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Angiogenic oligosaccharides of hyaluronan enhance the production of collagens by endothelial cells

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SUMMARY

The present study demonstrates a relationship between angiogenic oligosaccharides of hyaluronan (HA) and the production of collagens during the process of angiogenesis in vivo and in vitro. The addition of angiogenic oligosaccharides of HA to the chorioallantoic membrane of the chick embryo induced a deposition of collagen fibrils. The treatment of sub-confluent cultures of bovine aortic endothelial cells with the same oligosaccharides (1 μg/ml) increased the uptake of [^{3}H]proline by approximately 60%. SDS-polyacrylamide gel electrophoresis of treated cultures demonstrated the enhanced synthesis of type I and type VIII collagens. The production of type VIII collagen was confirmed by western blotting and immunocytochemistry using antibodies to sheep and bovine type VIII collagen. Type VIII collagen is a short chain collagen that has a high degree of homology to cartilage-specific type X collagen. The biological functions of type VIII and type X collagens are unknown. We have suggested that the two collagens play a role in the process of angiogenesis.

Key words: hyaluronan, collagen, angiogenesis

INTRODUCTION

Hyaluronan (HA) has been implicated in several developmental processes (Toole, 1982; Stern, 1984; Spooner, 1986). An HA-rich environment inhibits blood vessel formation, i.e. angiogenesis, in chick embryo limb buds and within granulation tissue (Balazs and Darzynkiewicz, 1973; Feinberg and Beebe, 1983; Dvorak et al., 1987), whereas, HA oligosaccharides stimulate angiogenesis on the chicken chorioallantoic membrane (CAM; West et al., 1985). Similar oligosaccharides of 3-10 disaccharide units in length enhance cell proliferation and migration by bovine aortic endothelial cells in vitro (West and Kumar, 1989; Sattar et al., 1992).

The role of collagen in angiogenesis is unclear. Ingber and Folkman (1988) have shown that metabolic reduction of collagen synthesis inhibits capillary formation on the CAM, suggesting that collagen might be a necessary substratum for the migration of endothelial cells. Furthermore, Maragoudakis et al. (1991) reported that the addition of a specific inhibitor of basement membrane collagen biosynthesis (GPA 1734) prevented angiogenesis on CAMs. Recently, type VIII collagen has also been proposed to play a role in angiogenesis (Iruela-Arispe and Sage, 1991; Iruela-Arispe et al., 1991a). Type VIII collagen is a short chain collagen, originally isolated from bovine vascular endothelial cells but, only those undergoing active cell proliferation (Sage et al., 1980; Sage et al., 1983). This collagen has since been found to be synthesised by and immunolocalised to several non-vascular endothelial cells and non-endothelial tissue (Alitalo et al., 1983; Sage et al., 1984; Benya and Padilla, 1986; Kapoor et al., 1988; Kittelberger et al., 1990). Type VIII collagen is very similar in size and chemical characteristics to type X collagen, which is a gene product unique to hypertrophic chondrocytes (Kielty et al., 1985; Kwan et al., 1986; Yamaguchi et al., 1989; Yamaguchi et al., 1991).

In this report we have investigated whether induced angiogenesis, in vivo and in vitro, is associated with collagen synthesis. Angiogenesis was induced by the addition of HA oligosaccharides to the CAM or to cultures of bovine aortic endothelial cells (BAEC). We report that angiogenesis in vivo is associated with the deposition of collagen fibrils on the CAM and with a specific, increased production of type I and type VIII collagens by BAEC. The enhanced synthesis of type VIII collagen by treated BAEC is demonstrated both in western blots and immunocytochemically using two different antibodies against type VIII collagen. Immunocytochemistry with a polyclonal antibody to chick type X collagen (Kwan et al., 1989), which is known to cross-react with type X-like collagens (Canfield and Schor, 1991), also demonstrates specific staining in activated cultures whereas control cultures do not stain significantly above background levels. These results reinforce

the similarity between type VIII and type X collagens and are discussed in terms of both collagens having a role in angiogenesis.

MATERIALS AND METHODS

CAM assay

Fertilised White Leghorn chicken eggs were incubated at 37°C for 3 days, then a portion of the eggshell was removed and the membranes allowed to collapse. The egg was sealed with Sellotape and returned to the incubator until the embryos were 10 days old. At this time, HA fraction F3 (see below) was added to the CAM, the egg was re-sealed and incubated for a further 4 days. At the end of this period, CAMs were fixed in situ for 30 min with 6% gluteraldehyde (in Sorenson's phosphate buffer) before excision and transfer to 2% gluteraldehyde in buffer.

