Quarterly Journal of Medicine, New Series 61, No. 235, pp. 985–990, November 1986

Editorial

Bones in Muscles: The Problems of Soft Tissue Ossification

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Extraskeletal osteogenesis, most often in muscles, is a rare and inexplicable event with serious clinical consequences [1]. It has many causes but there are two striking examples. The first is inherited, fibrodysplasia (myositis) ossificans progressiva (FOP), and the second acquired after neurological injury. In these cases ossification of the muscles is a clinical catastrophe which leads either to progressive rigidity from early life or compounds the disability of paresis.

Investigation of the cause of myositis ossificans has until recently been limited and unrewarding, but this is to be expected whilst we remain so ignorant about the process of normal (skeletal) bone formation and have little idea about what determines phenotypic expression of the bone-forming cell, the osteoblast. However there is now considerable interest, for physiological and possible therapeutic reasons, in identifying and isolating those factors which induce osteogenesis. This research has relevance to ectopic ossification, of which the most extreme form occurs in fibrodysplasia ossificans progressiva [2, 3]. In this very rare condition (less than one per million of the population), regarded as one of the heritable disorders of connective tissue, progressive ossification within muscles is associated with constant and characteristic skeletal abnormalities, especially of the toes and cervical spine [4]. The severe disability reduces biological fitness close to zero and nearly all patients represent new autosomal dominant gene mutations [5].

The abnormal toes are present though often unrecognised, at birth, and ossification of the major muscles, particularly around the neck and spine, begins in infancy to affect about half the patients by the age of three years and all by adult life. Progressive disability results from fixation of the major joints: ossification in the muscles around the hips, often in later childhood or adolescence, is a major setback and may usher in a wheelchair existence.

The cause of the ossification is unknown, but each event follows weeks after redness, swelling and pain in the affected muscle which initially suggests inflammation (hence the term 'myositis') or some rare alternative such as sarcoma. Limited histological studies show small round cell and fibroblastic infiltration of the oedematous muscle with myofibrillar fragmentation, followed by bone formation with both cartilage and bony elements. Because of the apparent involvement of the connective tissue within muscle the term 'fibrodysplasia' rather than 'myositis' is now a preferred alternative [1]. Although the episodes of 'myositis' may sometimes appear to follow injury, there is no other known reason for their occurrence. It is difficult to be certain that all such episodes are inevitably followed by ossification, but this is clearly the usual result. There is one obvious question; why in this heritable condition do the mesenchymal cells within the major muscles behave as if they are osteoblasts?

The same question is foremost in the acquired ectopic ossification which follows neurological injury. Although such injuries are diverse, ossification characteristically occurs in a person who has sustained a traumatic paraplegia (or quadriplegia) some one to four months previously who is noticed to be increasingly stiff around the uninjured hips and may have local symptoms with warmth, redness and swelling. Radiographs show new bone formation within the muscles. This ossification cannot be attributed to local injury (as can that for instance after hip replacement or road traffic accidents) and must in some way be related to the neurological insult. Since only a proportion (about 30 per cent) of those at risk develop ectopic ossification, there appears to be an individual susceptibility to this complication; and since immobility is an almost constant feature of neurological damage, this may be an important determining factor.

How far does our current knowledge of the experimental production of bone in normal and abnormal sites help to explain how such ectopic osteogenesis occurs?

The ability to induce undifferentiated mesenchymal cells to enter the osteogenic pathway is the basis for all bone formation, whether normal or ectopic. It has been known for many years that mesenchymal tissues respond to certain stimuli resulting in altered phenotypic expression. Experimentally, non-osseous mesenchymal cells can be induced to form bone by two methods; either by the implantation of certain living tissues (including bone); or by implantation of devitalised bone or material derived from it. Over 50 years ago Huggins [6, 7] demonstrated induction of bone tissue in non-skeletal sites of the post-natal animal. This was done by transplanting living urinary transitional epithelium into connective tissues in dogs and rabbits. Later it was found that other living epithelial cells shared this property [8, 9]. An observation apparently more relevant to bone physiology was that living mouse bone also secreted a morphogenetic agent [10], and it was subsequently found that certain mouse and human osteosarcomas synthesised a related substance [11–14].

The phenomenon that implanted acid-demineralised bone matrix can affect certain fibroblastic cells in muscle septa and other connective tissues so that they become chondrogenic and osteogenic was first observed by Urist [15] and since corroborated by many other groups. The factor (named by Urist as bone morphogenetic protein) or factors involved appear to be proteinaceous and diffusible, as their action is transmitted through bacterial filters. Further, in contrast to a variety of living tissues, only osteogenic or dentinogenic tissues retain the capacity to induce bone formation when devitalised. Whether or not the agent(s) responsible is accumulated by adsorption to bone mineral, as demonstrated for certain other proteins is unknown [16, 17]. There is also some evidence that this factor may be complexed to bone matrix, perhaps by the newly discovered matrix gla-protein [18]; in this situation its exposure during bone resorption could exert a feed-back loop to increase osteogenesis.

