Accumulation and Regulation of Elastin in the Rat Uterus (42965)

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Abstract. **The relative levels of elastin-specific mRNA were used as a measure of tropoelastin expression in uteri from pregnant Sprague-Dawley rats. The levels of elastin-specific mRNA were also correlated with values for net tropoelastin production and net deposition of mature, crosslinked elastin. The total content of uterine elastin increased throughout gestation, reaching maximal levels at Day 19 of gestation, which were three times those of nongravid tissue. Following involution, the elastin content decreased rapidly to near baseline values by 5 days postpartum. The content of soluble elastin, estimated using an enzyme-linked immunosorbent assay, paralleled in part the increase in elastin deposition and elastin mRNA levels. Uterine elastin metabolism appears to be unlike that in other elastic tissues, e.g., lung and large blood vessels. In most elastin containing tissues, the protein is synthesized during discrete developmental periods and is not readily degraded. However, uterine elastin is continuously expressed, and appears to be in a continual cycle of degradation and replacement.**

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The elastic characteristics of tissues, such as lung, blood vessels, skin, and elastic ligaments are dependent upon the deposition and configuration of elastin-containing fibers (1–3). Although the elastin gene has been cloned and sequenced (4–7), little is known regarding factors that are important to its overall regulation.

In lung, blood vessels, and elastic ligaments, elastin deposition occurs in discrete periods during perinatal development (8–16). Furthermore, once elastin deposition occurs, elastin turnover is not easily measured (17, 18). However, a possible exception is uterine elastin. Unlike other tissues, uterine elastin fibers appear to undergo continuous cycles of deposition and resorption (19–23). Although the content of elastin in the uterus increases severalfold during the course of pregnancy, the elastin content returns to near nongravid values at parturition (19–23). Elastin appears to play a role in controlling the expansion of the uterus and facilitates its patency and contraction during resorptive involution (19–23). Consequently, we chose this unique tissue as a system for the study of elastin metabolism and to assess the suitability of the uterus as a potential source of soluble elastin (tropoelastin) from adult rodents.

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Materials and Methods

All chemicals were of the highest grades chemically available and were purchased from: Sigma Chemical Co. (St. Louis, MO), Bethesda Research Laboratory (Bethesda, MD), Bio-Rad Co. (Richmond, CA), or Pierce Chemical Company (Cincinnati, OH). Nylon and nitrocellulose membranes used in Northern, Western transfers, and hybridization assays were purchased from Schleicher and Schuell (Burlington, VT). Radiolabeled ^{32}P-dCTP was purchased from New England Nuclear Co. (Boston, MA).

Forty female Sprague-Dawley rats (300 ± 23 g of body wt) were used as the source of tissue. They were bred at 9–12 weeks of age. Mating was confirmed by the presence of a vaginal plug, which was designated as Day 1 of pregnancy. The rats were killed at 0, 7, 10, 13, 15, 17, 18, 19, 20, or 21 days after conception and at 1 or 5 days after parturition. Uterine horns were removed, cleansed of adhering tissue and vasculature, weighed, and immediately frozen and stored in liquid nitrogen. Each uterine horn was considered a separate sample.

For extraction of soluble elastin and nucleic acids, samples were pulverized in liquid nitrogen using a mortar and pestle. About 0.5 g of the powdered uteri

were transferred into 5 ml of quanidine thiocyanate reagent (GTC): 5 *M* guanidine thiocyanate, 0.5% *N*-laurylsarcosine, 0.7% 2-mercaptoethanol, 10% Antifoam A, 0.05 *M* EDTA, 0.1 *M* Tris-HCl (pH 7.5). The suspended tissue was homogenized rapidly using a Tissuemizer (Tekmar Co.) at medium speed for 90 sec and extracted for 5 to 7 hr at 4°C.

The homogenates were centrifuged at 15,000*g* at 20°C (20 min), and the pellets saved for insoluble elastin purification. An aliquot (500 μl) of supernatant fraction was removed for DNA determination. The rest of the fraction was layered over 1.2 ml of CsCl-EDTA solution (5.7 *M* CsCl, 0.1 *M* EDTA, pH 7.0). The RNA was pelleted by centrifugation at 130,000*g*, 20°C for 12 hr (24, 25). The supernatant fraction was used for tropoelastin assays.

