THE SMOOTH MUSCLE CELL

I. In Vivo Synthesis of Connective Tissue Proteins

RUSSELL ROSS and SEYMOUR J. KLEBANOFF

From the Departments of Pathology and Medicine, School of Medicine, University of Washington, Seattle, Washington 98105

ABSTRACT

These studies have examined the ability of smooth muscle cells from developing aorta of the prepubertal rat to utilize amino acids in the synthesis and secretion of connective tissue proteins. Prepubertal rats, previously given either an alcohol carrier or estradiol-17-beta, were each given an intravenous injection of proline-^{3}H. The animals were sacrificed after 15 and 30 min, and 4 hr. Light and electron microscope radioautographs of the aortic smooth muscle and of the myometrial cells demonstrated that the aortic cells, in both groups of animals, and the myometrial cells, in the estrogen-stimulated animals, took up the proline and rapidly secreted it in both collagen and elastic fibers within 4 hr. In contrast, the myometrial cells of the nonstimulated animal took up relatively small amounts of proline and retained most of the amino acid within the cells. Electron microscope radioautographs demonstrated that the organelles involved in this activity were the rough endoplasmic reticulum and Golgi complex together with peripheral elements, presumed to be small vesicles. These studies have demonstrated that the smooth muscle cells of the developing aorta and of the estrogen-stimulated myometrium have a capacity to synthesize and secrete proteins associated with the extracellular connective tissue matrix.

INTRODUCTION

Smooth muscle has for many years been postulated to be capable of the synthesis of many of the extracellular connective tissue proteins in blood vessels, and in the uterus (1, 2, 5, 10, 11, 13, 15, 17, 18, 25, 26, 27). One of the most constant findings in the development of the atherosclerotic plaque in the aorta or in the coronary arteries is the proliferation of cells resembling smooth muscle cells, associated with the formation of increased amounts of connective tissue proteins, particularly collagen (3, 4, 6, 8, 9, 18, 19, 20, 21, 26). Similarly, the smooth muscle cells of the uterus undergo marked hypertrophy under the influence of estrogens. Particularly striking is the increase in rough endoplasmic reticulum, a phenomenon generally associated with an increased synthesis of secretory protein. The smooth muscle cells in these locations are in morphological juxtaposition to components of the extracellular matrix. However, no direct evidence for the synthesis and secretion of connective tissue proteins by these cells has been presented.

The studies reported here relate to the ability of smooth muscle cells of the aorta and of the estrogen-stimulated uterus to synthesize and secrete the proteins of the connective tissue matrix in vivo. In vitro studies have also established the ability of smooth muscle to synthesize elastic fiber proteins (28).

MATERIALS AND METHODS

Animals

Prepubertal Holtzman albino (Sprague-Dawley) rats weighing 53–64 g were divided into two groups.

Group A (controls) consisted of three animals. Each rat was injected subcutaneously with 0.1 ml of 20% alcohol twice daily for three days for a total dose of 0.6 ml.

Group B (experimental group) consisted of five animals. Each animal was given 0.1 ml of estradiol-17-beta (1 μg per ml) in 20% alcohol, twice daily for three days, for a total dose of 0.6 μg.

On the fourth day, both the control and experimental animals were injected intravenously by tail vein with proline-^{3}H (New England Nuclear Corp., Boston, Mass.) L-proline,3,4-^{3}H, SA 5Ci/mmole) at a dose of 20 μCi per g body weight.

Animals from each group were sacrificed by decapitation after 15 min (1 control, 1 experimental), 30 min (1 control, 2 experimentals), and 4 hr (1 control, 2 experimentals), after proline-^{3}H administration. The uterus and the abdominal aorta were dissected from each animal. One-half of the uterus was weighed to obtain a measure of the estrogenic response, and the remainders of the uterus and the aorta were prepared for examination by light and electron microscopy.

Tissue Preparation

The horn of each uterus was cut into short cylindrical segments. These were fixed in 2% osmium tetroxide buffered with *s*-collidine at pH 7.3 for 1 hr, rinsed, and postfixed in 10% neutral buffered formalin for 30 min. The aorta was fixed in 2% paraformaldehyde–2.5% glutaraldehyde (29) in cacodylate buffer (pH, 7.3) for 3–5 hr, washed overnight in buffer containing 0.2 M sucrose, and postfixed the next day in 2% osmium tetroxide for 1 hr. After fixation, both groups of tissues were dehydrated through a graded series of alcohols and embedded in epoxy resin (Epon 812).

The tissues were sectioned at either 1 μ for light microscope radioautography or at approximately 1000 A for electron microscope radioautography. (The electron microscope used was an AEI 6B.) Tissues prepared for light microscope radioautography were coated with Eastman Kodak NTB-2 emulsion with a dipping machine (30) and exposed for 6 wk. The radioautographs were then developed in Ektaflo developer (Eastman Kodak Co., Rochester, N. Y.), fixed and stained with azure 2 methylene blue (33).

Tissues prepared for electron microscope radioautography were processed according to a modified version of the method proposed by Granboulan (34). The radioautographs were exposed for 10 months and developed in either Microdol X or D-19B. The sections were stained with lead or uranyl acetate after clearing the emulsion with 0.1 N sodium hydroxide.

