Developmental regulation of the mRNAs for elastins a, b and c in foetal-calf nuchal ligament and aorta

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The data presented clearly suggest that relative amounts of mRNAs for elastins a, b and c are developmentally regulated in foetal-calf nuchal ligament and aorta and that this regulation is tissue-specific. In nuchal ligament, at earlier stages of foetal development, the relative amounts of mRNAs for elastins a and b are very low. After the foetal age of about 6 months the relative amount of mRNA for elastin b begins to increase. This is followed by an increase in the relative amount of mRNA for elastin a. In aorta, with increasing foetal age, the relative amounts of mRNAs for elastins b and c increase and decrease alternately. The relative amounts of mRNA for elastin a remain low, with only marginal increases with foetal age. A possible self-aggregation role of elastin a in elastogenesis is proposed.

INTRODUCTION

Elastin has the property of elastic recoil and is the major component of those tissues which require rapid extension and complete recovery (e.g. aorta and nuchal ligament). The polypeptide chains of elastin are cross-linked at suitable intervals and this generates an extensible three-dimensional network. It has been reported that the production of elastin is very low at early stages of foetal elastic-tissue development (nuchal ligament and aorta) and that there is a marked increase in the synthesis and appearance of elastin in these tissues at later stages of foetal development [1,2].

Very recently, we reported the occurrence of three mRNAs for elastin in foetal calf nuchal ligament, encoding three forms of elastin (a, b and c). These forms are the result of the presence, at a single position, of 102 addition nucleotides in the mRNA for elastin a and of 60 of these nucleotides in the mRNA for elastin b as compared with the mRNA for elastin c [3]. Since then, others have also reported the occurrence of multiple forms of mRNA for elastin [4,5]. We have now examined the relative abundance of these three mRNAs for elastin in nuchal ligament and aorta of foetal calves at various stages of foetal development. The results of this study are reported in the present paper.

EXPERIMENTAL

Materials

Chemicals. Guanidinium thiocyanate was bought from Fluka (Terrochem, Mississauga, Ont., Canada); guanidinium chloride (Ultra-pure) was from Gibco/BRL (Burlington, Ont., Canada). Biodyne nylon membrane, [γ - 32 P]ATP and poly(ethylene glycol) 8000 were from ICN (St. Laurent, Que., Canada). Dithiothreitol (DTT) and AG 501-X8(D) mixed-bed resin (analytical grade)

were from Bio-Rad (Mississauga, Ont. Canada). Sodium *N*-dodecylsarcosine, heparin (sodium salt), chloramphenicol, agar, ethidium bromide, Bromophenol Blue and ampicillin were from Sigma (St. Louis, MO, U.S.A.). Tris, calf thymus DNA, calf liver rRNA, bovine serum albumin, phage-T₄ DNA ligase, deoxynucleotides and dideoxynucleotides were from Boehringer-Mannheim (Dorval, Que., Canada). EDTA, β -mercaptoethanol, boric acid, diethyl ether, tryptone, yeast extract and glyoxal (technical grade) were from BDH (Etobicoke, Ont., Canada). Restriction endonucleases, DNA polymerase I, T₄ polynucleotide kinase, Klenow enzyme and M13mp¹⁸ vector were bought from Pharmacia (Etobicoke, Ont., Canada). X-ray film (XAR-5) was from Eastman Kodak Co. Nitrocellulose membrane (0.45 μ m pore size) was from Schleicher and Schuell (Keene, NH, U.S.A.). Nylon membrane (Hybond-N) and all other radiolabelled compounds were from Amersham (Oakville, Ont., Canada). GeneScreen Plus membrane was from NEN (Boston, MA, U.S.A.). Wheat-germ rRNA was a gift from Dr. B. Lane of this Department. Electrophoresis chemicals were from Bio-Rad or IBI (New Haven, CT, U.S.A.). All other chemicals were analytical grade and obtained from BDH or Mallinckrodt.

Foetal-calf tissues. Foetal-calf nuchal ligament and aorta tissues were obtained as a gift from Tenderlean Beef, a Burlington (Ont., Canada) abattoir. The age of each foetus was determined with the help of the veterinary officer in attendance and was based on body size, degree of tooth eruption, body and facial hair (whiskers and eyebrows). The indicated ages are believed to be within 2 weeks of the actual ages. In each case, the foetus was removed from the uterus within 30–35 min of the kill and the dissection of the tissues commenced immediately. After dissection, the tissues (nuchal ligament and aorta) were rinsed with cold water, frozen and stored in liquid nitrogen.