DNA was precipitated from the GTC extract with sodium acetate and ethanol. Interfering proteins were removed by digestion with pronase-sodium dodecyl sulfate (0.1 mg of pronase, 0.2% (w/v) sodium dodecyl sulfate, 0.6 ml of 0.5 *M* Tris-HCl, pH 7.2). To each 100-μl aliquot of extract, 0.1 volume of 3 *M* NaAc (pH 5.0) and 2 volumes of 95% ethanol were added and the mixture was allowed to precipitate overnight at −20°C. DNA in the precipitate was estimated fluorimetrically as described by Hinegardner (26).

Isolation of total RNA essentially followed the procedure of Chirgwin *et al.* (25). Pelleted RNA was resuspended in guanidine-HCl and precipitated overnight by the addition of sodium acetate and ethanol. The precipitate was recovered by centrifugation, washed with ethanol, and lyophilized to dryness.

Poly(A) mRNA was selected by oligodeoxythymidine cellulose chromatography (24). RNA from the polyadenylated fraction was then applied in serial dilution (starting concentration, 5 μg/well) onto a Nytran membrane which had been presoaked in a 20 × SSC (NaCl, 3 *M*; Na_3 citrate, 0.3 *M*, pH 7.0) solution. For purposes of characterization, other aliquots of the mRNA were electrophoresed in agarose gels (1%) under denaturing conditions (24, 25). This RNA was transferred to nitrocellulose in preparation for hybridization with an elastin-specific DNA probe (27). The DNA probe, which was used, was a 1.3-kb elastin gene fragment cloned into pUC9, which has been used in a number of studies dealing with mammalian elastin gene expression (9–11, 27–31). To prepare the DNA probe for the Northern and dot blot hybridization assays, conventional methods were used (24, 32, 33). The plasmid was cleaved with the restriction enzymes, *Bam*HI and *Eco*RI, and restriction fragments were separated by agarose electrophoresis (24).

Approximately 3 ng of DNA probe (>10^8 cpm/μg) were used for each of the hybridization assays (42°C for 24 hr, 5-ml total volume). This was followed by several brief washes at 25°C and two 30-min stringent washes at 60°C (cf. 27–31). Following autoradiography, the amount of radioactivity associated with each spot on the dot blot membrane was then quantitated by scanning densitometry.

The soluble elastin in the GTC-CsCl supernatant fraction was also estimated using a competitive enzyme-linked immunosorption assay system as previously described (34). Polyclonal antibodies against rat-insoluble elastin were prepared in rabbits. Tropoelastin from neonatal rat aorta was used to assess the specificity of the antibodies by Western blotting (34–37). For the enzyme-linked immunosorption assays and Western blots of guanidinium thiocyanate-extracted tissue, the guanidinium thiocyanate was first exchanged for 4 *M* urea by passage through a PD-10 column (Pharmacia Co.) preequilibrated with 4 *M* urea. The eluants were then electrophoresed in sodium dodecyl sulfate-polyacrylamide gels (36) and the proteins blotted onto nitrocellulose (35). The nitrocellulose was blocked with bovine serum albumin and then exposed to rabbit anti-rat elastin serum in preparation for immunostaining with goat anti-rabbit immunoglobin coupled to horseradish peroxidase (34, 36).

Insoluble elastin associated with the GTC residue was estimated after extraction with ethanol and hot alkali (45 min, 0.1 *N* NaOH at 90°C; ref. 16). Amino acid analysis was performed on the hydrolysates to confirm purity (16). Portions of the hydrolysates were also reacted with Ninhydrin and the amounts of elastin were estimated colorimetry using a hydrolysate of bovine ligamentum elastin as a standard (16).

Statistical tests and estimation of correlation coefficients were taken from Downie and Heath (38).

Results

Figure 1 depicts the change in uterine weight and DNA content during pregnancy. There was an excellent correlation ($r = 0.96$) between tissue weight and DNA content from Day 10 to Day 19 (2 days before parturition). Relative changes in uterine-soluble and insoluble elastin are shown in Figures 2 and 3. Absolute amounts of both soluble and insoluble elastin increased from Day 7 and peaked at Days 19 and 20. This was followed by a decrease to nongravid values by Day 5 of parturition. The increases in insoluble elastin and uterine weight were positively correlated ($r = 0.90$). Therefore, the concentration of uterine elastin remained relatively constant throughout pregnancy (Fig. 3). However, this was not the case for the soluble elastin concentration (Fig. 3). Soluble elastin, defined as tropoelastin plus elastin-derived peptides (Fig. 4, cf. ref. 24), increased sharply around Day 20 and then decreased at parturition.

