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Cell Kinetics of Aortic Smooth Muscle Cells in Long-Term Cultures Prepared From Rats Raised Under Conventional and SPF Conditions

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Summary

Migration and proliferation of smooth muscle cells (SMC) were studied in cultures prepared from the aorta of Wistar male rats (170–200 g b.w., 8 weeks old) raised under conventional (CC) or specific pathogen-free (SPF) conditions. In primary cultures, higher movement of cells from explants was found in CC raised donors, namely in samples cultured in serum incomplete medium. In the following subcultures (passage 3–16), the growth curves were steeper and the doubling time shorter in CC type of cultures. The faster growth of SMC population from conventional donors was found to be due to a shorter cell cycle and a higher proportion of dividing cells. As a consequence, the maximum population densities were also higher in the latter type of cultures. The differences in growth, that were dependent on raising conditions, were evident for 16 passages, i.e. 7 months after explantation of cells into culture. The data suggest that breeding conditions may affect the activation of growth of SMC in blood vessels *in situ*.

Key words

Vascular smooth muscle cells – Cell migration and cell proliferation – Cell cycle and growth fraction – Breeding conditions – Rat

Introduction

Migration of smooth muscle cells (SMC) from the tunica media into the intima, followed by their proliferation *in situ*, play a key role in the development of several vascular diseases, particularly atherosclerosis (Ross 1995). Several studies showed that growth of the vascular SMC in culture depends on the species, strain, sex, age and health of donors (Mogayzel *et al.* 1985, Hamada *et al.* 1990, Travo *et al.* 1980, Gutstein *et al.* 1991, Hadrava *et al.* 1995) as well as on the part of the vascular tree explanted into culture (Waldbillig and Pang 1992). The influence of breeding environmental conditions on the growth of SMC has not yet been sufficiently demonstrated. However, it has been shown that raising of animals under conventional and specific pathogen-free conditions (CC and SPF, respectively) can affect properties of other cell types. For instance, adherence of leukocytes to the endothelium damaged by commensual bacteria and viruses has been found to be relatively higher in the CC raised rats (Gabaldón and Capdevila 1989, Kefalides 1988). This is followed by penetration of chemotactic and mitogenic factors into the vessel wall which may stimulate SMC to migrate and proliferate. The SPF raised rats usually have lower body weight which may be caused, e.g. by difficulties in adequate substitutions of the biologically active molecules damaged by sterilization of food (Foster 1980). A difference in growth of SMC in cultures prepared from rats raised under conventional and SPF conditions, was suggested in our earlier study (Bačáková and Baudyšová 1990). The cultures were, however, assessed only by rough criteria which provided little insight into the growth kinetics of the populations. With respect to the pathophysiological importance of these findings, we have considered it worthwhile to re-investigate the impact of breeding conditions on the growth of the vascular SMC in cultures by using several independent cell kinetic criteria.

Material and Methods

1. Cell cultures

The cultures were prepared from the thoracic aorta dissected from Wistar rat males at the age of 8 weeks (170±8 g and 200±10 g for SPF and CC, respectively) raised under conventional or SPF conditions (Ipcv:WIST, Inst. Physiol. Acad. Sci. CR; Bondy *et al.* 1987, Greenhouse *et al.* 1990). Animals were fed a DOS2b-St diet (Velaz, Prague). In CC rats, *Pasteurella ureae, Staphylococcus aureus* and antibodies against Pneumonia virus of mice, Sendai virus and Kilham rat virus (titre 1:40 to 1:80, ELISA) were present without pathological clinical symptoms. In SPF rats, none of these or other pathogenic bacteria and viruses were detected. For each breeding group, cultures from 4 to 8 animals were prepared. Small pieces (cca 1 mm^3) of the intima-media complex of the aortas were digested by 0.1 % collagenase (Institute of Sera and Vaccines (SEVAC), Prague) in Eagle Minimal Essential Medium (MEM) at 37 °C (pH 7.4, 1 h) and seeded in Corning bottles (25 cm^2) coated with collagen. Each bottle contained about 200 microexplants in 2 ml of modified Eagle MEM (Baudyšová and Michl 1982) supplemented with gentamicin (40 μg/ml) and foetal calf serum (10 %) or Growth Promoting Proteins (0.5 %, SEVAC, Prague). The cultures were grown at 37 °C in 5 % CO_2 atmosphere. After reaching confluence, the cells were trypsinized (0.2 % trypsin, Sigma, in 0.02 % EDTA in phosphate-buffered saline and passaged in intervals of 7 to 14 days in split 1/2 or 1/3 (glass Müller flasks, 16 cm^2, 400 000 cells, 8 ml of modified Eagle MEM with 10 % foetal calf serum.

