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Collagen synthesis by cultured rabbit aortic smooth-muscle cells

Alteration with phenotype

Aik H. ANG,*†‡ George TACHAS,* Julie H. CAMPBELL,‡ John F. BATEMAN† and
Gordon R. CAMPBELL*§
Departments of *Anatomy and †Paediatrics, University of Melbourne, Parkville, Victoria 3052, Australia,
and ‡ Baker Medical Research Institute, Commercial Road, Prahran, Victoria 3181, Australia

Enzymically isolated rabbit aortic smooth-muscle cells (SMC) in the first few days of primary culture express a 'contractile phenotype', but with time these cells modulate to a 'synthetic phenotype'. Synthetic-state SMC are able to proliferate, and, provided that they undergo fewer than 5 cumulative population doublings, return to the contractile phenotype after reaching confluency [Campbell, Kocher, Skalli, Gabbiani & Campbell (1989) Arteriosclerosis 9, 633–643]. The present study has determined the synthesis of collagen, at the protein and mRNA levels, by cultured SMC as they undergo a change in phenotypic state. The results show that, upon modulating to the synthetic phenotype, SMC synthesized 25–30 times more collagen than did contractile cells. At the same time, non-collagen-protein synthesis increased only 5–6-fold, indicating a specific stimulation of collagen synthesis. Steady-state mRNA levels are also elevated, with $\alpha 2(I)$ and $\alpha 1(III)$ mRNA levels 30 times and 20 times higher respectively, probably reflecting increased transcriptional activity. Phenotypic modulation was also associated with an alteration in the relative proportions of type I and III collagens synthesized, contractile SMC synthesizing $78.1 \pm 3.6\%$ (mean $\pm$ S.D.) type I collagen and $17.5 \pm 4.7\%$ type III collagen, and synthetic cells synthesizing $90.3 \pm 2.0\%$ type I collagen and $5.8\% \pm 1.8\%$ type III collagen. Enrichment of type I collagen was similarly noted at the mRNA level. On return to the contractile state, at confluency, collagen production and the percentage of type I collagen decreased. This further illustrates the close association between the phenotypic state of SMC and their collagen-biosynthetic phenotype.

INTRODUCTION

Collagens are major structural components of the extracellular matrix of the artery wall, comprising 20–50% of the dry weight of tissue (Grant, 1967; McCloskey & Cleary, 1974; Bartos & Ledvina, 1979; Hosoda et al., 1984). The fibrillar collagen network is responsible for the overall integrity of the blood vessels, and, in addition, collagens are involved in haemostasis and thrombosis through their interaction with platelets after vessel injury (for reviews see Barnes, 1982, 1985).

A number of structurally related but genetically distinct collagen types have now been characterized (for reviews see Miller & Gay, 1987; Burgeson, 1988; Miller, 1988). In the vessel wall, the predominant collagenous species are the interstitial collagens types I and III (Chung & Miller, 1974; Trelstad, 1974), comprising 60–75% and 25–40% of all collagen respectively (Morton & Barnes, 1982; Hanson & Bentley, 1983; Halme et al., 1986; Leushner & Haust, 1986). Type V collagen is also present in relatively small amounts (Mayne et al., 1980; Leushner & Haust, 1984).

Smooth-muscle cells (SMC) are the major cell type present in the media and intimal thickenings of mammalian arteries (Pease & Paule, 1960; Wissler, 1968; Stary, 1989), are responsible for the synthesis of extracellular matrix (Ross & Klebanoff, 1971; Gerrity et al., 1975; Burke & Ross, 1979), and are capable of con-

traction (Campbell et al., 1988). To achieve this multiplicity of functions, the SMC are capable of existing in a range of phenotypes. Most SMC in the normal artery media and diffuse intimal thickenings have a high volume of myofilaments in their cytoplasm (Mosse et al., 1986) and are therefore referred to as being in the 'contractile phenotype' (Campbell & Campbell, 1985). Proliferating SMC, or those engaged in repair of artery wall, contain few myofilaments but large amounts of rough endoplasmic reticulum and free ribosomes (Manderson et al., 1989). These cells are therefore referred to as being in the 'synthetic phenotype' (Campbell & Campbell, 1985). Many of the SMC in diffuse intimal thickenings adjacent to human atheromatous plaques are in the synthetic phenotype (Mosse et al., 1985). We are therefore interested in the role that smooth-muscle phenotypic modulation (change from the contractile to the synthetic phenotype) plays in atherogenesis. Three important biological phenomena are associated with human atherosclerosis, a vascular disease affecting predominantly the intima of elastic and large muscular arteries. These are: (1) the proliferation of smooth-muscle cells; (2) the production of large amounts of extracellular matrix; (3) the accumulation of intracellular and extracellular lipid (Ross, 1986; Schwartz et al., 1986). Biosynthetic studies have shown that the accumulation of large deposits of collagen seen in atheroma is due to an increased rate of collagen synthesis (McCullagh & Ehrhart, 1974, 1977;

Ehrhart & Holderbaum, 1977, 1980; Fischer et al., 1980). Type III collagen was initially reported to be the major collagen type present in the normal arterial wall, with type I the predominant collagen in atherosclerotic lesions (McCullagh & Balian, 1975; McCullagh et al., 1980). Subsequent studies, however, did not support this contention, and type I collagen was consistently found to be the predominant collagen in lesions, as in normal tissue (for reviews see Barnes, 1985, 1988; Mayne, 1986, 1987).