Abbreviations used: oligo A and oligo B, synthetic oligodeoxyribonucleotides 5'-GTTTTGTAGGGCAGTCCATAGCCAG-3' and 5'-TCCCCGTTGGATACCCAGCCTTGCC-3' respectively; Pst:319, restriction-endonuclease-*Pst*I fragment of cDNA clone pcBE319 of bovine elastin mRNA [3]; M13:319, Pst:319 ligated into M13mp¹⁸; DTT, dithiothreitol.

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Methods

Isolation of total RNA. The total cellular RNA was isolated from the nuchal ligaments and aortas by slight modification of the previously described guanidinium thiocyanate/guanidinium chloride extraction method [6]. After the fourth precipitation with 0.7 vol. of ethanol, the pellet was resuspended in a minimum volume of resuspension buffer (6.0 M-guanidinium chloride/10 mM-DTT/1.0% sodium *N*-dodecylsarcosine/20 mM EDTA/0.1 M-Tris/HCl, pH 5.5 (no less than 3.0 ml) and extracted with an equal volume of chloroform/phenol (1:1, v/v). Before use, the phenol was water-saturated, then equilibrated with 10 mM-Tris/HCl/1.0 mM-EDTA, pH 8.0, and contained 0.5 mg/ml of quinolinol to give the phenol phase a yellow colour, which helped in revealing the two phases in the extraction mixture. The top aqueous phase was removed and the phenol phase was re-extracted with resuspension buffer. The aqueous phases from both extractions were pooled and extracted with an equal volume of chloroform. The aqueous phase from the chloroform extraction was then treated with an equal volume of water-saturated diethyl ether to remove traces of remaining phenol. The aqueous phase from the diethyl ether extraction was placed in a vacuum desiccator to remove residual ether by evaporation under reduced pressure. Finally, the RNA was pelleted and stored as described previously [7].

Assay of mRNAs for Elastin a, b and c. Probes. Two chemically synthesized oligodeoxyribonucleotides (Applied Biosystems 380B DNA Synthesizer), 25-mers (for sequences, see the abbreviations footnote), one complementary to a sequence unique to the mRNA for elastin a (oligo A) and the other complementary to a sequence present in the mRNAs for elastin a and b (oligo B), were used as probes to quantify the mRNAs for elastin a and elastins a and b respectively. The *Pst*I fragment from clone pcBE319 (392 bp) (*Pst*:319) [3], which carries the sequences common to all three mRNAs of elastin (a, b and c), served as a probe for these mRNAs.

Labelling. The synthetic oligomers (oligo A and oligo B) were labelled with the use of a T_4 polynucleotide kinase reaction as follows: 100 ng of the oligonucleotide were added to 70 mM-Tris/HCl (pH 7.5)/10 mM-MgCl₂/7 mM-DTT/[γ -³²P]ATP (80 μ Ci) and incubated at 37 °C for 30 min in the presence of 20 units of T_4 polynucleotide kinase. *Pst*:319 was nick-translated and labelled with [α -³²P]dCTP (specific radioactivity > 3000 Ci/mmol) as described in the literature [8]. *Pst*:319 was also inserted into vector M13mp¹⁸ and was labelled by a second-strand-synthesis reaction with the use of the hybridization probe primer (Biolabs, no. 1202) which produces a radiolabelled second strand [9]. The unincorporated radiolabel was removed by chromatography of the labelled probe on a Sephadex G-100 column pre-equilibrated with elution buffer [100 mM-NaCl/10 mM-Tris/HCl (pH 8.0)/1 mM-EDTA].

Dot-blot analysis. Various amounts of total RNA from nuchal ligament and aorta were made to a final volume of 120 μ l with 20 $\times$ SSC (1 $\times$ SSC is 150 mM-NaCl/75 mM-sodium citrate, pH 7.0), denatured by heating at 70 °C for 5 min and chilled on ice for 3 min before application on to nitrocellulose membranes. Nitrocellulose membranes were prepared as described in [10].