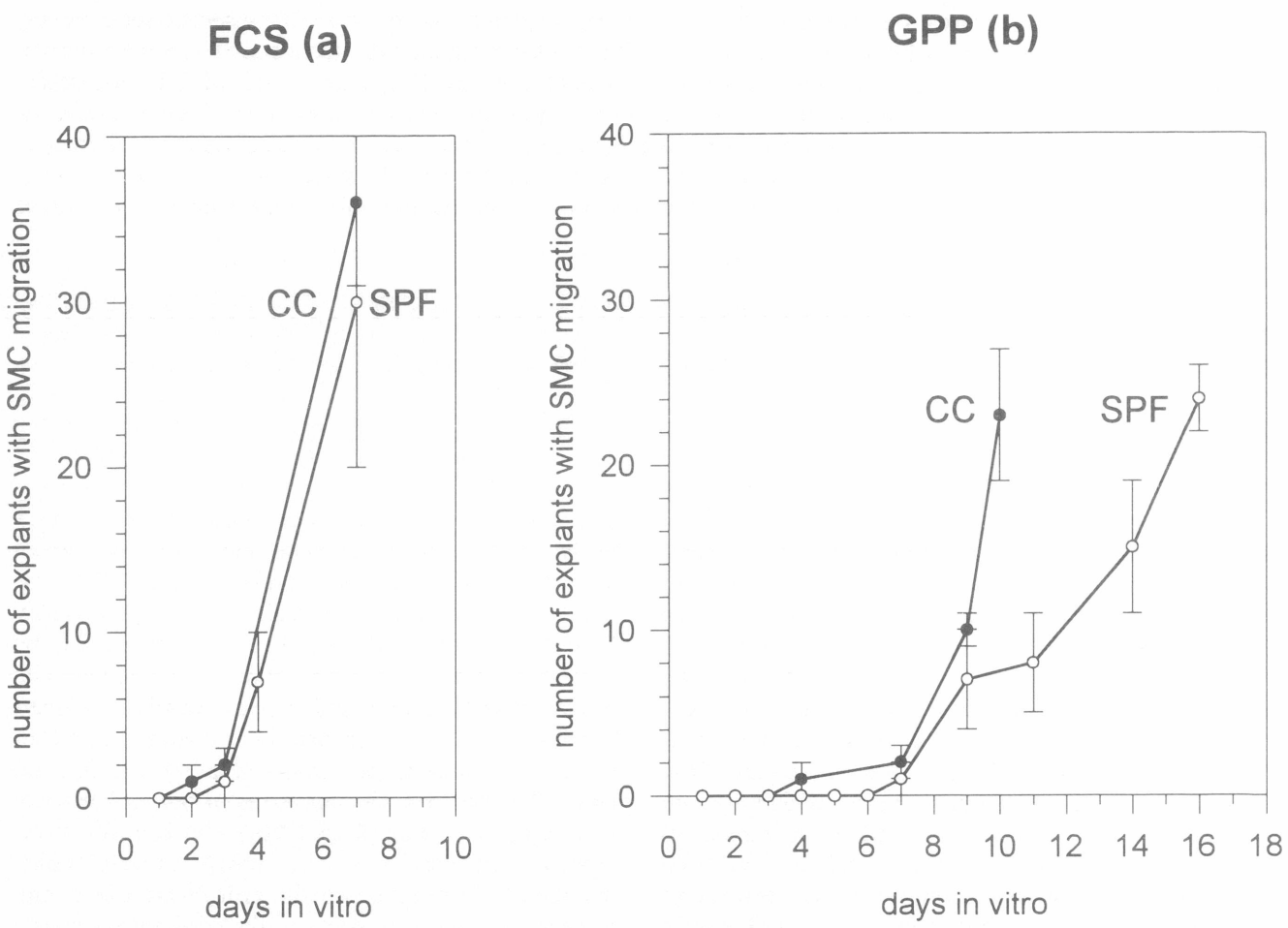

Fig. 1
Beginning of migration of SMC from explants of aorta in primary cultures prepared from rats raised under conventional (CC) and SPF conditions. The explants were cultured in modified Eagle MEM with 10 % foetal calf serum (FCS) or 0.5 % of Growth Promoting Proteins (GPP). Each point includes results from 8 animals in both breeding groups (mean ± S.E.M).