The current aim of a host of researchers is to identify and characterise the osteoinductive agents present in bone tissue. Inevitably this would allow investigation on the physiological controls of osteogenesis and give new information on the biochemical processes important in fracture healing and metabolic bone diseases, as well as on ectopic bone formation. Also inevitably the situation is complex and a number of factors affecting growth and differentation of osteogenic cells have been extracted from bone tissue. However, to identify unambiguously the bone morphogen it will be necessary to demonstrate the *in vivo* production of bone.

In the purification of any such factor, the method of assay is crucial. Until 1978 bone induction could be demonstrated only with 'solid phase' techniques by using, for example, decalcified bone particles implanted *in vivo* or muscle fibroblasts explanted on bone matrix *in vitro* [19, 20]. A potentially dramatic advance was the demonstration that the factors could be recovered in solution, after digestion of the bone matrix with partially purified bacterial collagenase [21], because this opened up the possibility of separation of the active moiety by biochemical procedures. It was subsequently shown that this activity could be obtained from

bone and dentine with a variety of solvents including aqueous 4M guanidinium chloride [22], 6M urea with added salts [23], sodium dodecyl sulphate [24], and ethylene glycol [25]. Guanidinium extracts seem to be most effective and this solution removes almost all the osteoinductive activity from the bone after demineralisation. Chromatographic column techniques and dissociating solvents could be used to further separate the osteoinductive agent with retention of its biological activity [22]. The osteoinductive agents from every source (mouse osteosarcoma, human, bovine and rabbit bone and dentine) are detected in the small molecular weight non-collagen bone protein fractions [22, 24, 26–28].

The main reason why further fractionation and purification of these osteoinductive agents has proved difficult is because there is no rapid and sensitive quantitative assay procedure. Until recently *in vivo* demonstration of bone formation was only possible by surgical implantation of 1–20 mg of material to responsive sites (i.e. intramuscular or subcutaneous positions) in isogeneic, allogeneic or xenogeneic animals, the incidence of detectable bone formation after three to four weeks depending highly on the amount implanted. Consequently in most cases only single chemical separations could be performed before too little material remained either to give a consistent bioassay or for further fractionation. Nevertheless by using xenogeneic implantation into mouse limbs Urist has reported the isolation and partial characterisation of bone morphogenetic protein [29]; but absolute proof that activity is associated with the major protein species and not some trace component is not possible with this assay.

Induction of bone formation *in vivo* begins a cascade of cellular reponses resulting in the differentiation of cartilage and subsequent bone formation. This can now be conveniently demonstrated *in vitro* [20] but only as far as the formation of cartilage, since true bone formation has not been proven with *in vitro* culture.

At present there are a number of *in vitro* systems for the detection and assay of osteoinductive factors based on the production of cartilage in tissue culture [30, 31]. Initially these assays were histological but the use of antibodies to cartilage proteoglycan and Type II (cartilage) collagen [31] and of cDNA probes to Type II collagen [32] enables early detection of the cartilage phenotype from non-cartilaginous precursors. At least the last origin is presumed, but the use of embryonic or neonatal tissues in the assays leads to the question whether this is actual induction of osteogenesis or stimulation of growth of already programmed cartilage precursors. Certainly with one assay which uses chick limb buds a factor has been characterised which stimulates cartilage growth [30]. Whether neonatal rat muscle cells also retain the capacity to differentiate permissively into cartilage has not been determined. The relationship of these studies to the ectopic formation of bone is at present difficult to determine. It is likely that a multitude of factors and cellular responses are important in the phenotypic change of fibroblastic cells towards osteogenesis. In this respect it is of interest that a cartilage-inducing factor is apparently identical to a transforming growth factor [33, 34]. It has been reported that TGFβ can restore osteoinductive activity to inactive bone matrix implants *in vivo*, but it is not known whether this is a direct effect of TGFβ on osteogenic induction or a potentiation of the minor residual matrix components.

Which cells respond to osteoinductive agents in extraskeletal (and skeletal) sites? Soft tissue connective tissue cells do not normally differentiate into bone, but certain cells removed mechanically with bone marrow and transplanted heterotopically differentiate into bone, even in the absence of any inducing agent [35, 36]. Such cells have been named determined osteogenic progenitor cells and are believed to be cells of the marrow stromal system [37]. Cells of connective tissue that differentiate to form bone only under the influence of an inducing agent are termed inducible osteogenic progenitor cells and these are also present in the marrow cell population [38]. Inducible osteogenic progenitor cells are present locally in experimental tissue sites surrounding the implanted inducing agent but are also found in populations of

lymphoid cells, including blood cells [39] and it is presumed that these inducible osteogenic progenitor cells migrate and circulate through the organism. The apparent difference in bone production by determined and inducible osteogenic progenitor cells may be in the cessation of bone production by the latter soon after removal of the inducer [37]. Whether this represents an effect of exposure time to an inducer and whether the inducible type can modulate into the determined osteogenic progenitor cell is not known, but by definition the latter are found close to bone tissue where an extremely inductive environment is likely to exist.