Electron microscopy

Treated and control CAMs were rinsed 3 times in Sorenson's buffer, post-fixed in 1% osmium tetroxide, rinsed, dehydrated through a graded series of ethanol, and embedded in Spurr resin. Semi-thin 1 μm sections were cut on a Reichart ultramicrotome and stained with toluidine blue to determine the exact area for ultrathin sections. Care was taken that this area of the CAM was within 2-3 mm of the implantation site. Sections were stained with uranyl acetate/lead citrate and examined on a Philips 400 electron microscope.

Angiogenic oligosaccharides of HA (F3)

Angiogenic fragments of HA were prepared as described previously (West and Kumar, 1989). Briefly, 500 mg of HA (Sigma) was dissolved in 50 ml of 0.1 M sodium acetate buffer (pH 5.4), containing 0.15 M NaCl and treated with 20000 units of bovine testicular hyaluronidase (Sigma) at 37°C. At regular intervals (2, 4, 6, 8 and 24 h), 10 ml aliquots were removed and 1 ml of 100% TCA was added to each. The mixtures were centrifuged and supernatants pooled, dialysed against distilled water for 24-48 hours, at 4°C, with at least 4 changes, in Spectra/Por 6 tubing (cut-off size, approximately 1000 Da), re-centrifuged and lyophilized. The powder was dissolved in 20 ml of 0.1% acetic acid and loaded onto a G50 Sephadex column (2.6×180 cm). 10 ml fractions were collected, assayed for uronic acid and individual fractions combined to give 3 pools viz F1, F2 and F3.

The size range of oligosaccharides in each pool was determined by incorporating [^{3}H]glucosamine-labelled HA and analyzing each fraction by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography. Successive bands differ on SDS-gels by one disaccharide unit, therefore, precise definition of the size range could by ascertained by comparing bands with labelled octa-, hexa- and tetrasaccharides of known molecular sizes. F1, F2 and F3 fractions consisted of oligosaccharides of >16, 10-16 and 3-10 disaccharide units, i.e. molecular masses of approximately >7200 Da, 4500-7200 Da and 1350-4500 Da, respectively.

The greatest angiogenic response was obtained when HA was digested with hyaluronidase for 2-8 h, no angiogenic response was observed when HA was digested for 24 h.

Cell culture

Cultures of BAEC were established and their endothelial nature determined as described previously (Kumar et al., 1987). They were maintained in Dulbecco's Modified Eagles Medium (DMEM) with 10% newborn calf serum and supplemented with L-glutamine, penicillin, and streptomycin. Cells were grown to semi-confluence in two 75 cm^2 tissue culture flasks (Falcon, Becton Dickinson), passaged in a 1% trypsin/EDTA solution, split 1:6 and plated out in twelve 35 mm tissue culture dishes (Sterilin) in DMEM as above. All dishes were incubated in control medium for 24 h, following which one group of 6 dishes was fed with DMEM supplemented as above plus 1 μg/ml HA oligosaccharide fraction F3, the remaining 6 dishes were maintained in control medium. After 48 h, the media were replaced by either F3 or control media containing [^{3}H]proline (25 μCi/ml; Amersham International) and incubated for a further 24 h. The procedure was repeated on 3 different batches of BAEC.

Collagen synthesis

The [^{3}H]proline-labelled culture media were removed and the protease inhibitors phenylmethylsulfonyl fluoride (PMSF; 2 mM), *N*-ethylmaleimide (NEM; 10 mM), EDTA (25 mM) and ethyleneamino-*n*-caproic acid (25 mM) were added. High molecular mass proteins, including collagens, were precipitated by the addition of ammonium sulphate to 30% saturation (176 mg/ml), collected by centrifugation (15,000 ***g*** for 1 h), resuspended in 0.5 M acetic acid and dialysed extensively against 0.2 M acetic acid. Radioactivity was assessed by counting in a TriCarb liquid scintillation counter (Packard) and samples of 25,000 c.p.m. were lyophilised.

