Cell volume and rate of proliferation, but not protein expression pattern, distinguish pup/intimal smooth muscle cells from subcultured adult smooth muscle cells

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Abstract. Smooth muscle cells from neonatal rats and from injured blood vessels grow with a characteristic cobblestone morphology that distinguishes them from adult smooth muscle cells. This has led to the proposition that there are two distinct types of smooth muscle cells with different proliferative capacity. Here we systematically compare the properties of subcultured adult smooth muscle cells in culture and clonal lines of cobblestone smooth muscle cells from both neonatal rats and injured vessels. The cobblestone smooth muscle cells have a significantly smaller average cell volume, estimated using two different flow cytometry measurements. However, the two types of smooth muscle cells have indistinguishable protein expression patterns when the levels of more than 20 different proteins (including cytoskeletal proteins, matrix proteins, cytokines, cytokine receptors, adhesion molecules and enzymes) are measured by quantitative immunofluorescence. Furthermore, in contrast to previous observations, we demonstrate that both types of smooth muscle cells secrete a powerful mitogenic activity. The higher cell density achieved by the cobblestone smooth muscle cells in culture was responsible for the earlier reports that this mitogenic activity was secreted only by cobblestone smooth muscle cells. We conclude that many of the differences seen between cobblestone smooth muscle cells and adult smooth muscle cells in vitro (proliferation rate, morphology, protein expression pattern, secretion of mitogenic activity) could be attributable to a stable difference in the median cell volume of the cultures.

INTRODUCTION

Inappropriate proliferation of vascular smooth muscle cells (SMCs) is a component of a variety of vascular diseases, including atherosclerosis, restenosis following angioplasty, as well as other forms of vascular trauma such as stenting, and transplant vasculopathy. In the healthy vessel wall, the SMCs are largely restricted to the medial layer of the vessel wall, where they express high levels of proteins involved in contraction (such as the smooth muscle-specific isoforms of

myosin and actin). As a result, they have been described as having a 'contractile phenotype' (Chamley-Campbell *et al.* 1979; Chamley-Campbell & Campbell 1981; Thyberg *et al.* 1985). Unfortunately, under certain circumstances, which are not well defined, SMCs migrate into the vessel intima and can proliferate there (Stary 1989; Schwartz *et al.* 1995a). The resulting expanded intima may then become inflamed and the site for deposition of lipoproteins sequestered from the circulation during the later stages of the atherogenesis process (Schwartz *et al.* 1995a).

Many excellent histological studies have unequivocally demonstrated that the intimal SMCs differ from the medial SMCs *in vivo* (Gabbiani *et al.* 1982, 1984; Kocher *et al.* 1985; Majack & Bornstein 1987). The intimal SMCs express lower levels of the proteins that compose the contractile apparatus (Gabbiani *et al.* 1982, 1984; Kocher *et al.* 1985) and higher levels of many matrix proteins and cytokines (Majack & Bornstein 1987). As a result, the intimal SMCs have been described as having a 'synthetic phenotype' (Chamley-Campbell *et al.* 1979; Chamley-Campbell & Campbell 1981; Kocher *et al.* 1985; Thyberg *et al.* 1985; Majack & Bornstein 1987).

Two hypotheses have been developed to describe the SMC population dynamics during the formation of an expanded intima: the first, termed the 'phenotypic transition hypothesis', was proposed almost 20 years ago by Chamley-Campbell and Campbell (Chamley-Campbell *et al.* 1979, 1981; Chamley-Campbell & Campbell 1981; Campbell & Campbell 1985). They proposed that all the medial SMCs in the contractile phenotype are functionally equivalent and are, at least in principle, capable of undergoing a phenotypic transition to the synthetic phenotype. This phenotypic transition, they suggested, is a necessary prerequisite for subsequent proliferation (Chamley-Campbell *et al.* 1981).

An alternative hypothesis was put forward by Majesky & Schwartz (1990): they postulated that the medial SMCs were structurally, and hence functionally, heterogeneous and that a subpopulation of medial SMCs were predisposed to proliferate in response to an appropriate stimulus. Consistent with this hypothesis they demonstrated that SMCs cultured from the neointima that forms 14 days after injury to the rat carotid artery have a distinct cobblestone morphology that resembles SMCs cultured from newborn, or pup, rats (Schwartz *et al.* 1990). Interestingly, these cobblestone SMCs were able to proliferate in the absence of exogenous mitogens. Majesky and Schwartz suggested that a small population of these neonatal cells persisted in the adult media, and that selective proliferation of these cells was responsible for the formation of an expanded intima (Majesky & Schwartz 1990; Schwartz *et al.* 1990, 1995a,b). This has been termed the 'stem cell hypothesis'.

A number of independent lines of evidence support each hypothesis. Much of the data supporting the phenotypic transition hypothesis has been derived from studies of medial SMCs from adult rat aorta when they are placed into cell culture. All of the cells that adhere to the culture plastic undergo a phenotypic transition that either precedes or accompanies proliferation (Chamley-Campbell *et al.* 1981; Grainger 1992). In contrast, studies of SMC culture lines established by dilute plating have provided evidence for both structural and functional diversity among SMCs (Hall *et al.* 1991; Grainger 1992; Bochaton-Piallat *et al.* 1993; Lemire *et al.* 1994).

