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A sex-related difference in the hypertrophic versus hyperplastic response of vascular smooth muscle cells to repeated passaging in culture

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Summary. Activation of growth of vascular smooth muscle cells (VSMC) in adults participates in pathogenesis of dysplastic diseases of the vascular system. In this study, we examined the impact of gender of rat donors on the degree of hyperplastic and hypertrophic responses of VSMC in cultures subjected to repeated passaging. The cells were derived from the outgrowth zone of explants of the thoracic aorta and were studied up to passage 45. Under these conditions, the cells undergo repeated growth stimulation by the serum growth factors mimicking some pathological situations in vivo. At lower passages (5-7), the cells from both sex donors did not differ significantly in their doubling time, maximum population density, protein content and ploidy. At higher passages (40-45), we found that the hyperplastic response, monitored by doubling time and BrdU-revealed DNA synthesis, was more intense in VSMC of male origin. In contrast, female-derived cells reacted by more prominent hypertrophic changes. The latter included a relatively higher increase in the volume and protein content of cells. As indicated by the DNA content histograms and chromosome numbers, these cells also showed a higher degree of passage-dependent polyploidization. In addition, the female-derived VSMC were found to be more effective in adhesion to the growth support evidenced by wider spreading and higher resistance of these cells to trypsin-mediated detachment as well as higher expression of some integrin and cytoskeletal molecules. These features could partly account for the slower proliferation and polyploidization of these cells. The results suggest that rat VSMC populations of male and female origin contain cells which are intrinsically different with respect to their capability of reacting to growth stimuli. The lower responsiveness of female-derived cells to growth stimuli may contribute to less frequent formation of hyperplastic vascular lesions in female organisms.

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Introduction

The growth of vascular smooth muscle cells (VSMC) in adult healthy mammals is generally very low. Actually, the frequency of cells in the S-phase in normal rat aorta in situ, as estimated by ^{3}H-thymidine autoradiography of enzymatically dispersed cells, is only 0.001% or less (Schwartz et al., 1982). Pathological activation of these processes leads to the development of severe dysfunctions of the vascular system (Schwartz et al., 1982, 1986; Lombardi et al., 1989; Owens, 1989; Su et al., 1998). Hyperplasia of VSMC can be induced, for instance, by acute severe hypertension or vascular surgery, i.e. by conditions entailing endothelial denudation and direct exposure of cells to blood-derived growth factors, mainly PDGF, FGF and EGF. Growth-stimulating molecules can also be produced autocrinely by mechanically stressed VSMC (Schwartz et al., 1982, 1986; Lombardi et al., 1989; Su et al., 1998). On the contrary, a hypertrophic response develops preferentially during mild chronic hypertension in large arteries, in which the endothelial barrier remains relatively intact and not permeable to blood-derived mitogens. In this case, the VSMC are stimulated by "incomplete growth factors", such as catecholamines, angiotensin II or vasopressin (released from nerve endings or produced autocrinely in VSMC) which enhance proteosynthesis and occasionally DNA synthesis and polyploidization (Schwartz et al., 1986; Yamori et al., 1987; Owens, 1989). Hypertrophy of VSMC may also occur as the result of physiological adaptation to prolonged mechanical, hormonal and neural stimulation in both adult and aged organisms (Schwartz et al., 1986; Yamori

et al., 1987; Lombardi et al., 1989; Owens, 1989; Agrotis et al., 1995; Devlin et al., 1995).

Pathological changes of blood vessels occur more often in male organisms (Espinosa et al., 1996; Moraghan et al., 1996; Vargas et al., 1996; Fitzpatrick et al., 1999). Nevertheless, sex-related differences in growth potential of VSMC in situ have only rarely been reported. Bucher et al. (1982, 1984) did not find significant differences in cell volume, DNA and protein content between male and female VSMC *in situ* in the thoracic aorta of 3- to 12-week-old normal and spontaneously hypertensive rats. Akishita et al. (1997) observed lower proliferation of intimal VSMC in experimentally damaged femoral arteries of female rats which was attributed to the growth inhibitory effects of estrogens. Proliferation of VSMC was also attenuated by estrogens or gestagens in vitro and this effect was more apparent in female VSMC (Espinosa et al., 1996; Moraghan et al., 1996; Vargas et al., 1996; Fitzpatrick et al., 1999). Some studies, however, showed that the VSMC of male origin were cycling more rapidly also in absence of physiological concentrations of gonadal hormones. In addition, these cells appeared to be less dependent on mitosis-stimulating factors provided by the serum supplemented in the culture medium (Travo et al., 1980; Bačáková et al., 1997a,b, 1999; Loukotová et al., 1998; Bačáková and Kuneš, 2000).

The response of male- and female-derived VSMC to external growth stimuli, especially as far as the participation of hypertrophy and hyperplasia is concerned, has attracted much less attention. For this reason, we examined the growth properties of VSMC populations in cultures prepared from male and female donors after repeated passaging. This treatment entails repeated proteolytic stimulation of plasma membrane by trypsin and re-exposures of cells to serum growth factors provided with the fresh culture medium; i.e. conditions which partially mimic the situation in endothelium-denuded vessels under some pathological conditions *in situ.* Special attention was paid to ploidy status of male- and female-derived cells, as an indicator of so-called "specific hypertrophy" (Schwartz et al., 1986; Yamori et al., 1987; Owens, 1989). This phenomenon has so far been studied only in hypertensive animals and man irrespective of the gender of donors (Barrett et al., 1983; Lombardi et al., 1989). Finally, because the proliferation of cells in culture depends on their attachment to the growth support, we also assessed the adhesion properties of VSMC from donors of both sexes by measuring the content and intracellular distribution of some plasma membrane adhesion- and cytoskeleton-associated molecules.