The phenomenon of phenotypic modulation of SMC as observed in vivo can be readily produced in enzymically isolated cultures of arterial SMC (Chamley et al., 1977; Chamley-Campbell et al., 1979; Kallioniemi et al., 1984; Thyberg et al., 1985). Enzyme-dispersed SMC resemble the SMC of the intact media, remaining in the contractile phenotype for the first few days in culture (G. R. Campbell & J. H. Campbell, 1987). If the cells are seeded at less than confluency, they undergo a phenotypic change after a variable number of days, depending on species, age and vessel, to the synthetic phenotype. If these cells remain in culture for less than 3 weeks, and undergo fewer then five cumulative population doublings, they will return to the contractile state after confluency. These cells are termed 'reversible synthetic' (RS). Cells that have been subcultured several times and undergo multiple population doublings are unable to return to the contractile state, and eventually lose their proliferative capacity. These cells are termed 'irreversible synthetic' (IRS) cells (Campbell et al., 1989). This system therefore provides an ideal model to determine the role of SMC in atherogenesis.

The present paper examines the amount and type of collagen synthesized by rabbit aortic SMC of differing phenotype in culture and attempts to answer the following questions. Does the amount of collagen produced by SMC change with phenotype and the state of the cells? Does the type of collagen produced by SMC change with phenotype? Do changes in levels of collagen synthesis directly reflect changes in the steady-state levels of collagen mRNA?

EXPERIMENTAL

Materials

L-[5-^{3}H]Proline (30 Ci/mmol), [α-^{32}P]dCTP (3000 Ci/mmol) and PCS liquid-scintillation solution were obtained from Amersham Australia Pty., Sydney, Australia. Cell-culture medium (M199), foetal-calf serum (FCS) and trypsin were purchased from Commonwealth Serum Laboratories, Melbourne, Australia. Chromatographically purified bacterial collagenase was obtained from Worthington Biochemical Corp., Freehold, NJ, U.S.A., and further purified (Peterkofsky & Diegelmann, 1971). Pepsin, β-aminopropionitrile fumarate and sodium ascorbate were purchased from Sigma Chemical Corp., St. Louis, MO, U.S.A. Oligo(dT)–cellulose was obtained from Pharmacia, Uppsala, Sweden, and Zeta-probe Nylon Membranes were from Bio-Rad Laboratories, Richmond, CA, U.S.A. Proteinase K and random-primed DNA-labelling kits were purchased from Boehringer Mannheim Australia Pty. Ltd., Melbourne, Australia. The human α2(I) cDNA clone (Hf32) was a gift from Dr. F. Ramirez (Chu et al., 1982) and a 2.5 kb human αI(III) cDNA clone a gift from Dr. R. Dalgleish (Miskulin et al., 1986; Mankoo & Dangliesh, 1988). Rabbits (9 weeks old) were obtained from a cloned colony maintained at the Baker Medical Research Institute, Melbourne, Australia, since 1961. All other chemicals were commercially available analytical-grade reagents.

SMC cultures

Thoracic and abdominal aortae of 9-week-old rabbits were cleaned of fat, and the intima-media was separated from the rest of the tissue with the aid of watchmaker's forceps. The intima-media was enzymically dispersed into single-cell suspension by a combination of collagenase and elastase (J. H. Campbell & G. R. Campbell, 1987). Cells were plated into primary culture at a constant density of 1.8×10^7 cells/90 mm dish, 8×10^6 cells/60 mm dish and 2×10^6 cells/30 mm dish in M199 supplemented with 5% (v/v) FCS and 2 mM-L-glutamine, in an atmosphere of air/CO_2 (19:1) at 37 °C. For subcultured RS cells, primary-cultured cells grown for 7 days were trypsin treated and seeded into first passage at the same density and grown for a further 3 days. IRS cells were third-passage cells (grown over a period of 4 weeks). RS cells were growth-arrested by replacing the normal medium with M199 supplemented with either 0.5% FCS serum or 0.23% (w/v) bovine serum albumin (BSA) for 3 days. At 24 h before any of the SMC cultures were used for analysis, the culture medium was replaced with fresh medium containing 0.15 mM-sodium ascorbate. Cell phenotype was assessed by electron microscopy, and cell counts were performed with a ZM Coulter Counter as described elsewhere (Campbell et al., 1989).

Determination of collagen and non-collagen-protein (NCP) synthesis

For biosynthetic protein radiolabelling, the growth medium was removed from triplicate dishes (60 mm) and replaced with 4.9 ml of warm serum-free M199 (M199-S) containing 0.15 mM-sodium ascorbate. After preincubation for 1 h, 100 μCi of L-[5-^{3}H]proline (100 μl of a 1 mCi/5 μmol/ml stock) was added and the cultures were labelled for 4 h (Bateman et al., 1984). After separation of the medium and cell-layer fractions, collagen and NCP synthesis were quantified by bacterial-collagenase digestion (Peterkofsky & Diegelmann, 1971; Bateman et al., 1984). Collagen synthesis was expressed as [^{3}H]proline d.p.m./10^6 cells per h of labelling. NCP synthesis was also expressed as [^{3}H]proline d.p.m./10^6 cells per h after the calculated value was multiplied by a correction factor of 5.4, since collagen has approx. 22.2% imino acids, compared with an average of 4.1% in NCP (Peterkofsky et al., 1982). Statistical difference was determined with the one-way analysis of variance and the Student Newman–Keul's (SNK) multiple-comparisons test (Zar, 1974).

