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SHORT COMMUNICATION

Sex-Dependent Differences in Growth and Morphology of Cultured Vascular Smooth Muscle Cells from Newborn Rats

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Summary

The morphology and proliferation of vascular smooth muscle cells (VSMC) were studied in cultures prepared from the aorta of newborn male and female Wistar rats. The doubling times (DT) of the male-derived population were 16.4±0.7 h and 30.0±2.2 h in the exponential and post-exponential growth phases, respectively. In the female donor cells, the corresponding DT values were significantly longer, i.e. 21.9±1.8 h and 38.0±2.2 h. In addition, the period of growth was shorter in the female-derived cultures. The percentage of ^{3}H-thymidine labelled cells in male cultures was 61.0±3.1, 92.8±1.9 and 98.7±0.6 % at 2, 27 and 52 h, respectively. In the female-derived populations, only 24.6±4.4, 66.1±3.8 and 82.8±2.0 % of cells were labelled at the corresponding incubation intervals. As a consequence, the final population density in male cultures was 5.6 times higher. In addition, the male-derived VSMC were mainly spindle-shaped and bulgy in appearance while those from female donors were flat and polygonal which means that the cells were adhering to the growth support to a different extent. The study revealed early determination and long-term persistence of lower adhesiveness as well as higher growth potential of male VSMC, i.e. properties which may be of importance for explaining the higher incidence of vascular wall disorders in males.

Key words

Rat aorta – Vascular smooth muscle cells – Cell proliferation – Cell adhesion – Cell culture – DNA synthesis – Sex-related differences

Activation of growth of vascular smooth muscle cells (VSMC) is of paramount importance for the development of vascular diseases, namely atherosclerosis and hypertension. The hyperplastic reaction of VSMC is also an undesirable complication of some pharmacological treatments and blood vessel surgery (Bagdade *et al.* 1985, Morimoto *et al.* 1993). It is well known that the morbidity and mortality of vascular diseases is higher in male than in female patients. The study on cultures prepared from the rat aorta showed that VSMC of male origin are endowed by higher migratory and growth potentials which can make the male blood vessel cells more prone to pathological hyperplasia (Travo *et al.* 1980, Bačáková and Kuneš 1995, Bačáková *et al.* 1997). As has been shown earlier, testosterone can stimulate growth of VSMC, e.g. by induction of more α_1-adrenergic and thromboxane A_2 receptors (Nakaki *et al.* 1990, Higashiura *et al.* 1996, for review see also Ely *et al.* 1994), stimulation of the conversion of angiotensinogen to angiotensin II (Chen *et al.* 1992) or potentiation of cholesterol and collagen accumulation (Fischer *et al.* 1985, Naseem and Heald 1987). Accordingly, testosterone also promotes the development of hypertension in rats (Takatsuka *et al.* 1992, Ely *et al.* 1994). Oestrogens, on the other hand, can inhibit VSMC proliferation by enhancing prostacyclin synthesis or by lowering calcium influx into the cells

(Chang *et al.* 1980, Stice *et al.* 1987). Tissue culture experiments, however, have shown that the sex-related differences in VSMC growth persist under *in vitro* conditions for more than one year, i.e they do not require the presence of gonadal hormones at levels occurring in the mature organism (Bačáková *et al.* 1997). The sex-related differences in proliferation potential of VSMC may therefore have been determined before sexual maturity of the organism. To test this hypothesis, we have examined the growth and morphology of VSMC in cultures prepared from the aorta of newborn male and female rats.

The VSMC cultures were prepared by the explantation method from the intima-media complex of the thoracic aorta dissected from aether-anaesthetized and decapitated newborn SPF Wistar rats (Ipcv:Wist, Inst. Physiol. Acad. Sci. CR, Prague). Twelve rats of either sex were used and four primary cultures were initiated from these animals. Thus, samples from three animals were pooled together in order to have enough material for initiation of each primary culture. The cells were maintained in the Dulbecco modified Eagle Minimum Essential Medium (SEVAC, Prague) supplemented with 10 % foetal calf serum and gentamicin (Sigma; 40 μg/ml). The cells were repeatedly passaged using 0.2 % trypsin (Sigma) in phosphate-buffered saline.