OBSERVATIONS

Aorta

Light Microscope Radioautography

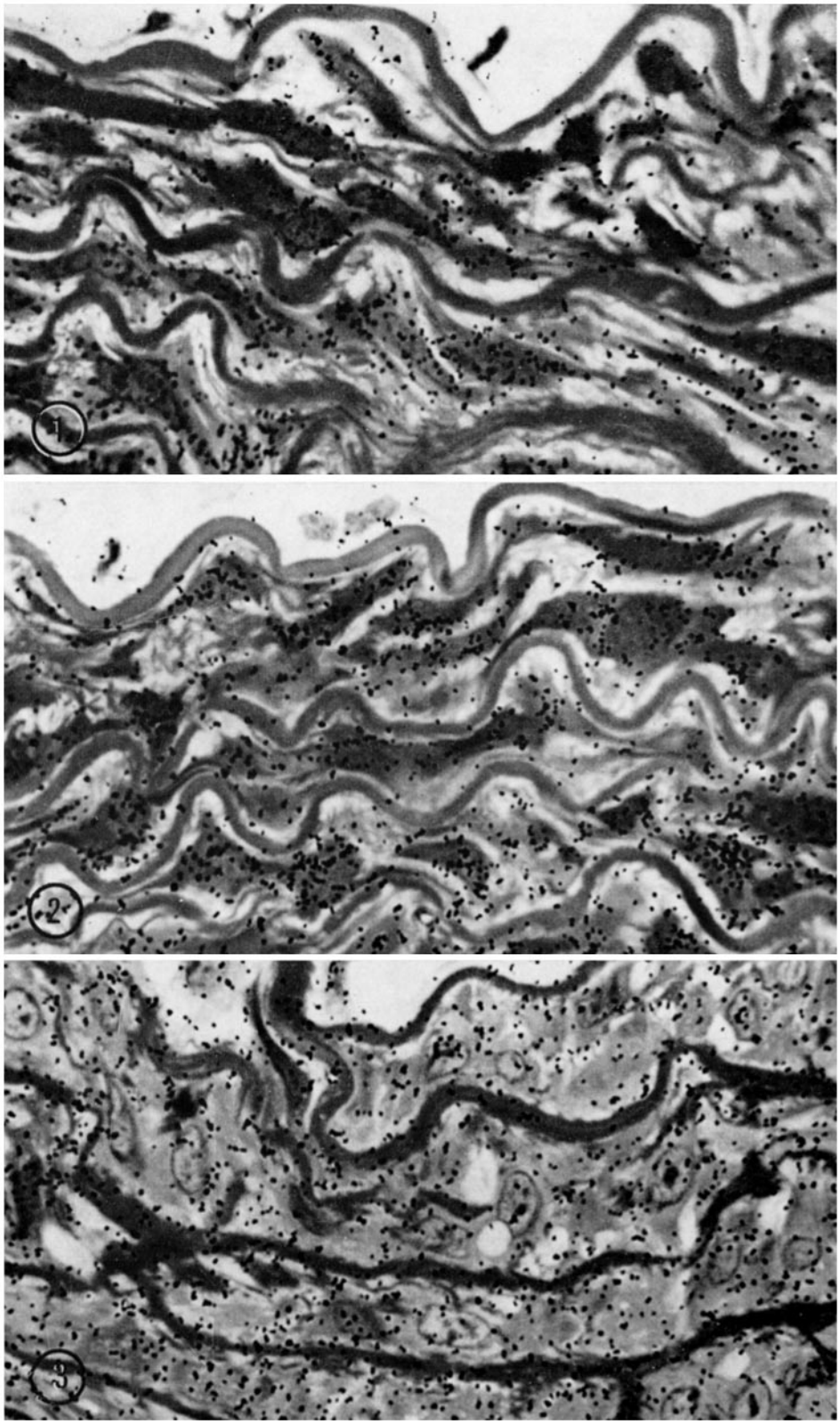
In this study, proline was localized in both medial and adventitial cells of the aorta. The media consists solely of smooth muscle, in contrast to the adventitia which consists largely of fibroblasts. The observations reported here relate only to proline-^{3}H localization in the media of the aorta.

No differences were observed in the patterns of proline-^{3}H utilization by the aortas of estrogen-treated and control animals. Within 15 min of the administration of proline-^{3}H, the smooth muscle cells of the media of the aorta were quite heavily labeled. Very little proline-^{3}H was present in the extracellular connective tissues (Fig. 1). The same was true after 30 min, at which time the smooth muscle cells were uniformly labeled. Most of the label was present within the cytoplasm, while relatively small amounts were also observed within the nucleus (Fig. 2).

FIGURE 1 This light microscope radioautograph demonstrates the incorporation of proline-^{3}H by the smooth muscle cells of the aortic media 15 min after intravenous administration of the amino acid. The majority of the proline has been incorporated into the smooth muscle cells at this time. × 1400.

FIGURE 2 This figure shows an incorporation of isotope similar to that demonstrated in Fig. 1. 30 min after proline injection, the smooth muscle cells of the aorta have retained the label within their cytoplasm. Both nuclear and cytoplasmic labeling are apparent. × 1400.

FIGURE 3 4 hr after intravenous administration of proline-^{3}H, the label in the aorta has been secreted from the smooth muscle cells to the extracellular connective tissue compartments. Silver grains are found over both elastic fiber lamellae and the intercellular spaces between the cells. × 1400.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In contrast, within 4 hr of the administration of proline-^{3}H, a marked difference was noted in the distribution of the amino acid. At this time the bulk of the labeled proline had been secreted by the cells. Silver grains were seen over the elastic fibers of the various elastic laminae of the media, and over the extracellular spaces located between the smooth muscle cells (Fig. 3).