Another important line of evidence stems from the observation originally made by Benditt & Benditt (1973) that the cells in human atherosclerotic plaques all express a single allele of the X-linked gene G6PDH in women heterozygous at this locus. They interpreted this observation as evidence that the intimal SMCs were derived by the proliferation of a single SMC. Initially, this observation was used to support the stem cell hypothesis, but two recent studies (Chung *et al.* 1998; Murry *et al.* 1997) have caused a substantial re-evaluation of the data generated by Benditt & Benditt (1973). Most importantly, Chung *et al.* (1998) concluded that the healthy artery was composed of a mosaic of developmental clones (up to 4 mm in size) all expressing a single allele of X-linked markers. The observation that all the SMCs in

the intima express the same X-linked marker therefore only implies that all the intimal SMCs were derived from a single developmental clone in the media. Since the developmental clones may be as large as the regions of expanded intima, the observations of Benditt & Benditt (1973) are consistent with both the phenotypic transition model and the stem cell model.

We have adopted an alternative strategy for comparing these two models of SMC population dynamics during intimal expansion. The two models predict that a different SMC culture type will be most similar to the intimal SMC *in vivo*. According to the phenotypic transition model, the subcultured adult medial SMC will be most similar to the intimal SMC *in vivo* (Chamley-Campbell *et al.* 1979, 1981), whereas according to stem cell model the cobblestone morphology SMC will be most similar to the intimal SMC (Majesky & Schwartz 1990; Schwartz *et al.* 1990, 1995b). We have therefore compared the two types of SMC culture *in vitro*, to determine which (if either) most closely resembles the published properties of the intimal SMC *in vivo*.

MATERIALS AND METHODS

Isolation and culture of adult rat medial SMCs

Adult rat medial SMCs were cultured after enzymatic dispersion of the aorta from three–six male Wistar rats, 12–20 weeks of age, exactly as previously described (Grainger *et al.* 1991). After dispersal, the cells were resuspended at 4×10^5 cells/ml in medium M199 + 10% FCS and plated out into culture flasks at 8×10^4 cells/cm². The cells were given fresh medium M199 + 10% FCS after 24 h and replaced every 48 h thereafter, until the culture became confluent 5–6 days after dispersal. The cells were subcultured every 4 days, following release with trypsin/EDTA, at a 1:2 dilution in DMEM + 10% FCS.

Culture of cobblestone morphology clonal cell lines

Cobblestone morphology clonal cell lines were established by dilute plating as described previously (Grainger 1992; Kemp *et al.* 1995). Frozen vials of each clonal cell line (frozen after at least 50 divisions in cell culture following the initial cloning) were thawed and the culture re-established in DMEM + 10% FCS. Cells were subcultured whenever they became confluent, as for the subcultured adult medial SMC cultures. The contents from each flask were diluted in the ratios 1:5 (BOB), 1:3 (BET) and 1:2 (YER) with DMEM + 10% FCS. The BOB and BET clones were divided every three days. The YER clone was divided every three to four days.

Cell counting

The number of cells in a flask, or well, was determined by releasing the cells with trypsin/EDTA and counting in a haemocytometer, exactly as previously described (Grainger *et al.* 1991). Unless stated otherwise, cells were cultured in 24 well plates at the same cell density as in flasks and triplicate wells were counted at each time point. For each well at least 100 cells were counted. Where cell counts were made at two different times during exponential growth, the cumulative population doubling time (CPD) was estimated according to the following formula:

$$\text{CPD} = (t_2 - t_1)(\log 2)/(\log n_2 - \log n_1),$$

where t_1 and t_2 are the times (in hours) after subculturing at which the first and second counts, respectively, were made and n_1 and n_2 are the mean numbers of cells per well present at the first and second counts, respectively. For most of the cell cultures described in this paper, it

has previously been shown that the fraction of cells that are actively progressing through the cell cycle is high (> 80%; for example, Grainger 1992). As a result, the CPD is an estimate of the average cell cycle transit time of the cells in the culture.

Estimation of cell volume by flow cytometry

Two different methods of estimating cell volume by flow cytometry were adopted. Cells (at least 2×10^4), released from the substrate as for subculturing, were added to an equal volume of DMEM + 10% FCS to inactivate the trypsin and analysed by flow cytometry within 10 min of release. The first method used relies on the fact that larger cells have (on average) a greater forward scatter. The height of the forward scatter pulse depends on both the cell size and the refractive index of the cells. Provided the refractive index remains constant, the height of the forward scatter pulse is proportional to cell size. The exact relationship between forward scatter pulse height and cell volume is given by the formula

$$\text{FS-H} = kd^{(3-w)},$$

where FS-H is the forward scatter pulse height, k is a constant of proportionality, w is a constant related to the width of the laser beam in the flow cytometer, and d is the diameter of the cell. Using spherical beads of known diameter, it was determined that under the conditions of our experiments, the condition $0.5 < w < 1$ applied, suggesting FS-H was approximately proportional to d^2 .

The second method used to estimate cell volume is a time-of-flight measurement, which determines the time taken for a cell to pass through the laser beam in the cytometer. Cells were incubated for 1 h with 1 $\mu\text{g/ml}$ freshly prepared fluorescein diacetate at 37 °C immediately prior to detachment for determination of cell volume. The signal pulse width (FL1-W) is related to cell diameter by the following relationship:

$$\text{FL1-W} = k'd + w',$$

where k' is a constant of proportionality, w' is a constant related to beam width and d is the cell diameter.

