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Cultured Human Atherosclerotic Plaque Smooth Muscle Cells Retain Transforming Potential and Display Enhanced Expression of the *myc* Protooncogene

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The proliferation of vascular smooth muscle cells (SMC) is critical to atherosclerotic plaque formation. The monoclonal hypothesis proposes that the stimulus for this SMC proliferation is a mutational event. Here we describe a procedure for growing human plaque smooth muscle cells (p-SMC) in culture. We show that p-SMCs derived from two patients differ from SMC cultured from normal vascular tissue in expression of the protooncogene* myc. *One p-SMC strain was extensively characterized; these diploid, karyotypically normal cells have a finite life span in culture. Ultrastructural examination revealed two populations, one with classic contractile SMC appearance, the other, modulated to a synthetic state. Northern blotting showed a 2- to 6-fold and a 6- to 11-fold enhanced expression of* myc *by p-SMC, compared to SMC derived from healthy human aorta (HA-SMC) and saphenous vein (HV-SMC), respectively. In contrast, the p-SMC and HV-SMC expressed similar levels of message for the genes* N-myc, L-myc, Ha-ras, fos, sis, myb, *LDL receptor, EGF receptor, IGF I receptor, IGF II, and HMG CoA reductase. Finally, although p-SMCs are not tumorigenic, DNA isolated from these cells is positive in the transfection-nude mouse tumor assay. Myc, however, does not appear to be the transforming gene because no newly introduced human* myc *gene was detected in the p-SMC–associated nude mouse tumor. Thus human atherosclerotic p-SMCs possess both an activated* myc *gene and a transforming gene that is retained throughout many cell passages. (Am J Pathol 1991, 138:765–775)

The atherosclerotic plaque is the principal lesion associated with cardiovascular disease. It is a complex structure that arises partly as a result of smooth muscle cell (SMC) proliferation in the intimal region of the artery wall. The plaque consists of a fibrous cap composed primarily of SMCs and connective tissue matrix, covering the atheroma, which contains large amounts of extracellular lipid as well as SMC, macrophages, and lymphocytes. The proliferation of these SMC in response to an unknown stimulus is considered one of the events indispensable for atherosclerotic plaque formation. The monoclonal hypothesis of plaque development proposes that the stimulus for SMC proliferation is a mutational event.[1] It has been suggested that plaque smooth muscle cells (p-SMC) are derived from a stably transformed and permanently altered cell population that is distinct from the bulk of arterial SMCs.

Previously we showed that DNA extracted from three groups of endarterectomized human coronary artery plaque samples contains a transforming gene(s).[2] That is, plaque-derived DNA, when transfected into NIH3T3 cells, gave rise to transformed foci, which elicited local tumors following injection into the subscapular area of nude mice. DNA from cloned foci was positive in second and third subsequent rounds of transfection, while DNA from a variety of normal human tissues, including coronary artery, was negative in these assays. Although one group recently reported an inability to identify transforming activity in human carotid plaques,[3] two other groups confirmed our earlier findings.[2] Dr. Renate Zwijsen and her colleagues at the Agricultural University at Wageningen, The Netherlands (written personal communication, September 1990), and Dr. A. Ahmed and colleagues at Baylor College of Medicine[4] all observed transforming activity in human plaque DNA.

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To study further the putative atherosclerotic transforming gene(s), as well as to study gene expression in p-SMCs, it was necessary to develop techniques for long-term growth of human atherosclerotic p-SMCs. Unlike the situation with normal vascular SMCs, which are readily grown and maintained *ex vivo*,[5–7] the culture of human p-SMCs has met with limited success and reports of long-term growth of these cells is rare.[5,7–10] A procedure recently was developed by Dr. Peter Libby and coworkers that permits reproducible growth of these cells. They used it to show that human plaque cells produce a mitogenic factor.[11]

In this work, we describe this procedure (and our modifications of it) for culturing human p-SMCs and we characterize extensively one of these strains (p-SMC 3-22 AA). In addition, we report that the p-SMC strains tested thus far (p-SMC 3-22AA and p-SMC 9-12AA) exhibited a 2- to 6-fold and a 6- to 11-fold enhanced expression of the *myc* protooncogene compared to SMCs derived from healthy human aorta (HA-SMC) and healthy human saphenous vein (HV-SMC), respectively. Furthermore DNA from these cultured plaque cells (like DNA extracted directly from human plaques) retains the capacity to transform NIH3T3 cells in the transfection-nude mouse tumor assay. Thus human atherosclerotic plaque SMCs possess both an activated *myc* gene and a transforming gene, which are retained throughout many cell passages.

