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Identification and partial characterisation of a low M_r collagen synthesised by bovine retinal pericytes

Apparent relationship to type X collagen

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Bovine retinal pericytes (BRP) in culture synthesise a low M_r collagenous polypeptide which appears similar, but not identical, to bovine type X collagen and which we have called 'BRP collagen'. This polypeptide displays the following characteristics: (i) it is sensitive to digestion by bacterial collagenase and is resistant to pepsin digestion; (ii) it has an apparent M_r of 45 kDa (pepsinised form); (iii) it is recognised by specific antibodies to type X collagen using immunoblotting; (iv) it is present in the cell layer/matrix but not in the medium of pericyte cultures; and (v) it is not disulphide-bonded into higher M_r multimers. The latter two properties distinguish BRP collagen from bovine type X collagen. We have recently shown that pericytes calcify in vitro. We now report that this calcification is associated with an increased synthesis of BRP collagen.

Collagen; Pericyte; Calcification

1. INTRODUCTION

Bovine retinal microvascular pericytes display a distinctive pattern of growth and differentiation in vitro. After reaching confluence, these cells form multilayered areas which retract away from each other resulting in the formation of multicellular nodules. Hydroxyapatite crystals deposit on the extracellular matrix of these nodules, leading eventually to their massive calcification [1]. Using a specific antiserum which recognises chick and bovine type X collagen [2,3] we have shown that pericyte calcification is accompanied by an apparent increase in the expression of type X collagen: no staining was detected in monolayer or sub-confluent areas, but positive immunoreactivity was observed in multilayered areas, sprouts and nodules [4]. To date, chondrocytes are the only cell type known to synthesise type X collagen (see [5,6] for recent reviews). This collagen is thought to be involved in endochondral ossification although its precise role remains to be determined [7–9]. Interestingly, Yamaguchi et al. [10] have recently cloned and sequenced α_1(VIII) collagen from rabbit corneal endothelial cells, and have shown that it has significant nucleotide sequence homology with chick α_1(X) collagen chains. These workers have therefore proposed that type VIII and X collagens belong to the same subclass of collagenous molecules.

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We have previously demonstrated that bovine retinal pericytes in culture synthesise various extracellular matrix components, including fibronectin, laminin, thrombospondin, tenascin and collagen types I, III and IV [4,11]. In this paper, we report the synthesis of an apparently novel low M_r collagen (M_r 45 kDa) which is deposited into the cell layer/matrix of pericyte cultures and which we have called BRP collagen.

2. EXPERIMENTAL

2.1. *Materials*

Culture medium, donor calf serum, sodium pyruvate, glutamine and non-essential amino acids were obtained from Gibco Bio-Cult (Paisley, Scotland, UK). Ascorbic acid was obtained from BDH Chemicals (Poole, Dorset, UK). 2-Mercaptoethanol, trypsin, pepsin (1:60000, from pig stomach mucosa), bovine serum albumin and silver enhancer were purchased from Sigma Chemical Co. (Poole, Dorset, UK). Highly purified bacterial collagenase (form III) was obtained from Advanced Biofactures (New York, USA). Auroprobe BL plus goat anti-rabbit immunogold reagent was supplied by Janssen Life Sciences (ICN Biomedicals Ltd., High Wycombe, Bucks., UK). [^{3}H]Proline (20–35 Ci/mmol) and [^{14}C]methylated protein standards (M_r 14300–200000) were purchased from Amersham International (Amersham, Bucks, UK).

2.2. *Cell culture*

Bovine pericytes were isolated from the adult retina and identified on the basis of several criteria [1,11,12]. Stock cultures were routinely maintained on plastic tissue culture dishes in Eagle's minimum essential medium supplemented with 20% (v/v) donor calf serum, 1 mM sodium pyruvate, 2 mM glutamine, non-essential amino acids, and ascorbic acid (50 μg/ml). This complete growth medium is referred to as 20% DCS-MEM. Cultures were incubated at 37°C in a humidified atmosphere consisting of 5% CO_2 and 95% air. In some experiments

fibroblasts isolated from human breast tissue were also used. These cultures were maintained in 20% DCS-MEM.

The studies presented here were conducted with six different batches of retinal pericytes between passages 4 and 11; BRP collagen was detected in five of these batches.