Immunofluorescence staining

The levels of 20 different proteins (selected to represent a broad range of protein markers including cytokines, cytokine receptors, extracellular matrix proteins, cytoskeletal proteins, enzymes and enzyme inhibitors) were measured by quantitative immunofluorescence analysis. Cells from various cultures were plated onto glass 16-well chamber slides by releasing the cells from confluent culture flasks with trypsin/EDTA, diluting the cells in DMEM + 10% FCS as for subculture and plating in a volume of 140 μl per well. Slides were then fixed for 90 s in ice-cold 70% ethanol/1% glacial acetic acid either in exponential growth or just as confluence was re-attained. All indirect immunofluorescence staining was carried out according to the protocols and recommendations made by Mosedale *et al.* (1996). Briefly, fixed cells on the chamber slides were rehydrated and non-specific binding of proteins to cells reduced by blocking with Tris-buffered saline (TBS; 10 mM Tris, 154 mM NaCl pH 7.4) containing 3% fatty-acid-free bovine serum albumin (TBS + 3% FAF-BSA) for 30–60 min at room temperature. The TBS + 3% FAF-BSA was aspirated and the cells incubated with 25 μl primary antibody at the concentration given below in TBS + 3% FAF-BSA at 4 °C overnight. Primary antibodies recognizing the following proteins were used: goat anti-monocyte chemoattractant protein-1 (MCP-1), goat anti-macrophage

inflammatory protein-1 α (MIP-1 α), goat anti-tumour necrosis factor- α (TNF α) and mouse anti-intercellular adhesion molecule-1 (ICAM-1) (all at 10 μ g/ml), chicken anti-transforming growth factor- β 1 (TGF β 1) 25 μ g/ml, goat anti-TGF β 2 and goat anti-TGF β 3 (50 μ g/ml) (all from R & D Systems Ltd, Abingdon, UK); Mouse anti-collagen II (21 μ g/ml), mouse anti-collagen IV (69 μ g/ml) and mouse anti-osteopontin (112 μ g/ml) (all from National Institute of Health Developmental Studies Hybridoma Bank, University of Iowa); mouse anti-calponin (44 μ g/ml) and mouse anti-smooth muscle alpha actin (10 μ g/ml) (Sigma-Aldrich, Poole, UK); Rabbit anti-collagen I (1:100 dilution of serum) (Chemicon, Harlow, UK); rabbit anti-TGF β Type I receptor (1 μ g/ml) (Santa Criz Biotechnology Inc., Santa Cruz, CA); rabbit anti-TGF β Type II receptor (10 μ g/ml) (Upstate Biotechnology, Lake Placid, NY); goat anti-plasminogen activator inhibitor-1 (PAI-1) (50 μ g/ml) (American Diagnostica, Greenwich, CT); sheep anti-tissue inhibitor of metalloproteinases-2 (TIMP-2) 1:100 dilution of serum (Biogenesis, Poole, UK). Other antibodies were generous gifts used at 1:100 dilution of the rabbit antisera: latent TGF- β binding protein (LTBP) (Dr L. Wakefield), ubiquitin conjugase M1 (ubc-M1) (Dr H.-P. Hauser), osteopontin (Dr L. Fisher), TIMP-2 (Prof. G. Murphy) and smooth muscle myosin heavy chain (SM-MHC) (56 μ g/ml) (Prof. U. Groschel-Stewart). Slides were washed 3 $\times$ 3 min with TBS and incubated with 25 μ l fluorescein-labelled second antibody in TBS + 3% FAF-BSA at room temperature for 6 h. The following FITC-labelled secondary antibodies were used: anti-chicken IgY (Jackson ImmunoResearch, West Grove, PA) at 50 μ g/ml; anti-goat IgG (Jackson) at 20 μ g/ml; anti-mouse IgG (Sigma) at 20 μ g/ml; anti-rabbit IgG (Sigma) at 50 μ g/ml; anti-sheep IgG (Jackson) at 20 μ g/ml. The slides were then washed as above followed by a short (less than 5 s) rinse with MilliQ water, allowed to air dry at room temperature and mounted in Citifluor AF1 within 1 h. All stained specimens were allowed to stand for at least 18 h in the dark following mounting prior to image capture.

For each primary antibody used, a replicate slide was prepared that was treated as above, with the exception that the primary antibody was omitted. Images of the stained cells (incubated with primary antibody) and control cells (with primary antibody omitted) were captured using an Olympus Provis AX electronic microscope connected to a Power Macintosh 8500, running OpenLab image analysis software (Improvision, Coventry, UK). All images were acquired under identical conditions. Four fields of view were captured from both the control and stained cells for each primary antibody used. The OpenLab software reported the mean pixel grey value (after background subtraction).

All cells (whether incubated with primary antibody or not) were incubated with a solution of secondary antibody containing propidium iodide (PI; 100 ng/ml final concentration) to stain the nuclei. When each field of view was captured under FITC illumination conditions (NIBA filter block: λ_{ex} = 470–490 nm, dichroic mirror = 505 nm, λ_{em} = 515–550 nm) the same field of view was also captured under TRITC illumination conditions (NG filter block: λ_{ex} = 530–550 nm, dichroic mirror = 570 nm, λ_{em} > 590 nm), which visualizes the PI-stained nuclei. For each image, the number of cells composing the field of view was estimated by counting the number of nuclei in the field of view. This assumes that polykaryosis is rare within the population. Previously, we demonstrated that polykaryotic cells compose less than 5% of the population in each of the cell cultures used (Grainger 1992).