SDS/PAGE

Labelled proteins were boiled for 2 min in Laemmli sample buffer and examined by SDS-PAGE using 8% slab gels under non-reduced conditions (Laemmli, 1970). In some cases, samples were partially purified by 0.86 M NaCl precipitation, to remove type I collagen, and the supernatants were digested in 100 μg/ml pepsin (Sigma), in 0.5 M acetic acid at 4°C for 4 h, prior to SDS-PAGE. All gels were processed for fluorography and exposed to X-Omat AR X-ray film (Kodak) (Bonner and Laskey, 1974; Laskey and Mills, 1975). Molecular masses were estimated by comparison with molecular mass standards and labelled collagen standards from chick embryo sternal chondrocytes (Kielty et al., 1985).

Collagenase digestion

Lyophilised samples were dissolved in 50 mM Tris-HCl, pH 7.4, containing 5 mM $CaCl_2$, 5 mM NEM, 1 mM PMSF, and digested with 30 units of highly purified bacterial collagenase form III (Advance Biofactures, New York) for 4 h at 37°C. Digested samples were lyophilised and analysed by SDS-PAGE.

Immunoblotting

Samples of labelled proteins were run on SDS gels and the polypeptides were transferred onto nitrocellulose sheets according to the method of Towbin et al. (1979). Briefly, polyacrylamide gels were equilibrated for 20 min in transfer buffer (20 mM Tris, 150 mM glycine, 20% methanol, pH 8.3) and transferred to cellulose nitrate membrane (Schleicher and Schuell, Germany) overnight at 40 V in a Transblot apparatus (Hoefer Scientific Instruments, USA). After 1 h in blocking solution, the membrane was incubated at room temperature overnight with either a polyclonal, rabbit anti-sheep type VIII collagen antibody (Kittelberger et al., 1990) or a polyclonal, guinea pig anti-bovine type VIII collagen antibody (Iruela-Arispe et al., 1991a) both at 1:50 dilution. Membranes were washed 3 times in PBS and incubated either with horseradish peroxidase-conjugated anti-rabbit IgGs or alkaline phosphatase-conjugated anti-guinea pig IgGs (Dakopatts) for 1.5 h at room temperature.

Both anti-type VIII collagen antibodies were raised against pepsin treated collagen extracted from the Descemet's membrane of sheep and bovine eyes, respectively. The specificity of each antibody was tested by ELIZA. The antibodies are highly specific and do not cross-react with human collagen types I, III, IV, V, VI, and VII; bovine collagen types I-VI and X, sheep collagen types III and V, or glycoproteins including fibronectin, laminin,

merosin, vitronectin, serum proteins, thrombospondin and SPARC (Kapoor et al., 1986; Kittelberger et al., 1990; Iruela-Arispe et al., 1991a).

Immunocytochemistry

Treated and control cultures were fixed in 3% paraformaldehyde and washed three times in PBS. Cells were digested with bovine testicular hyaluronidase (1 μg/ml in 0.05 M sodium acetate buffer, pH 5, with 25 mM NaCl) for 1 h at room temperature, washed in PBS and incubated with the polyclonal, anti-bovine type VIII collagen antibody for 1-2 h (Iruela-Arispe et al., 1991a). The primary antibody was washed off and the cells were then incubated with a secondary, alkaline phosphatase-conjugated anti-guinea pig IgG antibody (Dakopatts) for 1 h.

Some cultures were also incubated with a polyclonal antibody to chick embryo type X collagen (AS3) raised in rabbits (Kwan et al., 1989). Fixed cultures were processed as above using the same dilution of primary antibody followed by incubation with a secondary, peroxidase-conjugated, anti-rabbit IgG antibody (Dakopatts) for 1 h in PBS. This type X collagen antibody has been shown not to cross-react with collagen types I, II, V, VI, IX and XI, but does cross-react with a pericyte-derived collagenous protein, which is believed to be related to type VIII/X collagen (Canfield and Schor, 1991).

RESULTS

Addition of HA oligosaccharides to the CAM

HA oligosaccharide fraction F3 induced a marked angiogenic response, as indicated by the presence of whorls of newly formed capillaries surrounding the infiltration site, when added to the CAM of 10-day chick embryos. In treated CAMs, the observed angiogenesis was accompanied by the deposition of a large amount of collagen fibrils (Fig. 1) which were not present in such abundance in untreated or sham operated CAMs (Fig. 1b). No angiogenic response was observed when HA fragment F1 was added (not shown).

