# IN VITRO CHARACTERIZATION OF VASCULAR SMOOTH MUSCLE CELL HYPERPROLIFERATION IN SPONTANEOUSLY HYPERTENSIVE RATS

by

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**Abstract:** The proliferative phenotypes of cultured vascular smooth muscle cells (VSMC) derived from spontaneously hypertensive rats (SHR) aortae have been characterized in comparison to cells from normotensive Wistar-Kyoto rats (WKY). VSMC from SHR continue to proliferate at a higher rate in longterm culture. Using cell number determination, [$^3$H]thymidine incorporation, flow cytometry and Northern blot analysis, two distinct phenotypes of VSMC from SHR are demonstrated: 1. 4-hour accelerated entry into the S phase of the cell cycle in response to mitogens, including calf serum, epidermal growth factor and platelet-derived growth factor, and 2. altered contact inhibition accompanied by an earlier expression of protooncogene *c-fos*, preceded by an exaggerated expression of transforming growth factor $\beta_1$ (TGF-$\beta_1$) mRNA and paralleled by an abnormal response to exogenous TGF-$\beta_1$. Persistance of these intermediate phenotypes under culture conditions evokes their primary character and suggests their involvement in the pathogenesis of increased vascular resistance in hypertension.

**CARACTERISATION IN VITRO DE L'HYPERPROLIFERATION DU MUSCLE LISSE VASCULAIRE CHEZ LES RATS SPONTANEMENT HYPERTENDUS**

par Vratislav Hadrava

**Résumé:** Les phénotypes de prolifération des cellules du muscle lisse vasculaire (VSMC) en culture provenant des aortes des rats spontanément hypertendus (SHR) et des cellules de rats normotendus Wistar-Kyoto (WKY) ont été caractérisés. Les VSMC de rats SHR continuent à proliférer en culture prolongée à un taux plus élevé. En utilisant le comptage des cellules, l'incorporation de [$^3$H]thymidine, la cytométrie de flux et l'analyse par buvardage de type "Northern", deux phénotypes distincts ont été démontrés: 1) l'entrée accélérée de 4 heures dans la phase S du cycle cellulaire suite aux stimuli mitogéniques incluant le sérum de veau, le facteur de croissance épidermique et le facteur de croissance dérivé des plaquettes et 2) l'inhibition par contact altérée accompagnée par l'expression plus rapide du protooncogène *c-fos*, précédée par l'expression accrue de facteur de croissance transformant $\beta_1$ (TGF-$\beta_1$) ARNm, ceci en parallèle avec une réponse anormale au TGF-$\beta_1$ exogène. La persistance de ces phénotypes intermédiaires en culture évoque leur caractère primaire et suggère leur implication dans la pathogénie de la résistance périphérique augmentée dans l'hypertension.

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To Anne-Marie

To my parents

Zdenka and Vratislav

To my grand-parents

Johanna and Jan, Zdenka and Vladimir

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## LIST OF ABBREVIATIONS

[$^3$H]thymidine, methyl-$^3$H-thymidine
AMP, adenosine monophosphate
ANF, atrial natriuretic factor
ANOVA, analysis of variance
BrdU, bromodeoxyuridine
°C, degree Celsius
cdc, cell division cycle
Ci, curie(s)
cm$^2$, centimeter(s) square
cpm, counts per minute
DME, Dulbecco's modified Eagle's medium
DNA, deoxyribonucleic acid
DOCA-salt, deoxycorticosterone and salt induced
$EC_{50}$, 50% effective concentration
EDTA, ethylenediaminetetraacetate
EGF, epidermal growth factor
FACS, fluorescence-activated cell sorter
FGF, fibroblast growth factor
g, gram(s)
$G_0$, gap 0 of cell cycle
$G_1$, gap 1 of cell cycle
$G_2$, gap 2 of cell cycle
GTP, guanosine trisphosphate
H, histone
HDL, high density lipoprotein
hr, hour
hsp, heat shock protein
$ID_{50}$, 50% inhibition dose
IGF, insulin-like growth factor
IgG, immuno-globulin G
i.p., intraperitoneal
kb, kilobase
kD, kilodalton
LDL, low density lipoprotein
l, liter(s)
µl, microliter(s)
min, minute(s)
ml, milliliter(s)
M, molar
MPF, maturation-promoting factor
mRNA, messenger ribonucleic acid
n, number in study
N, normal (concentration)
NIH, National Institute of Health
NP-40, Nonidet P-40
NS, not significant
OD, optical density
%, (with numeral), percent
p, probability
PBS, phosphate-buffered saline

PDGF, platelet-derived growth factor
pH, negative logarithm of hydrogen ion
r, correlation coefficient
$R_{max}$, maximal response
RNA, ribonucleic acid
SD, standard deviation
SDS, sodium dodecyl sulfate
SEM, standard error of the mean
SHR, spontaneously hypertensive rat
TGF, transforming growth factor
U, unit(s)
V, volt(s)
VSMC, vascular smooth muscle cell
W, watt(s)
WKY, Wistar-Kyoto normotensive rat

## PREFACE

This work deals with the characterization of vascular smooth muscle cell hyperproliferation in spontaneously hypertensive rats. I have choosen the option provided in **Section 7** of the **Guidelines Concerning Thesis Preparation**, which allowed me to include as chapters of my thesis the text of original papers concerning the thesis research project.

