Effect of Culture Conditions on α-Smooth Muscle Actin Expression in Vascular Smooth Muscle Cell Lines Derived from H-2K^b-tsA58 Transgenic Mice

Elisabeth Ehler

Institute of Molecular Biology, Austrian Academy of Sciences, Salzburg and The Randall Institute, King's College London, Drury Lane, U.K.

Abstract

The expression of α-smooth muscle actin, a marker of smooth muscle cells, was studied in several smooth muscle cell lines derived from H-2K^b-tsA58 transgenic mice. These mice allow the establishment of stable cell lines since they have an insertion of a temperature sensitive mutant of the SV40 large T antigen under the control of the H-2K^b-promotor. Cells from these animals proliferate at permissive culture conditions (33°C, with γ-interferon) but can be induced to differentiate at non permissive culture conditions (39.5°C, without γ-interferon). To assess the expression of α-smooth muscle actin, the number of cells containing stress fibers composed of this actin isoform, as shown by immunofluorescent staining with a monoclonal antibody, was compared to the total cell number. Under proliferative culture conditions α-smooth muscle actin expression varied, depending on the age of the donor animal. Cell lines obtained from juvenile mice (newborn until 7 d. post partum) always displayed much higher percentages of α-smooth muscle actin-positive cells than cell lines from adult mice. Variations between the two different juvenile phenotypes were less pronounced; cell lines with spindle-shaped morphology tended to have slightly higher percentages than epithelioid ones. A shift to non permissive, differentiating conditions led to a dramatic increase in α-smooth muscle actin expression. Addition of heparin either alone or in combination with FGF-1 or cultivation on collagen I-coated culture dishes increased the proportion of α-smooth muscle actin-positive cells in all cell lines. These results indicate that H-2K^b-tsA58-smooth muscle cell lines display a plasticity in expression and incorporation into stress fibers of α-smooth muscle actin that is analogous to primary cultures of smooth muscle cells. Moreover, they respond in a similar way to known modulating agents thus providing a convenient model system in which to study smooth muscle in vitro.

Key words: H-2K^b-tsA58 transgenic mice, smooth muscle, α-smooth muscle actin, culture conditions.

BAM 6 (1): 37-45, 1996

One of the key events during the process of atherosclerosis is the phenotypic modulation of vascular smooth muscle cells [36, 38]. This modulation refers to a transition from a contractile to a synthetic phenotype, the cells lose their differentiated features, replace smooth muscle with non-muscle protein isoforms and return to the cell cycle. The cells are then able to migrate from their normal place in the media into the intima and, by rapid proliferation and extensive production of extracellular matrix proteins, become one of the basic founders of an atherosclerotic plaque [29, 39]. A similar process of phenotypic modulation happens when smooth muscle cells are taken into culture [7, 8]. After a few days they de-differentiate, down-regulate the contractile proteins and come to resemble fibroblasts.

Proliferation-inhibiting agents such as heparin can delay the de-differentiation process, as monitored by the expression of α-smooth muscle-actin [6, 8, 13], whereas cultivation on distinct extracellular matrix proteins can either accelerate or decelerate de-differentiation, (i.e. cultivation on laminin leads to a longer retention of the contractile phenotype [21]). However, different preparations of primary cultures exhibit large variations in their response to these agents [6, 33, 44] and investigations on

stable smooth muscle cell lines have been hampered by the varying degree of differentiation of existing lines.

In this study the influence of growth conditions on the expression of α-smooth muscle actin in cell lines derived from aortae of H-2K^b-tsA58 transgenic mice has been monitored. This mouse strain bears a temperature sensitive mutant of the SV40 large T antigen under the control of the interferon γ-inducible H-2K^b-promotor, enabling the establishment of cell lines derived from different organs of these animals [24]. The fate of the cells can be controlled due to the temperature sensitivity of the large T antigen: at 33°C in the presence of γ-interferon the promotor is induced and the large T antigen active, therefore the cells divide rapidly (termed permissive culture conditions), a shift to 39.5°C and withdrawal of γ-interferon leads to rapid degradation of the large T antigen and proliferation ceases, thus favouring differentiation (non permissive culture conditions). Two distinct phenotypes of vascular smooth muscle cells have been cloned depending on the age of the donor animal: cell lines with an epithelioid shape could only be established from newborn mice, whereas cell lines displaying the spindle-shaped morphology typical for primary cultures of smooth muscle were obtained from juvenile as well as from adult transgenic animals. The alteration of culture conditions led to effects on α-smooth muscle actin expression in all cell lines investigated.