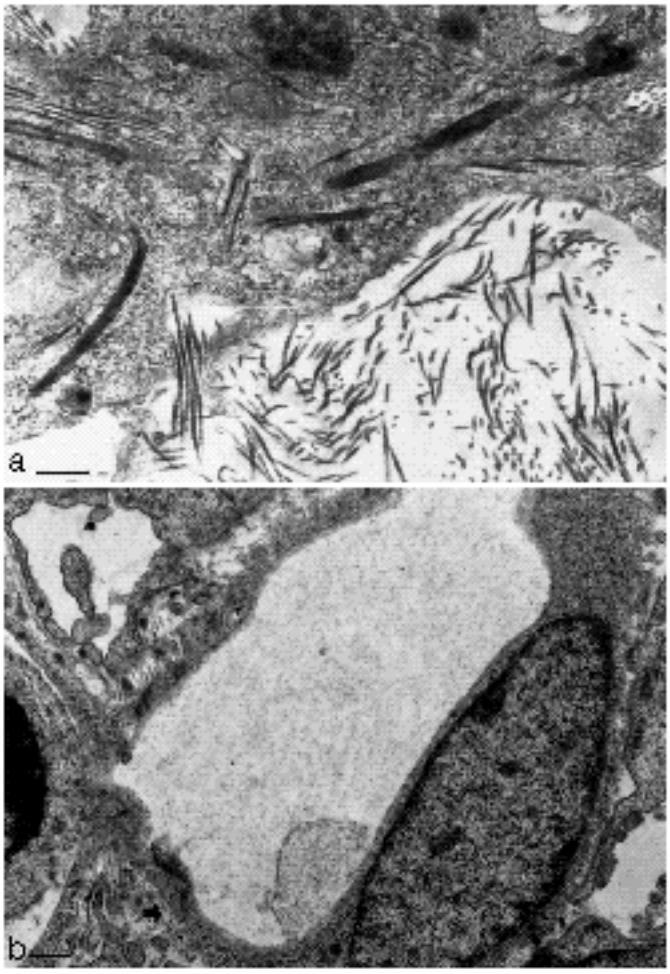

Fig. 1. Electron micrographs of a portion of CAM. (a) CAM treated with HA fraction F3. Many collagen fibrils can be seen. In contrast, in a control CAM (b), very few collagen fibrils are present. Bar, 0.1 μm.

Collagen biosynthesis by BAEC

The addition of HA oligosaccharide fraction F3 had no effect on the morphology of the BAEC as previously reported (West and Kumar, 1989; data not shown). The F3 oligosaccharide induced a 58% increase in [^{3}H]proline incorporation into precipitable proteins, in treated cultures when compared with controls (615,025 c.p.m. versus 388,125 c.p.m.). This matched the 50-60% increase in cell number already reported (West and Kumar, 1989), indicating that protein synthesis per se was not stimulated by the HA oligosaccharides.

When samples containing 25000 c.p.m. (i.e. from approximately 2×10^4 treated or control BAEC) of radiolabelled proteins were analysed by SDS-PAGE, fibronectin and type I collagen were synthesised by both treated and control cultures (Fig. 2, tracks 1 and 2). Both cultures also produced polypeptides with a molecular mass of approximately 60-62 kDa, which were collagenous in nature (not shown) and which became reduced in size to 50 kDa upon treatment with pepsin (track 3). There was a 4.5-fold enhancement of type I collagen and a 6-fold enhancement in synthesis of the 60 kDa polypeptides by treated BAEC. In treated cultures, these 60-62 kDa polypeptides accounted for approximately 25% of total collagen production as determined by laser densitometry (not shown).