2. Determination of growth rate and cell kinetics parameters

2.1. The doubling time (DT) and the maximum population densities (MPD). The DT values were calculated from growth curves constructed from cell numbers determined in cultures (passage 3 to 16, the cells counted in a Bürker haemocytometer after trypsinization at 1 to 3-day intervals) over a period of 10 days.

$$DT = (t - t_0) \log 2 / \log N - \log N_0$$

where t_0 and t are the time intervals (24 and 96 h, respectively) at which the numbers of cells (N_0 and N, respectively) were used. The maximum population densities (MPD) was determined by counting trypsinized cells in stationary cultures and expressed as the number of cells/cm^2.

2.2. The cell cycle parameters were determined in 2 day-old cultures of the 3rd passage cells grown on collagen-coated Corning cover slips in plastic Petri dishes (Sterilin, diameter 6 cm, 300 000 cells, 3 ml of modified Eagle MEM with 10 % foetal calf serum and 40 μg/ml of gentamicin). The cultures were labelled with 6-^{3}H-thymidine for 2 h (1 μCi/ml, specific activity 20 Ci/mmol, Institute for Research Development & Production of Isotopes – UVVVR). Then, some cultures were washed in phosphate-buffered saline, fixed by Carnoy solution, dried by 96 % ethanol and used for determination of the Labelling and the Mitotic Indexes (LI % and MI %, respectively). The remaining specimens were transferred into a temperature and CO_2 adjusted non-radioactive medium for another 4 to 30 h. The cover slips were sampled at 2–3 h intervals and used for construction of the curve of labelled metaphases. The duration of the cell cycle and its phases was read either graphically at the 50 % level of the curve of labelled metaphases or calculated according to Mendelsohn and Takahashi (1972). Since this approach mainly includes the cells dividing at a relatively fast rate (TC_{fast}, Hamilton and Dobbin 1983 a, b), another set of cultures was exposed to the ^{3}H-thymidine continuously for 2 to 54 h . The time of the break point of the curve of cumulative labelling, indicating length of G1+G2+M of all cells, was added to the S phase length determined by the curve of labelled metaphases in order to calculate the cell cycle of all dividing cells (TC_{all}, see also paragraph 2.4 below).

2.3. The Growth Fraction was determined from the percentage of labelled cells in cultures continuously exposed to ^{3}H-thymidine (1 μCi/ml, see above). The Growth Fraction of the fast cycling cells (GF_{fast}) was determined from the percentage of labelling observed at the time corresponding to the G1+G2+M phase value obtained previously from the curve of labelled mitoses. The Growth Fraction of all cells (GF_{all}) was determined from the final break point of the curve of cumulative labelling after correction for exponential growth (Mareš *et al.* 1970).

2.4. The autoradiograms were prepared by dipping the slides into Nuclear Emulsion Ilford K2 (diluted 1:1 with distilled water) and a routine photographic and staining procedure (exposure 7 to 14 days at 40 °C, staining with 0.1 % toluidine blue).

3. Statistics

The values are given as means ± S.E.M. Statistical significance was determined by Student's t-test of unpaired data.

Results

1. Migration and growth of cells in primary culture

In cultures with serum complete medium, migration of SMC from the explants prepared from aortas of CC and SPF rats started on the 2nd and the 3rd postplating day, respectively. In the following 4 to 7 days, the increase in total number of explants with migrating cells was similar in both breeding groups (Fig. 1a). On the other hand, in cultures with the serum incomplete medium (modified Eagle MEM with Growth Promoting Proteins) migration of cells started only on the 4th and 7th day in both types of cultures. In the following days, the number of explants with migrating cells was remarkably higher in CC derived cultures (Fig. 1b). The stage of confluence was reached sooner in the cultures from CC rats (days 12 and 18 in serum complete and incomplete medium, respectively) than in cultures from SPF animals (days 14 and 28 in serum complete and incomplete medium, respectively). In this stage of growth, the cells formed characteristic "hills and valleys" corresponding to multi- and monolayer parts of the SMC population (Fig. 2).

2. The growth curves, doubling time and maximum population density (cells in the passage 3 to 16)

2.1. Growth curves and doubling times. The cells started dividing after a one-day lag phase in both types of culture. The increase in the number of cells in the exponential phase of growth was remarkably higher in CC derived cultures (Fig. 3, passage 3). The calculated doubling time values were significantly shorter in cells from CC rats in all, except the 10th to the 12th passages (Table 1). A higher rate of duplication of this type of the SMC population was, therefore, evident at least 7 months after explantation of cells (Table 1).