> *"The candidate has the option, subject to the approval of the Department, of including as part of the thesis the text, or duplicated published text, of an original paper, or papers. In this case, the thesis must still conform to all other requirements explained in Guidelines Concerning Thesis Preparation. Additional material (procedural and designed data as well as description of equipment) must be provided in sufficient details (e.g. in appendices) to allow a clear and precise judgment to be made of the importance and originality of the research reported. The thesis should be more than a mere collection of manuscripts published or to be published. It must include a general abstract, a full introduction and literature review and a final overall conclusion. Connecting texts which provide logical bridges between different manuscripts are usually desirable in the interest of cohesion."*

The experimental parts of the thesis (chapters 2, 3, 4 and 5) consist of four papers which have been either already published or submitted for publication. The text of these papers appears in its original form with only minor modifications. Some of the references appear in duplicates, that is, they are cited in the Review of the Literature as well as in the relevant article. For easier orientation, the references are numbered and listed in order of appearance in

the text. The use of the terms "we", and "our" within the text refers to myself and my thesis director, Dr Pavel Hamet. Dr Rafick-Pierre Sekaly is a co-author of the third paper; Dr Sekaly provided us with the opportunity to use FACScan and FACStar flow cytometers and guided us during the Hoechst 33 342 - bromodeoxyuridine substitution studies. Ursula Kruppa is a co-author of the fourth paper; she collaborated in *c-fos* expression studies, which are in part included in her thesis. Dr Johanne Tremblay is a co-investigator in the work presented here.

Chapter 1 is a survey of the literature concerning aspects of cardiovascular structural changes in hypertension, regulation of vascular smooth muscle cells proliferation in normal and hypertensive states and the general control of cell proliferation. Chapter 6 summarizes the conclusions and claims to originality.

# CHAPTER 1.

# REVIEW OF THE LITERATURE

## PRIMARY HYPERTENSION

### Definition

Hypertension is a quantitative trait defined arbitrarily as a statistical deviation from the mean blood pressure of the whole population at a given age. Primary hypertension is a nosologic entity in which the cause of high blood pressure is unknown, and essential hypertension is a term reserved for human primary hypertension. Selective breeding for high blood pressure has produced several animal models of primary hypertension, termed "genetic" or "spontaneous" hypertension as opposed to "experimental" hypertension induced by surgical or pharmacologic intervention in normotensive animals. These artificially-induced hypertensive models, such as Goldblatt renal hypertension and DOCA-salt hypertension, serve as models of secondary hypertension.

### Spontaneously hypertensive rats

The most frequently used animal model of genetic hypertension is the spontaneously hypertensive rat (SHR), which was developed by Okamoto and Aoki in Kyoto, Japan, in 1963 [1]. This strain was established by mating one male spontaneously hypertensive rat with one female of the same Wistar-Kyoto (WKY) colony with blood pressure levels slightly above the average. Successive brother-sister matings of offspring with hypertension persisting over one month were then undertaken. After three generations, the incidence of spontaneous hypertension was approximately one hundred percent

as early as a few months after birth. These animals are prone to various hypertensive pathologic lesions (cerebral hemorrhage, myocardial infarction and angionecrosis). In many respects, the SHR model resembles human essential hypertension [2, 3]. Hypertension in SHR has a hereditary component and does not appear to be only of renal or simple neurogenic origin. Arterial blood pressure development, cardiac output and total peripheral resistance alterations follow a very similar course in both SHR and human essential hypertension. In both forms of hypertension, the disease is aggravated by stress and other environmental factors [4-7]. Also, both naturally-occurring diseases react to antihypertensive agents. The accompanying pathologic images of hypertension in SHR are similar to those observed in essential hypertension (cerebral damage, renal lesions, cardiovascular hypertrophy and hyperplasia).

The use of the SHR model of essential hypertension has not been totally free of criticism mainly because of the lack of a proper normotensive control strain. The original breeding stock of WKY rats was not maintained as parallel controls and animals with blood pressure at the upper limits were usually withdrawn from the WKY breeding program, which started several years later [8]. Differences observed between WKY and SHR may be due to a chance selection of genes unrelated to the regulation of blood pressure [9]. This is, however, a general problem in studies comparing normotensive and genetically hypertensive animals. Recently, using DNA

fingerprinting analysis of genomic DNA, genetic heterogeneity was noted in WKY but not in SHR distributed by different commercial suppliers [10, 11]. On the other hand, it has been demonstrated that SHR may differ in their sensitivity to high salt diets depending on the breeding origins [12]. Therefore, the relevance of the described abnormality in the hypertensive strain for its pathogenetic linkage with high blood pressure should not be overestimated. The breeding facility from which WKY and SHR originate should also be taken into account. Considered together, the SHR is generally accepted as an useful research model not only for studies on the pathogenesis and therapy of primary hypertensive disease but also on its prevention and prognosis, and WKY strain is still its most adequate control since both strains come ultimately from the same Wistar-Kyoto colony.

**Other research models of primary hypertension**

Several other models of genetic hypertension have been developed. The New Zealand strain of genetically hypertensive rats [13], the Lyon hypertensive strain [14], the Munster hypertensive strain [15] and the stroke-prone spontaneously hypertensive rat [16] are models of spontaneous primary hypertension. The Milan hypertensive strain [17] is a model of genetic hypertension due to renal hypoplasia. Two strains have been developed for their susceptibility to salt ingestion: Dahl salt-sensitive rats [18] and Sabra hypertensive rats [19]. Recently, a series of recombinant inbred strains were

developed between SHR and normotensive Brown-Norway (BN.lx) rats [20], providing a unique opportunity to distinguish between an abnormality due to a chance selection of genes unrelated to hypertension in SHR and an abnormality which segregates with high blood pressure.

Other models of primary hypertension used in research are the spontaneously hypertensive mouse developed by Gunther Schlager [21] and the primate model of Green Vervet monkeys [22, 23].

The development of transgenic animals carrying genes relevant to blood pressure regulation offers new genetic models to study the involvement of specific genes in hypertension [24-26].