Analysis of relative distribution of type I, III, and V collagens

The culture dishes were pulse-labelled as described above, with the inclusion of 0.15 mM-β-aminopropionitrile fumarate in the labelling medium to inhibit collagen cross-linking. The medium and cell-layer fractions were separated, and procollagens were precipitated with 25%-satd. $(NH_4)_2SO_4$ and then subjected to limited pepsin digestion (100 μg of pepsin/ml) at 4 °C for 6 h (Bateman et al., 1984). SDS/polyacrylamide-gel electrophoresis of collagen chains was performed on a 5% (w/v) separating gel with a 3.5% (w/v) stacking gel. The

preparation of samples and the electrophoresis conditions were as previously described (Laemmli, 1970; Bateman & Peterkofsky, 1981). Radioactivity of the collagen bands was determined by fluorography (Bonner & Laskey, 1974) and quantified by excision of the individual collagenous bands, mild-alkaline hydrolysis at 37 °C for 20 h and scintillation counting (Bateman et al., 1988). Significant difference was determined by using the one-way analysis of variance and the Student Newman–Keul's multiple-comparisons tests (Zar, 1974).

Isolation and quantification of poly(A)$^+$ RNA

Some $(1–3) \times 10^7$ cells were lysed in 6 ml of RNA lysis buffer (10 mM-Tris/HCl, pH 7.5, 0.1 M-NaCl, 2 mM-EDTA and 0.5% SDS) containing 200 mg of proteinase K/ml, with immediate shearing through a 21-gauge syringe needle followed by incubation at 37 °C for 1 h to destroy nucleases. Oligo(dT)–cellulose beads were added to the lysate and gently rotated for 24 h to bind the poly(A)$^+$ RNA. Material not bound to the beads was removed by washing away with several volumes of lysis buffer, and beads were loaded on a small chromatographic column. After unbound material had been completely washed from the beads, as determined by a zero A_{260} measurement, the poly(A)$^+$ RNA was eluted with 1 ml of elution buffer (10 mM-Tris/HCl, pH 7.5, 0.2 mM-EDTA and 0.5% SDS) pre-warmed to 60 °C. A_{260} measurements in combination with cell counts from three independent RNA purifications were used to quantify the amount of poly(A)$^+$ RNA per cell. Usually less than 10% variations were obtained in this procedure with freshly dispersed cultured cells. The variations were larger with different batches of enzymically dispersed cells from fresh tissue (results not shown). The eluted RNA was then ethanol-precipitated, and the amount of RNA remaining after precipitation and washing determined again by A_{260} measurements.

RNA hybridization analysis

Poly(A)$^+$ RNA isolated from cultured rabbit SMC and human skin fibroblasts was electrophoresed through a formaldehyde denaturing gel, transferred to a Zetaprobe membrane and probed with hexamer-primed ^{32}P-labelled cDNAs at 42 °C in 50% (v/v) formamide/5 × SSC/0.1% SDS/0.25% BLOTTO (non-fat milk powder; Johnson et al., 1984). By washing at stringencies between 1 × SSC/0.1% SDS at 30 °C and 0.1 × SSC/0.1% SDS at 60 °C, specific signals were generated with both $\alpha 2$(I) and $\alpha 1$(III) cDNA.

To quantify type I and III collagen mRNA levels, three equivalent membranes were prepared, each containing duplicate 0.05 μg samples of poly(A)$^+$ RNA. The RNA had been heat-denatured at 68 °C for 15 min in 4 × SSC containing formaldehyde and applied to a Zetaprobe membrane by using a slot-blot apparatus (Bio-Rad Laboratories). Two of the membranes were probed with either $\alpha 2$(I) or $\alpha 1$(III) under the conditions described above, and the third membrane was probed with a 30-residue deoxythymidine oligonucleotide (dT$_{30}$) in 15% formamide/6 × SSC/0.1% SDS/0.25% BLOTTO. All membranes were washed at a final stringency of 1 × SSC/0.1% SDS at 42 °C and assayed for the amount of radiolabel bound above background by scintillation counting. The signals obtained with the oligonucleotide dT$_{30}$ were used as a measure of the relative amount of poly(A)$^+$ RNA bound to each membrane.

RESULTS

Cell phenotype and growth characteristics of primary cultured rabbit SMC

Enzymically dispersed rabbit aortic SMC adhered to the culture dish within the first 24 h of seeding and spread out during the next 24 h. SMC at 2 days in primary culture were spindle-shaped and displayed ultrastructural features typical of contractile SMC (G. R. Campbell & J. H. Campbell, 1987).