Electron Microscope Radioautography

Only a qualitative evaluation of grain distribution was performed in these studies. 15 min after the intravenous administration of proline-^{3}H, the label was seen largely in two organelles of the smooth muscle cells: the extensively developed rough endoplasmic reticulum, so characteristic of

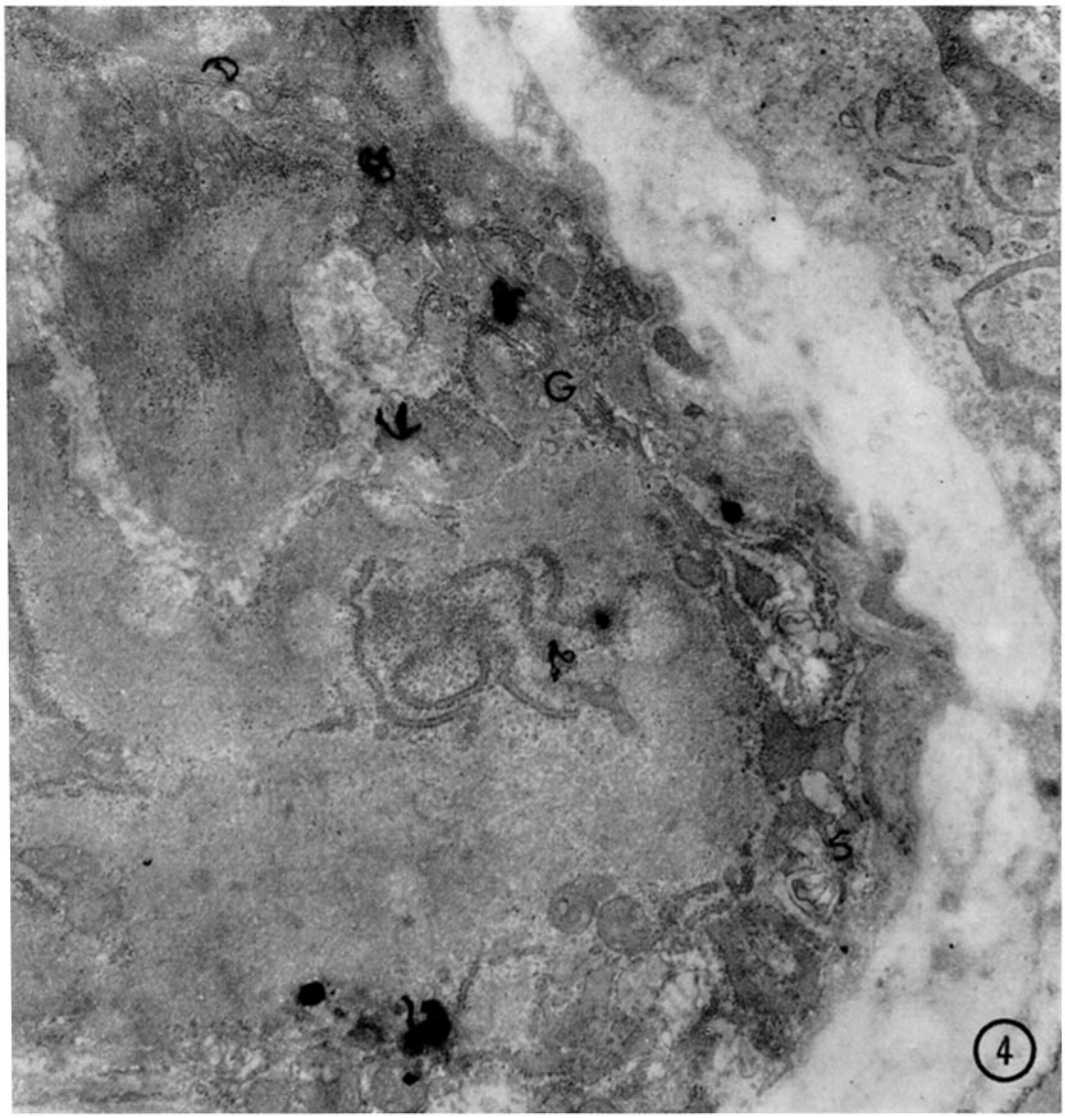


FIGURE 4 This electron microscope radioautograph demonstrates a portion of a smooth muscle cell from the media of the abdominal aorta 15 min after intravenous administration of proline-^{3}H. The silver grains are localized over elements of the rough endoplasmic reticulum and Golgi complex (*G*) at this time period. × 16,500.

these cells in the young growing animal, and the Golgi complex which is also well developed. A few of the cells had silver grains at the periphery of their cytoplasm in regions where numerous small vesicles and caveolae are located at the cell surface. Silver grains were also seen lying over myofilaments (Fig. 4) as well as over nuclei at this early time period.

A similar distribution of label was seen after 30 min; however, some silver grains were seen over extracellular elastic fibers and collagen fibrils, although the largest amount of label was still found in the rough endoplasmic reticulum and the Golgi complex of the smooth muscle cells (Fig. 5), together with peripheral vesicles and caveolae characteristic of smooth muscle. Unfortunately the resolution is such that it is not possible to assign individual silver grains to individual vesicles or caveolae.