## PATHOGENESIS OF PRIMARY HYPERTENSION

### General considerations

There is significant evidence that a long developmental period precedes the full manifestation of human essential hypertension beginning in early childhood. Hypertension is generally attributed to a mosaic interaction of genetic factors and a series of environmental influences. Studies of twins, familial aggregation of the disease and surveys of adopted children argue in favor of an inherited predisposition to essential hypertension [27-31]. Animal models of spontaneous hypertension, established by selective breeding, provide

further evidence that blood pressure is genetically determined. Estimates of heritability range from 20 to 90% for the SHR model [32]. Environmental factors, such as physical or emotional stress and dietary habits, play a significant role in determining the severity and possibly the time of onset of the disease [4, 33, 34]. The recent findings of our laboratory that thermosensitivity cosegregates with high blood pressure in $F_2$ and backcross progeny of sponatenously hypertensive mice suggest that the susceptibility to environmental stress is genetically determined and may be pathogenetically linked with hypertension [35]. Moreover, the genetic hypertension in mice and rats is characterized by an abnormal expression of major stress gene *hsp70* which is localized in the major histocompatibility complex [36, 37], a locus which apparently cosegregates with hypertension in recombinant inbred rats [20].

**Cardiac output**

Blood pressure is determined by cardiac output and total peripheral resistance. Cardiac output is dependent upon two basic variables: heart rate and stroke volume. An increased cardiac output has been reported in early or borderline hypertensives [38, 39], some of whom display a hyperkinetic circulation. However, the increased cardiac output does not persist since elevated peripheral resistance is the usual hemodynamic finding in established hypertension [40-42]. The role of cardiac output in the later development and

maintenance of sustained hypertension was studied by treating normotensive rats and SHR with ß-adrenergic-blocking drugs prior to and following conception (and then in the weaned offspring) in an effort to prevent any phase of increased cardiac output during in utero, postpartum or early development [43, 44]. Despite a reduced heart rate and hemodynamic evidence of ß-adrenergic inhibition of cardiac function, SHR and their normotensive controls presented the same body growth rate and arterial pressure increments as untreated hypertensive and normotensive rats. These studies provide strong evidence that: 1. even if there is a stage of increased cardiac output early in the development and elaboration of hypertension, it is not necessary for the later phase of sustained hypertension associated with a rise in total peripheral resistance; and 2. sustained hypertension can occur in the absence of elevated cardiac output.

**Total peripheral resistance**

Total peripheral resistance is the sum of the vascular resistance interactions of all organs and the regional circulations. Most of the resistance to blood flow is attributed to small diameter vessels. Muscular wall arterioles provide by far the major share of this resistance and are referred to as "resistance vessels". The remaining resistance is offered by large arteries, venules and veins. Increased total peripheral resistance is invariably observed in the established phase of the disease in SHR [41, 42, 45] and

humans [46].

Factors leading to increased total peripheral resistance can be divided into three categories:

1. **Vascular**, namely altered vessel design associated with a decrease in the lumen due to structural and functional changes, including excitation-contraction coupling;
2. **Extravascular**, including increased sympathetic activity and circulating humoral factors;
3. **Rarefaction**, a reduced number of vessels, some of which may be completely or permanently closed.

In both rats [47-50] and humans [51, 52], there is evidence that increased vascular reactivity may be partly related to altered vessel design. A 5% decrease in the average internal radius of resistance vessels would produce a 25% increase in total peripheral resistance.

Structural alterations resulting in greater wall thickness have been found in SHR [53] even before their blood pressure rises. Folkow's studies indicate that structural changes in the vessel wall are capable of increasing not only the absolute calculated resistance to basal blood flow but also the responsiveness of resistance vessels to constrictor agents [54, 55]. Enhanced vasculature reactivity to a variety of stimuli has been demonstrated in SHR [56-62]. Increased vascular reactivity to norepinephrine is a well-established

feature of essential hypertension [63, 64]. This heightened responsiveness is not observed in patients with renovascular hypertension [65] but is present in normotensive children of hypertensives [66] and in normotensive subjects with a familial history of hypertension [67]. The increased vascular reactivity to pressor agents seen in SHR is absent in one-clip, 2-kidney hypertension [68]. Vascular hyperresponsiveness is observed in SHR, even when blood pressure is controlled with antihypertensive drugs from the time of weaning, suggesting that these vascular changes are primary events [69]. Alterations of vascular reactivity seem to appear before the development of established hypertension in SHR [70, 71]. The oscillatory activity noted in stroke-prone SHR tail arteries in response to norepinephrine is pathogenetically associated with a significant increment of blood pressure [72] and is not influenced by antihypertensive treatment [73]. The response to manganese, cobalt and lanthanum occurs only in aortic strips from prehypertensive and adult SHR [74]. This abnormal reaction to non-physiologic cations has been demonstrated as a genetic locus (Hyp-2) co-segregating with blood pressure [75]. Thus, the vascular hyperresponsiveness appears to be an intrinsic trait of vascular tissue rather than the consequence of elevated blood pressure and indeed may be causally related to essential hypertension.

Enhanced extravascular neural participation in greater total peripheral resistance in essential hypertension is

suggested by the fact that several of the hemodynamic alterations detailed above can be produced by adrenergic mechanisms. Also, many available potent antihypertensive drugs operate through inhibition of adrenergic functions. Various degrees of elevated plasma and urinary catecholamines in hypertensive patients have been reported [76-81]. Hemodynamic and plasma catecholamine responses to mental stress are enhanced in adolescents with labile hypertension or with hypertensive parents [82]. Studies on SHR show: a greater than normal reduction of arterial pressure by surgical or pharmacological abolition of sympathetic nerve activity [83, 84]; prevention of the development of hypertension by immunosympathectomy [85, 86]; chemical sympathectomy [87] or by depletion of central catecholamines [88]; slowing of the development of high arterial pressure by renal denervation [89, 90]; increased sympathetic nerve activity by direct recordings [91, 92]; enhanced catecholamine release during brief immobilization [93]; and a positive correlation between renal sympathetic nerve activity and mean arterial pressure in hybrid SHR/WKY [94]. Therefore, although the exact nature and extent to which this system participates are presently unclear, an important role of the adrenergic nervous system in essential hypertension is evident.