The growth curve showed that the cell number remained relatively constant for the first 4 days in culture (Fig. 1). By day 4 in culture, most of the cells had modulated to the synthetic phenotype, and were broad rather than long and spindle-shaped. Myofilament bundles were absent, except for some which were located at the periphery of the cell, and, instead, the cytoplasm was filled with synthetic organelles such as rough endoplasmic reticulum and free ribosomes. These synthetic-state cells proliferated, and after 2 cumulative population doublings they reached confluency on day 10 (Fig. 1). By 2–3 days after confluency the SMC had returned to the contractile phenotype, as observed by morphometry (see Campbell et al., 1989).
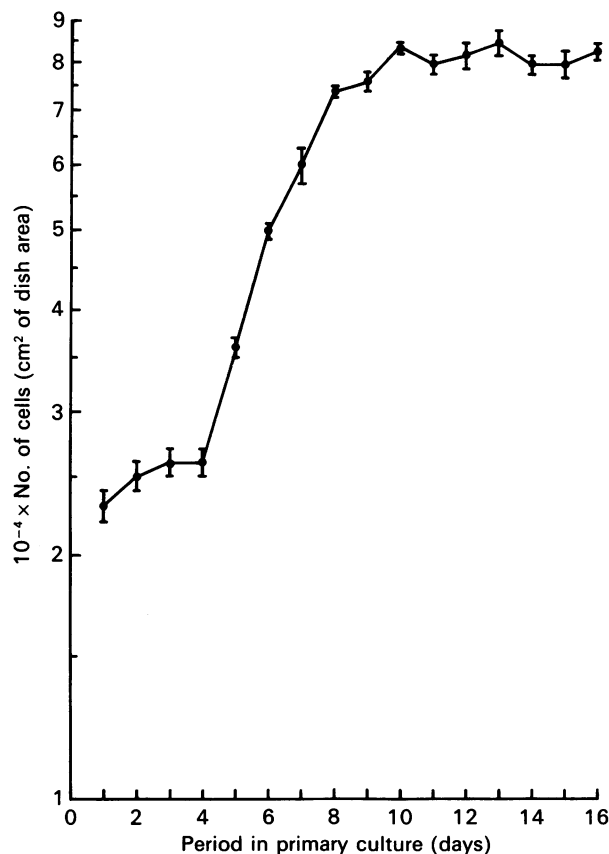

Fig. 1. Growth of SMC in primary culture

Enzymically isolated SMC were seeded at 2.7×10^5 cells/30 mm dish on day 0. Triplicate dishes were taken daily, trypsin-treated, and cell counts determined by a ZM Coulter Counter. Each value is the mean ± S.D. of cell counts of the triplicate dishes from one experiment.
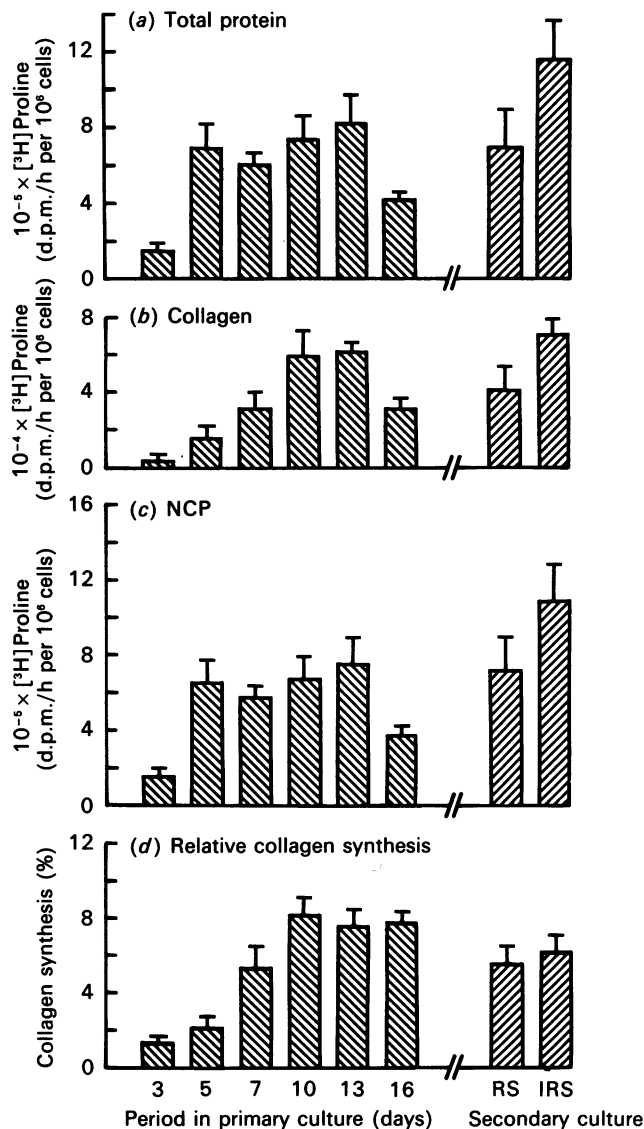
Fig. 2. Protein synthesis by rabbit SMC in primary and secondary cultures

Triplicate cell cultures were labelled with [5-³H]proline for 4 h, and synthesis of collagen and NCP was determined by the collagenase-susceptible-protein assay of Peterkofsky & Diegelmann (1971) (see the Experimental section for details). Each value is the mean±S.D. of six different experiments. (a) Total protein (collagen + NCP) synthesis; (b) collagen synthesis; (c) NCP synthesis; (d) relative collagen synthesis, determined by the formula:

Relative collagen synthesis =

$$\frac{\text{collagen d.p.m.}}{\text{collagen d.p.m.} + (\text{NCP d.p.m.} \times 5.4)} \times 100\%$$

Changes in total protein synthesis and poly(A)⁺ RNA

The rate of total protein (collagen + NCP) synthesis and amount of poly(A)⁺ RNA (mRNA) per cell were determined for freshly dispersed SMC (day 0) as well as cells in primary culture for 2, 3, 5, 7, 10, 13 and 16 days. RS cells after one subculture were also analysed to demonstrate that protein synthesis by synthetic cells was not dependent on the method from which they were

derived. Analysis of IRS cells, obtained by serial passaging, was also undertaken, since these cells resemble more closely the synthetic cells of atherosclerotic lesions.

