ULTRASTRUCTURAL AND IMMUNOCHEMICAL STUDIES
OF ELASTIN-ASSOCIATED MICROFIBRILS

by

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ABSTRACT

Elastic fibres consist of two morphologically distinct components, comprising an amorphous core of polymeric elastin surrounded by a peripheral mantle of microfibrils. Elastin-associated microfibrils possess different ultrastructural staining characteristics from amorphous elastin, on which basis they have been claimed to contain glycoprotein moieties. Many attempts have been made to differentially extract microfibrillar components, the products obtained ranging from heterogeneous protein mixtures to discrete glycoproteins. However, in no case has identity between these extracted materials and elastin-associated microfibrils been firmly established. As microfibrils precede the appearance of amorphous elastin in developing elastic tissues, a more precise understanding of the nature of microfibrils (and 'microfibrillar extracts') is essential if their role in elastogenesis is to be adequately delineated.

This study has used a combined biochemical, immunological and electron microscopic approach to study elastin-associated microfibrils. Experiments were carried out on foetal bovine tissues derived from animals in the third trimester (specifically of 200-220 days gestation) at which time elastogenesis has been shown to have reached its maximum rate. The specific aims were as follows:

i  A critical re-evaluation of the extractability of elastin-associated microfibrils with reductive guanidinium chloride (GuHCl) solutions.
ii  To raise an antiserum against such a 'microfibrillar antigen' extract, and study its tissue localization.
iii  To examine elastin fibrillogenesis in cultures of foetal calf ligamentum nuchae fibroblasts to assess their potential as a source of a soluble form of microfibrillar antigen(s).

Finely homogenized foetal calf ligamentum nuchae was subjected to a cyclical series of extractions with saline, 6M GuHCl and chromatographically purified bacterial collagenase, in the presence of protease inhibitors. Electron microscopic monitoring
showed that microfibrils were solubilized by 6M GuHCl throughout the extraction schedule, without the need for reduction. All remaining microfibrils could be removed by further GuHCL extraction. The product of this reductive extraction, 'microfibrillar antigen' (MAg), consisted of a heterogeneous mixture of proteins, the extreme insolubility of which limited biochemical characterization.

A rabbit antiserum raised against MAg produced only a single precipitin band on immunodiffusion, but was shown by a more sensitive ELISA assay to be polycpecific. No significant cross-reactivity was found toward elastin or collagen, but some antibody activity directed against fibronectin and components of foetal calf serum was present in the crude antiserum. It was possible to remove this contaminating antibody activity by affinity chromatography, whilst retaining high titres toward the original extract. The purified antibody was later shown, however, to contain low-level activity against a new collagen-like (CL) glycoprotein, which has been isolated from elastin-rich foetal bovine tissues by Dr M A Gibson in this laboratory.

Immunohistochemical experiments demonstrated specific localization of anti-MAg antibodies to elastin-associated microfibrils in both foetal calf ligamentum nuchae and aorta, with a small amount of binding to thin filaments which form a loose network in the extracellular matrix of these tissues. Comparative studies with a highly-specific anti-(CL glycoprotein) antibody preparation revealed specific localization to these thin matrix filaments, but not to elastin-associated microfibrils. These studies have shown unequivocally that MAg contains components derived from elastin-associated microfibrils. In addition, it is clear that microfibril-related antigens can be found in GuHCl extracts of elastin-rich tissues obtained with or without reducing agents. The antigenically active components in these extracts were, however, found to be quite insoluble if the GuHCl concentrations were reduced. This precluded isolation of antigen by immunoprecipitation with anti-MAg antiserum.

Attempts were made to circumvent this problem by growing nuchal ligament fibroblasts in tissue culture in order to obtain a soluble form of 'microfibrillar antigen'. Explant cultures of foetal calf ligamentum nuchae fibroblasts were shown to
accumulate large numbers of morphologically typical microfibrils in the extracellular matrix, but no amorphous elastin was observed even after extended periods in culture. Labelling of cultures with $[^3\text{H}]$-fucose and $[^3\text{H}]$-proline demonstrated that a large number of newly-synthesized macromolecules were secreted into the medium. However, attempts to isolate microfibrillar precursors from such medium by immunoprecipitation with anti-(MAg) antiserum were unsuccessful.

These findings indicate that the 'microfibrillar' glycoproteins MFP I and MFP II, isolated by Sear and his co-workers in similar immunoprecipitation experiments, may be related to contaminating non-microfibrillar antibodies contained within the antiserum on which their work was based.

During the course of these studies, claims were made in the literature that elastin fibrillogenesis occurred in ear cartilage without the participation of identifiable microfibrils. As this seemed of major importance to determining the role of microfibrils in elastogenesis, a comparative ultrastructural study of elastic fibres in foetal calf and rabbit ear cartilage in vivo and in vitro was undertaken in order to establish the presence of elastin-associated microfibrils in these tissues.

It was shown that elastin-associated microfibrils were visible at the periphery of elastic fibres in native foetal calf and neonatal rabbit (2-day-old) ear cartilage, but appeared to be masked by surface condensation of proteoglycan-like material in young rabbit (sixteen-day-old) ear cartilage. Cultures of enzymically dissociated foetal calf and neonatal rabbit auricular chondrocytes, when maintained under scurvy conditions, produced a scanty extracellular matrix of which microfibrils and amorphous elastin were the dominant components. In contrast, young rabbit auricular chondrocyte cultures accumulated an extensive, predominantly proteoglycan extracellular matrix with relatively few elastic fibres. As in native ear cartilage, elastin-associated microfibrils could not be identified due to the dense proteoglycan encrustation of the surface of these elastic fibres.

Ascorbate supplementation of foetal calf auricular chondrocyte cultures resulted in a massive increase in collagen fibrillogenesis with suppression of elastic fibre formation.
Immunofluorescent labelling of foetal calf auricular chondrocyte cultures with anti-MAg antibodies demonstrated the presence of fibrillar material in the extracellular matrix, implying that microfibrils produced by such cultures are antigenically similar to those found in intact tissues, and are present during elastogenesis in ear cartilage.

It is concluded that an affinity-purified polyclonal antibody preparation has been obtained which has good specificity for elastin-associated microfibrils, but which contains some residual contaminant activity, most notably against CL glycoprotein. Nevertheless, this antibody preparation provides a useful tool for monitoring tissue extracts in order to establish the presence of microfibrillar components. Further studies to elucidate the specific antigen(s) responsible for the observed anti-microfibrillar activity offer the possibility of substantial insights into the biology of microfibrils.

These studies have also shown that foetal calf ear cartilage chondrocyte cultures are a potentially useful model system for the study of elastogenesis in vitro, and have clearly demonstrated the presence of typical microfibrils in association with developing elastin in this tissue. Such cultures are technically simple to propagate and maintain, and readily produce both components of the elastic fibre, thus offering significant advantages over foetal calf ligamentum nuchae fibroblast cultures.