Materials and methods

Culture conditions, cell adhesion and doubling time

The VSMC cultures were prepared from thoracic aortas of 4 male and 4 female 8-week-old Wistar SPF rats (Inst. Physiol., Ac. Sci., Prague, Č.R.) by an explantation method (Bačáková et al., 1997a,b, 1999; Bačáková and Kuneš, 2000). Briefly, the rats were killed by exposition to 100% CO_2 atmosphere followed by decapitation. The intima-media complex was cut into small fragments, which were digested with 0.1% collagenase for 1 h at 37 °C in order to facilitate the migration of VSMC out of the explanted tissue. The explants were then seeded on 25 cm^2 collagen-coated flasks (Corning, Cambridge, MA, USA) in 2 ml of Dulbecco Minimum Essential Medium (DMEM, Institute of Sera and Vaccines, Prague, C.R.) supplemented with 10% of fetal calf serum (Sebak GmbH, Aidenbach, Germany) and 40 μg/ml of gentamicin (LEK, Ljubljana, Slovenia) at 37 °C in a humidified air atmosphere with 5% of CO_2. The majority of cells were of smooth muscle type, i.e. they contained the SMC-specific α-actin and were growing in the typical "hills-and-valleys" growth pattern (for details see Bačáková et al., 1997a, 1999). Starting with passage 3, the cells were seeded in 75 cm^2 polystyrene flasks at a density of 15,000 cells/cm^2 in 20 ml of DMEM supplemented as above. After reaching confluence, cells were trypsinized and re-seeded. The cells used at low (5 to 7) and high (40 to 45) passages are referred to as low- (LPC) and high-passaged cultures (HPC), respectively.

Adhesion of cells was evaluated by the number and degree of spreading of cells adhering to the growth support 24 h after seeding. In 1- and 3-day-old cultures, the strength of adhesion was measured by the time necessary for detachment of cells from the growth support by 0.2% trypsin (Sigma, St. Louis, MO, USA) in phosphate-buffered saline (PBS, at 37 °C). The number of released cells was counted in the Bürker haemocytometer. The doubling time (DT) was calculated using the following equation:

$$DT = (t-t_0)\log 2/\log Nt-\log Nt_0,$$

where Nt_0 and Nt were the numbers of cells in 1- and 3-day-old cultures, respectively.

Flow cytometry of DNA content and bromodeoxyuridine (BrdU) incorporation

Three-day-old cultures at the different passages were trypsinized, washed in PBS, fixed in 70% cold ethanol, and either immediately used or stored at 0 to 4 ºC.

For DNA staining, the cells were rehydrated in PBS and resuspended for at least 30 min in 50 μg/ml propidium iodide (PI) in distilled water containing 100 U/ml of RNase type A and 0.0015% Nonidet-P40 (all reagents from Sigma, St. Louis, MO, USA). To estimate the percentage of S-phase cells, some cultures were labelled with 4×10^{-5} M BrdU for 30 min. Cells were then washed with PBS, trypsinized and fixed as above, and treated with 3N HCl at room temperature for 30 min to denature DNA partially. BrdU incorporation was revealed by an anti-BrdU antibody (Exbio Praha a.s.,

Prague, Č.R., dilution 1:20) followed by an FITC-conjugated anti-mouse-IgG antibody (1:20, DAKO, Glostrup, Denmark, dilution 1:20). Both antibodies were diluted in PBS containing 0.1% bovine serum albumin and 0.5% Tween 20 (Sigma Chem. Co., St. Louis, MO, USA). The immunolabelled cell samples were counterstained for DNA with 5 mg/ml of PI in PBS, containing 100 U/ml of RNase type A and 0.0015% Nonidet-P40. For control of background fluorescence, the primary antibody was omitted in some samples.

Flow cytometric measurements were taken with a FACStar (Becton Dickinson, San José, CA, USA) equipped with a laser excitation (power 200 mW) at 488 nm, a 510-540 nm interference filter for the green fluorescence signals and a 610 nm long-pass filter for the red fluorescence signals.

The percentage of G_0/G_1-, S- and G_2/M-phase cells in DNA histograms was estimated by the Gaussian distribution fitting Friend's mathematical model adapted for a Hewlett-Packard computer. The percentage of BrdU-positive (S-phase) cells, and those of BrdU-negative in other cell cycle phases, was evaluated by rectangular-region analysis on dual-parameter scattergrams. At least 20,000 cells were contained in the gated regions used for calculations (Pellicciari et al., 1996).

Detection of binucleated and tetraploid cells

Using cells grown on coverslips, the presence of binucleated cells was assessed by bright-field microscopy (Axioplan, Opton, Germany or Docuval, Carl Zeiss, Jena, Germany) after toluidine blue staining (1% in distilled water for 3 min).