2.3. *Labelling of cell cultures and isolation of collagenous proteins deposited into the cell layer*

Pericyte and fibroblast cultures were incubated for 24 h at 37°C in 20% DCS-MEM containing [^{3}H]proline (20 μCi/ml). At the end of the incubation period the medium was collected and the attached cell layer/matrix was washed three times with cold Eagle's minimum essential medium. The cell layer/matrices were collected by scraping the dishes with a rubber policeman. Collagenous proteins present in this fraction were extracted by digesting with pepsin (200 μg/ml) in 0.5 M acetic acid for 24 h at 4°C. The samples were centrifuged (14000 × g, 20 min, 4°C) and the solubilised proteins were dialysed extensively against 0.5 M acetic acid at 4°C. Samples were stored at −20°C before analyses.

We have previously shown that pericytes isolated from the retinal microvasculature calcify in vitro [1]. In order to investigate a possible relationship between calcification and collagen deposition by the cells, pericyte cultures were radiolabelled at different stages of confluence and differentiation; namely when the cells were (i) just confluent (day 7–8), (ii) when they had formed small nodules (day 15–18) and (iii) when these nodules had calcified (day 35–36).

2.4. *Biochemical analyses*

Discontinuous SDS/polyacrylamide-gel electrophoresis was performed with or without reduction by 5% (v/v) 2-mercaptoethanol as previously described [13]. The same amount of radioactivity was applied to each track of the polyacrylamide gel. Newly-synthesised proteins were detected by fluorography [14,15]. Bovine and chick [^{3}H]proline-labelled type X collagen standards were generously provided by Dr J.T. Thomas (Department of Biochemistry and Molecular Biology, University of Manchester, Manchester, UK). The standards used generally comprised a mixture of collagen type II and X, and were prepared as previously described [9,16,17].

Newly-synthesised proteins which were resistant to digestion with pepsin were tested for their susceptibilities to digestion by bacterial collagenase as previously described [18].

Pepsin-resistant proteins were tested for their immunoreactivity with antibodies to type X collagen by immunoblotting. Samples were separated by electrophoresis in 8% polyacrylamide slab gels and transferred to nitrocellulose as previously described [19]. Remaining active sites were blocked by incubation in PBS/0.5% Tween-20 for 30 min and the nitrocellulose sheets were then washed (3 × 15 min) with PBS/0.05% Tween-20. The blots were incubated for 2 h with either polyclonal antibody raised against chick type X collagen [2] or normal rabbit serum, diluted 1 : 100 in PBS/0.05% Tween-20 buffer containing 0.1% BSA; they were then washed (2 × 10 min) in PBS/0.05% Tween-20 followed by a 10 min wash in TBS/NaN_3 buffer (20 mM Tris-HCl, 150 mM NaCl, 20 mM NaN_3, pH 8.2) containing 0.1% BSA. The blots were next incubated for 4 h with Auroprobe BL plus goat anti-rabbit IgG diluted 1 : 50 with TBS/NaN_3 buffer containing 0.4% gelatin and 0.1% BSA. After this time, the nitrocellulose sheets were washed (2 × 10 min) in TBS/NaN_3 containing 0.1% BSA and then in distilled water. The gold signal was enhanced by incubating the blots with silver reagent according to the manufacturer's instructions. Control experiments included the use of human skin fibroblasts and normal rabbit serum instead of immunoreactive serum; no immunoreactive polypeptides were detected.

3. RESULTS

3.1. *Identification of a low M_r collagen synthesised by retinal pericytes*

Bovine retinal pericytes cultured on plastic tissue culture dishes were incubated with [^{3}H]proline for 24 h and the newly-synthesised collagens deposited into the cell layer/matrix were isolated by pepsin digestion as described in section 2. Pepsin-solubilised samples were then analysed by SDS/PAGE under reducing and non-reducing conditions (Fig. 1). Fig. 1 clearly shows the presence of a pepsin-resistant polypeptide (indicated by an asterisk in Fig. 1, tracks 3, 6 and 9) which consistently comigrated with pepsinised chick type X collagen (track 2), just ahead of pepsinised bovine type X collagen (track 1). This electrophoretic behaviour suggests an apparent M_r of approximately 45 kDa (based on collagen standards). This polypeptide was sensitive to digestion by highly purified bacterial collagenase (Fig. 1b, tracks 9 and 10); it will subsequently be shown that this collagen exhibits some biochemical properties resembling type X collagen and others which make it distinct, it will therefore be referred to hereafter as 'BRP collagen'.