Within 4 hr, a marked shift in the distribution

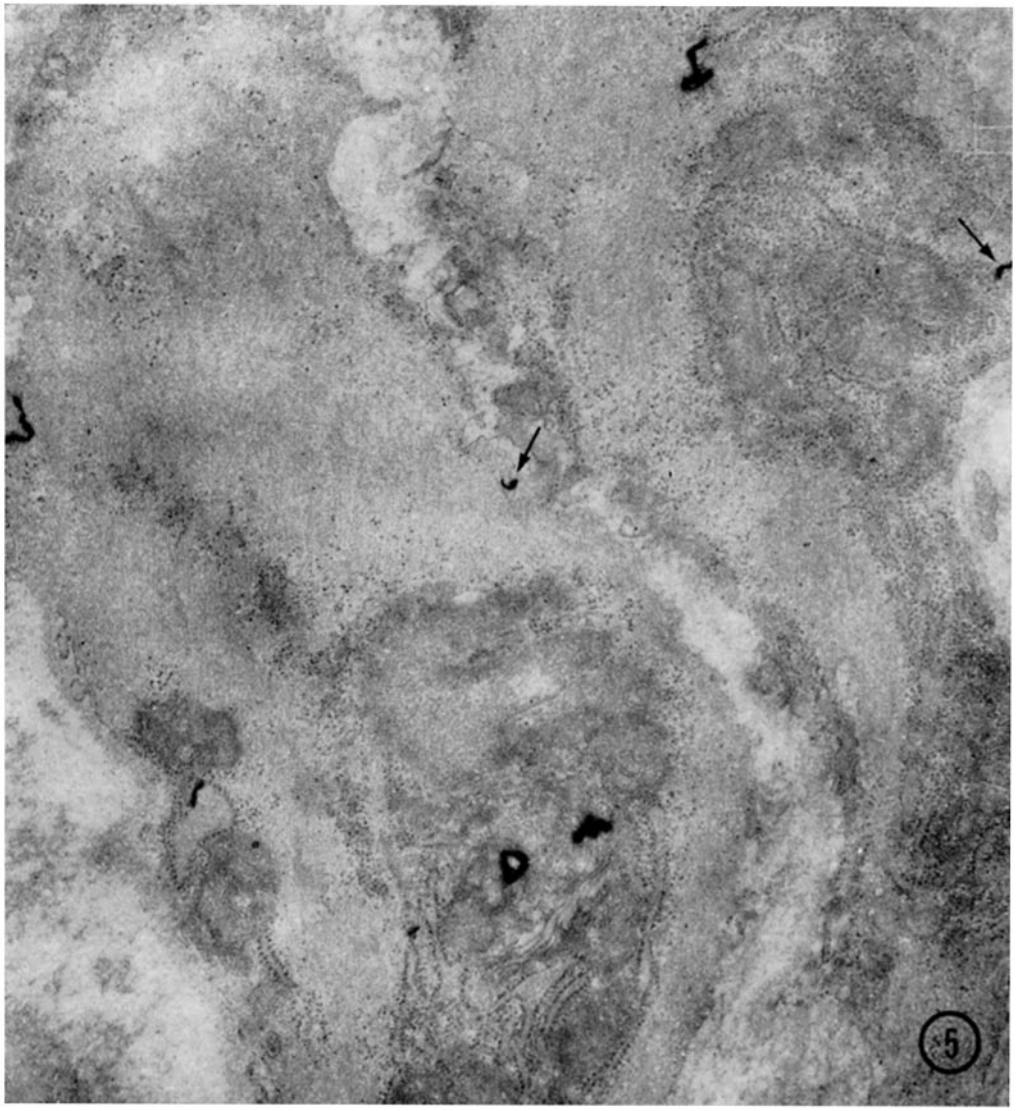


FIGURE 5 This electron microscope radioautograph demonstrates part of a smooth muscle cell 30 min after proline-^{3}H administration. Again the silver grains are located largely over cisternae of rough endoplasmic reticulum and Golgi complex, as well as the cell periphery (arrows). × 16,500.

of the proline-^{3}H had occurred. At this time the majority of the label was found either within the elastin component of the elastic fibers (35), the microfibrils of the elastic fibers grouped in aggregates, or small bundles of collagen fibrils. Relatively little label remained within the smooth muscle cells at this time. The label that did remain was found to lie either in myofilaments within the cytoplasmic matrix or, in a few cases, in the rough endoplasmic reticulum, Golgi complex, and nuclei of the cells (Fig. 6).

Uterus

Light Microscope Radioautography

CONTROL ANIMALS: In contrast to the aortic media, the uterine myometrium is a mixed population containing predominantly smooth muscle but