Methods

Cell Culture

Atherosclerotic plaques were obtained at surgery from the abdominal aortas of two patients. The patients had multiple plaques that were pooled. The use of this routinely discarded human tissue was approved by Parkview Hospital, Philadelphia, PA. Representative segments of each lesion were sectioned for light microscopy; frozen sections were stained with oil red O, and sections fixed in 10% buffered formalin were stained with hematoxylin and eosin. Each patient's surgical specimens were treated separately. They were rinsed, trimmed, and the plaque was separated from the underlying artery wall. Endothelial cells were scraped off gently with a scalpel after mild collagenase treatment. The plaques were minced into 1- to 2-mm fragments placed in 35-mm tissue culture dishes and suspended in 1.35 ml of Dulbecco's modified Eagle's medium (DMEM) with 25 mmol/l (millimolar) 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) (Sigma Chemical Co., St. Louis, MO), supplemented with 10% fetal bovine serum (FBS) (KC Biological, Lenexa, KS) and 1% penicillin/streptomycin. The tissue pieces then were cast into a collagen gel (Vitrogen 100ʳ; The Collagen Corp., Palo Alto, CA); 1.5 ml of ice cold collagen containing 0.15 ml of Earls' basal salt solution was added to the suspended minced tissue immediately after addition of 0.04 ml of 1N NaOH to the dish. The neutralized collagen was incubated for 15 minutes at room temperature, during which time a gel formed. Subsequently the cultures were fed with medium containing a variety of hormones and growth factors, including bovine pituitary extract (5.4 mg/ml) (BPE, Collaborative Research [CR]), epidermal growth factor (10 ng/ml) (EGF, [CR]), glucagon (35 nmol/l [nanomolar]) (Sigma Chemical Co.), insulin (100 nmol/l) (Sigma Chemical Co.), and platelet-derived growth factor (2 [half-maximal units] U/ml) (PDGF, [CR]). The collagen matrix in which the plaque pieces were embedded first favors migration of macrophages (which do not divide under these culture conditions) from the explants within 3 to 5 days, followed by SMC egress after 1 to 3 weeks. At that point, the matrix was digested with collagenase (2 mg/ml) (Type I, Worthington Biomedical, Freehold, NJ) and the cells were plated onto untreated 60-mm tissue culture dishes. When cells were subconfluent, remaining tissue pieces were removed with sterile forceps and cells were passaged into T-25 tissue culture flasks. At this stage, all cultures were fed with medium containing 10% FBS but no other growth supplements. Primary explant outgrowths were subjected to passage from 3 to 10 times to obtain sufficient material for experimental manipulations. This study presents data from two plaque SMC strains (p-SMC 3-22AA and p-SMC 9-12AA) derived from abdominal aortic plaques of two patients. Plaque smooth muscle cell strain 3-22AA was extensively characterized. Smooth muscle cell cultures HA-SMC and HV-SMC were gifts of Dr. Peter Libby (Brigham and Women's Hospital, Boston, MA) and Dr. David Hajjar (Cornell Medical School, New York, NY), respectively. HA-SMC and HV-SMC were cultured under conditions identical to those used for the p-SMC subsequent to passage in the collagen gel matrix. NIH3T3 cells were grown in DMEM (Gibco, Grand Island, NY) (without Hepes buffer) supplemented with 10% FBS. All cells were grown in a humidified 37°C 95% air/5% CO_2 incubator (Forma Scientific, Marietta, OH).

Growth Kinetics

To generate growth curves, $\sim 2.0 \times 10^5$ cells were plated into each of thirty 25-cm^2 tissue culture flasks, in DMEM-Hepes medium with 10% FBS. After 24 hours, three flasks were chosen randomly and the cells in each flask were counted on a Coulter electronic cell counter (Coulter Electronics, Hialeah, FL). The remaining flasks were fed with fresh medium. Every 48 hours for the next

17 days, cells were counted from three randomly chosen flasks. The three counts per day were averaged to give the number of cells for each time point. Generation times were calculated every 48 hours for log-phase cells.

Immunohistochemistry

Monoclonal antibody to SMC actin, anti-α-SM-1[12] (Sigma Chemical Co.) was used to visualize α-smooth muscle actin in plaque-derived cell cultures according to procedures provided by the suppliers.

Karyotyping

The human p-SMC 3-22AA strain was karyotyped by Dr. L. Alonzo at the clinical cytogenetics facility at Cornell University Medical Center, New York, NY. Twenty-six cells in mitosis stained by the Giemsa banding method[13] were analyzed.

Electron Microscopy

For ultrastructural analysis, p-SMC 3-22AA cultures were fixed briefly in a 2% glutaraldehyde–2% paraformaldehyde mixture in 0.1 mol/l (molar) Na cacodylate buffer, pH 7.3.[14,15] Cells were scraped gently from the culture dishes and transferred to 0.5-ml microfuge tubes in aliquots that yielded a pellet 0.5 to 1 mm thick when spun in a microfuge for 2 minutes. Pellets were removed from the tubes and placed into vials of fresh fixative for a minimum of 2 hours at room temperature, washed in buffer for 1 hour, and postfixed for 2 hours in 1% osmium tetroxide in s-collidine buffer (Structure Probe, West Chester, PA). After dehydration in a graded series of ethanols and infiltration with propylene oxide, the samples were embedded in poly/bed 812 (Polysciences, Warrington, PA). Thin sections were cut on a Sorvall MT-2B ultramicrotome, contrasted with uranyl and lead stains, and examined with a Philips 301 electron microscope (Philips, Eindhoven, The Netherlands).

Southern and Northern Blot Hybridization

Nude mouse tumors were pulverized in liquid nitrogen before DNA extraction, which was carried out *via* standard (phenol/chloroform and isoamyl alcohol) procedures.[16] DNAs from cultured cells and tumors were digested with restriction enzymes according to manufacturers' instructions. Hind III, Kpn I, and Xba I were not used because these restriction enzymes yield aberrant digestion patterns with tumor DNA.[17]

Poly A(+) RNA was isolated from subconfluent cultures of SMCs *via* the 'Fast Track' (Invitrogen, San Diego, CA) procedure.[18] Briefly, $\sim 10^8$ cells were lysed in 2% sodium dodecyl sulfate (SDS) buffer containing RNase inhibitors and proteases. Pooled lysed cells were subjected to proteolytic digestion at 45°C and oligo dT cellulose was added to the lysate. The poly A(+) RNA was eluted with low salt buffer.

Digested DNA and poly A(+) RNA were subjected to electrophoresis overnight in 0.7% agarose (BioRad) and 1% agarose–formaldehyde gels, respectively, at 20 V (15 mA) per gel and blotted onto nitrocellulose (Schleicher and Schuell, Keene, NH).