When the samples were electrophoresed under non-

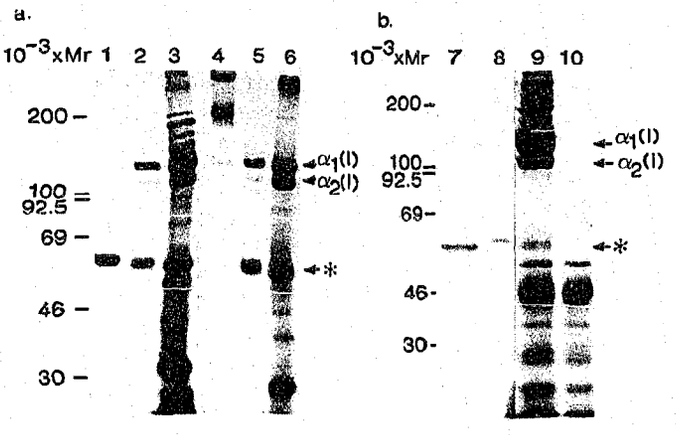

Fig. 1. SDS-PAGE of proteins extracted from pericyte cultures with pepsin. (a) Pericyte cultures containing small nodules were incubated with [^{3}H]proline for 24 h, and the proteins deposited into the cell layer/matrix were extracted with pepsin as described in section 2. Samples were separated by electrophoresis on 8% polyacrylamide slab gels and detected by fluorography. Samples in tracks 1, 2, 4 and 5 contain pepsinised type X collagen standards from cultures of bovine chondrocytes (tracks 1 and 4) and chick chondrocytes (tracks 2 and 5); samples in tracks 3 and 6 are from pericyte cultures. Samples were subjected to electrophoresis in the presence (tracks 1–3) and absence (tracks 4–6) of reducing agent. The position of BRP collagen is indicated by an asterisk. The migration positions of the α_1 and α_2 chains of type I collagen, and [^{14}C]methylated non-collagenous proteins of known M_r are also indicated. (b) In a different experiment, post-confluent cultures of pericytes containing dense cellular nodules were incubated with [^{3}H]proline. Pepsin-resistant proteins present in the cell layer/matrix were tested for their susceptibilities to digestion by highly purified bacterial collagenase, as described in section 2. Samples in the tracks are as follows: tracks 7 and 8 contain pepsinised type X collagen standards from chick (track 7) and bovine (track 8) chondrocytes; tracks 9 and 10 contain samples from pericyte cultures incubated with (track 10) or without (track 9) bacterial collagenase. The samples were electrophoresed under reducing conditions. BRP collagen is indicated by an asterisk. The samples in (a) and (b) are from different experiments; they were digested with pepsin and electrophoresed at different times.

reducing conditions, the mobility of this collagen was unaltered, suggesting that it does not contain either inter- or intra-chain disulphide bonds (Fig. 1a, compare tracks 3 and 6). This behaviour is similar to chick type X collagen (Fig. 1a, compare tracks 2 and 5), but clearly distinguishes BRP collagen from bovine type X collagen which does contain disulphide bonds (Fig. 1a, tracks 1 and 4; and [3]). BRP collagen was identified in the cell layer/matrix of five out of six batches of pericytes used in this study, although the amount synthesised was found to vary between different batches. We were, however, unable to detect this collagen in the medium suggesting that it is either preferentially or exclusively deposited into the cell layer/matrix by pericytes (results not shown). This characteristic also distinguishes BRP collagen from both chick and bovine type X collagen [9].

Fig. 2 demonstrates that a specific polyclonal antibody to type X collagen [2] reacted with chick type X collagen (track 1) and with a polypeptide of similar M_r in the pepsin extract of pericyte cultures (track 2). No immunoreactivity was detected in a pepsin extract of cultured fibroblasts (Fig. 2; track 3). Furthermore in control experiments using normal rabbit serum, no immunoreactive polypeptides were detected (not shown). These results suggest that BRP collagen and type X collagen share certain antigenic sites.