Swiss 3T3 fibroblast bioassay for transferable mitogenic activity

Swiss 3T3 murine fibroblasts (a gift from E. Rozengurt) were subcultured at 1:3 dilution in DMEM + 10% FCS and used between passages 12 and 20. These fibroblasts were selected because their responses to mitogenic stimulation have been extensively studied for more than a decade, and they are known to respond to a wide variety of different growth factors (for example, Rozengurt 1986). Cells were plated into 24 well plates (0.5 ml of diluted cells at

subculture per well), allowed to grow for 24 h in DMEM + 10% FCS at 37 °C, then washed once with SF-DMEM and incubated in fresh SF-DMEM for 16 h to render them quiescent. Triplicate wells were then re-stimulated with either DMEM + 10% FCS (positive control), SF-DMEM (negative control) or conditioned medium in the presence of 10 ng/ml IGF-1. After 8 h at 37 °C, 37 kBq or 1 µCi/ml [³H]thymidine (Amersham International, Little Chalfont, UK) was added to each well and the cells were harvested 30 h after stimulation. Cells were washed three times with ice-cold Dulbecco's PBS and then lysed in 0.25 ml 1% SDS and macromolecules were precipitated by the addition of 0.75 ml ice-cold 15% TCA. After incubation at 4 °C for at least 1 h, precipitated material was collected by ultrafiltration through GF/C filters, washing each well with four 1 ml washes of 7.5% TCA and passing the washings through the same filter. Filters were rinsed with 4 ml 96% ethanol and air dried before scintillation counting for incorporated tritium.

RESULTS

Medial SMCs were obtained from the thoracic and upper abdominal aorta of adult (12–20-week-old) Wistar rats by enzymatic dispersion (see Materials and Methods) and placed into cell culture in the presence of 10% FCS. By passage 7 in culture most, if not all, of the cells had a classical spindle morphology and the culture grew with a 'hills and valleys' appearance (Fig. 1). In all experiments, subcultured medial cells from three independent primary cultures were analysed.

The subcultured medial SMC cultures were compared with clonal SMC lines with cobblestone morphology. Three typical cobblestone clones (Fig. 1b–d), termed BOB (derived from the healthy media of the aorta of a 12-day-old Wistar rat), YER (derived from the healthy media of the aorta of a 12-week-old Wistar rat) and BET (derived from the neointima formed 14 days after balloon injury of the carotid artery of a 12-week-old Wistar rat) were analysed.

Here we characterize the six cultures (three of each type) for rate of proliferation, average cell volume, expression of a range of protein markers (including cytokines, receptors, matrix proteins, cytoskeletal proteins, enzymes and their inhibitors) and ability to produce a transferable mitogenic activity. Comparison of these properties should allow us to postulate which of the two cultures represents the best model of the intimal SMC *in vivo*.

Proliferation of two different SMC types in culture

The population doubling time of the six cultures was determined by cell counting, following release of the cells from the culture plastic using trypsin at various time points following subculture (Table 1). Consistent with previous data, at passage 7 the subcultured medial cells proliferated in 10% FCS with a population doubling time of 36 ± 3 h (Chamley-Campbell *et al.* 1981; Grainger *et al.* 1991; Kemp *et al.* 1991; Grainger 1992). For SMC culture C, as passage number increased the population doubling time gradually decreased (Table 1). With each successive passage a larger proportion of the cells in SMC culture C no longer had a spindle-shaped morphology but resembled cobblestone cells. SMC cultures A and B followed the same trend as SMC culture C, but cells resembling cobblestone SMC did not become evident until later passage numbers (> 12). For these two cultures, by passage 11 small patches of cells with cobblestone morphology were detectable. Beyond passage 12, the cells of cobblestone morphology begin to predominate, until by passage 20 all three cultures of subcultured medial SMCs were morphologically indistinguishable from the clones of cobblestone morphology cells. It should be emphasized that there was no possibility of cross-contamination between the cultures as separate bottles of media and trypsin/EDTA were used for each culture.

Figure 1. Morphology of adult subcultured medial SMCs and cobblestone SMC clones. Adult SMCs and SMC clones were subcultured into DMEM + 10% FCS. The figure illustrates typical morphologies at stationary phase. (a) Confluent subcultured medial SMC passage number 7, 96 h after subculture; (b) the BOB clone grown to confluence 72 h following subculture; (c) the BET clone grown to confluence 72 h following subculture; and (d) the YER clone 96 h after subculture (note that parts a–d are at the same magnification).

In marked contrast to the subcultured medial cells, two of the cobblestone cell clones (BOB and BET) proliferated much more rapidly with a population doubling time in the range of 18 ± 3 h in DMEM + 10% FCS, with no variation ($< 10\%$) from one passage to another. The third cobblestone culture (YER), however, did not behave stably when the culture was re-established from frozen vials. During the first three passages after re-establishing the culture, YER proliferated with a population doubling time of 40 ± 2 h. However, thereafter the rate of proliferation began to increase until the population doubling time reached 22 ± 4 h (similar to BOB and BET) after a further six passages (Table 1).