DNA probes (*Ha-ras*, *myc*, *L-myc*, *N-myc*, *fos*, *sis*, *myb*, HMG-Co-A reductase, β-actin, IGF II, EGF-receptor, LDL-receptor, IGF I receptor, and von Willebrand factor) were purchased from Oncor (Gaithersburg, MD) or ATCC (Rockville, MD). Filters were hybridized to labeled probes overnight. Filters were air dried and exposed with Cronex intensifying screens (DuPont, Wilmington, DE) to x-ray film (Kodak X-Omat, Eastman-Kodak, Rochester, NY) at −70°C. Quantitation of specific mRNAs was accomplished by means of scanning densitometry (LKB ultrascan XL, LKB Phamacia, Piscataway, NJ).

DNA Transfection

DNA from the p-SMC 3-22AA strain was tested in the NIH3T3 cell transfection assay. DNAs from T24 bladder carcinoma cells and untreated NIH3T3 cells were used as positive and negative controls, respectively. The DNAs were cotransfected with pSV_2 neo[19] using the standard calcium phosphate transfection protocol.[20] For each sample, 40 μg of DNA was transfected, along with 4 μg of pSV_2neo, into three T-25 flasks, each containing $\sim 1 \times 10^6$ NIH3T3 cells grown in DMEM with 10% FBS. Twenty-four hours later cells were split 1:4. Transfected cells were grown in the presence of gentamycin (G418).

Nude Mouse Assay

Three weeks after cotransfection, colonies were collected and $\sim 5 \times 10^6$ cells from each group were injected subcutaneously into the scapular area of 4- to 5-week-old athymic (nu^+/nu^+) female mice (Harlan, Sprague-Dawley, Indianapolis, IN). In addition, 5×10^6 p-SMC 3-22AA were injected into each of five nude mice to test directly for their tumorigenic potential. Animals were checked for the presence of tumors three times each week. Animals were killed when tumors were at least 10

mm in diameter and samples of tumors were taken for histologic observation, DNA isolation, and growth in cell culture.

Results

General Characteristics of p-SMCs

The human p-SMC strain, p-SMC 3-22AA, exhibited many characteristics reported for SMCs in a synthetic state.[21–23] Approximately 10% to 20% of the cells stained positively with the monoclonal antibody to SMC actin, α-SM-1 (Figure 1), an observation consistent with other reports in the literature (see Discussion). Four separate karyotypes revealed a normal male 46, XY chromosomal complement. No obvious chromosomal aberrations were apparent from G banding (not shown). Northern blot hybridization of p-SMC poly $A(^{+})$ RNA to a probe for von Willebrand factor was negative, indicating that the p-SMC 3-22AA strain lacked significant endothelial cell contamination.

Morphology

Lesions obtained at surgery had the appearance of fatty-fibrous atherosclerotic plaques. In each case a fibrous cap covered a necrotic core that contained remnants of foam cells and a moderate to marked amount of lipid as demonstrated by Oil red O stain (not shown). The core of some plaques had minor calcifications. Others were heavily calcified.

Following outgrowth of cells from collagen-embedded plaque explants and subculture in uncoated flasks, plaque cells exhibited typical SMC appearance as observed *via* phase-contrast microscopy (not shown). The SMCs were spindle-shaped and closely arranged in linear arrays. The p-SMC cultures exhibited the typical "hill and valley" configuration described by others.[5] Transmission electron microscopy of p-SMC 3-22AA revealed two morphologically distinct populations of SMCs. Some of the cells exhibited classic, contractile SMC appearance (Figure 2A and B). These cells had longitudinally oriented myofilaments within the cytoplasm; frequently dense bodies were observed associated with the myofilaments. Varying amounts of rough endoplasmic reticulum (RER) were observed in these cells, and β particles of glycogen, as well as caveolae, were present (Figure 2A and B). A larger number of cells showed characteristics of the modulated or synthetic SMC (Figure 2C). These cells lacked or had fewer myofilaments and dense bodies than typical SMCs and displayed extensive RER. The RER often was distended and the cells contained a well-developed Golgi apparatus (GA). Scattered pinocytotic vesicles and coated pits and vesicles were observed in the synthetic SMCs (Figure 2C).

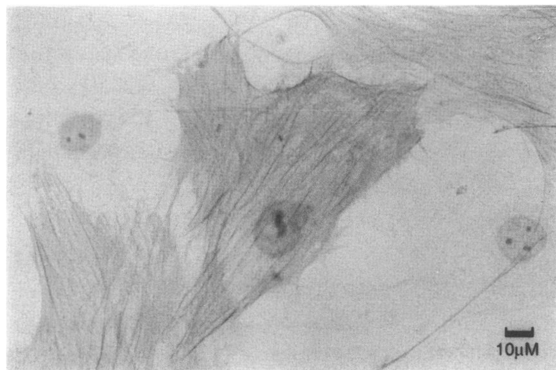


Figure 1. *p-SMC 3-22AA stained with anti-α-SM1. Formalin-fixed p-SMCs were blocked with 5% bovine serum albumin (BSA) before treatment (60 min; 37°C) with α-SM-1, diluted 1:400 in PBS, which contained 1% BSA. They were incubated (30 minutes, room temperature) and washed three times with PBS, 1% BSA. Myofilaments were clearly visible in 10% to 20% of the cells.*