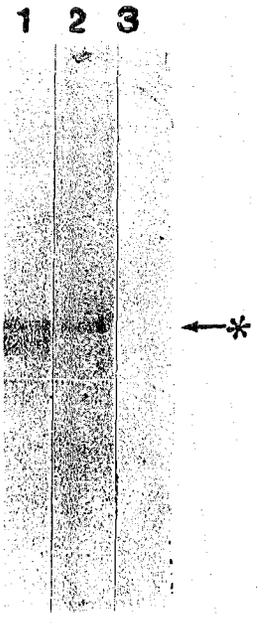

Fig. 2. Immunoreactivity of BRP collagen with type X collagen antisera. Cell layer/matrix extracts from pericyte and fibroblast cultures were separated by electrophoresis on a 8% polyacrylamide gel, transferred to nitrocellulose and immunoblotted with specific antibodies to type X collagen. The samples are as follows: track 1 contains pepsinised chick type X collagen; tracks 2 and 3 contain the pepsin-resistant proteins present in the cell layer/matrices of pericyte (track 2) and fibroblast (track 3) cultures. The major immunoreactive polypeptide is indicated by an asterisk.

We have previously demonstrated that pericytes calcify in vitro and that this process is associated with an increase in immunoreactivity with type X collagen antibody (see Introduction). In the light of these studies, an experiment was conducted to determine whether the secretion of BRP collagen was regulated by the state of differentiation of the pericyte cultures. Fig. 3a shows the results obtained by radiolabelling pericyte cultures at different stages of differentiation. Cells were incubated with [^{3}H]proline: (i) at confluence (day 7, track 2), (ii) when nodules had been formed (day 15, track 3), and two days later (track 4), and (iii) when the nodules had calcified (day 35, track 5). These tracks were scanned using a Schimadzu laser densitometer, and the areas under the curves were compared. The scans of tracks 2 and 5 (Fig. 3a) are shown in Figs. 3b and 3c, respectively. The amount of BRP collagen was measured in relation to the total collagen content of these cultures (predominantly types I and III [11]). The results indicated that cultures containing calcified nodules (Fig. 3c) secreted approximately twice as much BRP collagen as confluent cultures (Fig. 3b).

4. DISCUSSION

In this communication we describe the identification and partial characterisation of a low M_r 45 kDa polypeptide deposited into the cell layer by bovine retinal pericytes and which we have called BRP collagen. This polypeptide is sensitive to digestion by bacterial collagenase and is resistant to pepsin digestion (Fig. 1). In addition, it is recognised by specific antibodies to type X collagen (Fig. 2) suggesting that these two collagens share certain antigenic sites. However, in spite of this cross-reactivity, BRP collagen does not appear to be identical to type X collagen as (i) bovine type X collagen contains disulphide bonds whereas BRP collagen does not (Fig. 1; and [3]) and (ii) type X collagen can be identified in both the medium and cell layer/matrix of chondrocyte cultures [20] whereas BRP collagen has only been identified in the cell layer/matrix.

It is now becoming apparent that type X collagen belongs to a family of low M_r collagenous molecules which includes type VIII collagen [10] and may also include M_r 60 kDa smooth muscle cell collagen [21]. It is perhaps not surprising, therefore, that BRP collagen also shares certain characteristics with these other low M_r collagens, such as the M_r of the pepsinised forms (approx. 45–50 kDa) and the absence of intrachain disulphide bonds [22–24]. Moreover, M_r 60 kDa smooth muscle cell collagen and BRP collagen have only been detected in the cell layer/matrix, whereas type VIII collagen (like type X collagen) is secreted both into the medium and into the cell layer/matrix [22,23]. Of this family of collagens, only type X collagen has been associated with calcification in vivo [2,6,25]. Although