Immunoblotting

Western blot analyses of SDS-PAGE gels, using both anti-sheep and anti-bovine type VIII collagen antibodies, produced a positive reaction in media from both treated and control BAEC (Fig. 3, tracks 1 and 2). Only one positive band could be seen on these gels and the molecular mass of the reactive polypeptide was estimated to be approximately 62 kDa from molecular mass markers. The reactive polypeptide observed in control cultures was consistently of a lower intensity than that seen in treated cultures. Non-denatured samples (not boiled prior to loading) from treated cultures still produced an immunoreactive band, but with a molecular weight of approximately 180 kDa indicative of the 60-62 kDa polypeptides being derived from a trimeric molecule (not shown). The antibodies used in this study were produced prior to the molecular configuration of type VIII collagen being designated as [1(VIII)$_2$ 2(VIII)] (van

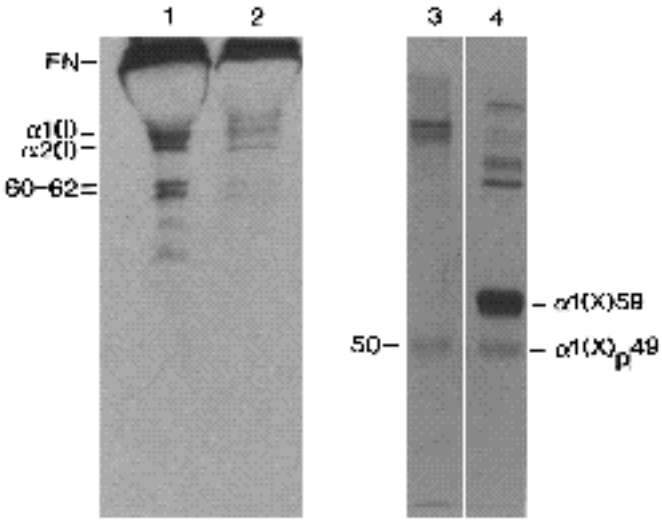

Fig. 2. Fluorogram of an SDS/PAGE gel of 30% ammonium sulphate precipitates, from [^{3}H]proline-labelled culture media, all non-reduced. 25000 c.p.m./lane. Relative molecular masses ($\times 10^{-3}$) are indicated. Lane 1, collagens synthesised by BAEC grown in the presence of HA oligosaccharides; lane 2, collagens synthesised by BAEC grown in control media; lane 3, partially purified and pepsin digested collagens synthesised by BAEC grown in the presence of HA oligosaccharides; lane 4, chick embryo sternal chondrocyte, type X collagen standard. Fibronectin (FN), type I and type X collagen chains are marked.

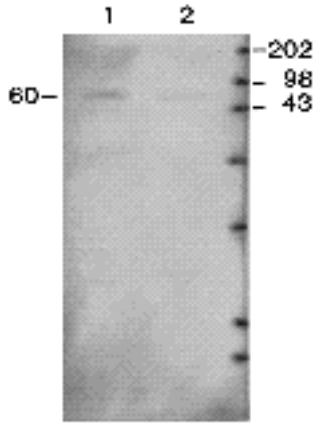

Fig. 3. Western blot of an SDS/PAGE gel of 30% ammonium sulphate precipitates from [^{3}H]proline-labelled media, all non-reduced. 50,000 c.p.m./track. Reactivity was observed using a rabbit anti-sheep type VIII collagen antibody. M_r markers are shown ($\times 10^{-3}$). Lane 1, BAEC grown in the presence of HA oligosaccharides; lane 2, BAEC grown in control medium.

der Rest and Garrone, 1991). Each antibody has been reported as cross-reacting with only one polypeptide chain and evidence suggests that this chain is 1(VIII) collagen (Kapoor et al., 1986; Kittelberger et al., 1990).

Immunolocalisation of type VIII/X collagen

Immunolocalisation of BAEC cultures with polyclonal antibodies to bovine type VIII collagen and chick embryo type X collagen produced an identical result. A specific, cytoplasmic reaction, of varying intensity, was observed in every cell of treated cultures (Fig. 4a,b). In contrast, control cultures demonstrated light staining in some cells with most cells exhibiting background staining or no reaction at all (Fig. 4c). Appropriate controls using treated cells and