The rate of total protein synthesis was elevated approx. 4-fold when SMC modulated to the synthetic phenotype on day 5 (Fig. 2a). This level of synthesis was maintained through day-7, -10 and -13 cultures, but decreased to almost half on day 16, when the cells had reverted to the contractile phenotype. Protein production by subcultured RS cells was comparable with the levels synthesized by primary-cultured RS cells, with IRS cells consistently synthesizing more protein.

The level of poly(A)⁺ RNA, in general, reflected the changes in protein synthesis (Fig. 3). Day-0, -2 and -3 contractile cells contained similar levels of poly(A)⁺ RNA, which increased approx. 2-fold by day 5 as the SMC acquired a synthetic phenotype. Such a level of poly(A)⁺ RNA was maintained through to day 16 of culture. Both subcultured RS and IRS cells also displayed similar high levels of poly(A)⁺ RNA.

Collagen and NCP synthesis

Synthesis of collagen and NCP were determined at days 3, 5, 7, 10, 13 and 16 in primary culture and in subcultured RS and IRS cells. Day-5 synthetic cells synthesized collagen at a rate which was approx. 7 times ($P < 0.01$) that of day-3 contractile cells (Fig. 2b), and NCP synthesis increased approx. 5-fold ($P < 0.001$) (Fig. 2c). Consequently the relative rate of collagen synthesis was not significantly altered between days 3 and 5 (Fig. 2d) when SMC have modulated to the synthetic phenotype. However, between days 5 and 10 there was an increase in relative rate of collagen synthesis, owing to a 4-fold increase ($P < 0.001$) in collagen production (Fig. 2b) without a concomitant increase in NCP production (Fig. 2c).

The levels of collagen and NCP synthesis on day 13 were not significantly different from the respective values
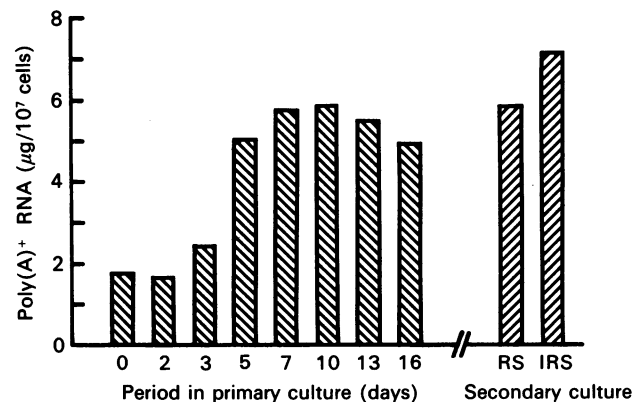

Fig. 3. Total cellular poly(A)⁺ RNA in rabbit SMC in primary and secondary cultures

Total cellular poly(A)⁺ RNA was prepared by lysing (1–3) × 10⁷ cells in RNA lysis buffer in the presence of proteinase K. Poly(A)⁺ RNA was extracted from the lysate with oligo(dT)–cellulose beads, eluted subsequently and the amount determined spectrophotometrically (see the Experimental section for details). Each value is the average of two different experiments.

Table 1. Effect of proliferation on collagen and NCP synthesis by rabbit synthetic SMC

Rabbit SMC which had been in primary culture for 7 days were subcultured; 24 h later, cultures were growth-arrested by replacing the normal culture medium with a medium supplemented with either 0.5 % FCS or 0.23 % BSA. Control cultures were grown in normal medium. Cultures were growth-arrested for 3 days after addition of fresh medium. They were then labelled with [^{3}H]proline, and syntheses of collagen and NCP determined as described in the Experimental section. Each value is the mean±S.D. of triplicate dishes.

Culture medium	Cumulative population doubling*	Synthesis (d.p.m./h per 10^6 cells)		Relative collagen synthesis
		Collagen	NCP	
M199+0.5% FCS	0.37	37899±318	463448±11800	7.6±0.1
M199+0.23% BSA	0.27	33953±721	384776±6515	8.1±0.3
M199+5% FCS +2 mM-glutamine	0.81	66170±1970	838989±38335	7.3±0.1

* log(final cell count/initial cell count)/log2.

on day 10, when the culture was confluent. However, when the cells had reverted to the contractile phenotype on day 16, collagen synthesis had declined to half of the day-13 level ($P < 0.001$) (Fig. 2b). Since NCP synthesis declined by a similar extent ($P < 0.001$) (Fig. 2c), the relative rate of collagen synthesis was the same as that of day 13 (Fig. 2d). Therefore, when synthetic cells returned to the contractile phenotype, there was a general down-regulation of protein synthesis.

Subcultured RS cells synthesized collagen at a rate which was 20 times higher ($P < 0.001$) than for contractile cells (Fig. 2b), with a concomitant 5-fold increase in NCP synthesis (Fig. 2c). This indicates a preferential stimulation of collagen synthesis over that of other proteins. However, as RS cells modulated to the IRS phenotype, a general stimulation of protein synthesis was observed (Fig. 2d), since both collagen and NCP syntheses increased 2-fold ($P < 0.001$) (Figs. 2b and 2c).

Effect of proliferation on collagen synthesis

Cell density has been shown to influence collagen synthesis. In SMC cultures rapidly proliferating low-density cultures synthesized little collagen, but as cells reached confluence collagen synthesis increased (Stepp et al., 1986; Liau & Chan, 1989). Since contractile SMC are non-proliferative, whereas synthetic SMC proliferate readily in response to serum mitogens (Chamley-Campbell et al., 1979), it was important to establish if the rate of cell proliferation had an influence on collagen synthesis by synthetic SMC.