The number of tetraploid cells was derived from the DNA histograms and the dual-parameter cytograms of BrdU- and DNA-stained samples (see also Results). The tetraploid status of cells (i.e. excluding binucleated 2C-2C or G2-2C DNA cells) was further confirmed by chromosome counting. Briefly, dividing cells in 2-day-old cultures were arrested in the metaphase by Colcemide (Ciba, 5 μg/ml, 120 min of exposure). Mitotic cells were detached by shaking the culture flasks, resuspended in hypotonic solution (0.56% KCl for 20 min at 37 °C), fixed (3:1 mixture of methanol and glacial acetic acid at -20 °C), spread onto glass slides and air dried. After staining with Giemsa solution (10% in 0.1 M phosphate buffer, pH 7.4) the number of chromosomes was determined by bright-field light microscopy.

Cell morphology, volume and protein content

The shape of cells and the degree of their spreading were monitored by phase-contrast microscopy (Axioplan, Opton, Germany). To estimate the volumes, cells from 3-day-old cultures were detached by trypsinization, and their diameters (d) measured by phase-contrast microscopy using an ocular microscale. The volume of cells (V) was calculated as $V = 4/3\pi(d/2)^3$. The protein content was determined by a colorimetric method (Lowry et al., 1951) in sonicated homogenates ($5x10^6$ cells/ml of distilled water, Ultrasonic Homogeniser Cole-Parmer Instrument Co, Chicago, Illinois, USA).

Immunofluorescence of the cell surface adhesion and cytoskeletal molecules

Three-day-old cultures of low and high passages were fixed by methanol (at -20 °C for 10 min) and air-dried at room temperature. The cells were pre-treated with 3% bovine serum in PBS-0.1% Triton-X100 solution (Sigma, St. Louis, MO, USA) for 30 min and incubated with primary mouse monoclonal antibodies against smooth muscle α-actin (clone 1A4, Sigma, St. Louis, MO, USA, dilution 1:200, incubation 60 min), or vinculin (Serotec Ltd., Oxford, England, dilution 1:50, incubation 60 min), or a rabbit polyclonal antibody against the α_v chain of human integrin (Chemicon International Inc., Temecula, CA, USA, dilution from 1:50 to 1:100, incubation overnight). FITC-conjugated goat anti-mouse or goat anti-rabbit IgG (Sigma, St. Louis, MO, USA, dilution 1:200, incubation 60 min) were used as the secondary antibodies. Both primary and secondary antibodies were diluted with PBS supplemented with 3% bovine serum or, in the case of intracellular antigens, also with 0.1% Triton X-100. The cells were mounted in PBS-glycerine (9:1) and observed by epifluorescence microscope (Axioplan, Opton, Germany) or by a Bio-Rad MRC600 confocal laser scanning microscopy (oil immersion objective x60, N.A.=1.4, excitation wavelength 488 nm).

Enzyme-linked immunosorbent assay (ELISA)

Aliquots of the cell homogenates corresponding to 1,000 to 50,000 cells in 50 μl of water were adsorbed in 200 μl wells of 96-well microtiter plates (GAMA, České Budějovice, Č.R., or Maxisorp, NUNC, Roskilde, Denmark) at 4 °C overnight. For demasking the epitopes of vinculin, the cell homogenates were treated by 0.1% Triton X-100 (Sigma, St. Louis, MO, USA) in PBS or by 0.1% Zwittergent 3-14 (Calbiochem, La Jolla, CA, USA) in 20 mM KCl at 4 °C overnight before adsorption. After washing twice with PBS (100 μl/well), the non-specific binding sites were blocked by 0.02% gelatine in PBS (60 min, 100 ml/well). The primary antibodies (listed above) were diluted by 3% bovine foetal serum in PBS and applied for 60 min at room temperature (50 μl/well). As secondary antibodies, peroxidase-conjugated goat anti-mouse $F(ab')_2$ IgG fragment (Sigma, St. Louis, MO, USA; dilution 1:2000) or peroxidase-conjugated goat anti-rabbit affinity purified antibody (Chemicon International Inc., Temecula, CA, USA, dilution from 1:10,000 to 1:40,000) were used. Both secondary antibodies were diluted in 3% bovine serum in PBS and applied for 45 min at room temperature (50 μl/well).

After double washing in PBS-0.1% Triton X-100 solution, the orthophenylendiamine reaction was performed (Sigma, St. Louis, MO, USA) using 0.05% H_2O_2 in 0.1M phosphate buffer (pH 6.0, dark place, 100 ml/well). The reaction was stopped after 10 to 25 min by 2M H_2SO_4 (50 μl/well). The absorbances were measured at 492 nm by the Titertek Multiskan MCC/340 (Finland). The values obtained in cells from female donors were expressed as percentage of those from male-derived cells.

Statistics

The results were expressed as means ± S.E.M. from 4 cultures for each sex and passage. Statistical significance was evaluated by Student's t-test for unpaired data.

Results

Cell proliferation and ploidy

The doubling time values measured for 1- to 3-day-old cultures of VSMC in low-passaged cultures (LPC) from both sex donors were similar, though the BrdU-labelling index was higher in male-derived cells (Table 1, Fig. 1). The doubling times were, however, calculated for the adhering part of the cell population and thus were possibly affected by a differential spontaneous detachment of cells. The latter was, indeed, higher in male type of cultures both in the present and our previous studies (Bačáková et al., 1997a, 1999). At higher passages (HPC), proliferation of cells of both sex donors increased. However, this increase in male-derived VSMC was substantially higher, as documented by a four-fold rise in the BrdU-labelling index and a significant shortening of the doubling time (-35%, $p<0.05$). In female-derived cells, the passage-dependent increase in BrdU incorporation was much lower (Table 1, Fig. 1) and reflected polyploidization rather than normal mitotic division (see the text below). As a consequence, the population density in the 3-day-old male-derived HPC was 1.5 times higher than in their female-derived counterparts (Table 1).