Materials and Methods

Cell culture

Smooth muscle cells were obtained by digesting freshly isolated aortae of H-2K^b-tsA58-transgenic mice [24] for 30 min with collagenase (1 mg/ml DMEM, Waymouth, U.K.). After centrifugation cells were seeded in tissue culture flasks (Falcon) and cultivated at 7% CO_2 in DMEM (Gibco) supplemented with 10% heat-inactivated Fetal Calf Serum (Gibco), 50 IU/ml penicillin (Gibco), 50 µg/ml streptomycin (Gibco) and 20 units/ml murine recombinant γ-interferon (Gibco). Permissive conditions were defined as growth at 33°C in the presence of IFN-γ, non permissive conditions as growth at 39.5°C in the absence of IFN-γ.

Cell lines were generated by cloning two times by limiting dilution in Terasaki-plates (Nunc, Denmark).

Immunofluorescence

Cells were grown on coverslips, fixed for 10 min with 4% PFA in PBS and permeabilized by a 30 s incubation in 0.5% Triton X-100 in PBS. After washing in PBS the immunofluorescent staining procedure was performed according to Rinnerthaler et al. [35]. Briefly, non-specific binding sites were blocked by incubation in 5% NGS in PBS, then the antibody layers were applied subsequently. FITC-phalloidin was used together with the Texas Red conjugated secondary antibody. After washing, coverslips were mounted in gelvatol containing 2.5 mg/ml n-propylgallate (Sigma; [15]) as anti-fading reagent and examined using a ZEISS Axioskop equipped with epifluorescence optics (Zeiss, Oberkochen, Germany). The monoclonal antibody against α-smooth muscle actin [41] was obtained from Sigma, the monoclonal antibody against SV40 large T antigen [16] was purchased from Santa Cruz Biotechnology Inc. Texas Red-conjugated anti-mouse immunoglobulins came from Vector Inc. (Burlingame, CA), the FITC-conjugated phalloidin was generously provided by Prof. Faulstich (Heidelberg, Germany).

To determine the percentage of α-smooth muscle actin expressing cells 20000-60000 cells were analyzed per culture condition and the number of cells containing α-smooth actin-stress fibers compared to the total cell number.

Induction of α-smooth muscle actin expression

The cells were seeded on coverslips and analyzed using immunofluorescence after 3 d under the respective growth condition. Heparin (Sigma) was added in a concentration of 100 µg/ml and FGF-1 (Collaborative Research) was used in a concentration of 20 µg/ml. To coat coverslips with collagen I a stock solution of 0.3% of collagen I (from calf skin, Sigma) in 0.1% acetic acid was diluted 1:10 and the coverslips were incubated for 3 hours at RT and then sterilized by UV irradiation after removal of the solution.

For the preparation of three-dimensional collagen I-matrices 17 ml of collagen I-stock solution were mixed on ice with 2.66 ml 10 x MEM (Gibco) and 1.10 ml 0.34 N NaOH and 1.5 ml was pipetted into each 35 mm-dish immediately. The cells were seeded on top of the gels after this had solidified. Matrigel (Collaborative Research) was used undiluted and handled using pre-cooled pipettes.

Results

Vascular smooth muscle cell lines were established from aortae of H-2K^b-tsA58 transgenic mice by cloning of primary cultures (Ehler *et al*, manuscript submitted for publication). The expression of the transgene could be demonstrated in all cells by staining with an antibody to SV40 large T antigen. At permissive culture conditions (33°C, with γ-interferon) the cells proliferate continuously and staining of large T antigen is restricted to the nucleus (Fig 1A); after a shift to non permissive culture conditions, the large T antigen is rapidly degraded, illustrated by diffuse nuclear and cytoplasmic staining, and the cells are growth arrested (Fig 1B).

The cell lines displayed two distinct morphologies depending on the age of the animal they were isolated from: spindle-shaped cells, growing in the hill and valley-pattern typical for smooth muscle cells in primary culture, could be established from adult (4 months, Fig 2E) as well as from juvenile (newborn - 7 d post partum, Fig 2C) animals, from the latter a second phenotype could be obtained, which displays a morphology reminiscent of epithelial cells and shows a tendency to grow in monolayers (Fig 2A). Despite their different phenotype, all cell lines were clearly smooth-muscle derived, as shown by their expression of the most commonly used marker protein, α-smooth muscle actin (Fig 2B, D, F). Since the number of