Collagen synthesis by RS cells which have been growth-arrested with either 0.5% FCS or 0.23% BSA was compared with that of RS cells grown in normal culture conditions (Table 1). Proliferation was minimal in cultures incubated with M199 supplemented with either 0.5% FCS or 0.23% BSA. In contrast, SMC cultures incubated with normal culture medium containing 5% FCS and 2 mM-glutamine underwent 0.81 cumulative population doubling. These proliferating cell cultures produced approximately twice as much collagen and NCP as did growth-arrested cells. Since both collagen and NCP synthesis increased equally, the relative rate of collagen synthesis was unchanged in the proliferating versus growth-arrested cell cultures.

Relative proportions of type I, III and V collagens

Electrophoretic analysis of pepsin-resistant radio-labelled proteins was used to determine the relative distribution of type I, III and V collagens as the SMC altered phenotype. Fig. 4 shows the resolution of the type
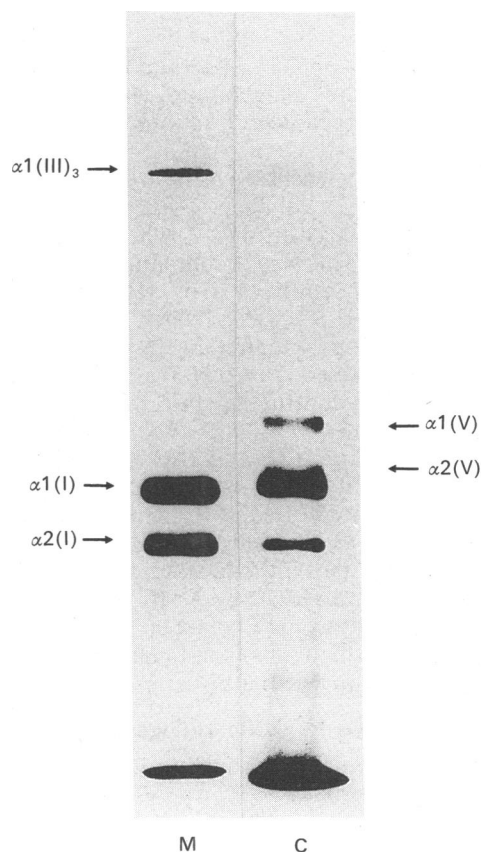

Fig. 4. Electrophoresis of pepsin-digested collagen species of cultured rabbit SMC

[5-^{3}H]Proline-labelled collagen species of medium (lane M) and cell-layer (lane C) fractions were subjected to limited digestion with pepsin and analysed by SDS/poly-acrylamide-gel electrophoresis on 5% (w/v) acrylamide separating gel (see the Experimental section for details).
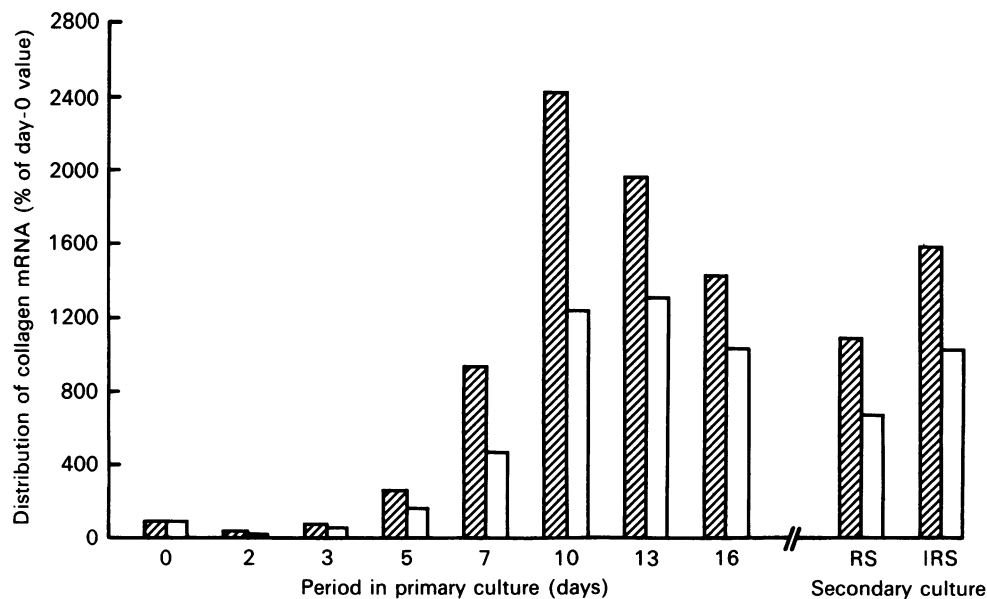
Fig. 5. Changes in the relative amounts of collagen-type mRNAs expressed by rabbit SMC in primary and secondary cultures

Relative collagen mRNA per cell was determined by multiplying the amount of poly(A)$^{+}$ RNA per cell (see Fig. 3) by the specific signal obtained with a constant amount (0.05 μg) of poly(A)$^{+}$ RNA. Results are expressed as percentages of the day-0 (freshly dispersed) cells, and each value is the average of two different experiments. Key: ▨, α2(I); □, α1(III).

I, III and V collagens on a 5%-polyacrylamide/SDS gel. The distribution of the collagen types was determined in both the medium and cell-layer fractions, and the findings were combined.