There is, however, no proof that excessive circulating pressor agents or decreased levels of depressor substances are directly linked to the pathogenesis of essential hypertension.

In studies of SHR in parabiosis with WKY, arterial pressure did not increase in the WKY partners, and there was no interference with the normal progression of hypertension in SHR [95, 96]. However, some investigators have demonstrated opposite results. Hypertension can be transmitted from SHR to normotensive animals by cross-circulation [97].

Even though the renin-angiotensin system undoubtedly plays a role in regulating normal blood pressure, its involvement in the pathogenesis of genetic hypertension is unclear. Indeed, patients with essential hypertension have been reported to have either normal, decreased or increased plasma renin activity, and in SHR, the results are equally conflicting [98, 99]. Transgenic rats harbouring the mouse *Ren-2* renin gene develop a fulminant hypertension with low plasma renin [25]. The inhibition of the conversion of angiotensin I to angiotensin II has been found to lower arterial blood pressure in humans with essential hypertension [100] and in SHR [101]. However, the mechanisms of action of these drugs may involve effects other than inhibition of the circulating renin-angiotensin system in both humans [102] and rats [103].

There are several reports of elevated plasma atrial natriuretic factor (ANF) levels in patients with essential hypertension [104, 105], but there are also studies in which no difference was found between hypertensive and normotensive subjects [106, 107]. Experiments on SHR have generally

confirmed the tendency of ANF secretion to rise with the development of hypertension [108, 109]. Transgenic mice carrying the ANF gene and expressing elevated ANF plasma levels have significantly lower blood pressure [26]. In response to different stimuli, higher plasma cyclic nucleotides are found in patients with labile hyperkinetic hypertension [110, 111] and in SHR than in their normotensive controls [112]. Exaggerated increase of cyclic AMP occurs early in hypertension and is followed by progressive diminution after the establishment of high blood pressure [113]. Endothelium-derived relaxing factor and endothelin are inactivated in the plasma and thus are unlikely to play a hormonal role [114, 115].

Steroids, such as aldosterone and 18-hydroxy-11-deoxycorticosterone, do not seem to be involved significantly in either essential or SHR hypertension [116, 117]. Essentially hypertensive men were reported to have lower levels of both free and total testosterone and androstenedione than controls and the converse was true for hypertensive women [118]. Estradiol levels were higher in both hypertensive men and women. The clinical and pathophysiological significance of these findings in increased total peripheral resitance remains unclear. Other possible humoral factors, such as prostaglandins, kallikrein-kinin and vasopressin, have been investigated, but their role in primary hypertension is not established.

In addition to the above mechanisms possibly responsible for increased vascular resistance and hyperreactivity, evidence of decreased arteriolar density in the skeletal muscle of SHR [119-121] and in the conjunctiva of humans [122] has been presented. However, the extent of this rarefaction in other tissues is unknown, and its contribution to augmented total peripheral resistance is uncertain. The potential mechanism may involve the bifunctional regulator of cell growth, the transforming growth factor $\beta_1$ (TGF-$\beta_1$). Its latent form secreted by endothelial cells is activated by a mechanism requiring contact between endothelial cells and pericytes, the two cell types playing a central role in microvascular growth [123, 124].

## CARDIOVASCULAR HYPERTROPHY/HYPERPLASIA IN HYPERTENSION

### Primary vs secondary events

#### Structural changes as a consequence of hypertension

The question of cardiovascular hypertrophy/hyperplasia being a primary event in hypertension or a secondary adjustment to elevated blood pressure is of major importance in respect to the etiology, prognosis and treatment of the disease. Cardiovascular hypertrophy/hyperplasia is usually believed to be a consequence of elevated blood pressure. Hypertensive patients show a high degree of cardiac pathology post-mortem, including concentric hypertrophy [125-127] and

thickened aortic media [128]. Concentric left ventricular hypertrophy is a classical change seen in the hearts of patients with an established phase of the disease [129, 130]. Cardiac hypertrophy is observed in adult New Zealand genetically hypertensive rats [131] and in experimental renal artery constriction or DOCA hypertension [132]. An 8-week or 15-month period of DOCA-salt hypertension combined with unilateral clipping of the renal artery in Carworth rats is associated with an increased diameter, thickness and cross-sectional area of the media of the thoracic aorta [133, 134]. A linear correlation is evident among vessel dimensions, the amount of medial elastin and collagen and calculated wall tension. The dominant change in rabbit elastic and muscular arteries seen after 8 months of hypertension induced by abdominal aorta constriction is an increase in arterial wall mass, including vascular smooth muscle cell (VSMC) proliferation [135]. In 6- to 8-week-old SHR, the minimal luminal diameters of almost all branches of jejunal small arteries and arterioles are significantly smaller than in normotensive control rats [47]. Increased media thickness in aortae [136-138] and resistance vessels [139-141] has been reported in SHR by many laboratories. Warshaw [140] demonstrated that the greater contractility of 6-week-old SHR mesenteric resistance vessels is due to their structural narrowing with thickening of the media caused by hyperplasia.