To determine whether or not the net increase in soluble elastin was due to an increase in tropoelastin synthesis, the relative change in elastin-specific mRNA

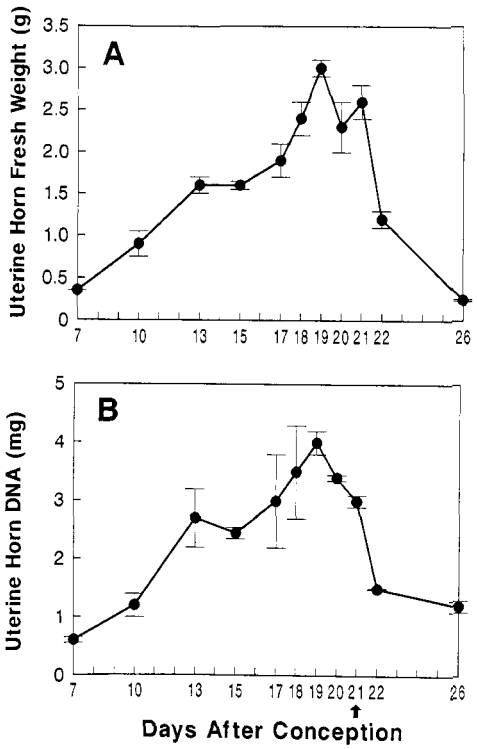

Figure 1. Changes in weight (A) and DNA content (B) of the rat uterus during pregnancy and early involution. Each point in this and all subsequent figures represents a minimum of three determinations. The bars denote the standard deviation. The arrow indicates parturition. Only horns containing more than eight pups were used in assays.

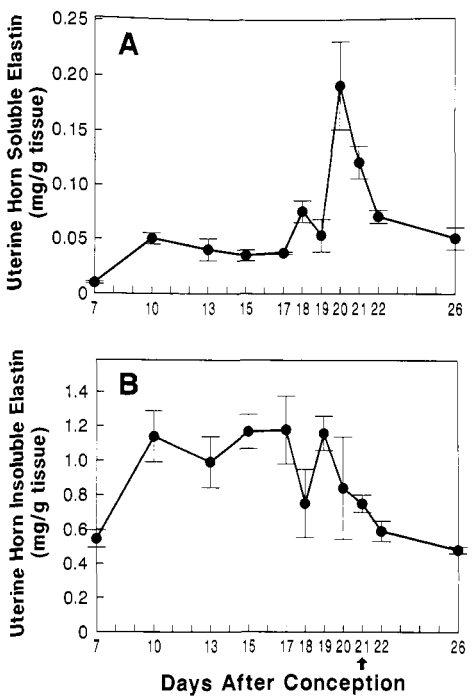

Figure 2. Changes in the soluble (A) and insoluble (B) elastin content of the rat uterus during pregnancy and early involution. Data are means of a minimum of three determinations ± SD, and are expressed on a fresh tissue basis. The arrow indicates parturition.

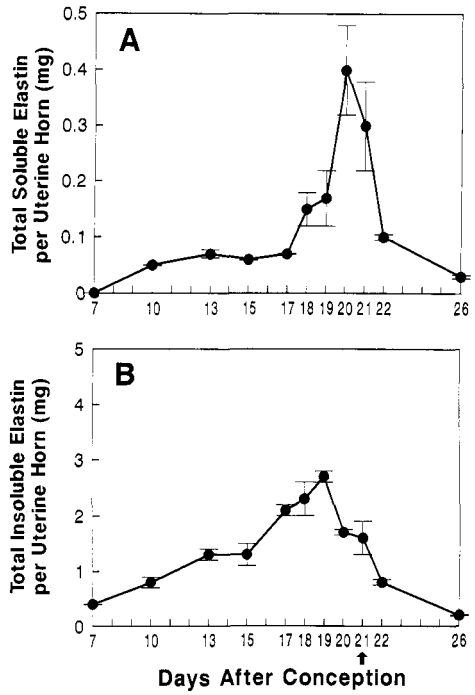

Figure 3. Net content of soluble (A) and (B) insoluble elastin in a rat uterine horn. Data are means of a minimum of three determinations ± SD. The arrow indicates parturition.

was estimated. Data for the relative change in elastin specific mRNA levels indicated that the increase in elastin-mRNA production paralleled both tropoelastin and elastin production (Fig. 5).