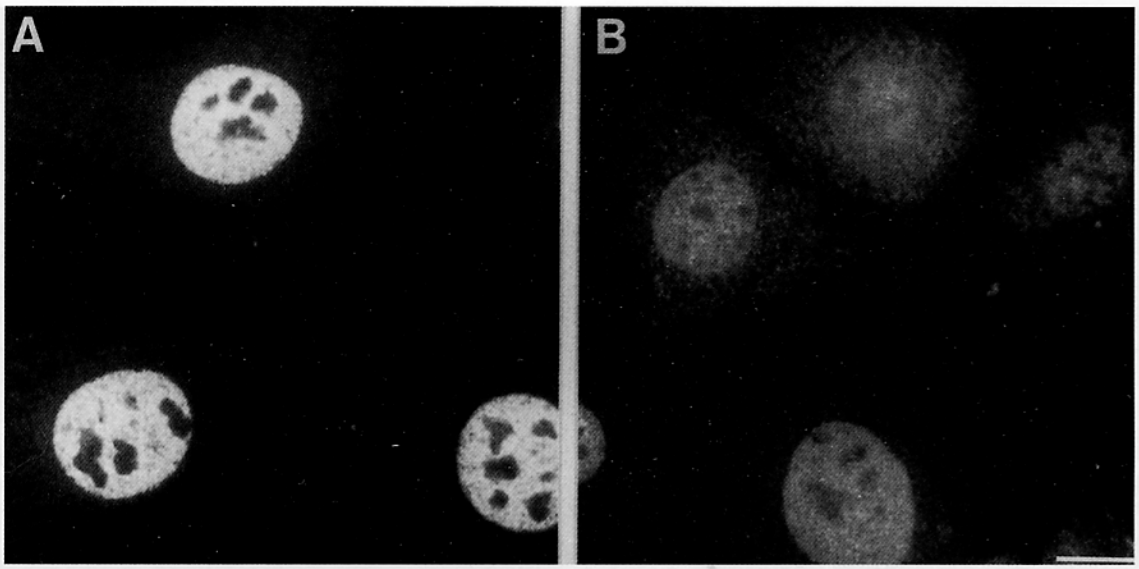

Figure 1. Expression of SV40 large T antigen in cell lines derived from H-2K^b-tsA58 transgenic mice at permissive (33°C, +γ-interferon, panel A) and non permissive culture conditions (39.5°C, -γ-interferon, panel B) as shown by immunofluorescence. Whereas at permissive culture conditions large T antigen is restricted to the nucleus it is rapidly degraded under non permissive culture conditions and staining can also be observed in the cytoplasm. Bar = 10 μm.

positive cells varied in the different lines, we tried to establish the amount of α-smooth muscle actin expression by comparing the number of cells showing stress fiber staining with a monoclonal antibody against this marker protein with the total number of cells labelled with FITC-phalloidin under permissive and non permissive culture conditions.

At permissive culture conditions, 36% of juvenile epithelioid cells were positive for α-smooth muscle actin. The amount of α-smooth muscle actin expressing spindle-shaped cells depended on the age of the donor animal. Whereas 52% of cells in cell lines established from juvenile animals expressed α-smooth muscle actin, only 1.6% of cells in cell lines from adult animals expressed this protein (Table 1). To study whether a shift to non permissive culture conditions could induce differentiation in our smooth muscle cell lines, we cultivated them for 3 d at 39.5°C without γ-interferon. The amount of α-smooth muscle actin-positive cells was increased from 36 to 58% in juvenile epithelioid, from 52 to 56% in juvenile spindle shaped and from 1.6 to 4.5% in adult spindle shaped cell lines (Table 1). Thus it can be concluded that non permissive culture conditions promote differentiation as far as the expression of α-smooth muscle actin is concerned.

Several groups have demonstrated that the addition of heparin can influence the expression of α-smooth muscle actin in primary cultures from smooth muscle [11, 13, 18, 34]. To investigate whether this holds true for our established cell lines we cultivated epithelioid and spindle-shaped cell lines for three days in the presence of 100 μg/ml heparin. A dramatic increase in α-smooth muscle actin-positive cells occurred in all lines at permissive as well as at non permissive culture conditions (Table 1). At 33°C with γ-interferon, 83% of the juvenile epithelioid, 75% of the juvenile spindle-shaped and 11% of the adult spindle-shaped cell lines could be shown to contain stress fibers composed of α-smooth muscle actin (control: 36%, 52%, 1.6%), these numbers rose to 89% for epithelioid or 84% and 31% for juvenile and adult spindle shaped cells respectively after three days at non permissive culture conditions (control: 58%, 56%, 4.5%). Heparin given in combination with FGF-1 lead to a further increase in α-smooth muscle actin-positive cells with a rate of 96% for the epithelioid and 24% for the adult spindle-shaped cell line at permissive culture conditions (Table 1).

In order to investigate whether the process of phenotypic modulation of primary cultures can be delayed by application of less artificial growth substrates than tissue culture plastic, we cultivated the individual cell lines on collagen I-coated dishes. The altered substrate had no detectable effect on the morphology of either epithelioid or spindle-shaped cells and there was also no obvious alteration in the growth pattern of the cultures. Epithelioid cells grew still in a strict monolayer whereas spindle-shaped cultures always showed a tendency towards the hill and valley pattern. However, when the percentage of α-smooth muscle actin-expressing cells was analyzed, a marked increase compared to the cultivation on plastic could be detected.