Growth of Plaque-derived Cells

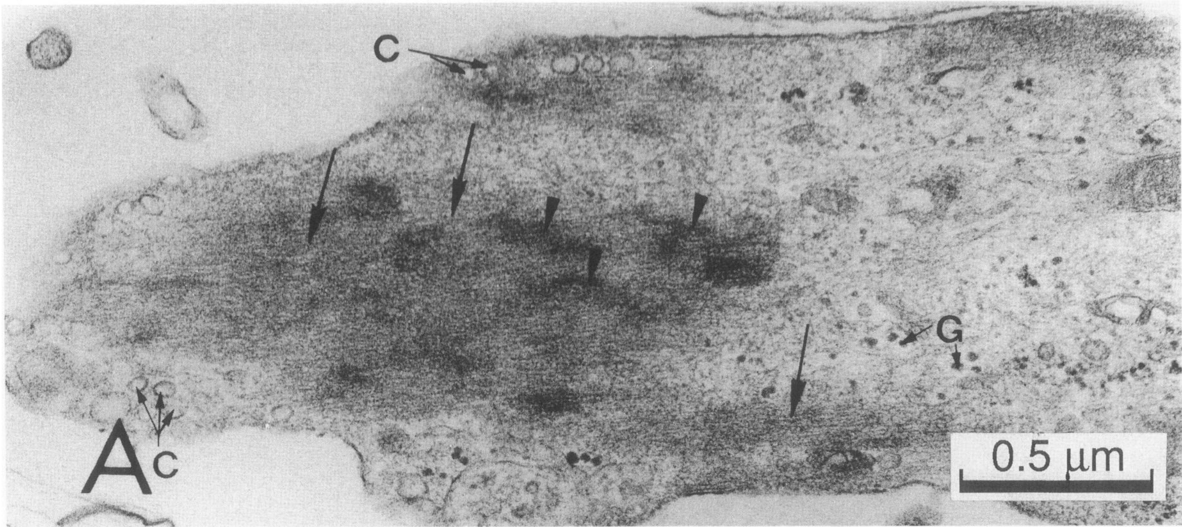
The p-SMC 3-22AAs were plated in T-25 flasks at an initial density of 2×10^5 cells/flask and grew in log phase from 24 to 216 hours. The doubling time for this strain was 65.7 hours (Figure 3). The cells underwent 25 population doublings from the time they were removed from the collagen gel.

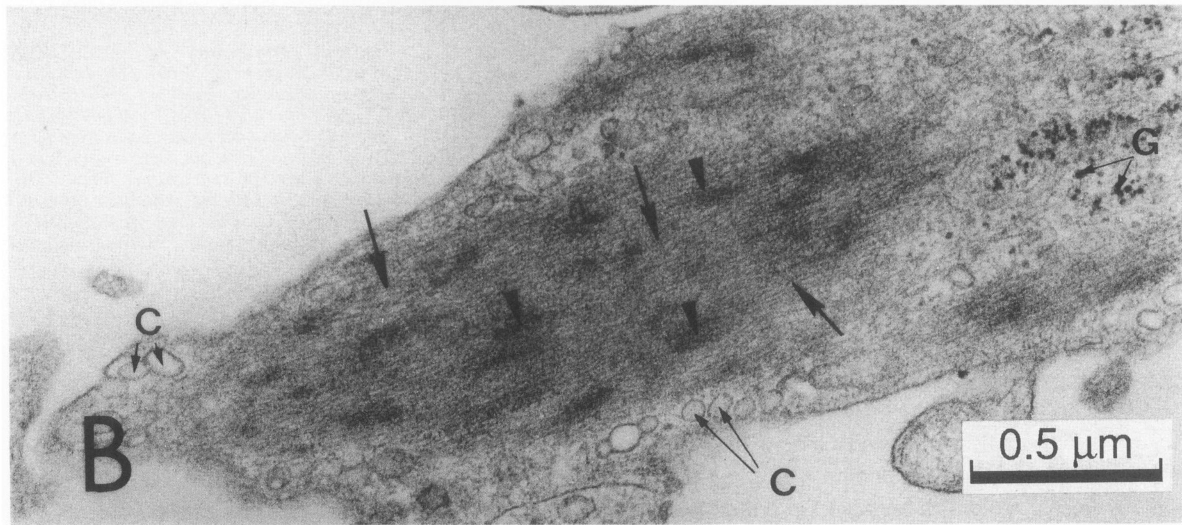
Serum Responsiveness

Both p-SMC 3-22AAs and HV-SMCs were grown in the presence of varying FBS concentrations (0%, 0.1%, 1%, 5%, and 10%) in the absence of any other macromolecular supplements. The p-SMCs showed a serum dependency comparable to that of the normal SMCs at serum concentrations between 1% and 10%; however at 0.1% serum the plaque SMCs had a slight growth advantage (not shown).

Transfection–Nude Mouse Tumor Assay

NIH3T3 cells cotransfected with pSV_2neo and DNA from p-SMC 3-22AA yielded an invasive tumor under the dorsal surface in one of five nude mice after injection with approximately 5×10^6 transfectant cells. The tumor grew