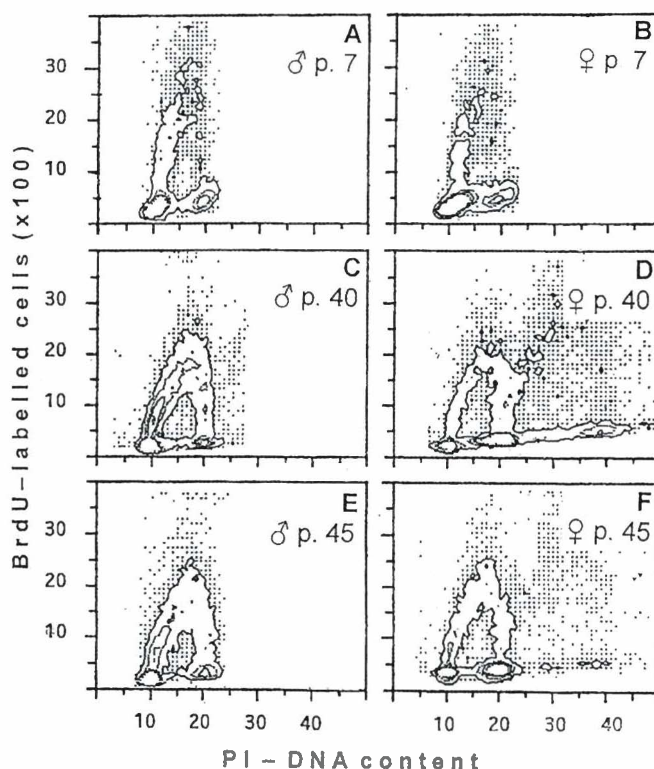

Fig. 1. Dual-parameter cytograms of BrdU immunopositivity vs. DNA content of VSMC of male and female origin at passage 7, 40 and 45. The percentage of BrdU-positive (S-phase) cells becomes higher in high-passaged cells, especially those of male origin (see also Table 1). It is apparent that in the high-passaged female-derived cultures the S-phase fraction of tetraploid cells is smaller than that of diploid cells.

The DNA content histograms in the exponential phase of growth (Fig. 2) demonstrated that cells in LPC of both sex groups were almost exclusively diploid; the percentage of cells with DNA content higher than 4C was found to be less than 1%. In HPC from both sexes, a progressive passage-dependent increase of cells with 4C or higher DNA amounts was observed which indicates an increasing proportion of the polyploid cells. The process of polyploidization was, however, much more prominent in female-derived cultures. In the latter, the percentage of tetraploid cells reached 30% and 70% at passage 40 and 45, respectively, whereas less than 5% of tetraploid cells were found in HPC of male origin (Fig. 1 C, E vs. F, D).

Interestingly, the dual-parameter cytograms of BrdU incorporation vs. the DNA content (Fig. 1) showed that in the polyploid fraction of cell population, present in

Table 1. Indicators of hyperplastic response of male- and female-derived VSMC in cultures of low and high passages.

CELL PARAMETER	PASSAGE 5-7		PASSAGE 40-45
a) Doubling time (hours)			
Males	18.7±2.4	p<0.05	12.2±0.8
	n.s.		p<0.05
Females	20.8±1.3	n.s.	17.1±1.8
b) Population density on day 3 (cells/cm²)			
Males	74,000±7,000	p<0.001	131,000±7,000
	n.s.		p<0.001
Females	73,000±3,000	n.s.	88,000±6,000
c) BrdU labeled S-phase cells (%)			
Males	9.2±0.1	p<0.001	34.1±2.8
	p<0.02		p<0.001
Females	7.9±0.4	p<0.01	16.4±2.0

female HPC samples, the BrdU-positive cells were relatively few or absent (Fig. 2D,F). The latter, together with the higher relative number of 4C DNA cells in HPC female-derived cultures (Fig. 1D,F) indicates that the number of cycling cells is lower in tetraploid than in the diploid compartment of the VSMC population.

The true tetraploid status of most 4C DNA cells (i.e. excluding binucleated or G2-phase diploid cells) was confirmed by chromosome counting in Colcemide-arrested metaphase cells (Table 2). In female-derived 40-passage cultures, about 30% of metaphase-arrested cells were tetraploid (78 to 84 chromosomes); the sub-tetraploid chromosome numbers were rare and were caused most probably by a loss of some chromosomes during hypotonic treatment and spreading of cells on glass slides.

Microscopic examination showed few binucleated cells in both male- and female-derived LPC. In HPC, they became more frequent in female populations (6.5% vs. 1.6% in males, Table 2). These low figures cannot account for the marked increase in the number of 4C DNA cells in DNA histograms of female-derived cultures.

Cell volume and protein content

In LPC, the volume of female-derived VSMC was 1.5 times larger than that of cells from male donors. After repeated passages, the volume of male-derived VSMC further decreased (by 20%, Table 2), whereas in female-derived cells it increased by 18% ($p<0.001$). As a result, the sex-related difference in cell size became more apparent in HPC in which the female-derived VSMC were about twice as large as the cells from males. The protein content per cell of LPC was similar for male- and female-derived samples, whereas in HPC it became 1.4 times larger in cells of female origin. This difference was mainly due to a decrease in protein content in male-derived HPC (Table 2).