2.2. Maximum population density. The densities of cells in the stationary phase cultures were significantly higher in populations grown from CC donors (by 50 to 56 %, $p<0.001$) in cells of passage 3 to 12; later, the differences were not significant (Table 1, passage 13 to 16, $p>0.05$).

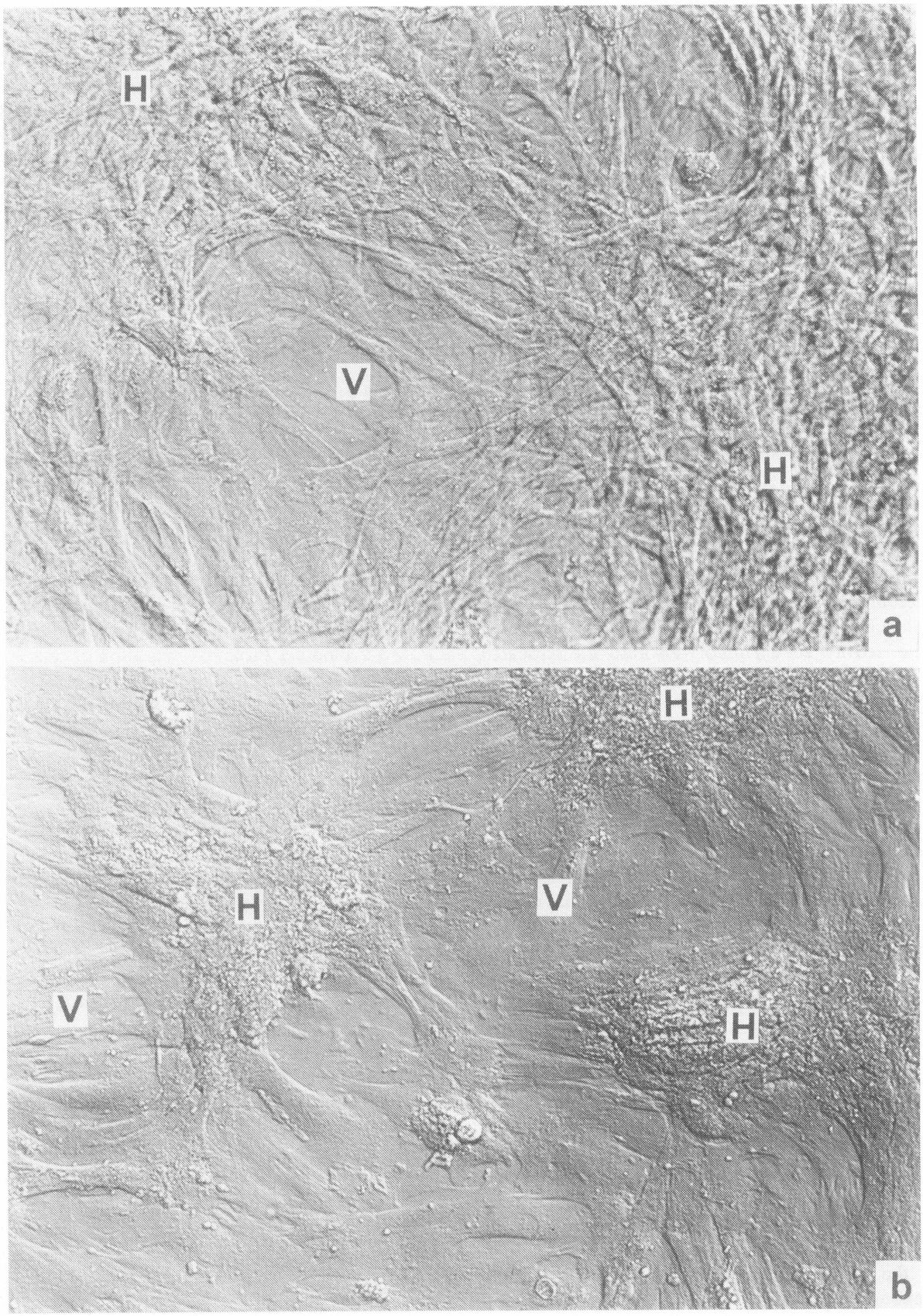

Fig. 2
Morphology of SMC cultures in stationary phase of growth. Cells were grown in plastic collagen-coated flasks in modified Eagle MEM with 10 % of foetal calf serum. (a) cells from conventional rats, 12 days *in vitro*; (b) cells from SPF rats, 14 days *in vitro*. The cells form characteristic "hills and valleys" (indicated by "H" and "V" symbols).