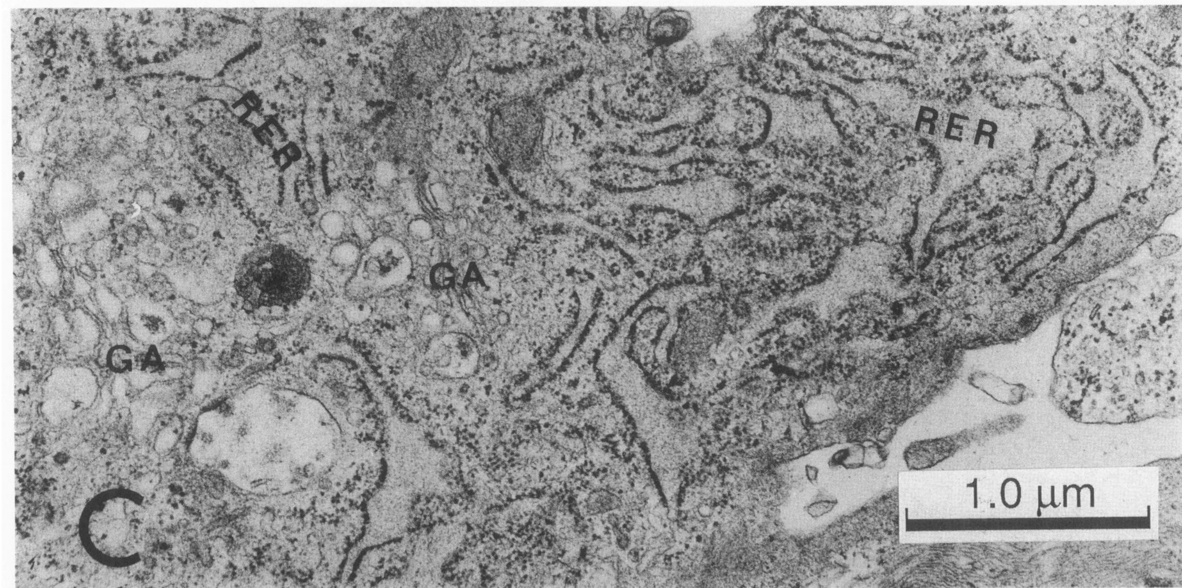


Figure 2. *p-SMC 3-22AA are categorized ultrastructurally as typical contractile* (**A** *and* **B**), *or modulated (synthetic) cells* (**C**). **A** *and* **B** *are serial sections of a portion of a cultured p-SMC showing myofilaments (arrows), dense bodies (arrowheads), caveolae (C), and glycogen (G), which are all characteristic of SMC.* **C** *shows a 'modulated' or 'synthetic' SMC. Such cells have extensive RER, a prominent GA, few or no myofilaments, and represent the majority of the cells in this culture.*

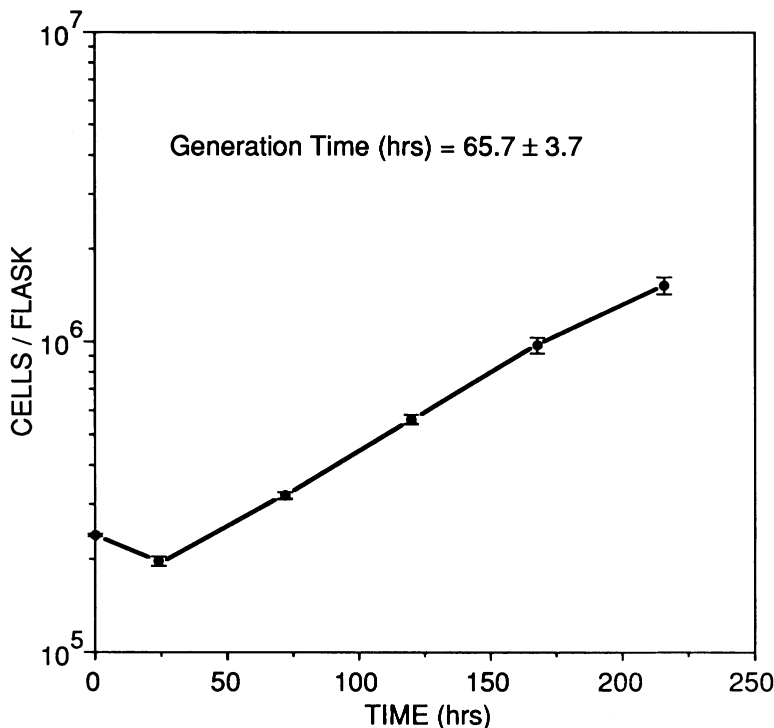


Figure 3. *Growth curve of p-SMC 3-22AA grown in the presence of 10% FBS. Cells were plated at an initial density of 2 × 10^5 cells/T-25 flask in DMEM-Hepes + 10% FBS. Starting at 24 hours after plating, cells from three flasks, chosen at random, were counted on a Coulter electronic cell counter (Model ZB). Cells were then counted at 48-hour intervals, from days 3 to 17. The data are expressed as mean cell counts/flask (±standard deviation).*

relatively slowly, first detectable 90 days after injection of cells, and reached approximately 10 mm in diameter 70 days later. The positive control, T24-human bladder carcinoma DNA transfectants, elicited tumors in all five mice within 23 days after injection, and grew to about 15 mm within 2 weeks; no tumors appeared in any of the five mice in the negative control group by 22 weeks (ie, mice injected with NIH3T3 DNA transfectants). The T24-associated tumors had a soft consistency and a smooth surface. They were histologically similar to fibrosarcomas (not shown).[2,20] In contrast, the p-SMC–associated tumor grossly had a hard consistency and an irregular surface

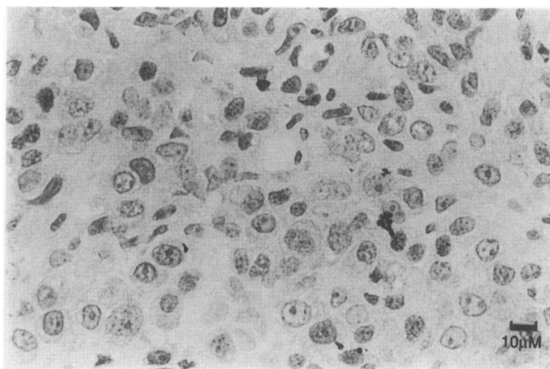


Figure 4. *Invasive tumor taken from a female nu^+/nu^+ mouse injected subcutaneously with 5 × 10^6 p-SMC 3-22AA DNA-transfected NIH3T3 cells. The tumor was fixed in formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.*

and adhered to the surrounding subcutaneous tissue and the overlying skin. Unlike the T-24–derived tumors, the plaque-derived tumor was invasive. Histologically the plaque-derived tumor was centrally necrotic; the periphery consisted of undifferentiated, loose, round to polygonal mesenchymal cells, displaying marked cellular and nuclear pleiomorphism (Figure 4).