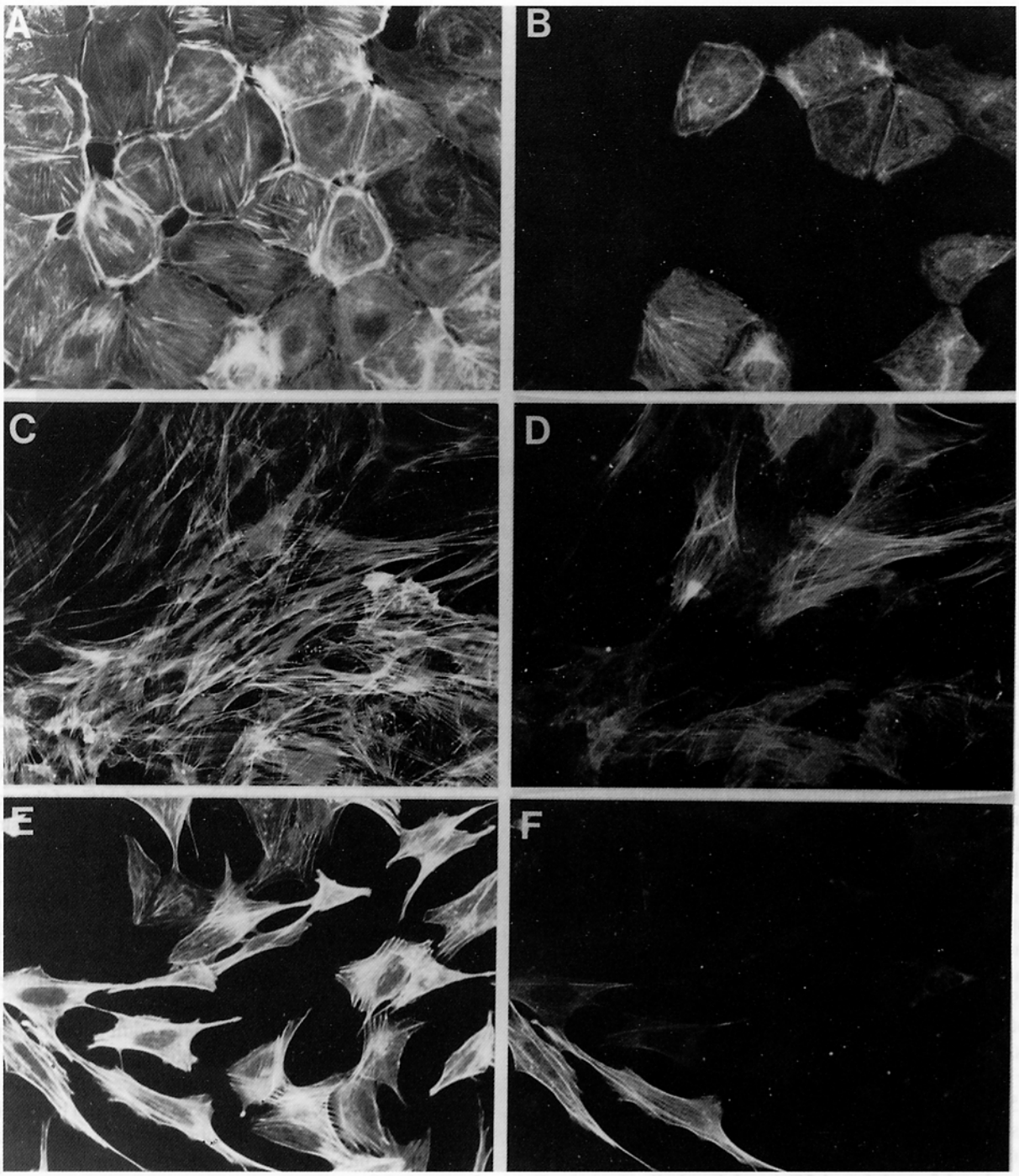

Figure 2. Cell lines from H-2K^b-tsA58 transgenic mice double-labeled with FITC-phalloidin (panels A, C, E) and a monoclonal antibody against α-smooth muscle actin (shown with a Texas Red secondary antibody, panels B, D, F). Juvenile epithelioid (panels A, B) as well as juvenile spindle-shaped (panels C, D) cell lines show a much higher degree of α-smooth muscle actin-positive cells than cell lines derived from adult animals (panels E, F). Bar = 10 μm.

Table 1: α-smooth muscle actin expression in smooth muscle cell lines derived from H-2K^b-tsA58 transgenic mice under different culture conditions.

	Cell line					
	Juvenile epithelioid		Juvenile spindle-shaped		Adult spindle-shaped	
Culture condition	perm.	non perm.	perm.	non perm.	perm.	non perm.
Standard	36%	58%	52%	56%	1.6%	4.5%
Heparin	83%	89%	75%	84%	11%	31%
Heparin + FGF-1	96%	n.d.	n.d.	n.d.	24%	n.d.
Collagen I	45%	58%	78%	80%	3.4%	5.3%

Cells were cultivated for 3 days at the respective culture condition and then analyzed for the percentage of α-smooth muscle actin positive cells. To facilitate the interpretation of the immunofluorescent staining only cells containing stress fibers brightly stained with a monoclonal antibody against this actin isoform were quantitated as positive and compared to the total cell number. Only cells between passage ten and twenty were used for this analysis. perm. = permissive, n.d. = not determined.