The specificity of the assay was based on subjecting the mRNA preparations to denaturing formaldehyde-agarose gel electrophoresis. When transferred to nitrocellulose and incubated with the recombinant probe labeled with ^{32}P, a band corresponding to about 3.7 kb was observed. This is in keeping with observations by Deak *et al.* (4) and Rich and Foster (39) for rodent elastin mRNA. However, the observation differs somewhat from that of Raghow *et al.* (31), who estimated the size of tropoelastin mRNA from hamster lung to be nearer to 4.1 kb.

Discussion

The uterus provides an unusual model for studies of elastin synthesis and degradation. Most elastin-enriched tissue express elastin during brief well-defined periods. However, our data along with those of Starcher and Percival (22, 23), Leppert *et al.* (20, 21), and Gunja-Smith and associates (40, 41) suggest that elastin metabolism in the uterus is dynamic, i.e., both elastin synthesis and degradation continues throughout tissue expansion and development. This is not the case for elastin expression in blood vessels, lung, or ligaments wherein elastin synthesis and degradation is difficult to measure in adult animals. For example, negligible quantities of elastin-specific mRNA are detected in

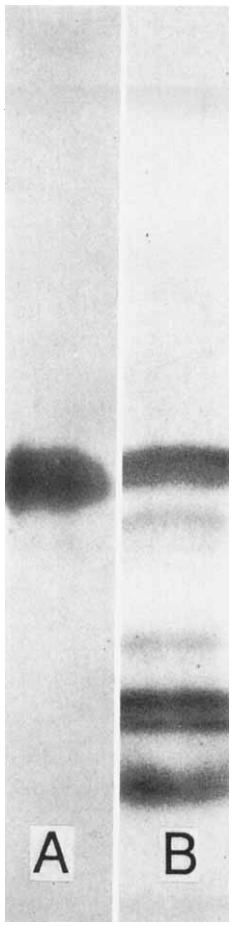

Figure 4. Western blots of protein extracted from rat aorta (Lane A) or uterus (Lane B). Aortas from 10-day-old rat pups or a uterus at Day 19 of pregnancy were used. Similar Western blots were obtained at each of the time periods given in Figures 2 and 3. For the period of Days 7 to 12 of pregnancy and at 5-day postconception, relatively less lower molecular weight material was observed than for the period of Days 15 to 21 of pregnancy. The arrow indicates parturition.

adult chick aorta (16), adult rat aorta (4), or lung and ligament from mature animals (8–18).

Uterine elastin deposition in the gravid tissue closely parallels the increase in uterine weight, except in late pregnancy. At 48–72 hr before parturition, the elastin content increases rapidly. At this time there is also an increase in the level of elastin-specific mRNA. The parallel increases in elastin production and elastin mRNA levels suggests that the elastin content in the rat uterus is primarily under transcriptional control. Since the concentration of elastin remained relatively constant upon expansion of uterine tissue and elastin mRNA levels were measurable throughout pregnancy, it may be inferred that elastin synthesis in the uterus is a continual process.

