IDENTIFICATION OF A MAJOR EXTRACELLULAR NON-COLLAGENOUS GLYCOPROTEIN SYNTHESISED BY HUMAN SKIN FIBROBLASTS IN CULTURE

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SUMMARY: The major protein released into the medium by human skin fibroblasts in culture has been shown to be a fucosylated glycoprotein (designated MFGP). Analysis by gel filtration chromatography and polyacrylamide gel electrophoresis demonstrated that under reducing conditions MFGP has a molecular weight of approx. 250,000, but occurs as a disulphide-linked aggregate in the medium. Three lines of evidence are presented to establish that MFGP is a non-collagenous molecule.

INTRODUCTION: Studies with cultured cells have contributed to our understanding of the synthesis and secretion of both collagenous and proteoglycan-related components of the extracellular matrix. However, there is an increasing amount of evidence to suggest that connective tissues also contain non-collagenous structural glycoproteins which, with few exceptions, have been very poorly characterised (1). In view of the possible importance of such macromolecules in the development of connective tissues (2) and in disease processes (3) we have established optimal culture conditions suitable for the study of glycoprotein biosynthesis by human skin fibroblasts (4). In this paper we demonstrate that such cells incorporate [^{3}H]fucose into a high molecular weight non-collagenous glycoprotein, which is the major protein released into the culture medium.

EXPERIMENTAL: Human skin fibroblasts were propagated as described previously (4). Confluent cultures maintained in a chemically defined medium, MAB 87/3 (5), were labelled for 12h or 24h with L-[1-^{3}H]fucose (10 μCi/ml). When [^{3}H]fucose-labelled glycoproteins were to be compared with secreted collagenous molecules both L-[1-^{3}H]fucose and [U-^{14}C]proline (1 μCi/ml) were

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Abbreviation: SDS, sodium dodecyl sulfate.

added to the maintenance medium, which was further supplemented with ascorbic acid (50 μg/ml) and β-aminopropionitrile (50 μg/ml).

Non-diffusible radiolabelled macromolecules in the medium and in the cell layer were analysed by SDS-agarose gel filtration chromatography either unreduced or after prior reduction and alkylation (6). The elution positions of collagenous polypeptides were determined by a specific radiochemical assay for hydroxy[^{14}C]proline following hydrolysis of the fractions (7). Columns were calibrated using the following standards: ovalbumin, bovine serum albumin and rat tail tendon collagen α- and β-chains.

The ^{3}H-macromolecules in the medium and cell layer were analysed by SDS-polyacrylamide gel electrophoresis (8) using a separating gel 10 cm long and containing 8% (w/v) acrylamide. The proteins in the medium were concentrated prior to analysis by precipitation with $(NH_4)_2SO_4$ at 75% saturation in the presence of 25 mM EDTA, 10 mM N-ethylmaleimide and 2 mM phenylmethylsulphonyl fluoride. Gels were stained with either Coomassie Brilliant Blue R or by the periodic acid-Schiff procedure (9), and the distribution of [^{3}H]fucose-labelled molecules determined by slicing and assaying for radioactivity.

RESULTS: Glycoprotein biosynthesis by human skin fibroblasts (line HF3 of our stocks) was examined by studying the incorporation of [^{3}H]fucose into non-diffusible macromolecules. After a 24h labelling period the ^{3}H-glycoproteins present in the medium were analysed by SDS-agarose gel filtration

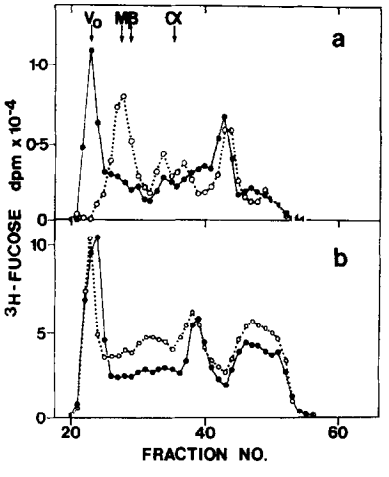

Fig. 1: Gel filtration on SDS-agarose (Bio-Rad A-5m) of macromolecules labelled with [^{3}H]-fucose synthesised by cultured skin fibroblasts. [^{3}H]-Glycoproteins (a) released into the medium, (b) present in the cell layer. The elution positions of collagen α- and β-chains (98,000 and 196,000 respectively), and the void volume of the column (57 ml) are arrowed. (● - ● - ●) unreduced sample; (o - o - o) reduced sample.

chromatography (Fig. 1a). In the absence of reducing agents the major peak, accounting for 25-35% of the total radioactivity, eluted at the void volume of the column. However, when the labelled medium proteins were chromatographed following reduction and alkylation the radioactivity at the void volume was markedly diminished and a new major peak appeared (peak M of Fig. 1a). The molecular weight of this major species was estimated from six experiments as 250,000 ± 25,000 by extrapolation from a calibration curve based on the elution positions of standard proteins. Similar results were obtained when the cells were labelled with [1-^{14}C]fucose and essentially identical chromatograms were obtained using six other human skin fibroblast cell lines.

Gel filtration analysis of the unreduced labelled macromolecules in the cell layer is presented in Fig. 1b. It is noteworthy that this profile was not significantly altered by reduction of the sample prior to chromatography. These results indicated that the peak M component, if present, represented a relatively minor proportion of the total fucosylated species in the cell layer.

