient source, employing the methods of de Duve for isolation of the lysosome-rich fraction in 0.25 M sucrose. It was found by Dingle that the addition of vitamin A alcohol to suspensions of lysosomes caused the release of soluble cathepsin; this reaction was temperature- and pH-dependent and exhibited a high degree of molecular specificity for vitamin A. The enzyme liberated from rat liver lysosomes produced rapid degradation of embryonic cartilage matrix *in vitro,* with liberation of chondroitin sulfate and loss of metachromasia (19).

Another model for the study of the action of vitamin A was introduced by Weissmann, based on the finding of Weber (20) that in the metamorphosing toad tadpole (*Xenopus laevis*), lysosomal enzymes become concentrated in the tail at the time when tail resorption occurs. Weber suggested that enzyme release might be the mechanism underlying resorption of this tissue. Weissmann postulated that in this case the feeding of excess vitamin A should lead to premature resorption of the tadpole tail before metamorphosis, and found that this was indeed the case (21). In addition, he observed a striking depletion of metachromatic material from the ground substance in other tissues, including the rostral tentacles, which collapsed after the administration of vitamin A in a manner reminiscent of the reaction in rabbits.

Prevention by Cortisone of the Effects of Vitamin A on Lysosomes

In view of the previously demonstrated inhibition by cortisone of the reconstitution of cartilage matrix following papain (1, 12), it was thought that the effects of vitamin A might be exaggerated or prolonged by steroid treatment. However, the contrary proved to be true. Treatment of rabbits with cortisone before the administration of vitamin A blocked completely the lytic effect on cartilage matrix (22). Injection of prednisolone into one knee joint resulted in selective protection of the articular cartilage of that joint, indicating that the action of cortisone was a direct one on cartilage itself. Similar findings were obtained in organ cultures of embryonic bone

by Fell and Thomas (23). The addition of hydrocortisone to the medium conveyed almost complete protection against excess vitamin A.

These observations suggested that cortisone might have the property of stabilizing lysosomes, *in vivo,* against the lytic action of vitamin A. Weissman and Dingle (24) provided evidence for the same property *in vitro.* They learned that exposure of rat liver lysosomes to ultraviolet light caused the release of cathepsin and other lysosomal enzymes; pretreatment of the rats with cortisone before preparing the lysosome suspensions caused a significant decrease in fragility to ultraviolet light. Similarly, Weissmann and Thomas (25) have found that hydrocortisone added to lysosomes *in vitro* will block the lytic action of vitamin A.

In the experimental model involving hypervitaminosis A in the toad tadpole, experiments with hydrocortisone at first yielded results which were quite contrary to those above. When tadpoles were fed excess vitamin in the form of vitamin A alcohol, and simultaneously given hydrocortisone, the effect was magnified and accelerated. These results seemed more in accord with those obtained by Selye (26) in rats; this author found that the skeletal fractures caused by prolonged treatment with vitamin A were made worse by steroids. However, it now seems possible that the paradoxical findings may be based on the fact that cortisone has two separate properties—to stabilize lysosomes and prevent cartilage breakdown (as in the vitamin A experiments) and to interfere with the reconstitution of matrix (as in the papain experiments). In a long term experiment with chronic hypervitaminosis A, it is conceivable that the effectiveness of cortisone in preventing lysis might become outweighed by its capacity to prevent repair, and the end result might thus resemble synergism between the two agents. The protective effect of cortisone is most readily demonstrated in an acute experiment, as has recently been shown with tadpoles fed vitamin A acid. The latter form of the vitamin is much more rapidly effective than the alcohol in the tadpole, and is not stored in the liver. Hydrocortisone provides complete protection of tadpoles against the action of vitamin A acid (27).

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