Table 1. Cumulative population doubling times and estimated median cell volumes for subcultured medial SMCs and cobblestone SMCs. The population doubling time and the median forward scatter pulse height were determined as described in Materials and Methods for three cultures of subcultured medial SMCs at passages 7, 9 and 11 after primary culture, and for three cobblestone morphology SMC clones at the second passage after re-establishing the culture from frozen cell stocks (*n*) and at each alternate passage thereafter

Intimal cell culture model	Passage number	Population doubling time CPD (h)	Cell volume (median FS-H)
SMC (A)	7	37	478
	9	38	435
	11	41	462
SMC (B)	7	31	436
	9	36	412
	11	32	438
SMC (C)	7	40	474
	9	37	453
	11	31	398
BOB	<i>n</i>	16	332
	<i>n</i> + 2	16	353
	<i>n</i> + 4	14	306
BET	<i>n</i>	22	370
	<i>n</i> + 2	22	378
	<i>n</i> + 4	19	409
YER	<i>n</i>	39	490
	<i>n</i> + 2	25	374
	<i>n</i> + 4	19	343

The effect of removing the exogenous growth factors (in the FCS) on SMC proliferation was also investigated. As anticipated, the adult medial SMC cultures ceased to increase in number after 48 h in serum-free medium (Fig. 2a,b). However, the cobblestone cells continued to proliferate, with a population doubling time of 79 ± 9 h in serum-free medium (Fig. 2c,d). Although they grew more slowly in the absence of serum than in the presence of 10% FCS and reached a lower saturation density due to an enlarged morphology, nevertheless, they continued to proliferate in the absence of exogenous mitogens (Fig. 2c,d). Consistent with previous reports (Schwartz *et al.* 1995b; Grainger 1992; Lemire *et al.* 1994), this suggested that the cobblestone clones could proliferate by producing an autocrine growth factor.

Analysis of cell size by flow cytometry

The cobblestone cells appear much smaller in culture than the medial SMCs. Furthermore, in each culture, the appearance of cobblestone cells coincided with an increased rate of proliferation. We therefore tested the hypothesis that the average size of the cells in the population was correlated with the proliferation rate of the culture.

To determine the relative volumes of each cell population, cells were released from the plastic and analysed by flow cytometry. Initially, the height of the forward scatter pulse was used to estimate cell volume, assuming each of the different SMC cultures had a similar refractive index (see Materials and Methods). Three replicate flasks of the BOB clone were analysed in this way to assess the reproducibility of the technique. The histogram of forward scatter pulse heights was very similar for all three flasks, with medians of 332, 353 and 306 (Fig. 3a,b shows a population

Figure 2. Proliferation of subcultured medial SMCs and the BOB clone. Subcultured adult medial SMCs and the BOB clone were subcultured into DMEM + 10% FCS. The medium was then removed and each well washed twice with SF-DMEM and incubated in either fresh DMEM + 10% FCS (10%) or SF-DMEM (SF). The cells in triplicate wells were then counted at 12–24 h intervals and the cell number per cm^2 plotted (squares: 10%; diamonds: SF). The data shown in (a) and (c) for proliferation in SF-DMEM are shown again in (b) and (d), respectively, with the y-axis expanded to demonstrate that proliferation continued for the BOB clone (d) but not for subcultured medial SMCs (b). Values are means $\pm$ SEM from triplicate determinations from each of two separate experiments.

with a median value of 306). This represents a coefficient of variation of 1.7%, suggesting the technique is highly reproducible.

The volume of BOB cells, estimated from the forward scatter pulse height, was analysed as confluence was attained at the end of each passage during which the population doubling time had been measured. The median forward scatter pulse height varied by approximately 10% between passages (see Table 1), suggesting that the cell volume of the cells composing the BOB clone were reasonably stable in cell volume through multiple passages. The volume of each of the other clones and the three adult medial SMC cultures were also estimated in the same way. The cells of the BET clone were similar in size to those of the BOB clone (Table 1), but the cells of the YER clone in the first few passages after the culture was re-established were very much larger (median forward scatter pulse height = 490) (Fig. 3c,d). However, through successive passages, the median forward scatter pulse height decreased until the cells were of a comparable size to the BET and BOB clones (Table 1).

For all three medial SMC cultures at passage 7, when almost all of the cells had a spindle morphology, the median forward scatter pulse height for all three populations was significantly larger than for the BOB and BET cells ($P < 0.01$, Student's *t*-test, Table 1). However, through the successive passages, the median forward scatter pulse height decreased for culture C,

Figure 3. Forward scatter pulse height (FS-H) distributions for the BOB and YER clones. Cells of the BOB clone (a,b) or the YER clone (c,d) were released from the culture plastic with trypsin/EDTA just as confluence was attained. The cells were diluted with DMEM + 10% FCS to give approximately 200 000 cells/ml and analysed for forward scatter pulse height (see Materials and Methods). For each clone 10 000 events were collected and examined on a two-parameter plot of forward scatter pulse height versus 90° scatter pulse height (SSC-Height) (b,d). The population of viable cells was clearly distinguishable from the debris, and the forward scatter pulse height distribution of this gated population is shown (a,c). Approximately 80% of the events fell within the gates shown for both clones. The median of the distribution was reported by the Lysys II software.

although by passage 11 the cells remained larger than the BOB and BET cells. For SMC cultures A and B the median forward scatter pulse height remained similar between passages 7 and 12.

Although the median forward scatter pulse height values shown in Table 1 agreed very well with the apparent cell size estimated by observation, it remained possible that the differences were due to differences in refractive index rather than in cell volume between the different cultures. An alternative flow cytometric method was therefore used to estimate cell volume, which relies on the time taken for the cell to pass through the laser beam of the flow cytometer (the median pulse width) and is independent of refractive index. There was a correlation between median forward scatter pulse height and median pulse width, suggesting that both are likely to be predominantly determined by cell volume (Fig. 4a). As expected, the pulse width was a less discriminating estimate of cell volume (being linearly related to cell diameter) than forward scatter pulse height (approximately related to the square of cell diameter).