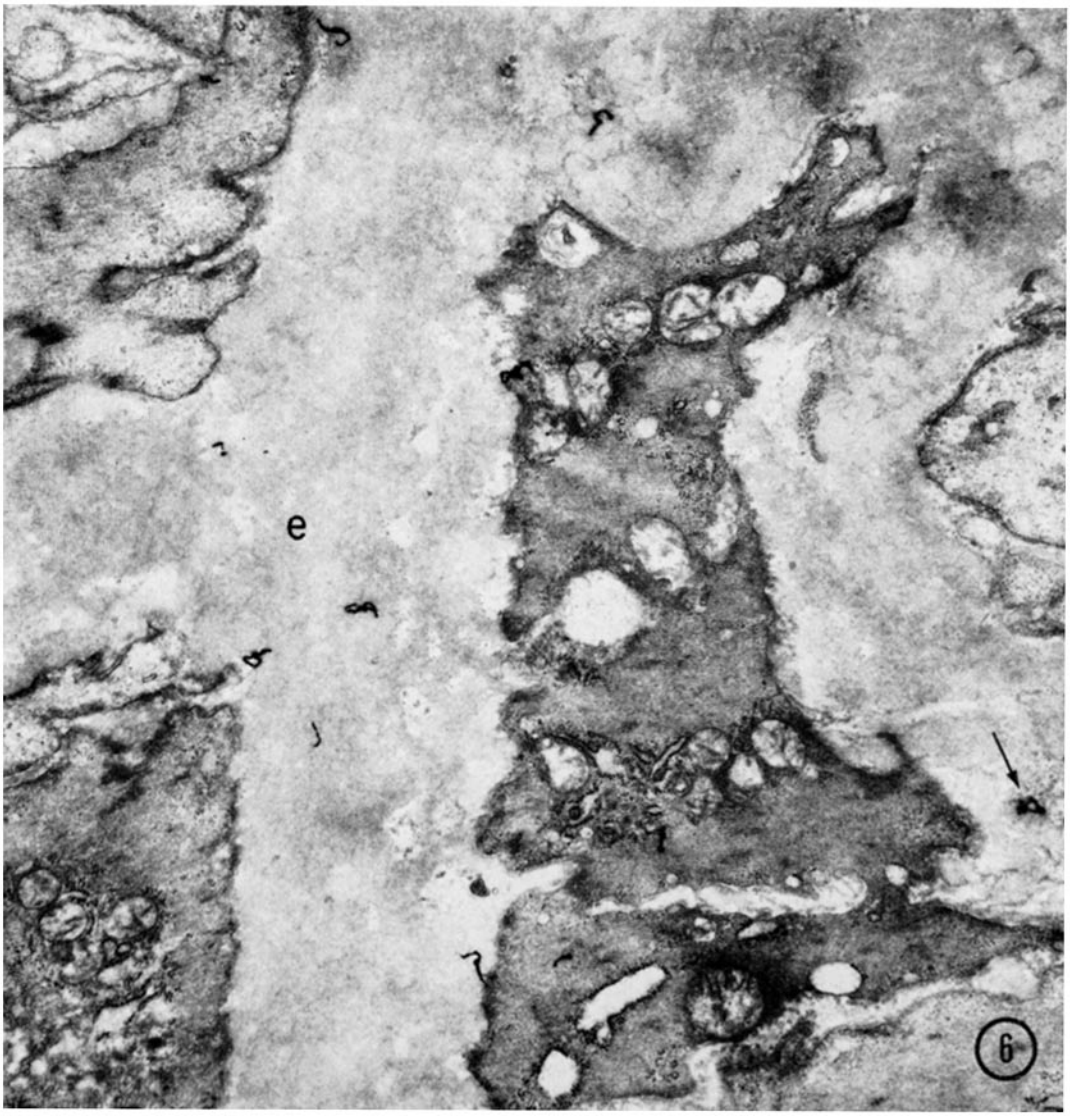


FIGURE 6 In this electron microscope radioautograph of aorta removed 4 hr after proline-^{3}H administration, the silver grains are seen to reside largely over the elastin components of the elastic fiber (*e*) as well as collagen fibrils (arrow). Some cytoplasmic labeling is still present as well. × 14,000.

also fibroblasts and macrophages. The observations reported here relate only to the localization of proline-^{3}H in the smooth muscle cell and the adjacent connective tissue that is rich in collagen and mucopolysaccharide and contains little to no elastic tissue.

Unlike the findings on the aorta, marked differences were observed in the uterus of the control animals versus those treated with estradiol. 15 min after the administration of proline, relatively small amounts of label had been incorporated into the myometrial cells. The label which was incorporated was randomly distributed in both the nucleus and cytoplasm of the smooth muscle cells (Fig. 7). At 30 min there was some increase in the amount of label in these cells, but the proline appeared to be randomly incorporated into both nucleus and cytoplasm (Fig. 8). At 4 hr the majority of the label appeared to remain within the smooth muscle cells in the same general locale as that seen at the earlier time intervals (Fig. 9).

ESTRADIOL-TREATED ANIMALS: In sharp contrast with the animals receiving the alcohol carrier, those receiving estradiol showed a greater amount of proline in many of the myometrial cells. The largest amount of proline appeared to be incorporated into cytoplasmic components, although many of the cells demonstrated extensive nuclear incorporation as well (Fig. 10). The distribution of the label shifted markedly within 4 hr, at which time a large amount of proline-^{3}H was seen in the extracellular spaces between the smooth muscle cells. Some label remained within the smooth muscle cells at this time as well (Fig. 11). This distribution of proline-^{3}H stood in sharp contrast to that seen in the uteri of animals which had received only alcohol.

Electron Microscope Radioautography

Relatively few silver grains were found overlying the smooth muscle cells of the alcohol-treated animals. This corresponded to the relatively small amount of label seen in the light microscope preparations after 15 and 30 min. The silver grains were dispersed both over nuclei and over various cytoplasmic organelles, including myofilaments, the sparse and poorly developed endoplasmic reticulum, mitochondria, etc.

In sharp contrast, the cells of the estradiol-stimulated myometrium showed a much larger amount of label in the rough endoplasmic reticulum and Golgi complex after both 15 and 30 min (Fig. 12), and a relatively large amount of label was present in the connective tissue spaces which contain both collagen and protein polysaccharides 4 hr after the administration of proline-^{3}H.

DISCUSSION

Aorta

The smooth muscle cell has for many years been implicated in the formation of connective tissue proteins (1, 2, 5, 10, 13, 15, 17, 18, 25, 26) because of the close morphologic association of these cells with the components of the connective tissue matrix.

The first ultrastructural observations suggesting that smooth muscle might form both elastic fiber proteins and, possibly, collagen were those of Karrer on the developing chick embryo aorta (12). He noted the close juxtaposition of these cells to collagen fibrils and suggested that fibroblasts might have differentiated to smooth muscle and subsequently formed collagen. In a later series of observations, Karrer and Cox (7) described microfibrils and elastin in intimate association with medial smooth muscle cells in the developing aorta. At that time they were not aware that both the microfibrils and the amorphous component were integral parts of the elastic fiber (35, 36). Karrer described the medial aortic cells as being reminiscent of smooth muscle, containing myofibrils, however, he thought that the cells did not secrete tropocollagen because the collagen fibrils did not appear to increase in diameter as did the collagen fibrils within the adventitia. Karrer also noted that the medial smooth muscle cells did not contain a well-developed rough endoplasmic reticulum and presumed that the collagen that was there had been secreted by fibroblasts before their development into smooth muscle. During this same period Pease and Paule (23) emphasized, in their examination of the thoracic aorta of the rat, that the smooth muscle cells must maintain the elastic fibers and collagen since they were the only cells present in the media of the vessel. Similar observations were presented by Paule in an extension of these studies in both newborn and adult rat aortas (22). Recently, both Cliff (32) and Stein et al. (31) have emphasized that in the immature rat the aorta contains smooth muscle rich in rough endoplasmic reticulum, and that this organelle dimin-

ishes with age and is replaced by numerous smooth membrane profiles which result in an increase in the surface area of the cells.