**Structural changes occur before hypertension is established**

In 1953, Grant [142] reported that, at autopsy, many patients with marked hypertension showed little or no cardiac hypertrophy, even though the duration and severity of the disease did not differ substantially from that seen in subjects who presented left ventricular hypertrophy, indicating that high blood pressure and the degree of hypertrophy may be dissociated. This is further supported by findings of hypertrophy and an enhanced contractile activity of veins and pulmonary arteries in SHR, despite similar right ventricular, central and portal venous pressures in WKY and SHR [143]. Moreover, several studies showing cardiovascular hypertrophy/hyperplasia before the development of high blood pressure suggest that the structural changes may represent a primary and perhaps independent event. Cardiac hypertrophy can be demonstrated very early in essential [144] and genetic hypertension [41, 145-147]. Several authors have reported an increased left/right ventricular weight and heart/body weight ratio in newborn SHR [148-151]. Thickened mesenteric vessel wall is observed in 3- to 5-month-old SHR in the prehypertensive stage [152, 153] and even in 15-day-old SHR [154]. Gray [155] found that the medial wall of the carotid artery is already thickened in 1-day-old SHR. Similarly, in 15-day old SHR, Nordborg and Johansson [154] noted that the cross-sectional area of the media in both extra- and intracranial segments of the internal carotid artery was larger in SHR than in WKY

rats. Aortic medial thickness and the number of laminae are significantly greater in SHR fetuses on the 22nd day of gestation [156]. Surprisingly, after stenosis of the renal artery in normotensive Wistar rats, a significant increase of [$^3$H]thymidine incorporation into aortic DNA had already occurred after 5 days whereas blood pressure rose over a 2-week period [157]. However, direct primary genetic programming for cardiovascular hypertrophy/hyperplasia in genetic hypertension still remains controversial since: 1) embryonic SHR hearts, when transplanted in the anterior eye chamber of adult SHR and WKY rats, do not grow differently from control WKY embryonic hearts [158]; and 2) slightly but significantly elevated blood pressure levels are found in newborn SHR [159]. Nevertheless, Walter and Hamet demonstrated higher [$^3$H]thymidine incorporation in the newborn SHR heart when compared to newborn WKY [160].

**Causal relationship of structural changes and hypertension**

An international effort has been initiated by our laboratory to approach this controversy by comparing several strains of genetically hypertensive rats and mice with their appropriate controls [9, 161]. Cardiac and renal hyperplasia is found to be present in newborns of four different spontaneously hypertensive rat strains and in spontaneously hypertensive mice but not in offspring of parents with renal or experimental hypertension and in normotensive controls [161],

thus providing evidence that cardiac and renal hyperplasia may indeed be causally related to spontaneous hypertension. In genetic segregating experiments, it was shown that the abnormal structure of SHR mesenteric resistance vessels is associated with one of the factors that produces hypertension [162]. In $F_2$ progenies, a correlation was observed between the mesenteric lumen to media ratio and blood pressure.

**Effect of antihypertensive treatment on structural changes**

The relationship between high blood pressure and cardiovascular hypertrophy/hyperplasia is investigated in hypertension through therapeutic modulation. The rationale for this investigation was that antihypertensive drugs preventing or normalizing high blood pressure should prevent or reduce the structural changes if they are only a secondary adaptation of the cardiovascular system to hypertension. Despite effective antihypertensive treatment, the impact on cardiovascular structure was dissimilar, depending on the age of the animals or patients, type of vessels investigated, duration and type of treatment. Combined treatment of SHR with several antihypertensive drugs [163-166] had a preventive and regressive effect on cardiac and aortic hypertrophy, if started at an early age (up to 5 months). No effect was observed after 52 weeks of age [163]. Resistance vessel hypertrophy was not reduced after 14 months of combined therapy in established human essential hypertension [52, 167].

Vasodilators (hydralazine, felodipine, isradipine) or ß-blocking agents (propranolol, metoprolol, timolol) alone had either no effect [168-172] or only a slight impact [171, 173] on cardiovascular structure in SHR, even when administered very early (42nd day after birth) [168]. Resistance jejunal arteries in SHR were unaffected by treatment with capsaicin, a drug releasing substance P, although blood pressure had been controlled at a normotensive level from birth [174]. In secondary (one-kidney, DOCA) hypertension, hydralazine largely prevented the structural changes of resistance in arterioles when compared to non-treated rats [175]. On the other hand, in SHR, administration of angiotensin-converting enzyme inhibitors (captopril, perindopril, cilazapril) was accompanied by prevention or regression of the structural cardiovascular alterations seen in non-treated hypertension [168, 173, 176, 177], and it appears that this effect transcends the blood pressure-lowering action, and persists after withdrawal of therapy [168, 173].

**Hypertrophy, hyperplasia and polyploidy**

Cardiomegaly in newborn [149, 159], and adult SHR [41, 147] as well as in induced hypertension is a well-known phenomenon. However, higher DNA content per gram of cardiac tissue has been reported only in newborns [148, 150, 151]. With [$^3$H]thymidine incorporation in SHR neonates *in vivo*, Walter and Hamet [160] demonstrated a higher specific activity (cpm per microgram) of DNA with no significant differences in the

synaptic α-adrenergic receptors have not provided evidence of specific alterations in SHR [203, 204]. The number of β-adrenergic receptors is reported to be reduced [205]. Nerve growth factor levels are elevated in mesenteric arteries and aortae of young SHR [206]. The vascular signalling system is abnormal in SHR [207]. Intracellular pH is more alkaline [208] and basal and noradrenaline-stimulated hydrolysis of inositol phospholipids is increased [209-211]. Thromboxane $A_2$ generation from SHR vessels is enhanced [212]. Hyperresponsiveness to noradrenaline is associated with an increased calcium influx [213]. Higher levels of cytosolic free magnesium, calcium and intracellular sodium have been demonstrated in the intact thoracic aorta of SHR by nuclear magnetic resonance spectroscopy [214]. Calmodulin levels are reported to be decreased or unchanged in SHR vessels [215, 216], but high amounts of a calmodulin activator have been recorded [217].