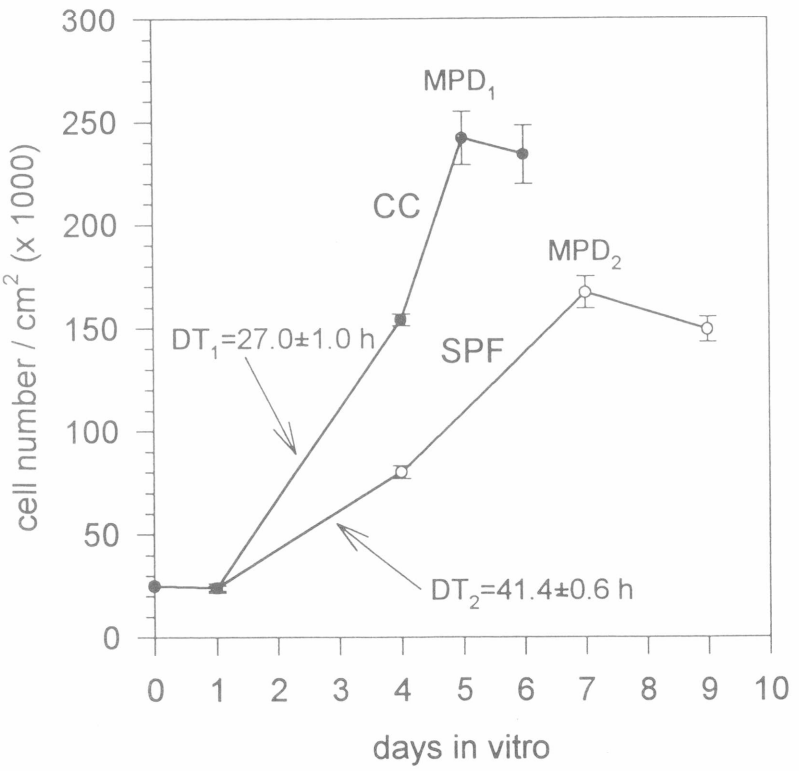

Fig. 3
Growth curves of SMC in cultures derived from conventional (CC) and SPF rats Passage 3, modified Eagle MEM with 10 % of foetal calf serum, Müller bottles. The calculated doubling time values (DT, calculated between day 1 to 4) and the maximum population densities (MPD) are given in Tab. 1. Each point includes results from 8 animals (mean ± S.E.M). Statistical significance: DT1 vs. DT2: $p<0.001$; MPD1 vs. MPD2: $p<0.01$.

Table 1
Doubling time in the exponential phase of growth and the maximum population density in stationary phase cultures of SMC from rats bred under conventional (CC) or specific pathogen-free (SPF) conditions

Growth Parameters	Breeding conditions CC		SPF
Doubling time (in hours)[a]			
passage 3–4	27.3±0.1	$p<0.001$	41.4±0.6
passage 10–12	24.8±0.5	n.s.	26.2±0.2
passage 13–14	23.3±1.6	$p<0.02$	28.6±0.6
passage 15–16	18.5±0.2	$p<0.001$	21.1±0.2
Maximal population density (cells/cm²)[b]			
passage 3–4	259 300±17 400	$p<0.001$	166 700± 7 800
passage 10–12	370 150±15 000	$p<0.001$	247 100±15 200
passage 13–14	353 500±12 300	n.s.	313 900±11 800
passage 15–16	308 900±19 600	n.s.	288 500±17 000

[a] cultures 1- to 4-day-old; [b] cultures 4 or 7 days old; the cells of the 3rd to 16th passage were grown in Müller bottles in modified Eagle MEM with 10 % of foetal calf serum. Both growth parameters were calculated from results obtained in 8 animals for each passage. Data expressed as mean values ± S.E.M.