DNAs of the human p-SMC–associated and T24-associated nude mouse tumors were digested with Bam HI, Southern blotted, and hybridized to human SMC genomic DNA. Hybridization patterns demonstrate the presence of human repetitive sequences in both sets of tumors (Figure 5). Hybridization of this blot to a *myc* probe did not detect a newly introduced human *myc* signal (not shown).

In contrast to the results with NIH3T3 cells transformed with DNA from p-SMC 3-22AA cells, no tumors arose in any of five nude mice injected with p-SMC 3-22AA cells.

Enhanced myc Expression by Human Plaque SMCs

Poly A(+) RNA from various types of SMC was Northern blotted and hybridized to a *myc* probe revealing a 2- to 6-fold and a 6- to 11-fold enhanced expression of *myc* by p-SMC, compared to HA-SMC and HV-SMC, respectively. The range reflects the facts that 1) *myc* expression for any given cell strain varied, depending on the partic-

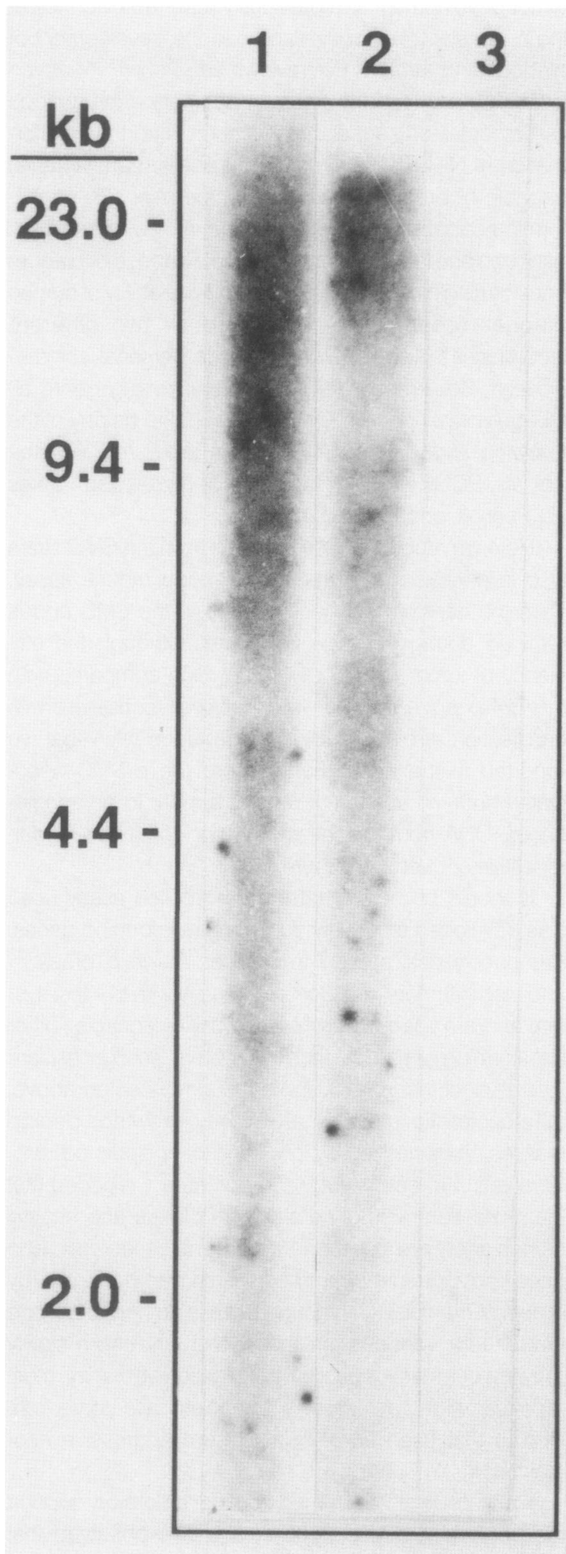


Figure 5. *Southern blot analysis of tumors arising in nude mice following injection of 5 × 10^6 NIH3T3 transfectants. Lanes: (1) p-SMC 3-22AA-associated tumor DNA; (2) T24-associated tumor DNA; (3) untransfected NIH3T3 cell DNA. Twenty micrograms of Bam HI-digested DNA was loaded per lane. Hybridization was to ^{32}P-labeled human SMC-genomic DNA in the presence of 50% formamide at 43°C.*