Day-3 contractile cells synthesized $78.1 \pm 3.5\%$ (mean $\pm$ S.D.) type I collagen, which increased to $90.3 \pm 2.0\%$ ($P < 0.001$) on day 7, when the cells were synthetically active. This was accompanied by a decrease in the relative proportion of type III collagen from $17.5 \pm 4.7\%$ to $5.8 \pm 1.8\%$ ($P < 0.001$). The enrichment of type I collagen (and concomitant decrease in type III) was significantly reversed ($P < 0.005$) at day 16 when the cells returned to the contractile state, with $83.3 \pm 2.8\%$ type I collagen and $13.1\% \pm 2.8\%$ type III collagen produced. Both subcultured RS and IRS SMC synthesized 91% type I collagen and 5–6% type III, which was similar to the primary cultured SMC in the RS phenotype.

Although there was enrichment of type I collagen as SMC modulated to the synthetic state, the predominant collagen species synthesized by SMC (irrespective of phenotype) was always type I collagen. Type V collagen constituted only 3–5% and was not significantly altered by the phenotypic state of the SMC.

Changes in the mRNA levels for collagen types I and III

mRNA was obtained from subcultured RS and IRS cells, and freshly dispersed SMC (day 0), as well as cells seeded in primary culture for 2, 3, 5, 7, 10, 13 and 16 days. Slot-blots containing a known amount (0.05 μg) of the mRNA were probed with ^{32}P-labelled cDNA probes for $a2$(I) and α1(III) under conditions shown to be specific by Northern-blot analysis. The α2(I) probe was used as a marker of type I collagen. Fig. 5 shows the changes in steady-state levels of mRNA per cell relative to day 0.

The α2(I)-mRNA level initially decreased during the first few days of primary culture and returned to the day-0 level by day 3. With modulation to the synthetic phenotype the α2(I)-mRNA level gradually increased, reaching a maximum on day 10, which was approx. 30 times that of day 3. As the SMC returned to the contractile phenotype (day 13–16) the steady-state levels of α2(I) mRNA declined.

The α1(III)-mRNA level also increased as SMC modulated to the synthetic phenotype, reaching a maximum on day 10, which was 20 times higher than the day-3 level. However, unlike α2(I) mRNA, the level of α1(III) mRNA on day 13 was similar to that of day 10 and was only noted to decline on day 16. Similarly to primary-cultured RS cells, subcultured RS and IRS cells expressed more of both α2(I)- and α1(III)-mRNA species than did day-3 contractile cells.

Although the levels of both α2(I)- and α1(III)-mRNA species were elevated and reduced simultaneously as SMC altered phenotype, the relative distribution of the two collagen mRNA species changed. As the SMC modulated from the contractile to the synthetic phenotype there was a significant increase in the proportion of the type I collagen α2(I) mRNA. These mRNA data are consistent with the protein data, which demonstrated a similar enrichment of type I collagen in the synthetic state.

DISCUSSION

Modulation of SMC from a contractile to a synthetic phenotype is directly associated with an increase in protein synthesis, in particular collagen. This increase in collagen synthesis is greatest (35 times) in those cells which have undergone more than five cumulative population doublings (IRS SMC). This increase in collagen synthesis from contractile to synthetic phenotype is

specific, since it is far greater than the increase in synthesis of NCP. Type I collagen is the predominant species synthesized by all SMC phenotypes. Synthetic-state SMC (both RS and IRS) produce more type I collagen (91 %) than do contractile-state SMC (78 %). Similarly to the protein data, there was an increase in the type I collagen $\alpha2(I)$ and type III collagen $\alpha1(III)$ mRNA levels in synthetic-state SMC as compared with contractile-state SMC. Likewise, IRS cells had higher collagen mRNA levels than did RS cells.

The studies of rabbit SMC at different times in primary culture provide a detailed picture of alterations in collagen and NCP synthesis with change in phenotype. Between 3 and 5 days in culture collagen synthesis is stimulated in the contractile SMC. This stimulus appears to be a generalized protein synthesis, since NCP synthesis increased to a similar extent. However, as the SMC become more synthetic after day 5, and then proliferate to reach confluency on day 10, collagen synthesis is preferentially stimulated over that of NCP. The maximal rate of collagen synthesis was achieved on day 10. mRNA studies performed in parallel were consistent with the protein studies, showing a similar increase over the day-3–10 period. Thus there appears to be increased synthesis of collagen in the first 10 days in culture, owing to increased steady-state levels of collagen mRNA. Cultures on day 13 still displayed the same collagen and NCP synthesis rates as on day 10, although the level of type I collagen $\alpha2(I)$ mRNA had declined. This apparent decrease in type I collagen mRNA without a corresponding alteration in collagen synthesis suggests that other post-transcriptional regulatory mechanisms may be involved. Such translational regulation of collagen synthesis has been demonstrated previously in SMC (Stepp et al., 1986), fibroblasts (Geesin et al., 1988) and chondrocytes (Allenbach et al., 1985). When most of the cells had returned to the contractile state by day 16, there was a general down-regulation in synthesis, with both collagen and NCP decreased by 50 % of day-13 levels. Similarly, this was reflected in mRNA levels, with type I collagen $\alpha2(I)$ and type III collagen $\alpha1(III)$ mRNAs decreased by 23 % and 20 % respectively.