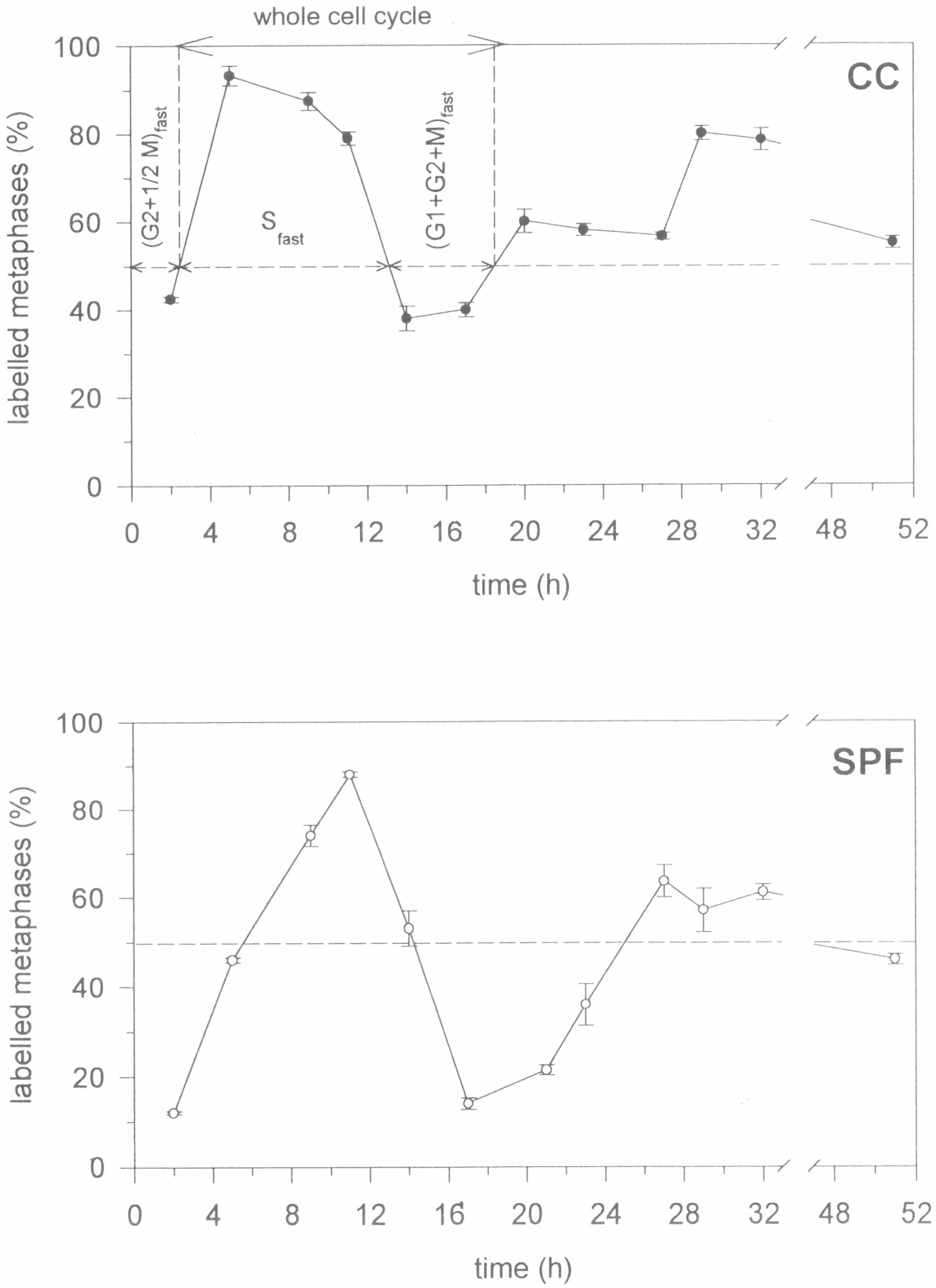

Fig. 4
The curves of labelled metaphases of SMC in cultures from conventional (CC) and SPF rats. Passage 3, modified Eagle MEM with 10 % of foetal calf serum; cells were grown on collagen-coated cover slips in plastic dishes. Duration of the cell cycle and its phases was determined at the 50 % labelling level (TC_{fast}). Each point includes results from 4 animals (mean ± S.E.M; 50 metaphases in each culture).

Table 2
Mitotic and Labelling Indexes, the cell cycle parameters and Growth Fraction values in SMC cultures derived from rats bred on conventional (CC) and SPF conditions (passage 3)