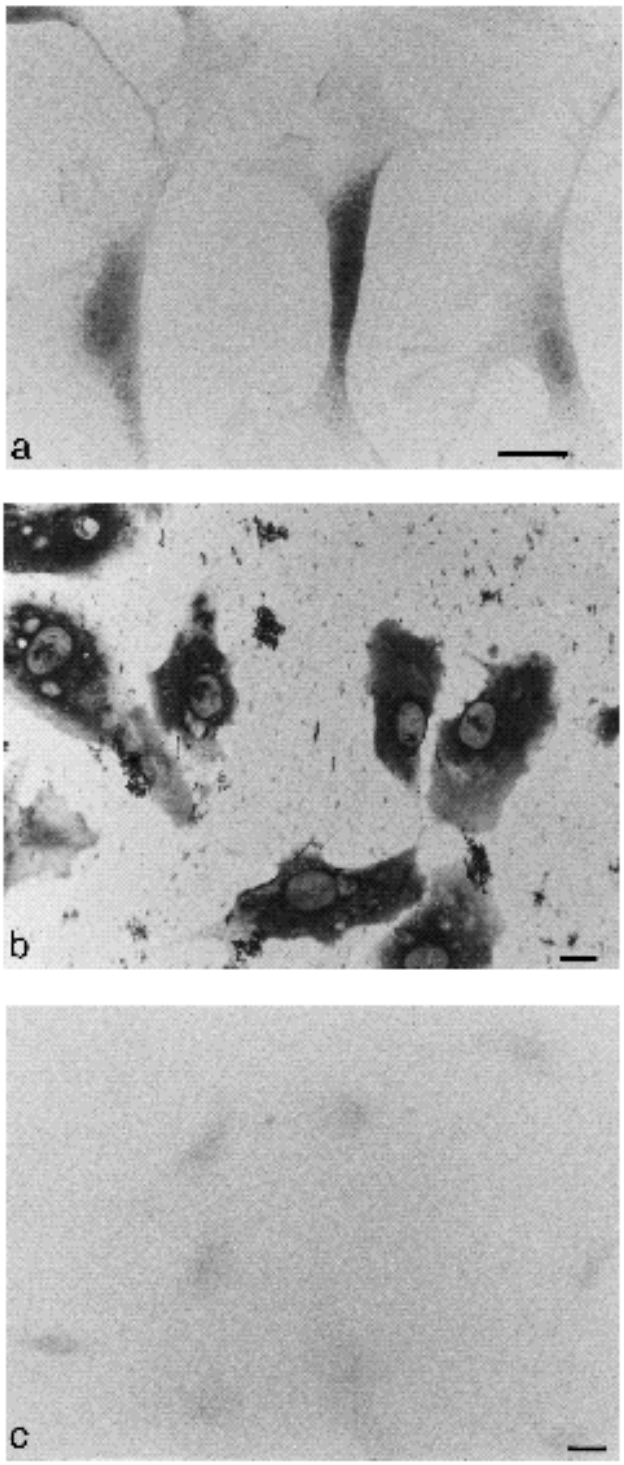

Fig. 4. Light micrographs of BAEC grown in the presence or absence of HA oligosaccharides then incubated with either the guinea pig, anti-bovine type VIII collagen antibody or the AS3, anti-type X collagen antibody. (A) Treated culture, anti-type VIII collagen. Every cell demonstrates positive cytoplasmic staining of varying intensities. Immuno-alkaline phosphatase staining. Bar, 10 μm. (B) Treated culture, anti-type X collagen. Every cell demonstrates positive cytoplasmic staining of varying intensity. Immunoperoxidase staining. Bar, 10 μm. (C) Control culture, anti-type VIII collagen. Faint staining is seen in very few cells whilst the remainder are negative. Immuno-alkaline phosphatase staining. Bar, 10 μm.

pre-immune serum demonstrated no positive staining (not shown).

DISCUSSION

The results presented here demonstrate that the addition of angiogenic HA oligosaccharides (F3) to the CAM in vivo and to BAEC in vitro induced an increased synthesis of collagen. In the case of BAEC the major collagen types have been identified as type I collagen and type VIII collagen by SDS-PAGE and immunoblotting. It is likely, therefore, that the collagen fibrils observed in treated CAMs may also be type I collagen. The increased proliferation of BAEC following addition of HA oligosaccharides is matched by a similar increase in incorporation of radiolabelled proline indicating that the four- to six-fold enhanced secretion of type I and type VIII collagens reported here is due to the specific up-regulation of these collagen genes. An enhancement of synthesis of type I and type VIII collagens has also been reported when endothelial cells undergo spontaneous angiogenesis, i.e. sprouting, in vitro (Iruela-Arispe et al., 1991a).