The increases in collagen and NCP observed with time in culture are not due to the proliferative state of the cells themselves, but are due to phenotypic change in the SMC. When RS SMC were grown in 5 % FCS and proliferated exponentially, there was only a doubling in the collagen synthesis rate as compared with cells kept relatively quiescent in 0.5 % FCS or 0.23 % BSA. This doubling in rate between quiescent and proliferating cells is relatively insignificant compared with the 28-fold increase in collagen synthesis when the cells modulate from the contractile to the RS state. Secondly, the IRS cells showed a 35-fold increase in collagen synthesis, yet these cells were undergoing less than 0.02 population doubling per day and thus were almost senescent.

Although collagen and NCP synthesis and collagen mRNA levels were down-regulated when synthetic-state SMC returned to the contractile phenotype, the levels did not return to those of day-2–3 contractile-state cells. The findings are in accordance with morphometric studies on primary-cultured SMC which have returned to the contractile phenotype after confluency. These SMC have a slightly lower volume fraction of myofilaments (V_v myo) than day-3 contractile cells ($35.4 \pm 1.5 \%$ versus $32.0 \pm 2.5 \%$) (Campbell et al., 1989), i.e. they do not fully

return to the contractile state, and therefore maintain increased levels of synthetic machinery.

Sjölund et al. (1986) have also shown that the transition of arterial smooth-muscle cells in primary culture from a contractile to a synthetic phenotype is associated with the activation of synthesis and secretion of collagen. Their data, however, differ from ours in several aspects. Using primary cultures of rat aorta, they found a biphasic stimulation of collagen synthesis, with maximal activity on days 4 and 6. Synthesis of collagen on day 5 was approximately one-third of that on days 4 and 6. It should be noted that rat aortic smooth-muscle cells in culture behave differently from those of rabbit. Morphometric studies indicate that the rat cells do not significantly return towards the contractile state after confluency is achieved (Thyberg et al., 1983). In fact, many of these cells are able to grow in an anchorage-independent manner (Sjölund et al., 1988), a property shared with tumour cells.

Rabbit SMC obtained from explants of atherosclerotic lesions were reported to have a non-specific 2-fold increase in collagen synthesis over similarly obtained SMC from normal tissue (Pietila & Nikkari, 1980), which is similar to the non-specific 2-fold increase between RS and IRS cells in the present studies. Biosynthetic studies of organ cultures of atherosclerotic and normal aortas are more comparable with the present study, since SMC organ cultures of normal aorta are invariably contractile, whereas SMC adjacent to lesions are in a synthetic phenotype (Mosse et al., 1986). This is borne out by the findings that, compared with normal tissue, lesions of dog (McCullagh & Ehrhart, 1974), pigeon (McCullagh & Ehrhart, 1977) and rabbit (Ehrhart & Holderbaum, 1977, 1980; Fischer et al., 1980) synthesize 2–20 times more collagen. An investigation in vivo by Opsahl et al. (1987) has also demonstrated that atherosclerotic aortae synthesize collagen at a higher rate than normal vessels.

Reports of the relative proportions of type I and III collagens synthesized by cultured SMC range from 35–75 % type I and 25–65 % type III (Barnes et al., 1976; Burke et al., 1977; Layman et al., 1977; Mayne et al., 1977, 1978; Scott et al., 1977). The cells for these studies were obtained from explants, and therefore likely to be synthetic. The present finding that rabbit synthetic SMC synthesize approx. 91 % type I and 6 % type III collagens emphasizes the heterogeneity of the results. This heterogeneity can be attributed to several factors: age (Layman & Titus, 1975; Schofield & Harwood, 1975; Uitto et al., 1976), cell density (Beldekas et al., 1982; Holderbaum & Ehrhart, 1984), different culture conditions and different methods used to quantify the collagen types (Burke et al., 1977). Pepsin may have also affected the quantification in some published data, since it can selectively degrade type III collagen at 15 °C (Burke et al., 1977).

As the cultured rabbit SMC modulated to the synthetic phenotype, an enrichment of type I collagen occurred. This trend was reversed when these synthetic SMC returned to the contractile phenotype upon reaching confluency. These changes in distribution of type I and III collagens were mirrored by comparable changes in collagen mRNA levels. These findings are in general agreement with studies in vivo of the type I/type III collagen ratio in normal and atherosclerotic arteries. Hanson & Bentley (1983) reported that normal media

had a composition of 58–63% type I and 37–42% type III collagen, whereas that of lesions was 88% type I and 12% type III collagen. Others have found a lesser enrichment of type I collagen in the lesion, with 76% type I and 24% type III collagen compared with 56% type I and 44% type III collagen in normal media (Morton & Barnes, 1982). Yet others have concluded that there is essentially no difference in composition of lesion and normal tissue, with 70% type I and 30% type III collagen (Leushner & Haust, 1986; Murata *et al.*, 1986, 1987). Similarly, Sjölund *et al.* (1986) showed that as the rat SMC modulated to the synthetic phenotype in primary culture, the type I/type III ratio was unaltered.

Thus the findings of the present paper lend further to the concept that synthetic-state smooth muscle in diffuse intimal thickenings adjacent to human atheromatous plaques (Mosse *et al.*, 1986) may contribute to the development of these lesions by their altered functional properties. Previous studies have shown that SMC must undergo a change in phenotype to the synthetic state before they proliferate in response to mitogens (Chamley-Campbell *et al.*, 1981) and that SMC in the synthetic phenotype have a substantially increased capacity to bind and accumulate lipoproteins such as β very-low-density lipoprotein (Campbell *et al.*, 1985). In the present paper we have shown that SMC in the synthetic phenotype produce increased amounts of collagen, and type I production is selectively enhanced. All these changes in function of the SMC with change in phenotype are observed in the SMC of the developing atheromatous plaque.

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