Cell cycle parameters	Breeding conditions CC		SPF
Mitotic and Labelling Indexes (MI % and LI %, respectively)			
MI %	8.1±1.1	n.s.	5.9±0.9
LI %	41.4±3.0	$p<0.02$	27.0±2.5
Cell cycle duration determined from CLM (hours)			
TC_{fast}	16.0±0.5	$p<0.05$	19.7±0.6
	(17.0±0.2)[a]	(n.s.)	(17.6±0.7)[a]
S_{fast}	11.0±0.2	$p<0.05$	8.8±0.3
$(G2+1/2M)_{fast}$	2.5±0.1	$p<0.001$	5.5±0.1
$(G1+1/2M)_{fast}$	2.5±0.7	$p<0.05$	5.4±0.9
	(3.5±0.2)[a]	(n.s.)	(3.3±0.6)[a]
Cell cycle duration determined by cumulative labelling and CLM (hours)			
TC_{all}	22.0[b]		40.0[b]
S_{all}	11.0[c]		8.8[c]
$(G1+G2+M)_{all}$	11.0[b]		31.8[b]
Growth Fraction (GF)			
GF_{fast}	0.45±0.02[b]	$p<0.02$	0.36±0.02[b]
GF_{all}	0.78±0.02[b]	n.s.	0.78±0.02[b]

Cells grown on collagen-coated cover slips in modified Eagle MEM with 10 % of foetal calf serum. For calculation of every growth parameter, results from 4 animals (mean±SEM) were used. [a] *values in brackets calculated according Mendelsohn and Takahashi (1972),* [b] *calculated from the curve of cumulative labelling (Fig. 5),* [c] *determined from the curve of labelled metaphases (Fig. 4), n.s.: non-significant.*

3. Cell kinetics parameters (the 3rd passage cells)

3.1. The ^{3}H-thymidine Labelling and Mitotic Indexes (LI %, MI %) and the cell cycle duration. The LI % values were higher by 53 % in cultures derived from CC animals ($p<0.02$; 41.4 % and 27.0 % in CC and SPF, respectively). The difference in MI % values was not statistically significant (Table 2, $p>0.05$).

The duration of the cell cycle of the faster dividing part of the population, evaluated by the graphic readings from the curve of labelled metaphases and calculation according to Mendelsohn and Takahashi (1972, TC_{fast}), ranged from 16.0 h to 17.0 h in CC derived cultures and from 17.6 h to 19.7 h in SPF cultures (Fig. 4, Table 2). The lesser drop of the first wave of the curve of labelled mitoses, as well as the steeper initial increase of the cumulative labelling curve (Fig. 5), suggest that population of CC derived cells contains a relatively higher number of cells with short G1 and G2 phases. Evaluation of the cell cycle length of all cells by a curve of cumulative labelling revealed a higher proportion of cells with a relatively longer cycle in cultures from SPF animals (TC_{all}, equal to 22.0 h and 40.0 h in CC and SPF type of culture, respectively; Fig. 5, Table 2).

3.2. Growth Fraction. The GF_{fast} value, referring to the relatively faster cycling part of the population, was found to be higher by 25 % in the CC animal cultures (0.45±0.02 and 0.36±0.02 in CC and SPF group, respectively). The proportion of all cycling cells was the same ($GF_{all}=0.78$) in both breeding group cultures (Fig. 5, Table 2).