**VASCULAR SMOOTH MUSCLE CELL GROWTH**

Blood vessels arise from mesenchyme as a budding network of small endothelial channels [218]. These channels become surrounded by locally-derived mesenchymal cells. With time, layers of these fibroblast-like cells become delimited and take on a more characteristic ultrastructural appearance of mature smooth muscle cells [219]. Extrinsic growth factors from the surrounding milieu and intrinsic regulatory factors play a role in these proliferation and differentiation processes. VSMC are responsible for maintaining both tension via

contraction-relaxation and vessel integrity by proliferation and synthesis of extracellular matrices. Therefore, VSMC possess multifunctional abilities and are capable of expressing a range of phenotypes, with contractile and synthetic phenotypes being at opposite ends [220, 221]. Smooth muscle cells in the media of fetal arteries express a synthetic phenotype whereas in the media of adult arteries, the majority of smooth muscle cells present a contractile phenotype. Thus, the normal development of an artery involves smooth muscle cells evolving from a proliferative synthetic state to a differentiated contractile state.

**Skeletal muscle paradigm of differentiation**

Little is known about smooth muscle differentiation. The recent identification of a family of muscle-specific regulatory factors that can convert fibroblasts to skeletal muscle has contributed significant insights into events that regulate myogenesis. A similar situation may be expected for smooth muscle cells. Rhabdomyoblasts, the precursor cells of skeletal muscle, appear to be similar to fibroblasts [222]. These undifferentiated cells require growth factors for proliferation [223]. When growth factors are removed from the medium, the cells rapidly change phenotype. Genes for characteristic skeletal muscle proteins are turned on and proteins are produced. Four mammmalian myogenic regulatory factors have been identified: MyoD [224], myogenin [225], myf-5 [226], and MRF4 [227]. They are expressed exclusively in skeletal

muscle and have the ability to activate myogenesis in transfected mesodermal stem C3H 10 T1/2 cells. Their proteins share 4 domains of high homology. Two of them are necessary and sufficient to induce myogenic differentiation: 1. the helix-loop-helix (HLH) domain with homology to the *myc* family of proteins [228], enabling dimerization; and 2. the basic domain adjacent to the HLH domain, together forming approximately a 70 amino acid region which is a prerequisite for binding DNA [229-231]. HLH dimers bind to a consensus region, CANNTG (E-box), found in regulatory *cis* regions of most but not all muscle-specific genes [230, 232, 233] and in certain muscle-nonspecific genes [234, 235]. It is suggested that even though these regulatory proteins may have a direct effect, they may rather act through transactivation of other genes regulating muscle-specific genes or form complexes with not yet identified proteins which bind regulatory sequences other than E-box in muscle-specific genes. These four factors autoregulate their own and activate one another's expression [236]. There is an evidence that other factors influence myogenesis. The fact that the expression of activated *ras* and *fos* oncogenes extinguishes the expression of MyoD and myogenin [237, 238] and that avian retroviruses expressing *v-ski* [239] can activate myogenesis in quail embryo fibroblasts supports this notion. The decision to differentiate appears to be determined by a balance between signals elicited by members of the MyoD family and signals generated by exogenous growth factors. Commitment to differentiation occurs during $G_1$ phase and may

be repressed by growth factors [240]. A high concentration of serum, fibroblast growth factor (FGF) or TGF-ß blocks the expression of muscle-specific genes in cells that express myogenic factors constitutively [240, 241]. TGF-ß has been also shown to act indirectly via upregulation of type I collagen expression. A type I collagen layer inhibits myoblast differentiation without blocking myogen in expression [242]. High levels of MyoD can suppress cell growth and lead to differentiation, even in the presence of a high concentration of mitogens or activated oncogenes [237, 238]. Myoblast differentiation is accompanied by a marked decrease of *c-myc* expression [243]. Interestingly, in $BC_3HI$ cells (an established smooth muscle cell-like line from mouse brain tumors), the expression of smooth muscle specific Á-isoactin can be increased at confluency when there is extensive cell-to-cell contact by serum deprivation [244]. The linkage of expression of a differentiated phenotype and loss of replication resembles the situation seen in rhabdomyoblasts deprived of growth factors.

**Contractile and synthetic phenotypes of VSMC**

The antagonism between the proliferation and the action of growth factors on the one hand and the differentiation on the other is also observed in VSMC *in vivo* and *in vitro*. Fetal smooth muscle cells expressing the synthetic phenotype are highly proliferative. Smooth muscle cells from adult vessels expressing the contractile phenotype proliferate only at a

very slow rate, being mostly in a quiescent $G_0/G_1$ state [245]. The two distinct phenotypes are also characterized at the biochemical level by the expression of different isoforms of structural and contractile smooth muscle-specific and smooth muscle-nonspecific proteins [246, 247]. VSMC during fetal life predominantly present $\beta$-actin isoform with low levels of $\alpha$-actin. No new isoactins appear in corresponding adult tissue but a significant increase occurs in $\alpha$-actin relative to decreased levels of $\beta$-actin. $\gamma$-actin isoform is present in minor amounts and does not seem to correlate with phenotype.

In addition to contractile proteins, the cytoplasm contains structural proteins some of which also change with differentiation [246, 248, 249]. Vimentin is found in 87% of medial cells in fetal aorta; the remaining 13% of cells contain both vimentin and desmin, and none contains desmin alone. With development, there is a gradual decrease in cells containing vimentin alone (51%) and an increase in cells in which both vimentin and desmin co-exist (48%) with a low occurrence (1%) of cells containing only desmin.