The doubling time (DT) was determined from growth curves of passage 2 cells cultured in Nunclon Multidishes (Nunclon, Ltd., Denmark; diameter 1.5 cm; 20 000 cells per 1.5 ml of medium). The cells were counted in a Bürker haemocytometer at 2- to 3-day intervals. The DT values were determined by formula DT = $(t\text{-}t_o)\ \log 2/\log Nt - \log Nt_o$, where Nt and Nt_o were the numbers of cells at the beginning and end of the exponential or post-exponential growth phases. DNA synthesis was assessed by incorporation of ^{3}H-thymidine (1 μCi per ml of medium, specific activity 20 Ci per mM; UVVVR, Prague, CR) administered to 2-day-old cultures (passage 2, Corning coverslips in plastic Petri dishes, GAMA Ltd., České Budějovice, CR; diameter 5 cm; 300 000 cells per dish in 3 ml of medium) for 2, 27 and 52 hours. Autoradiograms were prepared from Ilford K2 Nuclear Emulsion by a routine procedure.

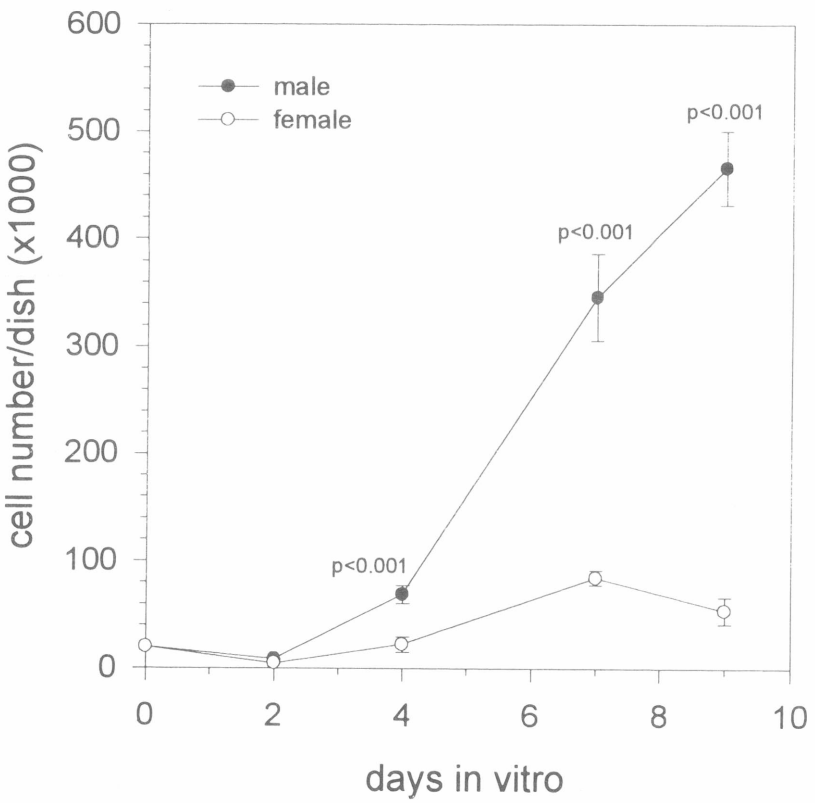

Fig. 1. *Growth curves of aortic smooth muscle cells in cultures derived from newborn male and female rats. Cells in passage 2, cultured in Dulbecco MEM with 10 % of foetal calf serum. P values refer to the differences between population densities in male and female cultures.*