Most of the observations implicating smooth muscle cells in the formation of connective tissue have derived from studies of the development of the atherosclerotic plaque. In an early series of investigations, Haust et al. (37) and Haust and More (5) implied that the smooth muscle cell was responsible for fibrinogenesis in atherosclerosis. In their studies they suggested that the collagen fibers formed from elements derived from the basement membrane surrounding these cells. A number of investigations of this phenomenon have continued to imply this association as seen in rabbits fed high cholesterol diets (20), in pigeon aorta (6), rabbit aorta (8), chicken aorta (3), and in the rhesus monkey (9), this association is also implied in a continuation of the earlier studies by Haust et al. (38).

Other vascular systems studied included various forms of injury to arterioles. In a study of double stenosis of the aorta, Kunz et al. (14) examined intimal cell proliferation and the uptake of isotopically-labeled thymidine or proline by the cells. They were not certain of the origin of the cells in the proliferative lesions that they produced, although they suggested that the cells might be related to smooth muscle. In a similar series of studies, Esterly et al. (15) established systemic-to-pulmonary artery shunts to produce hypertension and associated focal luminal obliterations in the arteries. They suggested that there was invasion of the subendothelial space by medial smooth muscle cells and they thought that these cells "became differentiated." They saw no fibroblasts in the lesion and hypothesized that the increase in collagen was attributable to a compensatory function of these modified smooth muscle cells.

The presence of actomyosin-like proteins has been observed in the cells of the atherosclerotic plaque labeled with fluorescein coupled to anti-actomyosin sera by Knieriem et al. (19) who suggested that the smooth muscle cell was responsible for connective tissue formation in these plaques. These observations have subsequently been confirmed and extended by Becker (39).

Other forms of injury which have been studied include surgical incisions in the media of dog brachial and femoral arteries. Murray et al. (26) observed smooth muscle cells with an extensively-developed rough endoplasmic reticulum in these wounds. They suggested that the smooth muscle cells produced collagen and could "dedifferentiate" in the direction of fibroblasts. A similar development of the organelles associated with the synthesis of a secretory protein was described by Cooke and Smith (6) in smooth muscle cells in the atherosclerotic plaque.