Fig. 1. Specificity of probes used for dot-blot and Northern-blot analysis

(a) Dot-blot hybridization: 5.0 ng of cDNA clones pcBE308, pcBE319 and pcBE320 [3] were blotted on to nitrocellulose filters and hybridized with radiolabelled probes as indicated: 1, pcBE308; 2, pcBE319; 3, pcBE320; 4, pBR322. Clone pcBE308 contains 102 additional nucleotides (corresponding to elastin a) and pcBE320 contains 60 of these nucleotides (corresponding to elastin b) as compared with clone pcBE319 (corresponding to elastin c). (b) Northern-blot analysis: 10 μ g of total RNA was denatured, electrophoresed on a 1.0%-agarose gel and transferred to hybridizing membranes as described in the Experimental section. Lanes 1, 3 and 5 contain RNA from the nuchal ligament of an 8-month-old foetus; lanes 2, 4 and 6 contain RNA from the aorta of a 9-month-old foetus. Lanes 1 and 2 were transferred on to GeneScreen Plus membrane by capillary blot and hybridized with oligo A at 51 °C; lanes 3 and 4 were transferred to Biodyne membrane by electroblot and were hybridized with oligo B at 55 °C; lanes 5 and 6 were hybridized *in situ* with *Pst*:319 at 68 °C.

RNA was blotted on to nitrocellulose with the use of the BRL Hybridot apparatus. The filters were dried and baked *in vacuo* at 80 °C for 2 h. With each probe and for each experiment, serial dilutions of standard, pcBE308, were also made to a final volume of 120 μ l in 20 $\times$ SSC and were blotted as described above. The DNA was denatured by laying the filters on to 3MM filter paper soaked with 1.5 M-NaCl/0.5 M-NaOH, then neutralized, as above, with 1.5 M-NaCl/0.5 M-Tris, pH 8.0, dried and baked *in vacuo* at 80 °C for 2 h [11]. To enhance the binding of RNA to the membranes, simple capillary suction was used by placing five sheets of 3MM filter paper into the dot-blot apparatus under the nitrocellulose membrane.

Pre-hybridization and hybridization. Hybridization temperatures for oligo A and oligo B were calculated as described by Zeff & Geliebter [12] and for *Pst*I fragment as described by Maniatis *et al.* [11] and were then experimentally confirmed by hybridization of each probe with pcBE308 at various temperatures. The filters were pre-hybridized for 16 h at 50 °C (oligo A), 55 °C (oligo B) and 65 °C (*Pst*:319 and M13:319) in their appropriate hybridization solution. The hybridization solution for oligo A and oligo B was 5 $\times$ SSC/0.1% SDS/sodium heparin (50 μ g/ml) and for M13:319 and *Pst*:319 it was bovine serum albumin (0.5 mg/ml)/sodium heparin (50 μ g/ml) / 5 $\times$ SSC / 2.5% poly(ethylene glycol) 8000/0.1% SDS. After pre-hybridization the solution was removed, fresh hybridization solution containing the desired radiolabelled probe was added and the filters were hybridized overnight. After hybridization the filters were rinsed twice in 2 $\times$ SSC/0.1% SDS with moderate shaking for 5 min at room temperature. The filters were then washed at the hybridization temperatures, first in 2 $\times$ SSC/0.1% SDS for 90 min with four changes of the wash buffer and then in 0.2 $\times$ SSC/0.1% SDS for 90 min with three changes of the buffer.

The filters were dried to dampness, sealed in plastic wrap and were exposed to Kodak X-Omat AR X-ray film for 16 h at room temperature or with the use of a Corning intensifying screen at -80 °C.

A Bio-Rad 620 Video Densitometer was used to estimate the intensity of the hybridization product signals on the autoradiographs. The area under each standard and RNA peak was integrated by a Hewlett-Packard HP 3392A integrator. With each probe and for each experiment a standard curve was generated with the integrated areas of the standard peaks (serially diluted blots of pcBE308) with the use of the Cricket Graph (for Macintosh SE) linear-regression program (the coefficient of correlation, *r*, in each case was 0.98 or greater). The relative amounts of mRNA for elastin present in each dot were calculated with the use of this standard curve and expressed as fmol of mRNA for elastin per 1.0 μ g of total RNA.

After autoradiography, each dot on the nitrocellulose membranes was cut into equal-sized squares, placed into a scintillation vial and the radioactivity of each dot was measured in the Searle Liquid Scintillation System 6892 counter. The radioactivity of the dots of the serially diluted pcBE308 was used to generate the standard curve and this curve was used to determine the amounts of mRNA for elastin in the various RNA preparations. Both of these techniques were used on the dot-blot hybridizations to verify the reported values.