Forty-eight hours after seeding, i.e. at the end of the lag phase, the number of cells which adhered to the growth support did not differ significantly in male- and female-derived cultures. Later, the number of cells in male cultures increased more rapidly. The DT values of male-derived populations in the exponential and post-exponential growth phases were shorter by 25 and 21 %, respectively (Table 1) when compared to female cells. Moreover, as follows from the growth curves (Fig. 1), the total period of growth was remarkably shorter in female-derived populations. As a consequence, the population density reached 5.6 times higher value by day 9 in male-type cultures (Fig. 1, Table 1). Similar results were also obtained in an independent experiment carried out on cultures from 3-day-old donors (Bačáková *et al.*, unpublished data).

Faster growth of male-derived VSMC was also characterized by a higher percentage of ^{3}H-thymidine labelled cells at all labelling intervals (Tab. 1). The ^{3}H-thymidine data suggest that the cell cycle is shorter and the number of dividing cells (the growth fraction) in male-derived populations is higher. The cells of both sex donors also differed in their morphology. The male-derived cells were usually spindle-shaped and of bulgy appearance while those from females were more flat and polygonal, i.e. they adhered to the growth support by relatively smaller and larger areas, respectively. In stationary cultures (day 9), the male cells formed a "hills and valley" growth pattern characteristic for VSMC (Chamley-Campbell *et al.* 1979).

Table 1. Doubling time, maximum population density and ^{3}H-thymidine incorporation in aortic smooth muscle cells from newborn male and female rats

Growth indicators	Male	Females	Significance
Doubling time (h)			
Exponential growth phase (days 2 to 4)	16.4±0.7	21.9±1.8	$p<0.05$
Post-exponential growth phase (days 4 to 7)	30.0±2.2	38.0±2.2	$p<0.05$
Final population density (cells x 1000/cm^2)	264.3±19.6	30.4±7.0	$p<0.001$
^{3}H-thymidine labelling (%)			
2-hour exposure	61.0±3.1	24.6±4.4	$p<0.001$
27-hour exposure	92.8±1.9	66.1±3.8	$p<0.001$
52-hour exposure	98.7±0.6	82.8±2.0	$p<0.001$

Data are mean ± S.E.M.

The data on growth and morphology of VSMC from male and female newborn rat aortas are similar to those obtained in cultures from adults (Travo *et al.* 1980, Bačáková and Kuneš 1995, Bačáková *et al.* 1997). Moreover, our study provides evidence that the sex-related differences in growth and adhesiveness of vascular VSMC in rats are already present on the day of birth. A long-term impact of pulse elevation of gonadal hormone levels in newborn or early postnatal rats were demonstrated on the growth of bones and the brain (Ošťádalová 1976, Mack *et al.* 1993) as well as on the development of genetically determined hypertension (Cambotti *et al.* 1984, Ely *et al.* 1994). The mechanisms responsible for early determination of growth properties of VSMC by gonadal hormones remain to be specified. In addition to those mentioned in the Introduction, they may also include alterations in the expression of growth factor receptors, autocrine synthesis of mitogens or expression of cell surface molecules, namely integrins, which take part in adhesion of cells to the extracellular matrix.

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References

BAČÁKOVÁ L., KUNEŠ J.: Sex-dependent differences in growth of vascular smooth muscle cells from spontaneously hypertensive rats. *Physiol. Res.* **44**: 127–130, 1995.

BAČÁKOVÁ L., MAREŠ V., LISÁ V., BOTTONE M.-G., PELLICCIARI C., KOCOUREK F.: Sex related differences in the migration and proliferation of rat aortic smooth muscle cells in short and long term culture. *In Vitro Cell. Dev. Biol. Anim.* **33**: 410–413, 1997.

BAGDADE J.D., SUBBAIAH P.V., HINTZ R.L., WILSON D.M.: The measurement of arterial smooth muscle cell mitogens in the blood of oral contraceptive users. *Atherosclerosis* **56**: 149–155, 1985.