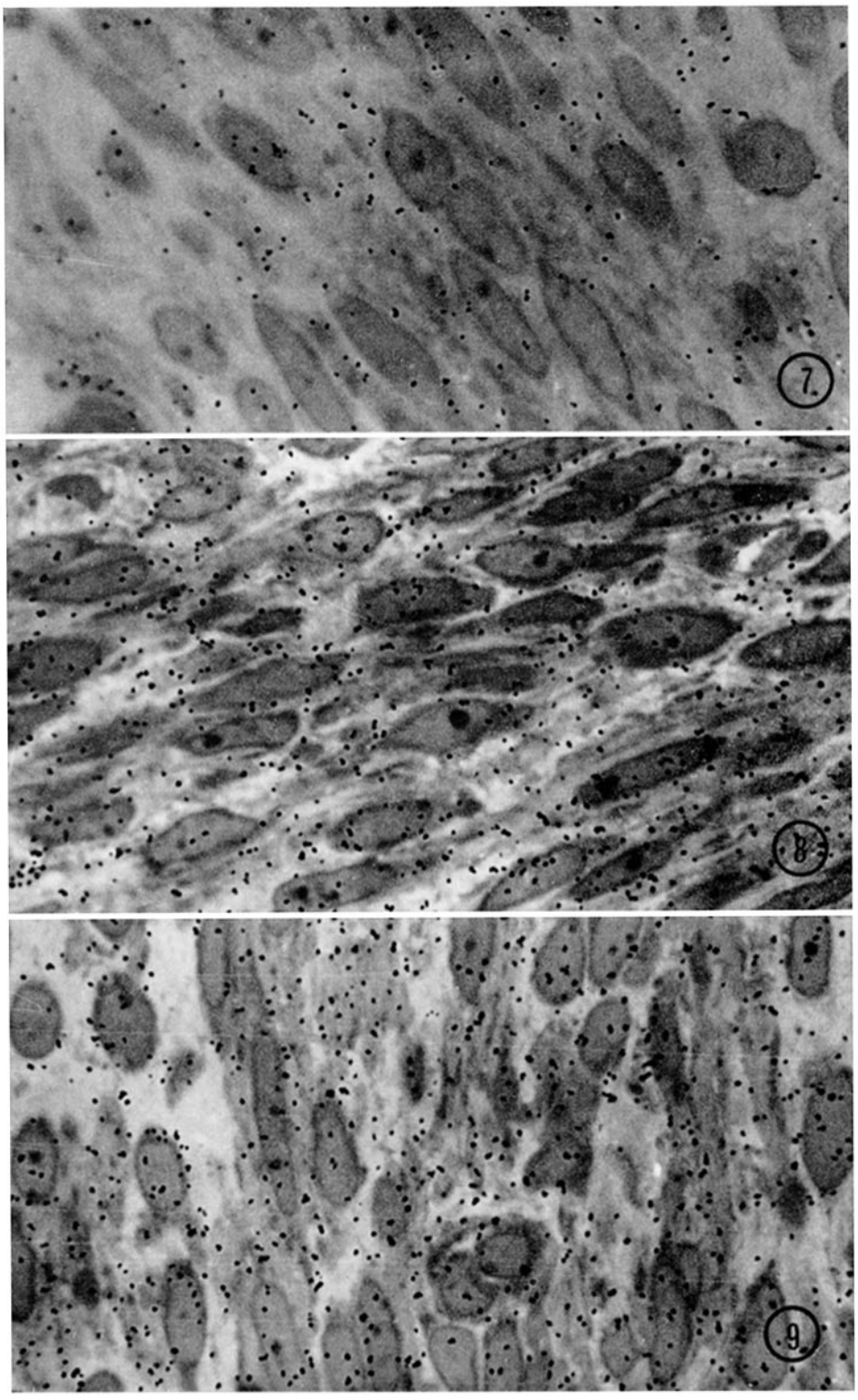
Uterus

Observations of a highly-developed rough endoplasmic reticulum and Golgi complex in the smooth muscle of the myometrium of the rat uterus of mature animals in estrous, or in prepubertal animals primed with estradiol, were described by Ross and Klebanoff (10) and subsequently confirmed in studies by Bo et al. (24, 25), Bergman (16), and Tokuoka (40). Tokuoka (40) demonstrated that the cells of the estrogen-stimulated myometrium utilized tritium-labeled proline, as indicated in a series of light and electron microscope radioautographic studies. He exam

FIGURE 7 This is a light microscope radioautograph of the uterus removed from a control, prepubertal animal given a previous injection of the alcohol carrier. The proline-^{3}H had been administered 15 min before the animal was sacrificed. Silver grains are seen to reside in small numbers over both nucleus and cytoplasm of the cells. × 1,400.

FIGURE 8 This is a light microscope radioautograph of myometrial cells from a control prepubertal animal given an injection of alcohol. The tissue was removed 30 min after intravenous administration of proline-^{3}H. There is an increased amount of label over the myometrial cells, but the distribution is similar to that seen after 15 min, in which both nuclei and cytoplasm are labeled. × 1400.

FIGURE 9 This is a light microscope radioautograph of myometrial cells of a control animal 4 hr after administration of proline-^{3}H. The localization of proline is the same as that seen after 15 and 30 min (Fig. 7 and 8), demonstrating retention of the label within the cells. × 1400.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ined the ability of these cells to use this amino acid in the formation of extracellular proteins. The cells took up the proline within 15 min of intraperitoneal administration and began to secrete it sometime after 3 hr. These observations are similar to those in the present study.

The radioautographic observations in the present study suggest a similarity between the estrogen-stimulated myometrial cell and the medial smooth muscle cells of the developing aorta. Both types of cell have a well-developed rough endoplasmic reticulum and Golgi complex, and both convert amino acids into protein which is secreted relatively rapidly into the extracellular

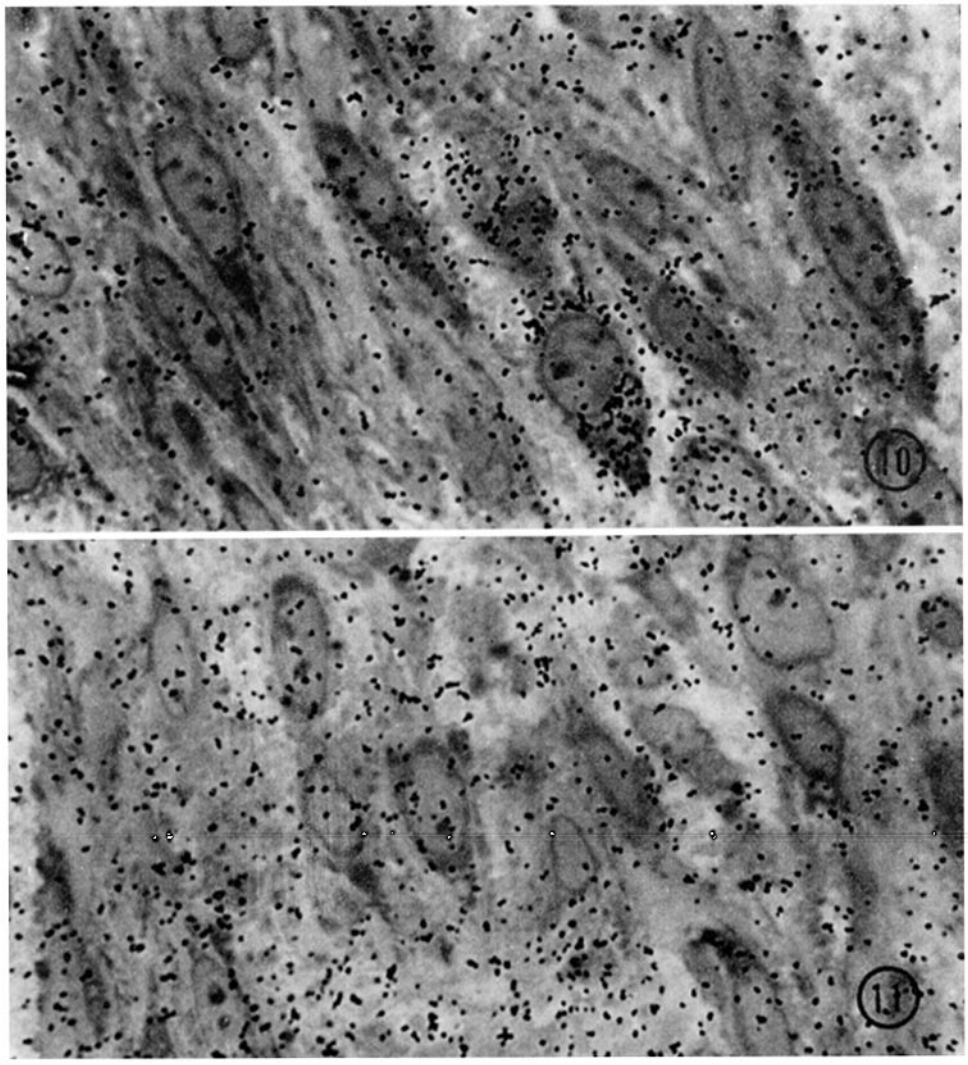

FIGURE 10 This is a light microscope radioautograph of myometrial cells of a prepubertal animal which had been previously injected with estradiol. 30 min after the administration of proline-^{3}H, a relatively large amount of cytoplasmic label can be seen in several of the myometrial cells. × 1400.