Further experiments were conducted to determine whether the high molecular weight [^{3}H]fucose-labelled macromolecule present in the medium represented a major constituent released by the cells. Polyacrylamide gel electrophoresis of the medium macromolecules after reduction with dithiothreitol demonstrated that the major protein present had an apparent molecular weight of approximately 200,000, stained strongly with the PAS reagent and co-electrophoresed with the major [^{3}H]fucose-labelled species (Fig. 2). It was also established that the [^{3}H]fucose-labelled Peak M material recovered from the SDS-agarose column (Fig. 1a) electrophoresed with the same mobility. Analysis of the cell layer proteins under reducing conditions revealed a complex series of Coomassie Blue-staining bands including high molecular weight species which did not penetrate the separ-

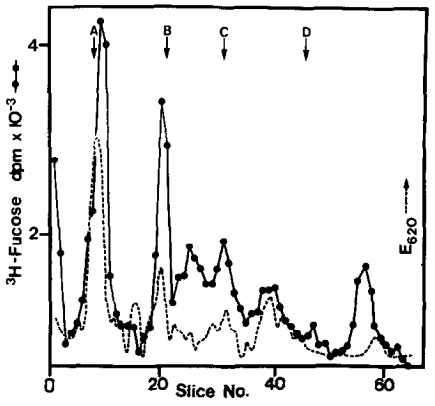

Fig. 2: SDS-polyacrylamide gel electrophoresis of [^{3}H]fucose-labelled macromolecules released into the medium of skin fibroblast cultures. Proteins were detected by scanning a Coomassie Blue-stained gel at 620 nm, and the presence of [^{3}H]glycoproteins detected in a duplicate gel by slicing and scintillation counting. The mobilities of the following standards are indicated: bovine serum albumin (A) trimer (204,000), (B) dimer (136,000), (D) monomer (68,000); (C) phosphorylase (100,000).
(● - ● - ●) [^{3}H]fucose ; (- - - -) E_{620}.

ating gel. Several protein bands having apparent molecular weights ranging from 180,000 to 220,000 were observed, and further work is in progress to determine whether or not any of these is related to the high molecular weight glycoprotein identified in the medium.

In order to determine whether the major fucosylated glycoprotein in the medium (hereafter referred to as MFGP) was related to the precursors of Type I and Type III collagens, which are known to be synthesised and secreted by human skin fibroblasts (10), cultures were dual-labelled with [^{3}H]fucose and [^{14}C]proline. The macromolecules released into the medium were applied to the SDS-agarose column without reduction and the material eluting at the void volume (fractions 22-25 in Fig. 1a) was reduced, alkylated and rechromatographed. The results presented in Fig. 3 show two included peaks of [^{14}C]-proline-labelled polypeptides, the first coincident with the [^{3}H]fucose-labelled peak of MFGP, and the second eluting in a position between standards of tendon collagen β- and α-chains. A small proportion of the radioactivity

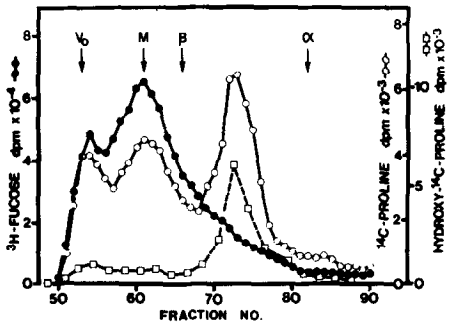

Fig. 3: Gel filtration on SDS-agarose (Bio-Rad A-5m) of reduced, high molecular weight medium macromolecules labelled with [^{3}H]fucose and [^{14}C]-proline. Dual-labelled proteins were initially chromatographed under non-reducing conditions, and the void volume fractions (see Fig. 1a and the text) pooled, concentrated and rechromatographed after reduction and alkylation. Fractions (1.05 ml) were collected, aliquots (0.05 ml) assayed for ^{3}H and ^{14}C, and the remaining samples pooled in pairs, hydrolysed and analysed for hydroxy-[^{14}C]proline. Elution positions of standards are indicated as in Fig. 1. (● - ● - ●) [^{3}H]fucose; (o - o - o) [^{14}C]proline; (□ - □ - □ -) hydroxy-[^{14}C]proline.

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remained at the void volume, and is probably attributable to the marked tendency of MFGP to aggregate even in 0.1% SDS. Analyses of the fractions for hydroxy[^{14}C]proline demonstrated the collagenous nature of the second included [^{14}C]proline peak, which was thus identified as reduced polypeptides of procollagen molecules. By comparison, the peak of [^{3}H]MFGP contained only basal levels of hydroxy[^{14}C]proline, suggesting it to be a non-collagenous glycoprotein. Furthermore, the [^{3}H]MFGP peak was resistant to digestion with a highly purified bacterial collagenase (11), whereas the hydroxy[^{14}C]proline-containing procollagen peak was reduced to background level by this enzyme. Pertinent to these observations was the finding that the synthesis and secretion of procollagen peptides were much reduced when the cells were cultured in ascorbate-deficient medium whereas the amount of radiolabelled-MFGP released into the medium was unaltered.

DISCUSSION: We believe that this study establishes for the first time that the major protein released into the medium by human skin fibroblasts in

culture is a non-collagenous fucosylated glycoprotein (MFGP). The molecular weight of MFGP has been determined as 250,000 ± 25,000 by SDS-gel filtration chromatography. The value of 200,000 obtained by SDS-polyacrylamide gel electrophoresis is probably an underestimate, as glycoproteins are known to behave anomalously in such systems. The behaviour of MFGP before and after reduction of disulphide bonds suggests that this glycoprotein may possess quaternary structure or be part of a larger heterologous protein complex.

During the preparation of this manuscript, Muir et al.(12) reported that smooth muscle cells in culture synthesise a high molecular weight glycoprotein which is presumed to represent a subunit of the microfibrillar protein found in elastic tissue. The function of MFGP synthesised by cultured fibroblasts is unknown but its apparent similarity to the smooth muscle cell molecule raises the possibility that it is also a precursor or subunit of a structural glycoprotein laid down in the extracellular matrix.

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