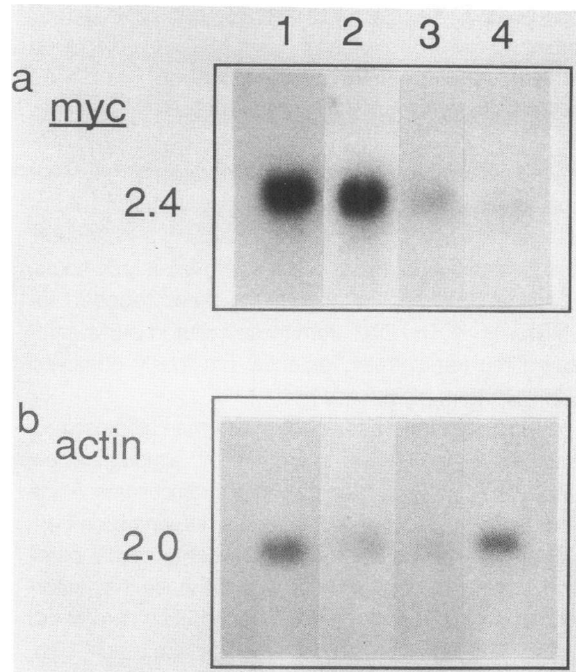


Figure 6. *Northern blot analysis of p-SMC 3-22AA (lane 1); p-SMC 9-12AA (lane 2); HA-SMC (lane 3); and HV-SMC (lane 4). Polyadenylated RNAs (2 μg) were size fractionated by agarose gel electrophoresis and transferred to nitrocellulose.* **a** *is an autoradiogram of a blot probed with human* c-myc *exon III in the presence of 45% formamide at 43°C.* **b** *is an autoradiogram of the same blot rehybridized to a β-actin probe after the* myc *signal was washed off. Hybridization conditions were the same as above. When normalized to β-actin, the p-SMC 3-22AA and p-SMC 9-12AA show a 2- and 3.7-fold enhanced expression of* myc *over HA-SMC, respectively, and a 6- and 11-fold enhanced expression over HV-SMC, respectively; as determined by scanning densitometry.*

ular RNA isolate assayed, and 2) p-SMC 9-12AA cells exhibited a higher level of *myc* mRNA than p-SMC 3-22AA. Figure 6a shows a twofold enhancement of *myc* expression by p-SMC 3-22AA cells (lane 1) and a 3.7-fold enhancement by p-SMC 9-12AA (lane 2) compared to that of HA-SMCs (lane 3), when normalized to expression of β-actin (Figure 6b). Expression of *myc* by p-SMC 3-22AA and p-SMC 9-12AA cells was 6- and 11-fold greater than by HV-SMCs (lane 4), respectively. Southern blot analysis of p-SMC DNA indicated that the *myc* gene was not amplified or rearranged, and the normal karyotype showed the absence of any gross chromosomal translocations in p-SMC 3-22AA cells (data not shown). In contrast to *myc*, there were no detectable differences between p-SMC 3-22AA and HV-SMC cells in expression of the genes for *Ha-ras*, *N-myc*, *L-myc*, *fos*, *sis*, *myb*, IGF II, IGF I receptor, EGF receptor, LDL receptor, or HMG-Co-A reductase (not shown).

Discussion

We describe a reliable, reproducible method for culturing SMCs derived from human atherosclerotic plaque. Data

from two p-SMC strains are presented here. Both p-SMC strains (p-SMC 3-22AA and p-SMC 9-12AA) displayed overexpression of the *myc* protooncogene. The p-SMC 3-22AA strain was characterized extensively. The cells were diploid, exhibited a normal karyotype, and displayed no obvious chromosomal aberrations. They were contact inhibited, had a finite life span in culture of approximately 25 doublings before reaching senescence, and exhibited a doubling time of approximately 66 hours, which is consistent with doubling times reported for SMCs.[5,6,21,23] The DNA from these cultured p-SMCs retained the transforming potential previously observed with DNA from plaque tissue.[2]

Historically successful culture of human atherosclerotic p-SMCs has proved to be difficult, although a few laboratories[5,7–10] reported growth of such cultures. Aside from the difficulty in obtaining viable human tissue from surgery, plaque-derived SMCs present technical problems when grown in tissue culture. Because they are irregularly shaped and often lipid laden, they tend not to attach to the substrate but rather float off the culture dish. This problem has been circumvented by embedding plaque explants in a collagen matrix, which encourages outgrowth of SMCs from the tissue while offering solid three-dimensional support.[11,24]

The results of staining with antibody to SMC α-actin were consistent with the ultrastructural findings. Approximately 10% to 20% of the cultured p-SMCs stained with anti-α SM-1 (Figure 1). When SMCs are placed in tissue culture they undergo modulation from a contractile to a synthetic state, at which time there is a decrease in the number of myofilaments and an increase in organelles involved with synthesis.[21,23,25,26] A switch in actin isoform expression also occurs.[21,23,27–29] *In vivo*, plaque SMCs differ morphologically from normal SMCs in a similar manner, ie, the SMC of the plaque contain a more-developed RER and GA and a less-developed microfilamentous apparatus.[25,28,30] The actin isoform profile of *in vivo* plaque SMC is also different from healthy artery SMCs. Actin of nondiseased SMCs shows a predominance of the α isoform, whereas in intimal thickening and in atheromatous plaque there is a typical switch to the β form with small amounts of the γ form.[27–30] In one study of human atheromatous plaques, the α isoform accounted for only 10% of total actin.[28] Our observations that the human p-SMC cultures exhibit modulated morphology is probably the result of both their plaque origin and growth *ex vivo*.

Proliferation of SMCs is a key event in atherosclerotic plaque development. The monoclonal hypothesis[1] proposes that plaques, which behave as monoclonal growths, are benign SMC tumors of the artery wall and that chemical mutagens would be expected to play a role in plaque formation comparable to the role they play in tumor development. We previously reported evidence at the molecular level for similarities in the development of plaques and tumors[2] (for reviews see Penn[31,32]) as follows. DNA isolated from human coronary artery plaque tissue contained sequences that completed the transformation of NIH3T3 cells, resulting in cells (transfectants) capable of producing tumors in nude mice. The transforming capability was shown to be serially transmitted. Although one laboratory reported no evidence of plaque-transforming elements,[3] transforming capability of human plaques recently was confirmed by two different laboratories[4] (also R. Zwijsen, written personal communication, September 1990). The transforming gene, although not yet identified, was shown not to be any of the following: *N-ras*, *K-ras*, *Ha-ras*, *erb A*, *erb B*, *fes*, *src*, *mos*, *abl*, *sis*, PDGF A-chain, *fos*, *myb*, or *myc*[2] (also Parkes JL, Penn A, unpublished results).