Based on the median forward scatter pulse height as an estimate of cell volume, a very strong correlation between cell size and population doubling time ($r = 0.914$, $P < 0.0001$, Fig. 4b) was seen. This correlation holds equally tightly between the different culture types

Figure 4. Association between cell volume and rate of proliferation. (a) One 25 cm² flask of each culture (SMC cultures A–C and the BOB, BET and YER clones) was treated with fluorescein diacetate at 1 µg/ml final concentration (from a 1-mg/ml freshly prepared stock solution in acetone) at 37 °C for 1 h. The cells were then released with trypsin and the median forward scatter pulse height (FS-H) determined as described in the legend for Fig. 3. In addition, the median pulse width (FL1-W) was determined on the same gated population. The correlation between FS-H and FL1-W is shown, with Pearson's R correlation coefficient. Although the correlation coefficient does not reach statistical significance (due to the small number of data points), the estimated volumes using the two different methods follow an identical rank order. (b) Median forward scatter pulse height (FS-H) and cumulative population doubling time (CPD) values from Table 1 are plotted as estimates of cell volume and proliferation rates, respectively.

(small rapidly proliferating cobblestone cells versus larger slower proliferating spindle-shaped cells) and also across several passages of the same culture (the adult medial SMCs in culture C became progressively smaller and proliferated more rapidly with each successive passage). It should be emphasized that these observations provide no indication of whether rapid proliferation leads to small cell size or whether small cell size results in a more rapid proliferation rate.

Protein expression pattern of SMC cultures

Three separate cultures of both subcultured medial cells (cultures 1, 2 and 3, different from A, B and C analysed above) and cobblestone cells were analysed for their contents of various proteins. The YER clone was excluded from this analysis because of the unexpected instability in

proliferation rate over the first 10 passages following re-establishment of the culture from frozen stocks. A number of protein markers were selected for analysis, representing various classes of secreted proteins, cellular components and matrix molecules. In each case, the antibody used was known to react with either the rat or the mouse protein. The markers selected include growth factors and cytokines (MIP-1 α , MCP-1, TGF- β 1, 2 and 3 and TNF- α), cytokine receptors (Type I and Type II TGF- β receptors), cell adhesion molecules (ICAM-1), extracellular matrix proteins (Type I, II and IV collagens, LTBP and osteopontin), protease inhibitors (PAI-1 and TIMP-2), intracellular structural proteins (SM- α -actin, SM-MHC and calponin) and enzymes (ubcM1). The general metabolic enzyme ubcM1 (which conjugates ubiquitin onto cellular proteins destined for degradation) was included as a control, since this enzyme is expressed at constant, high levels in every cell type examined to date (data not shown). To assess accurately the relative amounts of each protein per cell, the technique of quantitative immunofluorescence (QIF) was used, as previously described for tissue sections (Mosedale *et al.* 1996). QIF was used to examine protein expression because it detects the proteins in their native conformation (as compared to Western blotting, which detects denatured proteins). As a result, QIF is more likely to pick up any reproducible differences in the two cell types, since post-translational modifications and differences in protein:protein interactions can be detected, in addition to changes in the protein concentration that would be detected by Western blotting.

All three cultures (1, 2 and 3) of subcultured medial SMCs showed a similar pattern of expression of all 20 proteins analysed. The cells expressed detectable levels of osteopontin, PAI-1, TIMP-2, type I TGF- β receptor, TGF- β 1 ligand, collagen type I and ubcM1. With the exception of ubcM1, all these proteins have been reported to be expressed at higher levels in intimal SMCs than in medial SMCs. Of these markers, type I TGF- β receptor, TGF- β 1 ligand and type I collagen are also expressed by healthy medial SMCs *in vivo*, at lower levels than in intimal SMCs. All these protein markers of intimal SMCs were expressed at somewhat higher levels during exponential growth than at confluence, except for ubcM1.

Consistent with previous reports, the subcultured medial cells contained detectable levels of the contractile proteins SM- α -actin and SM-MHC and very low levels of calponin (Kocher *et al.* 1985; Grainger 1992). These proteins are all expressed at high levels by medial SMCs *in vivo* but at lower levels by intimal SMCs *in vivo*. All three proteins were expressed at higher levels at confluence than during exponential growth.

A variety of proteins associated with the intima *in vivo* were not expressed at detectable levels by subcultured medial SMCs in culture. None of the cultures contained significant levels of MIP-1 α , MCP-1, ICAM-1 or TNF- α . All of these proteins are cytokines and cell adhesion molecules involved in regulating the inflammatory response. It is possible that the association of these proteins with the intima *in vivo* is due to the presence of infiltrating leucocytes, which are obviously absent during SMC culture *in vitro*. This staining pattern is summarized in Fig. 5.

With the exception of SM- α -actin, the two cobblestone cultures analysed had very similar protein expression patterns. Furthermore, the pattern shown by the cobblestone cultures was almost indistinguishable from that shown by the subcultured medial cells (Fig. 5). Strong expression of the intimal markers osteopontin, PAI-1, TIMP-2, type I TGF- β receptor and TGF- β 1 was noted, just as for subcultured medial cells, as well as high levels of ubcM1.

Levels of SM-MHC and calponin were also low but detectable, similar to subcultured medial cells. The cells of the BET clone contained SM- α -actin, at similar levels to the subcultured medial cells, but the cells of the BOB clone contained very low, scarcely detectable levels of SM- α -actin. As for subcultured medial cells, no expression of any of the cytokines and cell adhesion molecules associated with the inflammatory response was detected.