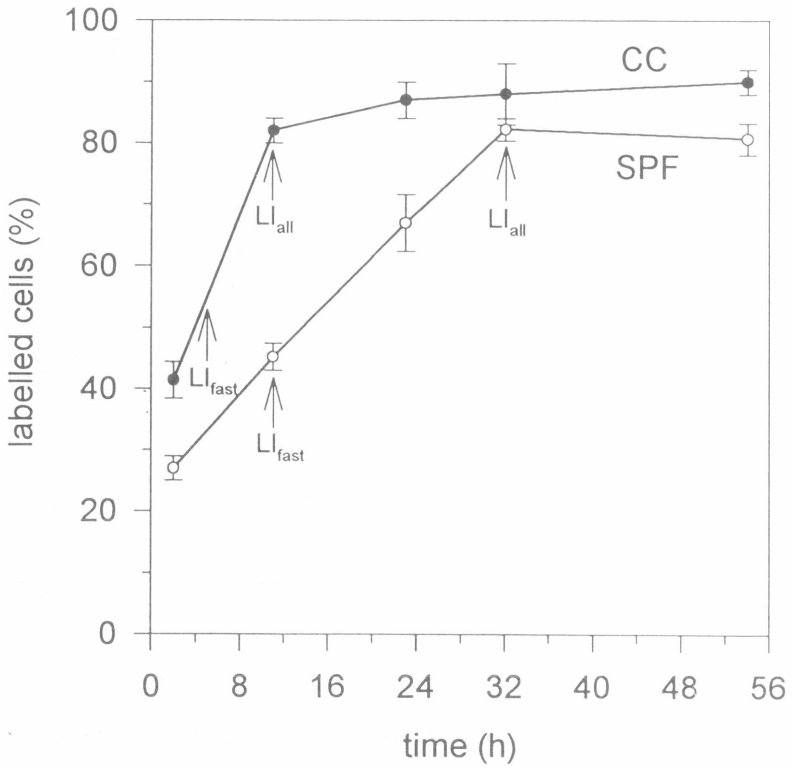

Fig. 5
The cumulative labelling of SMC in cultures from conventional (CC) and SPF rats. Passage 3, modified Eagle MEM with 10 % foetal calf serum, collagen-coated cover slips in plastic dishes. The arrows indicate the cumulative ^3H thymidine labelling values (LI fast, all) from which Growth Fractions of the fast cycling and all dividing cells were calculated (Table 2, GF_{fast} and GF_{all}, respectively; see also Material & Methods, paragraph 2.3). The times of the break points of the curves also correspond to the length of G1+G2+M phases of all dividing cells in cultures of both breeding groups. Each point includes results from 4 animals (mean ± S.E.M). For statistical significance see Table 2.

Discussion

The present study showed significant differences in migration and growth of the SMC obtained from aortas of CC and SPF rats. The cells from the CC animals started to move out of the primary culture explants earlier, and in the subsequent days, the number of explants with migrating SMC was increasing more rapidly. The latter was better evident in cultures with serum incomplete medium supplemented with Growth Promoting Proteins. In the subsequent passages, the cells from CC rats grew to higher population densities and their doubling times were significantly shorter. The cell kinetics analysis revealed that one of the reasons of the faster growth of these cells in subcultures was a shorter cell cycle. More rapid cycling of cells from CC animals was due to a shorter G1 and G2 phases (Fig. 4, Table 2). The cells from CC animals need, therefore, less time for crossing the main cell cycle checking points and can start a new cycle more easily. Another reason responsible for the differences in growth of cultures from animals of both breeding groups was the higher proportion of cell cycling at a higher rate (GF_{fast}) in the CC derived population. The presence of clones of SMC endowed with a different proliferation ability in cultures prepared from the aorta of individual rats was demonstrated earlier by Hall *et al.* (1991).

The physiological reasons for the differences in growth of SMC derived from rats of these two breeding groups are difficult to explain unequivocally. The body weight of SPF age-matched animals was slightly lower in this, as well as in other studies. First, it is considered to be due to the sterilization of food and possible incomplete compensation of heat-damaged biologically active molecules. Furthermore, the activity of several tissue enzymes and the some other biologically important molecules were reported to be altered in germ-free animals as a consequence of the deficiency of intestinal microorganisms and their products (Foster 1980). In addition, in CC animals, the presence of some pathogens (Bondy *et al.* 1987, Gabaldón and Capdevila 1989) may have caused microinjuries of the blood vessel endothelium followed by increased adherence of leukocytes. These cells, as well as the damaged or regenerating endothelial cells themselves, may release growth factors penetrating into the vessel wall and stimulate migration and proliferation of SMC (Nomoto *et al.* 1988, Gabaldón and Capdevila 1989, Kefalides 1988, Ross 1995). The importance of serum growth factors for migration of SMC is evidenced in this study by the later beginning of migration of SMC from explants cultured in serum incomplete medium (supplemented with Growth Promoting Proteins). Viruses, such as the Kilham rat virus, occasionally present in CC animals, may also act directly *via* alterations of the expression of genes regulating cell division (Nachtigal *et al.* 1987, Csonka *et al.* 1989, Kaner and Hajjar, 1992).

As is indicated by the shorter doubling time values, the better growth of CC derived SMC in our study persisted for 16 passages, i.e. for almost 7 months in culture. A long-term persistence of a higher proliferation capacity was reported earlier for aortic SMC derived from rats with spontaneous heritable hypertension (Hadrava *et al.* 1989), but not for the cells obtained from animals with experimentally induced hypertension (Hamada *et al.* 1990). Better growth of SMC derived from rats with heritable hypertension was accompanied by enhanced expression of c-myc and c-fos genes and autocrine production of growth factors (Hamada *et al.* 1990).

Irrespective of the difficulties in evaluation of the actual physiological reasons responsible for different proliferation of SMC in our study, the data showed that cells from the aorta of conventionally raised rats have a higher growth capability than those from the SPF donors. The data may be of importance for explanation of the vascular wall disorders in some individuals. This assumption is supported by recent findings on SMC in cultures from rats with spontaneous hypertension where the cell cycle, mainly the G1 phase, was remarkably shorter than in cells of normotensive donors (Hadrava *et al.* 1995).

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Reprint Requests

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