In normal skeletal osteogenesis the most likely role of the inducing agents presumably formed by the osteoblastic cells is the recruitment of undifferentiated but potentially osteogenic cells into the osteogenic pathway but the relative contribution of determined and inducible osteogenic progenitor cells to this process is unknown.

Exactly how and why bone forms in muscles in certain clinical states remains quite obscure, but it is probably the result of a number of separate mechanisms (Fig. 1). Ectopic ossification after orthopaedic surgery such as total hip replacement could be due to the release of an inducing factor from damaged normal bone, or to the liberation of determined osteogenic progenitor cells into extra-osseous sites during the operation. Myositis ossificans at a distance from the initial injury (as in paraplegia) or genetically determined requires a different explanation. In these situations there are a number of plausible hypotheses based on the experiments discussed above. The inducible osteogenic progenitor cells in particular soft tissue sites may be stimulated to differentiate by increased production of a circulatory factor, such as bone morphogenetic protein, as the special environment in the tissue site develops or exists as a result of trauma or prolonged immobility.

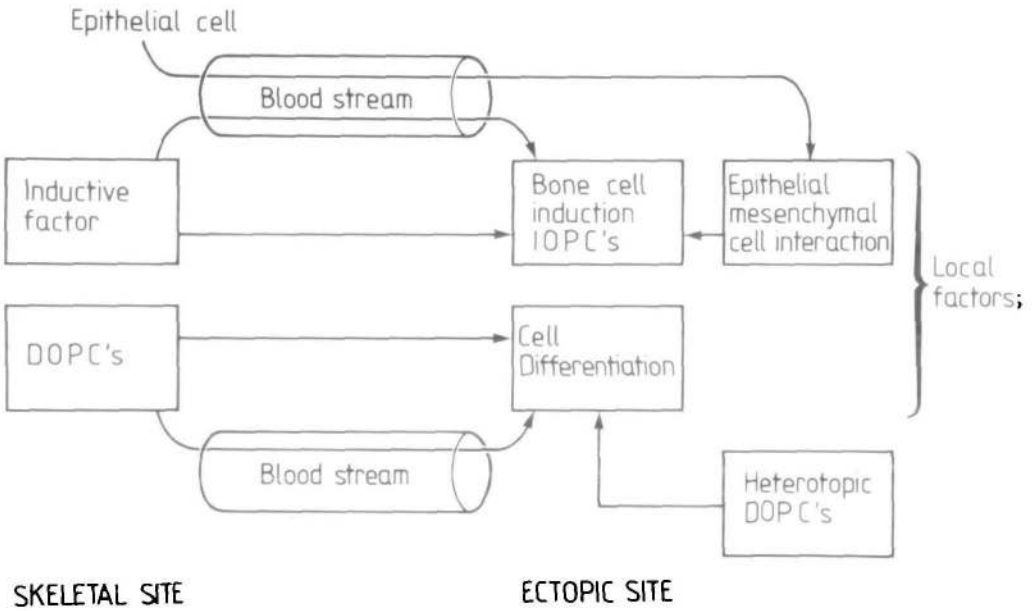

FIG. 1. To demonstrate possible mechanisms for the formation of ectopic bone. It is proposed that inductive factors may act locally or *via* the bloodstream. Determined osteogenic progenitor cells may initially be present at the heterotopic site or reach it from a local bone marrow site (as after orthopaedic surgery) or possibly *via* the bloodstream. Circulating epithelial cells could also stimulate ectopic bone formation. Local factors such as injury and immobilisation are important determinants of the site of ossification. DOPC=determined osteoprogenitor cell: IOPC=inducible osteoprogenitor cell.

Inductive factors may act locally or through the bloodstream to be effective at distant sites. Cells determined for osteogenesis (determined osteogenic progenitor cells) are considered not to be migratory under normal circumstances [40] but in pathological conditions it is possible that their adhesive characters may be altered and possibly after entering the bloodstream, may be targeted to particular tissue locations. Thus ectopic osteogenesis (not related to local damage) could be due to the effect of inducers on inducible osteogenic progenitor cells, or to the abnormal presence of the determined type either derived from a distant site or genetically present (as in fibrodysplasia ossificans progressiva). There is also the possibility that stimulatory epithelial cell migration may initiate this process. Local factors, at the site of osteogenesis, should not be disregarded, since neurological damage is associated with prolonged immobility and the 'myositis' of fibrodysplasia ossificans progressiva may be precipitated by trauma. Such conditions could trigger a dormant differentiation of cells that retain chondrogenic potential.

Some of these possibilities are shown in Fig. 1. Whilst our ways of investigating them remain relatively crude (and in man are limited by the availability of tissue), preliminary measurements have shown that the circulating levels of bone morphogenetic protein (and of prostaglandins) in patients with fibrodysplasia ossificans progressiva may be increased tenfold [41].

Much work remains to be done to characterise abnormal osteogenesis. The current explosion of interest in bone research should go some way to solve these problems and their distressing clinical outcome.

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