Figure 3. Juvenile epithelioid cells growing in a three-dimensional collagen I-matrix display an elongated morphology and form linear arrangements. Bar = 50 μm.

Juvenile epithelioid cell lines showed 45%, juvenile spindle-shaped 78% and adult spindle shaped 3.4% α-smooth muscle actin-positive cells, after three days at non permissive culture conditions these numbers were increased to 58%, 80% and 5.3%, respectively. Cultivation in a three-dimensional matrix of collagen I instead of on coated dishes had dramatic effects on the morphology of epithelioid shaped cells. They became elongated and started to form linear arrangements (Fig. 3). This alteration of morphology was less pronounced in spindle-shaped cell lines (data not shown). However, the use of three-dimensional Matrigel, which is composed of several constituents of basal lamina and has been reported to induce or retain the differentiated status of various cell types lead to death of all cells of our smooth muscle cell lines within 24 hours (data not shown).

Comparison of responses to agents which elevate the number of α-smooth muscle actin-expressing cells in lower and higher passages of our cultures revealed that the epithelioid cell lines undergo an aging process. The initial percentage of cells containing α-smooth muscle actin positive stress fibers dropped to 7.7% at permissive and 34% at non permissive culture conditions in older cultures (> passage 25). Also the increase following treatment with heparin and FGF-1 or cultivation on collagen I was much less pronounced than in lower passages. In the presence of heparin and FGF-1 the amount of α-smooth muscle actin-expressing cells could be raised to 13% at permissive and 33.4% at non permissive culture conditions, cultivation on a collagen I substrate resulted in α-smooth muscle actin percentages of 12.4 and 34.8%, respectively (Table 2). These signs of senescence could not be demonstrated in cells derived from adult animals (data not shown).

Discussion

The expression of smooth muscle marker proteins, including α-smooth muscle actin, in cultivated smooth muscle cells has been shown to be influenced by various parameters and an inverse correlation between the expression of contractile proteins (i.e. differentiation) and the rate of proliferation has been assumed [9, 12, 31, 37]. Several groups have demonstrated that prevention of proliferation in culture by either the addition of heparin or contact inhibition leads to an up-regulation of smooth muscle specific proteins [2, 4, 11, 13, 18, 31, 37, 40]. This inverse correlation provides an explanation for the markedly increased percentage of α-smooth muscle actin-positive cells in our cultures at non permissive compared to permissive culture conditions. The influence of γ-interferon on the proliferation of primary cultures of smooth muscle cells remains to be established, since conflicting reports about its mitogenic activity exist [19, 46], but it has been clearly demonstrated that this cytokine downregulates α-smooth muscle actin expression in smooth muscle

Table 2. Comparison of α-smooth muscle actin expression in juvenile epithelioid cells of different passages.

	Standard		Heparin + FGF-1		Collagen I	
Juvenile epithelioid cell line	perm.	non perm.	perm.	non perm.	perm.	non perm.
Passage 12	36%	58%	96%	n.d.	45%	58%
Passage 25	7.7%	34%	13%	33.4%	12.4%	34.8%

The number of cells containing α-smooth muscle actin stress fibers was counted and compared with the total cell number. perm. = permissive n.d. = not determined.

cells [19] as well as in fibroblasts [14]. Withdrawal of γ-interferon at non permissive conditions led to a pronounced increase in α-smooth muscle actin-positive cells in our study. However, the specific approach that was used to immortalize the cells in our system makes it impossible to attribute the upregulation of α-smooth muscle actin solely to the lack of γ-interferon. It seems more likely that the shift towards differentiation is due to the absence of mitogenic large T antigen.

In addition to the proliferation-inhibiting effect of heparin [6, 10, 11, 13, 17, 18, 23, 34] it has also been demonstrated that heparin degrading enzymes can induce the process of phenotypic modulation in smooth muscle cells *in vitro* [5]. Heparin does not show any effect on the total expression of α-smooth muscle actin per cell [13], but exerts its effect by increased incorporation of available α-smooth muscle actin into the stress fibers [18]. This change in stress fiber composition could also be observed in our cell lines upon addition of heparin. Since our evaluation system classified cells as positive, whose stress fibers could be stained with a monoclonal antibody against α-smooth muscle actin, this resulted in more cells to be counted as positive for this actin isoform.