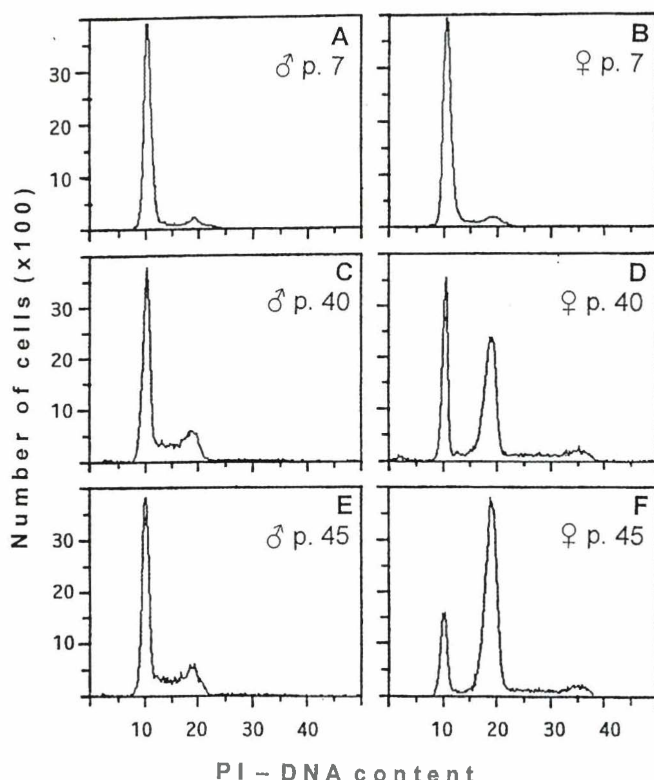

Fig. 2. DNA content distribution after propidium iodide (PI) staining of male- and female-derived VSMC at passage 7, 40 and 45. At low passages (7), the cells of both sex donors are almost diploid. At higher passages (40 and 45), the cells of female origin become more often tetraploid whereas the male-derived populations remain mostly diploid.

Adhesion and expression of cell surface and cytoskeletal molecules

The number of cells which adhered to the growth support within 24 h after seeding (i.e. the plating efficiency) was similar in male and female-derived LPC (12,500±1,300 vs. 14,700±700 cells per cm^2, $p<0.2$). In HPC, the plating efficiency in female-derived cultures was higher by 47% (12,500±1,500 vs. 8,500±1,200 cells/cm^2 in cultures from males, $p<0.05$). In addition, the female-derived HPC were flatter and more polygonal, whereas the cells from males were spindle-shaped and bulging in appearance. The female-derived HPC also exhibited stronger adhesion to the growth support as shown by the longer time necessary for their detachment by trypsin before each passage (15 min in female- and 5 min in male-derived cultures at passage 45).

Table 2. Indicators of hypertrophic response of male- and female-derived VSMC in cultures of low and high passages.

CELL PARAMETER	PASSAGE 5-7		PASSAGE 40-45
a) Tetraploid metaphases* (%)			
Males	5.0±1.9	— n.s. —	7.7±3.1
	n.s.		p<0.01
Females	2.0±2.0	— p<0.001 —	30.8±3.1
b) Binucleated cells (%)			
Males	1.2±0.4	— n.s. —	0.9±0.2
	n.s.		p<0.001
Females	1.6±0.2	— p<0.001 —	6.5±0.2
c) Cell volume (µm³)			
Males	1,640±20	— p<0.001 —	1,320±10
	p<0.001		p<0.001
Females	2,480±30	— p<0.001 —	2,920±30
d) Protein content (mg/10⁶ cells)			
Males	0.22±0.04	— n.s. —	0.16±0.01
	n.s.		p<0.001
Females	0.22±0.02	— p<0.02 —	0.32±0.01

*: based on counting of chromosomes in Colcemide-arrested metaphases.