An analogous situation has been noted in cell culture [250, 251]. Enzyme-dispersed aortic smooth muscle cells seeded into primary culture are morphologically and functionally similar to those of intact vessels. They either contract spontaneously or can be induced to contract by electrical, mechanical or chemical stimulation. They also synthesize only

small amounts of extracellular matrix material. However, if these cells are seeded below a critical cell density, after about one week, they lose the capacity to contract, gain the capacity to divide, and synthesize 4 to 5 times more extracellular matrix than do contractile-state cells [252, 253]. These cells therefore resemble immature smooth muscle cells. Cultured VSMC growing in their logarithmic phase incorporate [$^{35}$S]sulfate into chondroitin/dermatan sulfates containing proteoglycans at a higher rate than do non-dividing cells. This process is accompanied by a decrease in the activity of chondroitin sulfate-synthesizing enzymes [254]. However, not all components of the extracellular matrix are synthesized in a lower scale as cells reach contractile phenotype under confluent conditions. Changes from the synthetic to the contractile state, induced by dimethylsulfoxide, are accompanied by the formation of basement membranes and increased type IV collagen synthesis [255]. Collagen type I and III formation is also greater as VSMC approach confluency and growth slows while their mRNA increases markedly under serum deprivation [256]. The analogy of confluent cells and *in vivo* situations has also been seen at the membrane potential level [257]. The membrane potential of rat aortic smooth muscle cells in high density culture (-51 to -58 mV) closely matches the value of -55 mV recorded for the normal rat *in vivo*. The membrane potential of low density culture is -30 mV due to a continuous process of hyperpolarization during proliferation.

Cells can regain the contractile phenotype when they return to a quiescent $G_0/G_1$ state, one or two days after achieving confluence and presenting a "hill-and-valley" morphology [258, 259]. This is observed in primary culture as well as in subculture when cells are plated at subconfluent but at sufficiently high densities. In contrast, cells that require more than two to three weeks to achieve confluence do not return to the contractile state [253]. Changes from contractile to synthetic phenotype can be correlated with alterations at the biochemical level [260-262]. In primary VSMC culture, $\alpha$-actin is a preponderant isoform; with time, it decreases with a loss of desmin, whereas vimentin increases with $\beta$-actin, which becomes the most abundant isoform. Changes in the expression of various isoactins are paralleled by the differential expression of smooth muscle and non-smooth muscle-specific myosin heavy chains [263, 264]. During the lag and early log phase after seeding, more than 90% of VSMC react positively to specific smooth muscle myosin antibody. The relative number of smooth muscle myosin-positive cells reaches a minimum at the end of the log phase and then rises during the plateau phase, at confluency. The minimum level of smooth muscle myosin-positive VSMC reached is correlated with seeding density and is lowest in sparsely-seeded cultures [265]. The process of phenotypic modulation is therefore reversible, depending, as stated above, on seeding densities and on how long it takes the cells to reach confluency. Synthetic and contractile phenotypes are only two points on a continuous

spectrum of smooth muscle cells and some cells may have both contractile and synthetic state features.

Modulation from contractile to synthetic phenotype and vice versa also occurs *in vivo* under such conditions as arterial injury and repair. One to two weeks after endothelial damage, medial cells migrate to the intima, where they proliferate and are structurally equivalent to the synthetic state of cultured cells [266, 267]. A slight transient decrease in the cellular content of smooth muscle-specific myosin occurs during the development of experimental vascular lesions *in vivo* [268]. $\alpha$-actin and desmin are reduced, vimentin increases and $\beta$-actin becomes predominant. When the endothelium is completely restored (10-11 weeks later), these neointimal cells regain the contractile phenotype and proportions of their cytoskeletal elements become similar to undamaged medial cells. Consistent with this finding is the observation of Gabbiani [261] that, like sparse VSMC in culture or cells *in vivo* after denudation, human atheromatous plaques and surrounding diffuse intimal thickenings show a predominance of $\beta$- and $\gamma$-actin isoforms.

**Regulation of VSMC proliferation**

Most of our knowledge about VSMC proliferation comes from *in vitro* studies of cultured cells and *in vivo* investigations of artery regeneration. To proliferate, VSMC need to be attached to substrates. The presence of growth factors and an

extracellular matrix is essential.

Platelets whose contents are released during clotting at sites of damaged endothelia or when serum is prepared contain high amounts of platelet-derived growth factor (PDGF), TGF-$\beta_1$ and epidermal growth factor (EGF) [269-272]. Cultured VSMC maintained on uncoated tissue culture plastic dishes supplied only by plasma remain in a quiescent state [273]. When whole blood serum or platelet extracts are added to the culture medium they start to multiply, indicating that growth factors from platelets are essential for VSMC proliferation [195]. The major mitogenic component is identified as PDGF [274]. PDGF-AA and PDGF-BB homodimers are equally active on VSMC cultures from newborn human aortae [275]. Genes for PDGF-A and PDGF-B chains and PDGF receptors ($\alpha$ and $\beta$ subunits) are expressed in normal rat and balloon-injured aortae and are independently regulated during regeneration [276]. After binding to its specific receptor on VSMC [277, 278], PDGF intiates a series of events leading to DNA synthesis and proliferation. Even though few studies have been directed toward the elucidation of specific intracellular events that occur in VSMC after PDGF stimulation, similarities to the general paradigm described for fibroblasts are plausible. In the cultured embryonic smooth muscle A-10 cell line, PDGF stimulates $Na^+$ influx via an elevation of intracellular $Ca^{2+}$ [279]. Platelet extract stimulation of DNA synthesis in cultured VSMC from Wistar rats is preceded by adenylate cyclase activation [280]. Serum

stimulates *c-fos* and *c-myc* expression in VSMC [281].

VSMC from aged (8-month-old) rats need a higher concentration of PDGF to promote their growth, even though they respond to serum stimulation better than cells from 5-day-old rats. Antibodies against PDGF partially inhibit the stimulatory effect of serum in VSMC from young rats and have no influence in cells from aged animals, suggesting that in the latter case, VSMC are stimulated preferentially by other growth factors [282].