The distinct smooth muscle cell lines used in this study show marked differences in their initial content of α-smooth muscle actin positive cells as well as in the response to differentiation promoting agents, always depending on the morphology and the age of the animal from which they were isolated. Whereas cells derived from juvenile transgenic mice (up to 7 d p.p.), start with an initial α-smooth muscle actin expression between fifty and sixty percent and can be stimulated to show almost 100% α-smooth muscle actin stress fibers, cells from adult animals (4 months) never reach more than 31% of α-smooth muscle actin-positive cells. The same age-dependent variability has been reported with comparable percentages from primary cultures of smooth muscle cells [3]. It seems to be a common feature of smooth muscle cells to display a decrease in differentiation features and a higher replicative activity with increasing age of the donor animal. The comparison of the proliferation rates of our distinct cell lines revealed far shorter doubling times for adult than for juvenile cell lines (Ehler *et al*, manuscript submitted for publication).

Not only additives to the culture media but also the substrate itself can influence the rate of phenotypic modulation in primary cultures of smooth muscle cells. Cultivation on fibronectin or collagen I has been shown to promote phenotypic modulation [20, 21, 45], whereas laminin coatings are able to retain primary cultures longer in a differentiated state until this effect is overridden by endogenous fibronectin production [21]. In contradiction to the model of inverse relationship between proliferation and differentiation, the evaluation of α-smooth muscle actin-positive cells on collagen I shows increased percentages in primary cultures of smooth muscle cells [45] as well as in our cell lines. At the moment it can only be speculated whether this elevated α-smooth muscle actin content is due to an increased migratory activity of smooth muscle cells in vitro on substrates, which compose the interstitial matrix in vivo, such as collagen I and fibronectin, compared to basement membrane components (e.g. laminin, collagen IV) or plastic. This could favour the expression of "motile" actin isoforms rather than cytoplasmic ones.

The striking effect of cultivation in a three-dimensional collagen I-matrix on the morphology of epithelioid cells draws specific attention. It has been claimed that smooth muscle cells cultivated in this system do not undergo phenotypic modulation [1, 42]. However, a thorough evaluation of the fine structure and protein expression pattern is essential in order not to overinterpret this observation, since comparable linear arrangements and alterations in the morphology have also been observed with endothelial cells and hepatocytes [25, 27]. Thus, the possibility of a specific tissue culture artefact can at present not be ruled out. The cultivation in Matrigel, a reconstituted gel of basement membrane proteins, has been reported to have a differentiating effect on smooth muscle cells [32]. We have been unable to demonstrate this property of Matrigel: although our cells did arrange themselves in tubular-like structures immediately after seeding, but then contracted and died within 24 hours. Whether this is a specific feature of our cell lines or caused by the relative high amount of transforming growth factor β (TGF-β), inhibiting smooth muscle cell proliferation in this matrix, remains to be established.

The comparison of reactivity towards agents promoting differentiation between lower and higher passages of our smooth muscle cell lines reveals that the epithelioid phenotype but not the spindle-shaped one undergoes an aging process in vitro. It has been postulated that the epithelioid phenotype might have potential stem cell properties [26, 43] and could have a role in repair processes in vivo [28, 30]. This intrinsic clock *in vitro* is puzzling and requires further attention.

This study demonstrates that smooth muscle cell lines, obtained from H-2K^b-tsA58 transgenic mice, respond to altered growth conditions in an analogous way to primary cultures of smooth muscle cells. This is further evidence that these cell lines can be used as a substitute for primary cultures to study the influence of agents affecting phenotypic modulation.

Acknowledgements

I am indebted to Dr. Annette Draeger, Maria Schmittner, Andreas Weber and Dr. Simon Hughes for help and encouragement. This study was supported by a grant from the Austrian Research Council and the Austrian Ministry of Science.

Address correspondence to:

Dr. Elisabeth Ehler, The Randall Institute, King's College London, 26-29 Drury Lane, London WC2B 5RL, UK, tel. 0171 8368851, fax 0171 4979078

References

[1] Akita M, Murata E, Kaneko K, Ghaida J, Merker HJ: Cell shape and arrangement of cultured aortic smooth muscle cells grown on collagen gels. *Cell Tissue Res* 1993; 274: 91-95.

[2] Blank RS, Thompson MM, Owens GK: Cell cycle versus density dependence of smooth muscle α actin expression in cultured rat aortic smooth muscle cells. *J Cell Biol* 1988; 107: 299-306.

[3] Bochaton-Piallat ML, Gabbiani F, Ropraz P, Gabbiani G: Age influences the replicative activity and the differentiation features of cultured rat aortic smooth muscle cell populations and clones. *Arteriosclerosis Thromb* 1993; 13: 1449-1455.

[4] Campbell JH, Kocher O, Skalli O, Gabbiani G, Campbell GR: Cytodifferentiation and expression of α-smooth muscle actin mRNA and protein during primary culture of aortic smooth muscle cells: correlation with cell density and proliferative state. *Arteriosclerosis* 1989; 9: 633-643.

[5] Campbell JH, Rennick RE, Kalevitch SG, Campbell GR: Heparan sulfate-degrading enzymes induce modulation of smooth muscle phenotype. *Exp Cell Res* 1992; 200: 156-167.