Figure 5. 'QIF Fingerprints' of subcultured medial SMCs and cobblestone SMCs. 'QIF fingerprints' were constructed for medial subcultured SMCs (a,c) and cobblestone SMCs (b,d) from the raw QIF data. For each culture type, a separate 'fingerprint' was constructed for the cells stained during exponential growth ('subconfluent') and at confluence. The 'fingerprint' consists of bars representing the strength of staining for each of the 20 protein markers analysed by QIF. The height of each bar represents the strength of staining (in arbitrary units) calculated as the mean staining per cell for the three subcultured medial SMC cultures (or two cobblestone cultures) analysed. To emphasize the pattern, error bars are not shown. The bars are in the following order along the x-axis: MCP-1, MIP-1 α , TGF- β 1, TGF- β 2, TGF- β 3, TNF- α , T β R-type I, T β R-type II, ICAM-1, Collagen I, Collagen II, Collagen IV, LTBP, osteopontin, calponin, SM- α -actin, SM-MHC, PAI-1, TIMP-2, ubcM1.

Effect of cell size on protein levels

It is interesting to note that while the pattern of expression of all the markers analysed is very similar between the cobblestone cells and the subcultured medial cells, the amount of protein per cell is lower for the cobblestone cells than for the subcultured medial cells for all of the markers analysed (where a given protein was detectable in either cell type), particularly as confluence is attained (Fig. 5). This is consistent with the cell volume data, which indicated that subcultured medial cells have a volume between 1.5- and 2.0-fold larger than the cobblestone cells just as confluence is attained. Correcting for cell volume, the level of each of the proteins is very similar in the subcultured medial SMCs and cobblestone SMCs.

Both culture types secrete an autocrine SMC mitogen

Cobblestone SMCs, but not subcultured medial SMCs, have previously been reported to produce an autocrine growth factor, which is also able to stimulate proliferation of Swiss 3T3 fibroblasts (Schwartz *et al.* 1990; Grainger 1992). Several batches of conditioned media from each culture were tested for the presence of growth factor activity. Media conditioned on all three cobblestone SMC cultures contained a high level of growth factor activity, consistent with the continued proliferation of the cobblestone cells in the absence of exogenous mitogens. Media conditioned on subcultured medial SMCs also stimulated DNA synthesis of 3T3 fibroblasts but to a lesser extent than the media conditioned on the cobblestone clones (Fig. 6a). This was

Figure 6. Transferable mitogenic activity secreted by subcultured medial SMCs and cobblestone SMCs. (a) Mitogenic activity of medium conditioned on two cobblestone SMC clones (BOB and BET) and one culture of subcultured medial SMCs (SMC). 5 μl of 100-fold concentrated conditioned medium was added to quiescent Swiss 3T3 murine fibroblasts in a total volume of 500 μl SF-DMEM in the presence of 10 ng/ml IGF-1. DNA synthesis was measured as the incorporation of $[^3\text{H}]$ thymidine into the TCA cold precipitable fraction during a pulse from 8 to 30 h after stimulation. (b) Conditioned medium was prepared on the four cultures shown and concentrated 100-fold, then adjusted in volume with SF-DMEM so the total protein concentrations were equal. Mitogenic activity was then tested as in (a) using the volumes shown in a final volume of 500 μl of SF-DMEM. All values are means $\pm$ SEM from triplicate wells.

unexpected, because subcultured medial cells cease to proliferate within 48 h when exogenous mitogens are withdrawn (Fig. 2a,b).

The cell density attained by the medial cells during the incubation with serum-free media was approximately 3-fold less than the density of the cobblestone cultures. This is consistent with the smaller size and protein content of the cobblestone cells noted above. Thus, the same volume of serum-free media was being conditioned by many fewer cells in the subcultured medial cell cultures than in the cobblestone cultures. The total protein concentrations of the concentrated conditioned media from both types of cultures were therefore measured using the Coomassie Plus binding assay. Consistent with the lower number of cells conditioning the media

in the subcultured medial SMC cultures, the total protein concentration in the conditioned media was lower (5.23 µg/ml versus 10.59 µg/ml).

Several batches of conditioned media from the cobblestone cultures were pooled and compared to pooled batches of media conditioned on subcultured medial cells. The two pools of conditioned media were then adjusted in volume until they had equal total protein concentrations. Media conditioned on the two different culture types then had comparable mitogenic activity for 3T3 fibroblasts (Fig. 6b). We conclude that the amount of mitogenic activity made by each cell per unit of time is similar between the two types of SMC culture.

DISCUSSION

We have compared two different culture models of the intimal smooth muscle cell, examining the proliferation rate, size and protein expression pattern of both spindle-shaped subcultured adult medial SMCs and cobblestone SMCs. Surprisingly, despite the obvious difference in morphology between the cultures, they had remarkably similar protein expression patterns (based on an analysis of more than 20 proteins including extracellular matrix components, intracellular structural proteins, cytokines and cell adhesion molecules). It is important to note that differences between the methods used to prepare the cultures we have compared (whole populations versus clones, for example) do not undermine our conclusions that the two populations are strikingly similar.

The protein expression pattern common to both culture models shares a number of features with the intimal SMC *in vivo*: high levels of several proteins known to be associated with the intima [osteopontin (Giachelli *et al.* 1993), PAI-1 (Schneiderman *et al.* 1992), TGF-β1 (Nikol *et al.* 1992), type I TGF-β receptor (McCaffrey *et al.* 1995) and type I collagen (Newby 1997)] and lower levels of contractile proteins associated with the healthy vessel media [SM-α-actin, SM-MHC and calponin (Gabbiani *et al.* 1982, 1984)].