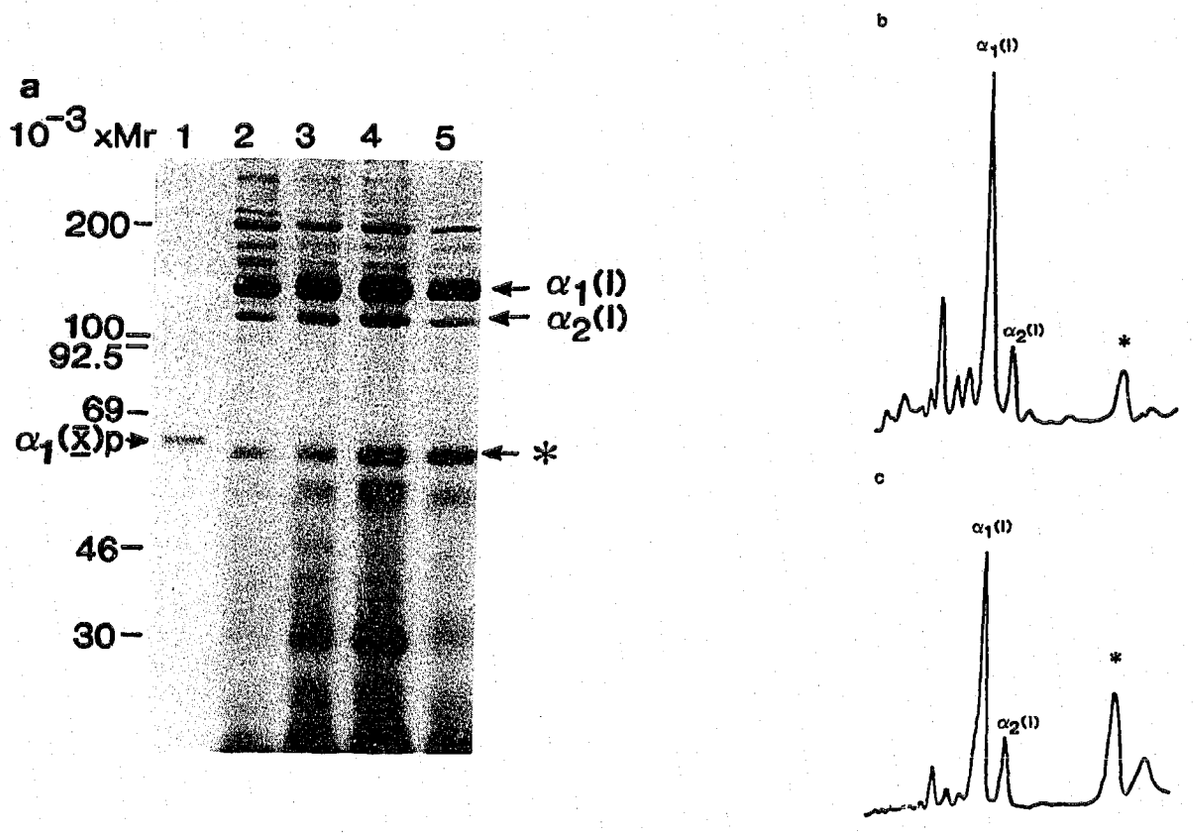

Fig. 3. Fluorogram of pepsin-resistant proteins extracted from the cell layer/matrices of pericytes at different stages of confluence. (a) Retinal pericytes were plated on plastic tissue culture dishes and were incubated with [^{3}H]proline for 24 h when they were just confluent (day 7, track 2); when small nodules were present (day 15, track 3); 2 days later (track 4) and when the cellular nodules were calcified (day 35, track 5). Newly-synthesised pepsin-resistant proteins present in the cell layer/matrices were separated by electrophoresis on an 8% polyacrylamide gel and detected by fluorography. Track 1 contains pepsinised bovine type X collagen (α_1(X)p). The migration positions of the α_1 and α_2 chains of type I collagen and non-collagenous proteins of known M_r are indicated. The migration position of BRP collagen is indicated by an asterisk (b), and (c) shows laser densitometric scans of tracks 2 and 5 (respectively) of the fluorogram shown in (a). The areas under the curves were measured, and the amount of BRP collagen was assessed relative to: (a) the total collagenous proteins, and (b) the α_1 chain of type I collagen. With both methods, it was found that there was approx. twice as much BRP collagen in calcified (c) as in confluent (b) cultures.

its specific role remains to be determined, it has been proposed that type X collagen may provide a permissive matrix for calcification [8] or may be related to the invasion of vascular elements which precedes calcification [7]. By contrast, no relationship between either type VIII collagen or M_r 60 kDa smooth muscle cell collagen and calcification has been proposed. It is therefore of particular interest that retinal microvascular pericytes undergo calcification in vitro [1,11] and synthesise a collagen which appears to be similar, but not identical to, type X collagen (this communication). Furthermore, the synthesis of this collagen appears to increase during pericyte calcification as detected biochemically (Fig. 3) and by immunofluorescence [4]. It is also of interest that the levels of synthesis of BRP collagen appear to vary between different batches of pericytes. Heterogeneity in the level and types of collagens synthesised has previously been documented for endothelial cultures [26,27]. It is not surprising that similar heterogeneity also appears to be found amongst pericytes. The function of BRP collagen in the microvasculature remains to be determined. However, it is possible that this collagen may play a role in calcification in vivo which is normally associated with angiogenesis.

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