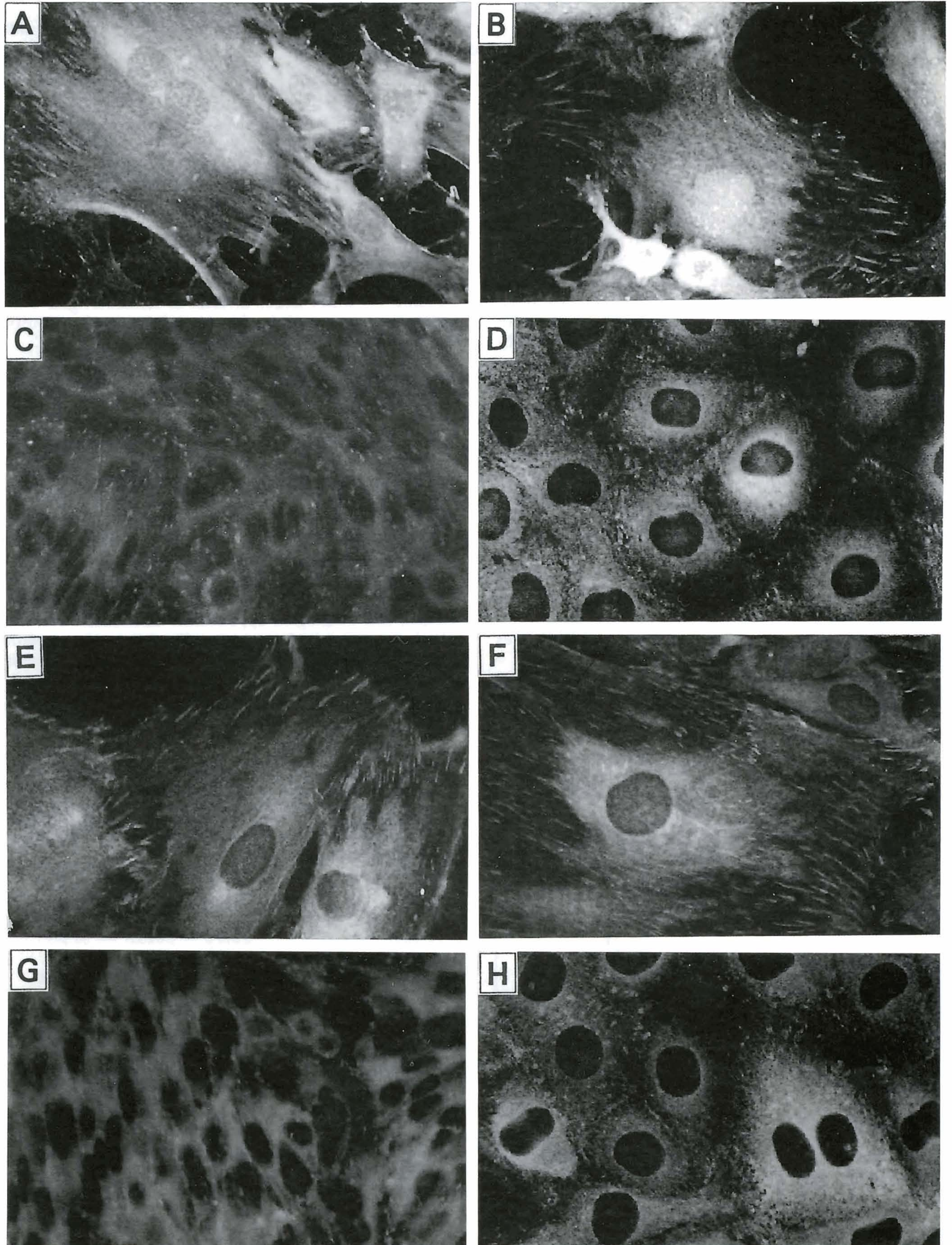

Fig. 3. Immunofluorescence staining of α_v integrins **(A-D)** and vinculin **(E-H)** in 3-day-old cultures of male **(A, C, E, G)** and female **(B, D, F, H)** VSMC in low **(A, B, E, F)** and high **(C, D, G, H)** passages. Confocal laser scanning microscope. x 560

As revealed by immunofluorescence, the α_v integrins and vinculin in LPC samples, were grouped in abundant streak-like focal adhesion plaques regularly distributed over the cell surface. The confocal microscopy data showed that these plaques were predominantly located on the cell surface in contact with the growth support. In most cells, both molecules also occurred in a diffuse, and/or fine granular form scattered throughout the cytoplasm (Fig. 3A,B,E,F). In HPC of both sex donors, the streak-like pattern of staining disappeared. The plaques became smaller, less frequent and rather of a dot-like appearance. The drop in staining was more apparent in male-derived HPC. In addition, the fine granular and/or diffuse cytoplasmic fluorescence became more conspicuos in the HPC, especially in female-derived material (Fig. 3C,D,G,H). The ELISA data revealed a higher total content of both α_v integrin and vinculin molecules in female-derived cells of both low- and high-passaged populations (Fig. 4).

The cultures of both sex- and passage groups contained alpha-actin-stained cells (Fig. 5A-D). In each sex group, the immunopositivity for this molecule decreased with repeated passaging. This decrease was relatively lower in female-derived samples (Fig. 4). In fact, light-microscope immunocytochemistry showed that most cells of male HPC cultures contained only threshold amounts of this molecule. The cells with brightly alpha-actin-positive microfilaments were less frequent than in female-derived cultures (Fig. 5C,D). As a result, the relative prevalence of alpha-actin content per cell, as measured by ELISA, appeared higher in female HPC samples (Fig. 4).

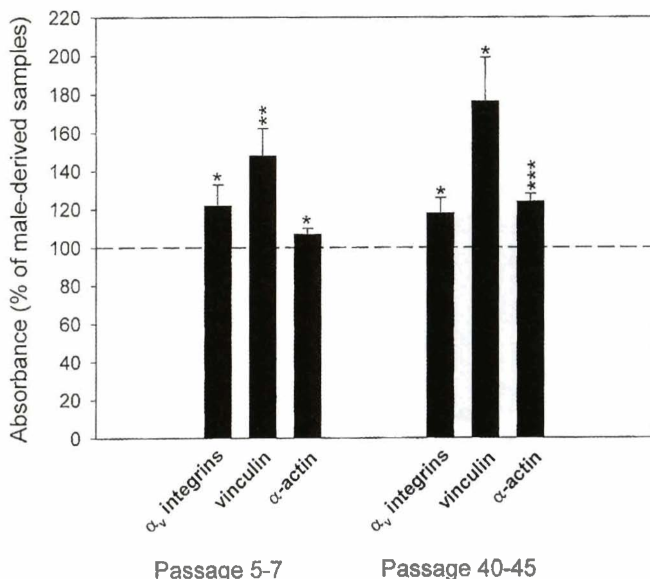

Fig. 4. The content of adhesion and cytoskeletal molecules in 3-day-old cultures of male- and female-derived VSMC in low and high passages. Measured by ELISA; absorbances of female-derived cell samples are expressed as a percentage of the values obtained in male-derived material. Means±S.E.M. from 4 experiments; Student's t-test for unpaired data, *: $p \leq 0.05$, **: $p \leq 0.01$ and ***: $p \leq 0.001$ compared to the control.