Evidence of elastin degradation was also observed. Starcher and Percival (22) reported previously that uterine elastin appears to undergo rapid degradation prior to parturition. Furthermore, Gunja-Smith and Woessner (40) and Guinja-Smith *et al.* (41) report that the number of intermolecular crosslinking amino acids in uterine elastin is less than for other tissue elastins. In the uterus, the formation of intermolecular crosslinks does not appear to keep pace with elastin synthesis during pregnancy. Uterine elastin contains 2.4 residues of desmosines per 1000 total residues in the early stages of gestation, but this value decreases to 0.95 residues per 1000 residues at term (40, 41). The desmosine isomers are derived from condensation of other lysine-derived crosslinks, e.g., dehydrolysinonorleucine and aldol condensation products (1–3). The condensation step(s) take place extracellularly in a time-dependent manner. For example, the results from pulse-chase experiments, in which [^{14}C]lysine is used to label the desmosines, suggest several days may be required before the full equilibration and condensation of peptidyl lysine to desmosine occurs (cf. refs 2 and 16 for review). This may have important ramification to elastin degradation in the uterus. The relative decrease in elastin crosslink content may facilitate remodeling of the "postgravid" elastin fibers. Partially crosslinked elastin is very susceptible to degradation by proteinases that are normally not elastolytic in nature (34). As shown in Figure 4, the elastin-derived peptides of different sizes were extracted from the uterus toward the end of pregnancy. Such an observation is predicted based on (i) the evidence for decreased elastin crosslinking in late pregnancy (40, 41), (ii) our previous observations that partially crosslinked elastin is a substrate for a broad spectrum of proteinases (34), and (iii) the actual in-

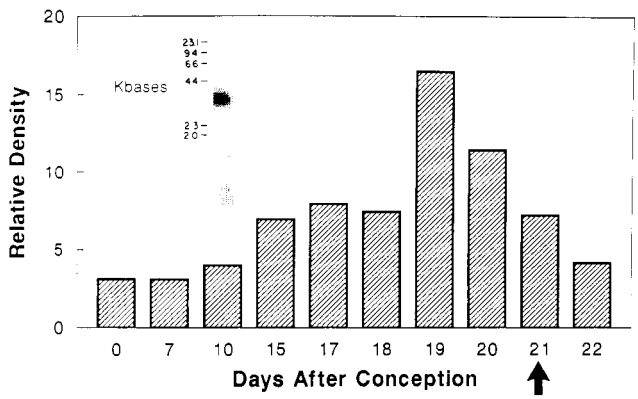

Figure 5. Relative quantities of rat uterus tropoelastin mRNA present throughout pregnancy. The figure insert is a Northern transfer. The ^{32}P-cDNA probe used in the assay is described in (26). The hybridizations were performed at 42°C for 24 hr. The hybridization solution consisting of 5 × SSC, 5 × Denhardt's solution, 0.1 *M* sodium phosphate (pH 7.6), 250 μg/ml herring sperm DNA, and 50% deionized formamide. Final stringency washes were 0.5 × SSC, 0.1% sodium dodecyl sulfate at 60°C (see text for other details). Hybridization of the cloned elastin gene to elastin mRNA was visualized by autoradiography on Kodak X-AR film in cassettes containing intensifying screens at −70°C. Dot hybridization on nitrocellulose was used to quantitate elastin mRNA levels. Serial dilutions of RNA (starting concentration, 5 μg/well) was applied to nitrocellulose previously treated with 20 × SSC solution. Other hybridization conditions and probe preparation are described in the text. Each point is the average of at least two determinations. The arrow indicates parturition.

crease in uterine elastolytic activity associated with late term (22, 23).

Regarding other aspects of the work, the DNA probe that was used to measure elastin mRNA levels has been shown to also hybridize with a 4.8-kb form of mRNA that apparently shares homology with the 3′-untranslated region of elastin-specific mRNA. Deak *et al.* (4) have observed that a 4.8-kb mRNA hybridizes with elastin DNA probes derived from reverse transcription of the 3′-untranslated sequences of human elastin mRNA. This form of mRNA persists into adulthood, whereas to date only 3.5- to 4.1-kb elastin-specific mRNAs have been observed in neonatal tissue (cf. 4, 29, 30, 39). Of interest, we did not detect a 4.8-kb mRNA species in uterus. The size of the mRNA identified by us was near 3.7 kb using as a probe DNA isolated from a sheep genomic library (27–29).

As a final point, the uterus appears to be a practical and good source of rodent-soluble elastin. At Day 21, 140 to 240 μg of soluble elastin could be extracted per gram of uterus. In contrast, the aortas from 40 to 50 neonatal rat pups would be required for a comparable yield of soluble elastin. Indeed, the uterus may be able to serve as an unusual model for studies of elastin synthesis and degradation. Variations in the physiologic state of the uterus during pregnancy offers the opportunity to study *in vivo* factors that may be important to the overall regulation of elastin.

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