In contrast, neither culture type contained detectable levels of any of the pro-inflammatory cytokines or cell adhesion molecules examined, all of which are known to be selectively expressed in the diseased intima *in vivo* (Van der Wal *et al.* 1997). It is possible that these proteins accumulate in the intima *in vivo* as a result of expression by the infiltrating leucocytes rather than by the intimal SMCs. Alternatively, intimal SMCs *in vivo* may express these pro-inflammatory proteins in response to other factors secreted by the leucocytes. It is therefore unclear whether or not the lack of expression of these proteins indicates that both types of SMC cultures are imperfect models of the intimal SMC *in vivo*.

Although the protein expression patterns of the subcultured medial SMCs and cobblestone SMCs were very similar, the cultures differed in the amount of each protein per cell. As confluence was re-attained, the subcultured medial SMCs had approximately twice as much of each protein analysed as the cobblestone SMCs. This difference is likely to reflect the larger cell volume of the subcultured medial SMCs compared to the cobblestone SMCs estimated from the forward scatter pulse height distribution.

Another consequence of the smaller cell volume of the cobblestone SMCs is the higher cell density the cells can achieve in culture. At confluence, the cell density is more than three times greater for the BOB and BET clonal cell lines than for the subcultured medial SMC cultures. This higher cell density is presumably responsible for the higher total protein concentration in serum-free media conditioned on the cell cultures, since the same volume of media is incubated with many more cells in the case of the cobblestone cultures. Comparison of total protein

concentration and transferable mitogenic activity suggested that both culture types secreted a mitogenic factor but that this factor reached a higher concentration in media conditioned on the cobblestone cells simply as a consequence of the higher cell density during conditioning.

Although we noted few differences in the protein expression patterns of the two culture types, nonetheless, several possible differences have already been reported in the literature. For example, Schwartz and colleagues have demonstrated that cobblestone SMCs synthesize PDGF-BB (Majesky & Schwartz 1990), and suggested that this represents a difference from subcultured medial SMCs. Unfortunately, in many of these studies, including the report by Majesky & Schwartz (1990), cobblestone SMCs and subcultured medial SMCs were not compared directly. Even when direct comparison is made, the experimenter would have to be very careful about the normalization method used because of the different cell volumes. For example, normalizing by cell number gives a very different answer to normalizing by total cell protein. Even if differences in the expression of certain genes do exist (for example, see Giachelli *et al.* 1992; Lemire *et al.* 1996), however, this may be an artefact of the culture method: since the cell volumes are different and the densities achieved in cell culture are different, we have already noted that the concentration of total cell protein in media conditioned on the cell cultures is different. In particular, the concentration of the factor responsible for mitogenesis when transferred onto 3T3 fibroblasts is different. Thus, from the earliest time in culture, cobblestone SMCs and subcultured medial SMCs will be subject to differing concentrations of autocrine signalling molecules. It is therefore possible that any stable differences in protein expression pattern between the culture types is the consequence of exposure to different concentrations of autocrine signalling factors.

For both types of SMC cultures, the rate of proliferation was tightly correlated with cell size (estimated from the forward scatter pulse height distribution). We have no evidence to indicate whether small cell size causes more rapid proliferation or whether more rapid proliferation causes small cell size. Indeed, a third factor may be responsible for both small cell size and rapid proliferation. Studies of the impact of cell size on proliferation rate have mostly been conducted on yeast, and several investigators have reached the opposite conclusion for yeast compared with our observations for mammalian cells: larger yeast cells divide faster (Alberghina *et al.* 1984; Sveiczer *et al.* 1996). This apparent paradox can readily be resolved, however, by examining in detail the design of the experiments. For yeast, the investigators took an asynchronous population of yeast cells and asked how cell size related to time to mitosis. They observed that the largest cells underwent mitosis sooner than the smaller cells and concluded that, for yeast, there was a critical size that had to be attained before the cell division 'checkpoint' could be passed. As the larger cells were almost of threshold size they could rapidly enter mitosis, whereas the smaller cells took longer because they had to grow for a period until the threshold size was reached. These experiments, however, did not examine the relationship between average size of the whole population and average population doubling time over a number of cell cycles. It is impossible to predict what the outcome of this comparison will be from observations of individual cells within the population made over a single cell cycle. In the experiments described in our report, average cell size and average population doubling time for mammalian cells were compared. For cells with very similar protein expression patterns, populations with the smallest average cell size have the shortest average population doubling time. This is not inconsistent with the idea that even within the cell populations we studied during asynchronous growth the largest cells within the distribution would reach mitosis earliest. Instead, it suggests that for some cell populations the threshold size at which mitosis occurs is smaller than for other populations. This being the case, with a similar rate of cell growth for cells in both populations, the population with the smallest threshold size for entry into mitosis will have the shortest cell cycle time.

In summary, comparison of subcultured medial SMCs and cobblestone SMCs suggests that the two types of SMC are very similar, and both are equally suitable as models of the rat intimal SMC *in vivo*. Application of Ockham's razor would indicate that average cell volume need be the only difference between the two types of cultures, with all the other differences (morphology, concentration of autocrine factors in conditioned media, population doubling time and any minor differences in protein expression pattern) being ramifications of the difference in cell size. Our observations do not preclude other stable differences in properties we have not examined, but indicate that careful consideration of the impact of cell volume must be made before such conclusions can be drawn.

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