Here we report that DNA isolated from a p-SMC strain also is positive in the transfection-nude mouse assay. Thus the transforming gene resides in the SMC and is retained during passage in culture. Although the efficiency of tumor formation was not high compared with the T24-positive controls, it is, however, consistent with transfection efficiencies of human plaque DNA that we reported in the past.[2] Furthermore the p-SMC–associated nude mouse tumor did not appear to be spontaneous; DNA from this invasive tumor contained human repetitive sequences (Figure 5).

It should be noted that the transfection assay used was developed to identify dominant transforming genes. *Ras* oncogenes, present in bladder,[33] skin,[34] breast,[35] and lung[33] tumors, are identified readily with this test system and are used most often as positive controls. Other transforming genes tested in this system, such as *hst*, are less efficient than *ras*.[36] Unlike the tumors noted above, atherosclerotic plaques are slowly growing, benign masses characterized by low levels of episodic cell proliferation. In a recent study, Gordon et al[37] reported that the replication rates of cells in both plaque and healthy human artery are less than 1%. They note that replication rates in malignant human neoplasms can be higher by orders of magnitude. Although there is no hard evidence that directly supports the theory that DNA from slowly growing cell masses has low transfection efficiency, most reports of high transfection efficiencies have come from studies that used DNA isolated from aggressive neoplasms.

Data previously published from our laboratory support the observations of Gordon et al.[37] We observed that small but significant increases in SMC proliferation precede plaque-size increases in carcinogen-injected cockerels.[38,39]

Northern blot hybridizations showed a two- to sixfold enhancement of *myc* expression (when normalized to β

actin) by p-SMCs when compared to healthy human aortic SMCs, and a 6- to 11-fold enhancement of *myc* expression when compared to healthy human saphenous vein SMCs (Figure 6). The level of *myc* expression for any given cell strain varied (up to two times), depending on the particular RNA isolate assayed. However p-SMC 9-12AA cells always expressed more *myc* mRNA than p-SMC 3-22AA cells, and both p-SMC strains always expressed at least two times more *myc* mRNA than HA-SMCs. This is of particular interest because this protooncogene is overexpressed by tumor cells of some leukemia and lymphoma patients,[40] as well as by cells from human breast[41] and prostate carcinomas.[42] Recently a modest enhancement in *myc* expression was observed in cultured aortic SMC of spontaneously hypertensive rats compared with Wistar-Kyoto normotensive rats.[43] Enhanced expression of the *myc* gene in these and other systems has been related to 1) rearrangements involving insertion of new DNA sequences upstream from the c-*myc* 5' coding region, replacing the normal c-*myc* transcriptional promoter,[40] 2) *myc* gene amplification of 2 to 15 times,[41,42] or 3) chromosomal translocations whereby an immunoglobulin enhancer activates the translocated *myc* gene.[44] In many cases, however, no obvious genetic changes were detected. The mechanism of the p-SMC–enhanced *myc* expression is being studied in this laboratory. The normal karyotype of p-SMC 3-22AA suggests the absence of any gross chromosomal translocation. In addition, Southern blot analysis of both p-SMC strains indicates the absence of *myc* amplification or rearrangement (not shown).

A few notes of caution should be mentioned. First, although mRNA was isolated from cells grown in medium supplemented only with 10% FBS, the initial outgrowth of SMCs from the plaque tissue explants occurred in medium supplemented with a hormone-growth factor cocktail. Because the control cells, HA-SMC and HV-SMC, were not similarly treated, a possible influence of these hormones and growth factors on *myc* expression cannot be ruled out. Second, because it was not possible to procure healthy artery samples from the same patients from whom plaque was obtained, one cannot rule out the possibility that individual variation might account for the difference observed between p-SMC and control cells. However both p-SMC strains, which were derived from different patients, expressed more *myc* mRNA than either HA-SMC or HV-SMC derived from two other patients. The difference in *myc* expression between HA-SMC and HV-SMC probably reflects differences between arterial and venous SMCs, as well as individual variation. Third, a problem inherent in cell culture studies is that culture conditions may result in artifactual selection of a unique population of cells with specific growth characteristics not necessarily representative of the original population. Although this cannot be precluded as a factor contributing to the unique properties of the p-SMC, these strains have a finite life span of ~25 population doublings, and studies were performed with early passage cells.

Although the *myc* protooncogene is overexpressed by the p-SMC, it does not appear to be the atherosclerotic plaque-transforming gene because no newly introduced human *myc* sequences were detected in the p-SMC DNA-associated nude mouse tumor. Similarly *myc* is not responsible for transforming potential present in cockerel arteriosclerotic plaque DNA (Penn et al, manuscript submitted for publication).

Cell transformation is a multi-step process involving two or more distinct genetic events (for reviews see references 45 to 47). Thus it should be emphasized that the p-SMCs are not themselves fully transformed. They have a finite life span in culture, exhibit a serum requirement consistent with normal SMCs and most importantly, do not form tumors when injected into nude mice. However they show an enhancement in mRNA for the protooncogene *myc* and their DNA can complete the transformation of NIH3T3 cells, which subsequently elicit tumor formation in nude mice. Thus, these p-SMC cells, with their modulated morphology and reduced α- actin content are, as the monoclonal hypothesis predicts, at least partially transformed.

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