Discussion

Earlier studies performed in cultures of low-passaged VSMC showed that the male-derived cells proliferated more rapidly than those from females and also differed in their gross morphology. These differences became more pronounced with an increased number of passages (Travo et al., 1980; Bačáková et al., 1997a, 1999). Consistently, we found more cells in S-phase in both male LPC and HPC in the present study. In HPC, the sex-related growth difference was also manifested by significant changes in the doubling time, which reflects not only cell proliferation but also spontaneous cell loss. The novelty of the present study consists of showing that the female-derived HPC also exhibited a marked hypertrophy, as demonstrated by increased cell volume, larger total protein content and higher number of tetraploid cells. The latter was revealed by two independent approaches, i.e. the DNA content and chromosome numbers.

Under normal conditions, the number of tetraploid VSMC in the rat aorta in situ ranges from 1 to 13.5% depending on the strain and age of animals and the method used for their detection (for a review see Lee et al., 1992). The number of these cells increases up to 20 to 50% in rats with spontaneous or experimentally-induced hypertension (Lombardi et al., 1989; Owens, 1989; Lee et al., 1992; Conyers et al., 1995) and some other pathological conditions, such as nitric oxide deficiency or diabetes (Devlin et al., 1998; Vranes et al., 1999). Similar changes also occur in human blood vessels (Barrett et al., 1983). *In vivo* studies have shown that the increase in protein synthesis and polyploidization are due to an incomplete growth stimulation exerted e.g. by the "partial growth factors", such as angiotensin or catecholamines. A relatively higher sensitivity of female VSMC to these factors could be responsible for the increase in cell size in lower passages, and in HPC, also for the higher incidence of tetraploid cells. Differential responsiveness of male and female VSMC has so far been shown in cultures from SHR rats in which angiotensin shortened the population doubling time in male- but not in female-derived cultures (Loukotová et al., 1998). The observed sex-dependent differences could also be a result of a differential sensitivity of VSMC to growth inhibitors, such as TGF-beta. Compared to quickly proliferating spindle-shaped cells, lower sensitivity to PDGF and higher autocrine secretion of TGF-beta has been observed in polygonal and slowly proliferating human aortic VSMC (Kirschelohr et al., 1995), i.e. the cells of a similar morphology as those in female-derived cultures in the present study. Promotion of hypertrophy and polyploidization of VSMC by TGF-beta has been shown by Owens et al. (1988) and Agrotis et al. (1995).

Proliferation of cells in culture depends on their attachment to the growth support, being optimal at intermediate adhesion strength (Fernandez et al., 1993: Palecek et al., 1997; Podestá et al., 1997;). In our experiments, the adhesion of female-derived cells was

relatively stronger, and this probably contributed to the slower proliferation of these cells. Better adhesion of polyploid cells was also observed in cultures isolated from the aorta of normal and hypertensive rats (Yamori et al., 1987; Owens, 1989; Owens et al., 1988; Lee et al., 1992). However, these studies did not compare these phenomena in male- and female-derived VSMC. As has been demonstrated in the present study, better adhesion of VSMC of female origin may be due to the higher expression of α_v integrin and vinculin. However, the α_v integrin group includes several molecular forms with a differential effect on the migration and proliferation of cells (Dufourcq et al., 1998; Woodard et al., 1998). Therefore, further analysis of the individual members of the α_v integrin family in VSMC of both sex origins will be essential. Better adhesion of female-derived VSMC could also be a result of a different amount and spectrum of extracellular matrix molecules, the counterparts of plasma membrane proteins, synthesized by the VSMC (Owens, 1989; Glukhova and Koteliansky, 1995;