CAMBOTTI L.J., COLE F.E., GERALL A.A., FROHLICH E.D., MACPHEE A.A.: Neonatal gonadal hormones and blood pressure in the spontaneously hypertensive rats. *Am. J. Physiol.* **247**: E258–E264, 1984.

CHAMLEY-CAMPBELL J., CAMPBELL G.R., ROSS R.: The smooth muscle cell in culture. *Physiol. Rev.* **59**: 1–61, 1979.

CHANG C.W., NAKAO J., ORIMO H., MURITA S. I.: Stimulation of prostacyclin biosynthetic activity by estradiol in rat aortic smooth muscle cells in culture. *Biochim. Biophys. Acta* **619**: 107–118, 1980.

CHEN Y.F., NAFTILAN A.J., OPARIL S.: Androgen-dependent angiotensinogen and renin messenger RNA expression in hypertensive rats. *Hypertension* **19**: 456–463, 1992.

ELY D.L., FALVO J., DUNPHY G., CAPLEA A., SALISBURY R., TURNER M.: The spontaneously hypertensive rat Y chromosome produces an early testosterone rise in normotensive rats. *J. Hypertens.* **12**: 769–774, 1994.

FISCHER G.M., BASHEY R.I., ROSENBAUM H., LYTTLE C.R.: A possible mechanism in arterial wall for mediation of sex difference in atherosclerosis. *Exp. Mol. Pathol.* **43**: 288–296, 1985.

HIGASHIURA K., BLANEY B., MORGAN E., MATHUR R.S., HALUSHKA P.V.: Inhibition of testosterone 5 alpha-reductase: evidence for tissue-specific regulation of thromboxane A_2 receptors. *J. Pharmacol. Exp. Ther.* **279**: 1386–1391, 1996.

MACK C.M., FITCH R.H., COWELL P.E., SCHROTT L.M., DENENBERG V.H.: Ovarian estrogen acts to feminize the female rat's corpus callosum. *Dev. Brain Res.* **71**: 115–119, 1993.

MORIMOTO S., YAMADA K., HIRAMITSU S., UEMURA A., KUBO N., KIMURA K., YAMAGUCHI T., WATANABE S., MIZUNO Y.: Fragmentation of internal elastic lamina and spread of smooth muscle cell proliferation induced by percutaneous transluminal coronary angioplasty. *Jpn. Circ. J.* **57**: 388–394, 1993.

NAKAKI T., NAKAYAMA M., YAMAMOTO S., KATO R.: Alpha 1-adrenergic stimulation and beta 2-adrenergic inhibition of DNA synthesis in vascular smooth muscle cells. *Mol. Pharmacol.* **37**: 30–36, 1990.

NASEEM S.M., HEALD F.P.: Sex mediated lipid metabolism in human aortic smooth muscle cells. *Biochem. Biophys. Res. Commun.* **144**: 284–91, 1987.

OŠŤÁDALOVÁ I.: The effect of single dose of oestrogens administered during the early postnatal period on the DNA content of rat bone epiphyses. *Physiol. Bohemoslov.* **25**: 97–102, 1976.

STICE S.L., FORD S.P., ROSAZZA J.P., VAN ORDEN D.E.: Role of 4-hydroxylated estradiol in reducing Ca^{2+} uptake of uterine arterial smooth muscle cells through potential-sensitive channels. *Biol. Reprod.* **36**: 361–368, 1987

TAKATSUKA D., UCHIDA N., YAMAMOTO R., TSUJI M., TERADA N., MATSUMOTO K.: Enhancement by androgen of the angiogenic ability of androgen-responsive Shionogi carcinoma. *Anticancer Res.* **12**: 2001–2004, 1992.

TRAVO P., BARRETT G., BURNSTOCK G.: Differences in proliferation of primary cultures of vascular smooth muscle cells taken from male and female rats. *Blood Vessels* **17**: 110–116, 1980.

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