FIGURE 11 This is a light micrscope radioautograph of the myometrial cells of an estrogen-treated animal whose uterus was removed 4 hr after intravenous administration of proline-^{3}H. A large amount of the label that had previously been intracellular has been transported to the extracellular spaces surrounding these cells. × 1400.

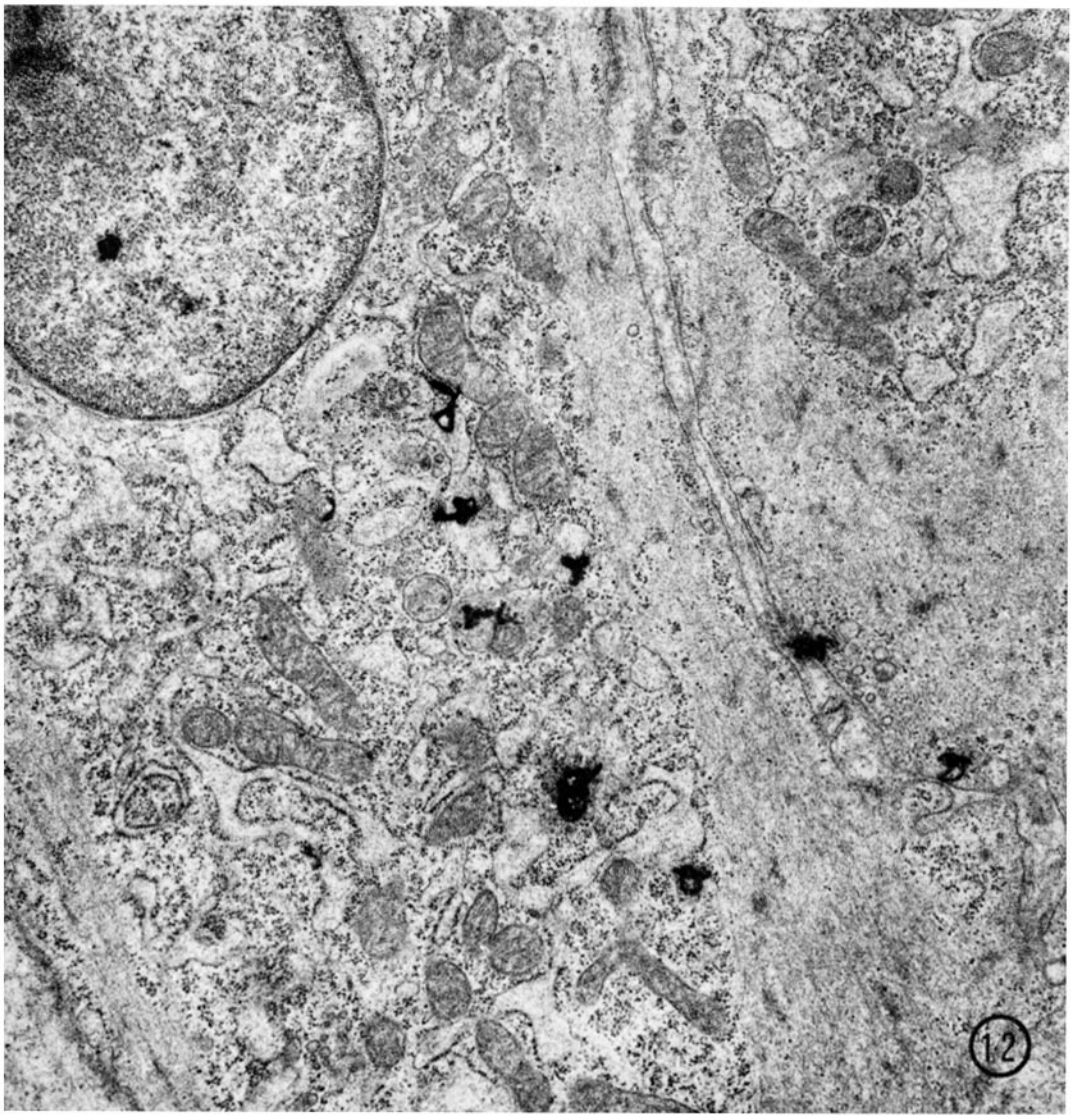

FIGURE 12 This electron microscope radioautograph is representative of the myometrium of a prepubertal animal given estradiol-17 β. The proline had been administered 30 min before sacrifice, and the label can be seen to reside largely in the cisternae of rough endoplasmic reticulum and peripheral cellular elements. × 16,500.

environment. In the uterus, the silver grains are found to overlie collagen fibrils, whereas in the case of the aorta they are found over both collagen fibrils and elastic fibers. Although radioautographic studies do not establish the proteins being synthesized, the presence of silver grains over collagen fibrils and over the components of the elastic fibers strongly suggests that the smooth muscle cells are actively synthesizing the proteins present in these extracellular structures. These observations, together with recent observations in smooth muscle cells grown in culture (28), provide clear evidence that the smooth muscle cell must be classified together with the fibroblast, osteoblast, and chondroblast as cells capable of the synthesis and secretion of collagen, elastic fiber proteins, and presumably the proteoglycans associated with these fibrous proteins.

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