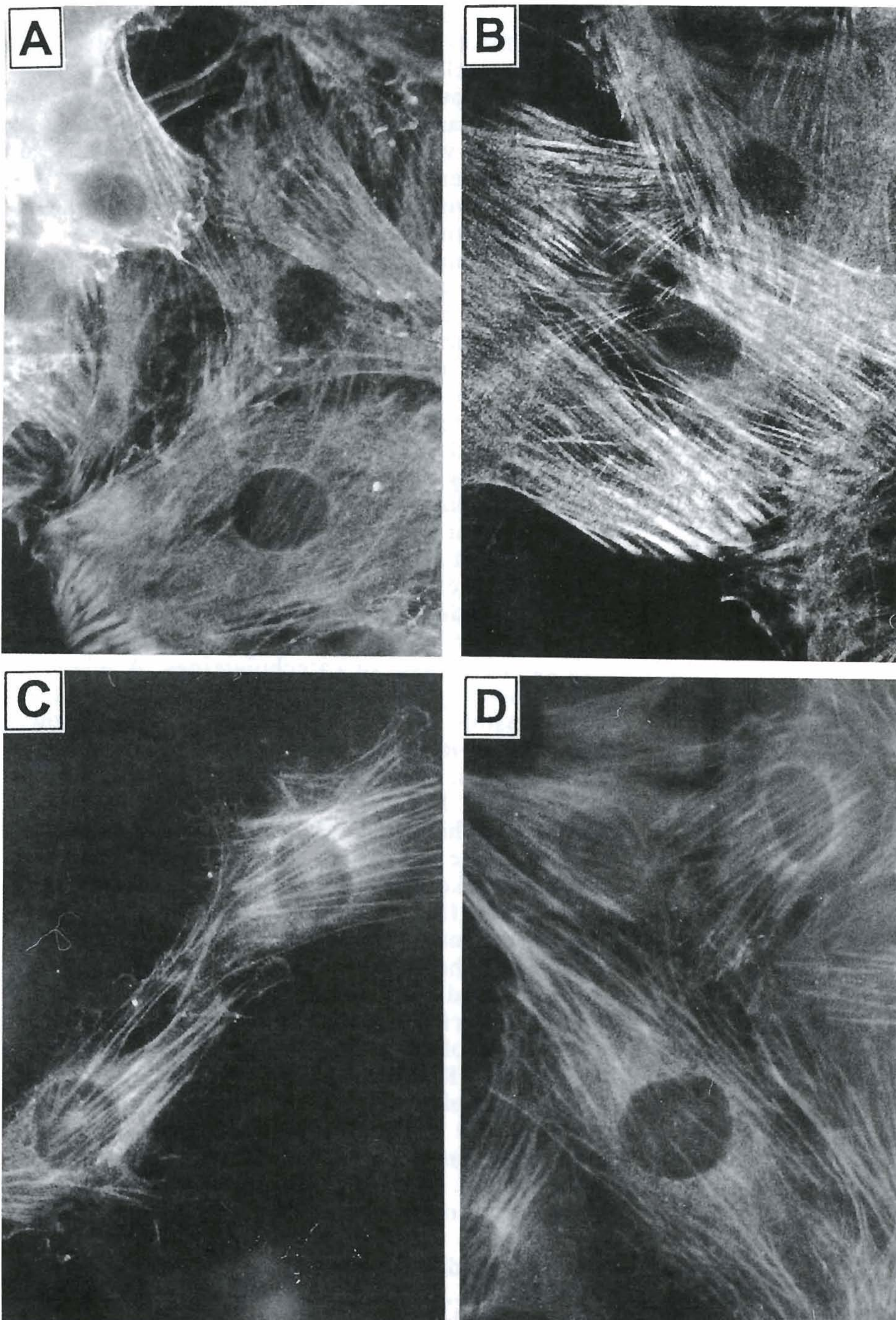

Fig. 5. Immunofluorescence of α-actin in 3-day-old cultures of male **(A, C)** and female **(B, D)** VSMC in low- **(A, B)** and high- **(C, D)** passaged cultures. Axioplan, Opton. x 1,000

Dufourcq et al., 1998).

Polyploidization of VSMC is considered as a sign of a specific hypertrophic reaction of VSMC in vivo (Owens et al., 1988; Owens, 1989). In agreement with this, female-derived cells in the present study were larger and richer in proteins. The latter included a higher content of α-actin, a marker of VSMC differentiation, in both female LPC and HPC cultures. It should be mentioned that polyploidization can be a hallmark of cell senescence (Kaji and Matsuo, 1981; Molenhauer and Bayreuther 1986; Fujisawa et al., 1998; etc.). However, cell senescence is accompanied by decreased proliferation not observed in the high-passaged female-derived VSMC cultures in our study. Polyploidy also often occurs in transformed immortalized cell lines (Fujisawa et al., 1998), including those of the VSMC type. These types of cells in culture are regularly fast proliferating (Csonka et al., 1984, Blaes et al., 1991; Fujisawa et al., 1998) which is contrary to the relatively low proliferation of the female-derived tetraploid-rich population in our study. Furthermore, viable and slowly proliferating polyploid VSMC without signs of senescence or transformation have been described in some studies in which, however, the impact of gender of donor animals was not analyzed (Goldberg et al., 1984; Rosen et al., 1985).

The process of polyploidization can occur via replication of DNA without mitosis (i.e. endoreduplication, Owens, 1989) or incomplete mitosis (Schwartz et al., 1982; 1986; Yamori et al., 1987; Owens, 1989; Devlin et al., 1995). In addition, this process may include a transient stage of binucleation observed e.g. in the liver (Brodsky and Uryvaeva, 1977). Because of the absence of enlarged chromosomes and the low incidence of binucleated cells in both LPC and HPC cultures of either sex group in the present study, the process of incomplete mitosis seems to be mainly responsible for polyploidization of cells in HPC female-derived cultures.

As has been shown previously, atherosclerotic plaques are formed mainly by multiplied diploid cells (Barrett et al., 1983, Schwartz et al., 1986). These plaques more often occur in blood vessels of males, including man (Espinosa et al., 1996; Vargas et al., 1996). The higher proliferation capacity of male-derived cells repeatedly exposed to serum mitogens during multiple passaging, described in the present study, could be related to the higher incidence of hyperplastic disorders in males, including man. The higher formation of polyploid cells in female-derived cultures could, on the contrary, act protectively against damage-induced proliferation. The polyploid cells are considered to be more resistant to physical and chemical mutagens as well as to oxidative damage (Uryvaeva, 1981; Fujisawa et al. 1998). Nevertheless, these cells could also enhance the stiffness of blood vessels and lower their compliance which can facilitate secondary pathological changes e.g. during hypertension (Schwartz et al., 1982, 1986; Owens, 1989). Therefore, the final understanding of the biological significance of the differential sex-dependent reactivity of VSMC to repeated passaging or other growth simulation requires further studies.

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