[6] Castellot JJ, Favreau LV, Karnovsky MJ, Rosenberg RD: Inhibition of vascular smooth muscle cell growth by endothelial cell-derived heparin: possible role of a platelet endoglycosidase. *J Biol Chem* 1982; 257: 11256-11260.

[7] Chamley JH, Campbell GR, McConnell JD, Groschel-Stewart U: Comparison of vascular smooth muscle cells from adult human, monkey and rabbit in primary culture and subculture. *Cell Tiss Res* 1977; 177: 503-522.

[8] Chamley-Campbell JH, Campbell GR: What controls smooth muscle phenotype? *Atherosclerosis* 1981; 40: 347-357.

[9] Chamley-Campbell JH, Campbell GR, Ross R: Phenotype-dependent response of cultured aortic smooth muscle to serum mitogens. *J Cell Biol* 1981; 89: 379-383.

[10] Clowes AW, Karnovsky MJ: Suppression by heparin of smooth muscle cell proliferation in injured arteries. *Nature* 1977; 265: 625-626.

[11] Clowes AW, Clowes MM, Kocher O, Ropraz P, Chaponnier C, Gabbiani G: Arterial smooth muscle cells in vivo: relationship between actin isoform expression and mitogenesis and their modulation by heparin. *J Cell Biol* 1988; 107: 1939-1945.

[12] Corjay MH, Thompson MM, Lynch KR, Owens GK: Differential effect of PDGF-versus serum-induced growth on smooth muscle α-actin and non-muscle β-actin mRNA expression in cultured rat aortic smooth muscle cells. *J Biol Chem* 1989; 264: 10501-10506.

[13] Desmouliere A, Rubbia-Brandt L, Gabbiani G: Modulation of actin isoform expression in cultured arterial smooth muscle cells by heparin and culture conditions. *Arteriosclerosis Thromb* 1991; 11: 244-253.

[14] Desmouliere A, Rubbia-Brandt L, Abdiu A, Waltz T, Macieira-Coelho A, Gabbiani G: α-smooth muscle actin is expressed in a subpopulation of cultured and cloned fibroblasts and is modulated by γ-interferon. *Exp Cell Res* 1992; 201: 64-73.

[15] Giloh H, Sedat JW: Fluorescence microscopy: reduced photobleaching of rhodamine and fluorescein protein conjugates by n-propyl gallate. *Science* 1982; 217: 1252-1255.

[16] Gurney EG, Tamowski S, Deppert W: Antigenic binding sites of monoclonal antibodies specific for Simian virus large T antigen. *J Virol* 1986; 57: 1168-1172.

[17] Guyton JR, Rosenberg RD, Clowes AW, Karnovsky MJ: Inhibition of rat arterial smooth muscle cell proliferation by heparin: in vivo studies with anticoagulant and non-anticoagulant heparin. *Circ Res* 1980; 46: 625-634.

[18] Hamati HF, Britton EL, Carey DJ: Inhibition of proteoglycan synthesis alters extracellular matrix deposition, proliferation and cytoskeletal organiz-

ation of rat aortic smooth muscle cells in culture. *J Cell Biol* 1989; 108: 2495-2505.

[19] Hansson GK, Hellstrand M, Rymo L, Rubbia L, Gabbiani G: Interferon γ inhibits both proliferation and expression of differentiation-specific α-smooth muscle actin in arterial smooth muscle cells. *J Exp Med* 1989; 170: 1595-1608.

[20] Hedin U, Thyberg J: Plasma fibronectin promotes modulation of arterial smooth muscle cells from contractile to synthetic phenotype. *Differentiation* 1987; 33: 239-246.

[21] Hedin U, Bottger BA, Forsberg E, Johansson S, Thyberg J: Diverse effects of fibronectin and laminin on phenotypic properties of cultured arterial smooth muscle cells. *J Cell Biol* 1988; 107: 307-319.

[22] Hedin U, Bottger BA, Luthman J, Johansson S, Thyberg J: A substrate of the cell-attachment sequence of fibronectin (Arg-Gly-Asp-Ser) is sufficient to promote transition of arterial smooth muscle cells from a contractile to a synthetic phenotype. *Dev Biol* 1989; 133: 489-501.

[23] Hoover RL, Rosenberg R, Haering W, Karnovsky MJ: Inhibition of rat arterial smooth muscle cell proliferation by heparin. *Circ Res* 1980; 47: 578-583.

[24] Jat PS, Noble MD, Ataliotis P, Tanaka Y, Yannoutsos N, Larsen L, Kioussis D: Direct derivation of conditionally immortal cell lines from an H-2K^b-tsA5B transgenic mouse. *Proc Natl Acad Sci USA* 1991; 88: 5096-5100.

[25] Karst W, Merker HJ: The differentiation behaviour of MDCK cells grown on matrix components and collagen gels. *Cell Differ Dev* 1988; 22: 211-224.