When BAEC treated with HA oligosaccharides are incubated with a polyclonal antibody to bovine type VIII collagen, an intense, cytoplasmic staining is observed. Similar intracellular staining has been reported in endothelial cells undergoing spontaneous angiogenesis using the same antibody (Iruela-Arispe et al., 1991a). The staining observed in cells incubated with the type X collagen antibody (AS3) is **not** due to the synthesis of type X collagen since bovine type X collagen is disulphide bonded and would only be present on SDS gels at a higher molecular weight, without prior reduction (Thomas et al., 1991). It seems likely, therefore, that the AS3 antibody is cross-reacting with type VIII collagen, which is known to have a high degree of homology to type X collagen. It is also likely that the type X-like collagen produced by pericytes will be more closely related to type VIII collagen (Canfield and Schor, 1991).

The role played by collagens during the angiogenic process is unclear. Type I collagen synthesis is known to be initiated de novo when BAEC undergo capillary sprout formation and, prevention of collagen synthesis inhibits capillary formation on the CAM (Ingber and Folkman, 1988; Iruela-Arispe et al., 1991b). Taken together, these observations suggest that type I collagen may play a role as a substratum for endothelial cell migration during capillary sprout formation (Iruela-Arispe et al., 1991a) and would be supported by the 4.5-fold increase in type I collagen synthesis reported here. Recently, type VIII collagen has also been implicated in angiogenesis where it is thought to interact with extracellular matrix components such as HA or some other polyanionic glycosaminoglycan (Yamaguchi et al., 1989; Sage and Iruela-Arispe, 1990; Iruela-Arispe and Sage, 1991; Iruela-Arispe et al., 1991a). It seems possible, therefore, that in vivo breakdown of native, high molecular weight HA within the extracellular matrix could result in the production of angiogenic oligosaccharides capable of inducing cell proliferation, type I and type VIII collagen synthesis and initiation of cell migration and differentiation. It has been postulated that the role of type VIII collagen may be to facilitate the assembly of endothelial cords and tubes (Iruela-Arispe et al., 1991a).

The biological functions of type VIII collagen are unknown. During the angiogenic process, endothelial cell proliferation, migration and differentiation are localised events, possibly regulated by changes in the extracellular matrix microenvironment. The controlling factors in this local activation are unknown; it is possible that the synthesis of type VIII collagen, only by the proliferating cells, may trigger the matrical changes initiating the activation process.

Similarities in size, chemical characteristics and gene structure have been well documented between type VIII and type X collagens (Yamaguchi et al., 1989) and have been reinforced in this report by the cross-reaction observed between type VIII collagen and a type X collagen antibody. However, no explanation has been put forward for these similarities. We would suggest that both collagens work in a similar fashion by altering an existing extracellular matrix, allowing angiogenesis to proceed.

Type X collagen is only produced by hypertrophic chondrocytes, the cells whose extracellular matrix mineralises to be replaced by bone marrow and subsequently bone in the epiphyseal growth plate (Ham and Cormack, 1979). This has led to type X collagen being associated with the process of mineralisation, however, in the developing cartilage long bone rudiment, particularly of the chick embryo, type X collagen is synthesised in the tibia at 8-9 days of incubation (Schmid and Linsenmayer, 1985) whilst mineralisation does not begin until at least 1 week later (Fell, 1925). It is interesting to note that, in chick embryos, vascular invasion and bone marrow cavity formation also begin in the centre of the hypertrophic zone at 8-9 days (Fell, 1925; Caplan and Pechak, 1987). In contrast, the time interval between chondrocyte hypertrophy, type X collagen synthesis, mineralisation and marrow cavity formation in developing mammalian embryos is less than 48 hours and it has not been fully elucidated whether mineralisation or vascular invasion occurs first (Hinchliffe and Johnson, 1980; Caplan and Pechak, 1987).

Like type VIII collagen in the Descemet's membrane of the eye, type X collagen produces an hexagonal structure within the cartilage extracellular matrix (Sawada, 1982; Kwan et al., 1991). Formation of this hexagonal structure will alter the existing properties of the matrix and this alteration may make the hypertrophic region angiogenic, triggering endothelial cell proliferation, type VIII collagen synthesis and cell migration. It is possible, therefore, that the production of type X collagen may influence angiogenesis rather than mineralisation.

The results presented here do not suggest that type VIII or type X collagens are angiogenic molecules, rather they imply that both molecules may play a role in the process of angiogenesis by modifying the extracellular matrix and stimulating cell migration. During angiogenesis in cartilaginous long bone rudiments, it is possible that type X collagen and type VIII collagen work in tandem.

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