Northern-blot analysis. The total RNA preparations from nuchal ligament of an 8-month-old-foetus and aorta of a 9-month-old foetus were denatured with the use of the dimethyl sulphoxide/glyoxal method [13]. Briefly, 10 μ g of RNA were incubated in 10 mM-sodium phosphate buffer (pH 7.0)/50% dimethyl sulphoxide/1.0 M deionized glyoxal at 50 °C for 60 min. The glyoxal was deionized with the use of Bio-Rad AG 501-X8(D) mixed-bed resin (analytical grade) until the pH was

Fig. 2. Autoradiograph of dot-blot hybridization analysis of mRNAs for elastin from nuchal ligament and aorta at different stages of bovine fetal development

(a) Amounts blotted for nuchal ligament: 9 months, 2.0 μ g; 8.5 and 8 months, 4.0 μ g; 7.5 and 7 months, 10 μ g; 6.5 months, 15 μ g; 6 months, 20 μ g; 5.5 months, 30 μ g; 5 and 4.5 months, 15 μ g; 4 and 3 months, 20 μ g. (b) Amounts blotted for aorta: 9–5.5 months, 10 μ g; 5–4 months, 20 μ g; 3–2 months, 10 μ g. The RNA dot-blot were hybridized with the radiolabelled probes as indicated. Exposure times were adjusted to get the best relative signals [oligo A (18 h) and oligo B (14 h), overnight with intensifying screen; nick-translated *Pst*:319 (14 h), overnight without screen].

between 6.9 and 7.1 before use. The RNA was cooled on ice, mixed with agarose-gel load buffer to a final concentration of 10% glycerol, 10 mM-sodium phosphate and 0.08% Bromophenol Blue and electrophoresed at 70 V on a 1.0%-agarose gel containing 10 mM-sodium phosphate, pH 7.0, for 3 h at room temperature. The running buffer was circulated between the two electrophoresis-buffer wells. The lanes containing the wheat-germ rRNA size markers [14] were removed, stained with Acridine Orange and destained as described by McMaster & Carmichael [15].

The specificity of each probe for the mRNAs of elastin a, b and c was demonstrated by one of three ways. (i) Capillary transfer of RNA: the RNA-containing gel was soaked in 0.25 M-NaOH/1.5 M-NaCl for 30 min, then neutralized in 0.5 M-Tris/HCl buffer, pH 7.0, for 30 min [16] and transferred to nitrocellulose of GeneScreen Plus overnight as described by Maniatis [11]. The filters were air-dried and baked under vacuum at 80 °C for 2 h. (ii) Electroblotting of RNA: electrophoretic transfer of RNA to nylon membrane was performed in 25 mM-sodium phosphate buffer, pH 7.0, according to the manufacturer's (Bio-Rad) instructions. The apparatus was pre-chilled to 4 °C and the transfer was carried out at 4 °C with 0.22 A for 2 h and then with 0.45 A for 4 h. The filters were pre-hybridized and washed as described for the dot-blot analysis. (iii) Hybridization *in situ*: Hybridization was carried out essentially as described in [17]. After hybridization, the membranes and gels were covered in plastic wrap and exposed to Kodak X-Omat AR film for 3–5 days at –80 °C with the use of a Corning intensifying screen.

RESULTS AND DISCUSSION

The specificity of the three probes {oligo A, 5'-GTTTGTAGGGCAGTCCATAGCCAG-3', complementary to a sequence unique to the mRNA for elastin a; oligo b, 5'-TCCCCGTTGGATACCCAGCCTTGCC-3', complementary to a sequence present in mRNAs for elastin a and b; the *Pst*I fragment of pcBE319 (Pst:319), complementary to the sequence common to all three forms of mRNA for elastin (a, b and c) [3]} was examined by dot-blot hybridization with clones pcBE308, pcBE320 and pcBE319 and also by Northern-blot analysis of total RNA isolated from nuchal ligament and aorta. The results are shown in Fig. 1. As Fig. 1(a) shows, oligo A hybridizes with clone pcBE308 only; oligo B hybridized with pcBE308 and pcBE320, but did not hybridize with pcBE319. *Pst*:319 hybridized with all three clones, but not with pBR322. As Fig. 1(b) shows, only one radioactive band was observed in the RNA preparations from both tissues (with the mobility corresponding to the mobility of elastin mRNA [3]) with each of the three probes. Thus it is safe to assume that the probes are specific for the complementary sequences present in the mRNAs for elastin a, b and c. The results in Fig. 1(b) also show that the mRNAs for elastin were essentially intact.