[26] Lemire JM, Covin CW, White S, Giachelli CM, Schwartz SM: Characterization of cloned aortic smooth muscle cells from young rats. *Am J Pathol* 1994; 144: 1068-1081.

[27] Lilja S, Merker HJ, Ghaida J: The influence of matrix components on the morphological differentiation of a proliferating hepatocyte line from liver of newborn mice. *Histol Histopathol* 1988; 3: 249-262.

[28] Majesky MW, Giachelli CM, Reidy MA, Schwartz SM: Rat carotid neointimal smooth muscle cells reexpress a developmentally regulated mRNA phenotype during repair of arterial injury. *Circ Res* 1992; 71: 759-768.

[29] Munro JM, Cotran RS: The pathogenesis of atherosclerosis: Atherogenesis and inflammation. *Lab Invest* 1988; 58: 249-261.

[30] Orlandi A, Ehrlich HP, Ropraz P, Spagnoli LG, Gabbiani G: Rat aortic smooth muscle cells isolated from different layers and at different times after endothelial denudation show distinct biological features in vitro. *Arteriosclerosis Thromb* 1994; 14: 982-989.

[31] Owens GK, Loeb A, Gordon D, Thompson MM: Expression of smooth muscle specific α-isoactin in cultured vascular smooth muscle cells: relationship between growth and cytodifferentiation. *J Cell Biol* 1986; 102: 343-352.

[32] Pauly RR, Passaniti A, Crow M, Kinsella JL, Papadopoulos N, Monticone R, Lakatta EG, Martin GR: Experimental models that mimic the differentiation and dedifferentiation of vascular cells. *Circulation* 1992; (suppl III): 11168-11173.

[33] Reilly CF: Rat vascular smooth muscle cells immortalized with SV40 large T antigen possess defined smooth muscle cell characteristics including growth inhibition by heparin. *J Cell Physiol* 1990; 142: 342-351.

[34] Reilly CF, Fritze LMS, Rosenberg RD: Heparin inhibition of smooth muscle cell proliferation: a cellular site of action. *J Cell Physiol* 1986; 129: 11-19.

[35] Rinnerthaler G, Geiger B, Small JV: Contact formation during fibroblast locomotion: involvement of membrane ruffles and microtubules. *J Cell Biol* 1988; 106: 747-760.

[36] Ross R: The pathogenesis of atherosclerosis. An update. *N Eng J Med* 1986; 314: 488-500.

[37] Rovner AS, Murphy RA, Owens GK: Expression of smooth muscle and nonmuscle myosin heavy chain in cultured vascular smooth muscle. *J Biol Chem* 1986; 261: 14740-14745.

[38] Schwartz SM, Campbell GR, Campbell JH: Replication of smooth muscle cells in vascular disease. *Circ Res* 1986; 58: 427-444.

[39] Schwartz SM, Heimark RL, Majesky MW: Developmental mechanisms underlying pathology of arteries. *Physiol Rev* 1990; 70: 1177-1209.

[40] Shirinsky VP, Birukov KG, Koteliansky VE, Glukhova MA, Spanidis E, Rogers JD, Campbell JH, Campbell GR: Density-related expression of caldesmon and vinculin in cultured rabbit aortic smooth muscle cells. *Exp Cell Res* 1991; 194: 186-189.

[41] Skalli O, Ropraz P, Trzeciak A, Benzonana G, Gillesen D, Gabbiani G: A monoclonal antibody against α-smooth muscle actin: a new probe for smooth muscle differentiation. *J Cell Biol* 1986; 103: 2787-2796.

[42] Stadler E, Campbell JH, Campbell GR: Do cultured vascular smooth muscle cells resemble those of the artery wall? If not, why not? *J Cardiovasc Pharmacol* 1989; 14: S1-S8.

[43] Van Neck JW, Medina JJ, Onnekink C, van der Ven PFM, Bloemers HPJ, Schwartz SM: Basic fibro-

blast growth factor has a differential effect on MyoD conversion of cultured aortic smooth muscle cells from newborn and adult rats. *Am J Pathol* 1993; 143: 269-282.

[44] Wright T, Castellot J, Petitou M, Lormeau J, Choay J, Karnovsky M: Structural determinants of heparin's growth inhibitory activity. *J Biol Chem* 1989; 264: 1534-1542.

[45] Yamamoto M, Yamamoto K, Noumura T: Type I collagen promotes modulation of cultured rabbit arterial smooth muscle cells from a contractile to a synthetic phenotype. *Exp Cell Res* 1993; 204: 121-129.

[46] Yokota T, Shimokado K, Kosaka C, Sasaguri T, Masuda J, Ogata J: Mitogenic activity of interferon gamma on growth-arrested human vascular smooth muscle cells. *Arteriosclerosis Thromb* 1992; 12: 1393-1401.