Autoradiographs of dot-blot hybridization analysis of RNA preparations of nuchal ligament at various stages of foetal-calf development are shown in Fig. 2(a) and those of aorta in Fig. 2(b). The line labelled 'oligo A' measures the relative amounts of mRNA for elastin a, the 'oligo B' line measures the relative amounts of mRNA for elastin a and b, and the line 'Pst:319' measures the amounts of mRNA for all three forms of elastin at

Fig. 3. Relative amounts of mRNAs for elastin in foetal-calf nuchal ligament at various stages of development

(a) Total amount of mRNAs for elastin (a + b + c) / μg of total RNA. (b) Relative amounts of mRNAs for elastin a (○), b (◇) and c (●). The values were obtained by densitometric scanning. Each age point in the curves is from a single foetus.

the indicated foetal ages. In order to obtain reliable quantitative results, the amount of total RNA blotted varied with age (see the Figure legend). The quantitative determination of mRNAs for elastin was carried out by densitometer scanning of the autoradiographs of the hybridized dot-blot and also by measuring the radioactivity of the dot-blot. Each RNA sample was analysed with each probe a minimum of three times. The results are shown in Figs. 3 and 4.

As shown in Fig. 3(a), the relative amounts of total mRNAs for elastin (a + b + c) in nuchal ligament was low until the foetal age of about 6.5 months and began to rise rapidly thereafter. This pattern is similar to that observed for the appearance of insoluble elastin in foetal-calf nuchal ligament [1]. Thus our data confirm an earlier report that elastin synthesis and deposition in nuchal ligament parallel the amounts of mRNA for elastin [18].

Fig. 4. Relative amounts of mRNAs for elastin in foetal-calf aorta at various stages of development

(a) Total amount of mRNAs for elastin (a+b+c)/μg of total RNA. (b) Relative amounts of mRNAs for elastin a (○), b (◇) and c (●). The values were obtained by densitometric scanning. Each age point in the curves is from a single foetus.

The relative amounts of elastin a, b and c at various developmental stages of bovine nuchal ligament are shown in Fig. 3(b). At earlier stages of foetal development the relative amounts of mRNAs for elastin a and b are very low. After the foetal age of about 6 months the relative amount of mRNA for elastin b begins to increase. This is followed by the increase in the relative amount of mRNA for elastin a (after foetal age of about 7 months). The insertion sequence present in elastin a (missing from elastin c) has one very interesting feature: it is relatively rich in tyrosine residues [3]. Four of the total of seven tyrosine residues, encoded by the mRNA for elastin a, occur in this insertion sequence. It is tempting to speculate that this region may promote intermolecular aggregation and thus may play a role in elastogenesis. At early stages of development, elastin molecules appear around microfibrillar components [19] and thus need to avoid self-

aggregation. Elastin c will fulfil this requirement. At later stages of foetal development the elastin fibres in the nuchal ligament increase in diameter by depositing elastin on the existing fibres [19]. This requires self-aggregation, and elastin a may be involved in this process. The data shown in Fig. 3(b) are consistent with this hypothesis.

As shown in Fig. 4(a), there was a noticeable increase in the relative amounts of the total mRNAs for elastin a, b and c in aorta after the foetal age of about 5 months. After this increase only small differences in the amounts of total mRNAs for elastin were observed. Fig. 4(b) shows the relative amounts of mRNAs for elastin a, b and c at various stages of foetal development. At about 3 months the relative amounts of mRNA for elastin c is higher than those of the mRNA for elastin a and b. With increasing foetal age the relative amounts of mRNAs for elastin b and c increase and decrease alternately. The relative amounts of mRNA for elastin a remains low, with only marginal increases with foetal age. It may be mentioned that the nuchal ligament is a relatively simple structure with its structural elements aligned with the longitudinal axis of the tissue and its elastin component constitutes about 80% of the total dry weight of tissue [20]. In contrast, the structure of aorta is more complex. In this tissue, elastin is arranged in cylindrical layers in the form of fenestrated sheets and fibres [21] and constitutes about 40% of its dry weight [22]. The degree of self-aggregation required for the formation of these structures would be significantly lower than that for nuchal ligament. This may explain the lower amounts of mRNA for elastin a observed in aorta. In any case, the data clearly suggest that the expression of the mRNAs for elastin a, b and c is developmentally regulated and that this regulation is tissue-specific.

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