The second requirement for vascular smooth muscle cell cycle progression is the availability of EGF and insulin-like growth factor-I (IGF-I) [283-285]. VSMC, coated on an extracellular matrix and primed by a 6-hour presence of serum, respond well to EGF, FGF, insulin or IGF-I [286]. EGF and FGF may substitute for each other as well as may insulin and IGF-I. EGF markedly stimulates VSMC proliferation in plastic dishes in the presence of 1% calf serum, indicating that PDGF and EGF are important stimuli for VSMC proliferation. Cultured VSMC present a specific receptor for EGF [287] functional in inducing receptor phosphorylation, $Ca^{2+}$ fluxes and DNA synthesis [283, 288]. PDGF and EGF work together with other growth factors and extracellular matrices in complex interactions leading to the proliferation of sparse cells and the formation of the multilayered hill-and-valley pattern of confluent cells.

FGF exerts an additional stimulatory effect on the growth rate of VSMC cultured in plasma- or serum-supplemented medium in uncoated dishes but not in dishes coated with an extracellular matrix [289].

TGF-$\beta_1$ on its own acts as a VSMC growth stimulator but only on confluent cells [290]. Moreover, this growth factor has been shown to potentiate serum or PDGF stimulation of VSMC at high cell densities and to inhibit it at low cell densities [291, 292]. A combination of these growth inhibitory and stimulatory effects is believed to be responsible for the establishment and maintenance of the hill-and-valley morphology common to VSMC in culture. TGF-$\beta_1$ promotes the expression of mRNA for the PDGF-A chain and thrombospondin [290]. TGF-$\beta_1$ expression of the thrombospondin message and protein is delayed relative to their induction by PDGF. Experiments using cycloheximide and antibodies to PDGF suggest that the induction of thrombospondin depends on the de novo synthesis of PDGF-like activity. The growth stimulatory effects of TGF-$\beta_1$ may, therefore, be mediated by PDGF-like activity. The ability of TGF-$\beta_1$ to inhibit proliferation correlates with the expression of unique TGF-$\beta_1$ binding sites on the surface of spare VSMC of 75,000 kd [292]. TGF-$\beta_1$ inhibition is accompanied by VSMC hypertrophy [293].

Besides growth factors from platelets, other blood-borne

cell factors may play a role in modulating VSMC proliferation. Monocytes-macrophages and neutrophils adhere to and invade sites of arterial injury. PDGF is chemotactic, not only for VSMC [294] but also for monocytes and neutrophils [295]. Neutrophil-conditioned medium stimulates [$^3$H]thymidine incorporation into DNA as well as the number of rabbit VSMC in culture [296].

Interleukin-I stimulates [$^3$H]thymidine incorporation into cultured human VSMC [275]. This effect appears to be indirect and mediated by induction of the PDGF-A chain gene and PDGF-AA activity. Interleukin-6 induces *c-myc* and stimulates cultured rat VSMC proliferation [297]. PDGF enhances that effect.

$\gamma$-Interferon has been shown to inhibit exponentially-growing rat VSMC and calf serum-stimulated quiescent cells *in vitro* [298]. $\alpha$-Interferon supresses PDGF-induced competence as well as plasma- or FGF-induced progression [299].

Among the plasma components that affect VSMC proliferation are insulin, high (HDL) and low (LDL) density lipoproteins, endothelin and ANF. Insulin presents mitogenic activity on VSMC only at pharmacological doses and its effect may be mediated through its interaction with IGF-I-binding sites [286, 300]. HDL have been shown to stimulate VSMC proliferation in extracellular matrix-coated dishes in a dose-dependent manner, but stimulation by HDL, even with FGF and

insulin, never reaches the level of serum-induced stimulation [286]. Although some evidence of the mitogenic effect of LDL has been reported [301], they have been demonstrated to be rather cytotoxic, even at low concentrations, on human and bovine VSMC grown in lipoprotein-deficient serum [286, 302].

Endothelin clearly stimulates VSMC proliferation, but its effect is inferior to that of EGF and serum [303].

ANF has little influence on the proliferation of VSMC grown in medium supplemented with 2% fetal calf serum alone but exhibits a dose-related inhibition of PDGF-stimulated [$^3$H]thymidine incorporation [304] with a concomitant reduction of cell size [305].

Other agents required for VSMC homeostasis *in vivo* have been demonstrated to induce growth-related events in cultured cells. Angiotensin II activates $Na^+/H^+$ exchange [306], raises cytosolic free $Ca^{2+}$ [307, 308], and induces PDGF-A chain, *c-myc* and *c-fos* mRNA [309, 310]. It also enhances proliferation [311], even though there are studies reporting only hypertrophy due to augmented protein content with no changes in DNA synthesis and cell number [312, 313]. Norepinephrine and epinephrine increase the frequency of polyploid VSMC in culture as well as *in vivo* independently from elevated blood pressure [314]. Arginine vasopressin enhances [$^{35}$S]methionine incorporation into proteins and protein content but has no effect on VSMC

proliferation *in vitro* [315]. Intraperitoneal injection of phenylephrine or vasopressin or intravenous administration of angiotensin II leads to the induction of *c-fos*, *c-myc*, *hsp 70* and *hsp 68* genes in the rat aorta [316].

VSMC replication may also be controlled by factors intrinsic to the vessel wall. PDGF-like activity is produced by VSMC *in vitro* [317] and *in vivo* [318]. VSMC of synthetic phenotype obtained from injured parts of the artery, elicit 5-fold greater amounts of PDGF-like activity when placed in culture than their contractile counterparts from healthy arteries [319]. VSMC conditioned medium is reported to contain another stimulatory growth factor distinct from PDGF, FGF, EGF or IGF-I [320]. VSMC secrete an extracellular matrix whose important contribution to the integrated control of VSMC proliferation is well-recognized. In general, it decreases the requirement for growth factors [286, 289]. Changes in the proportion of its components may accelerate or inhibit VSMC growth, as shown by studies of cells grown on different substrates. Fibronectin and laminin potentiate VSMC attachment, spreading and proliferation [321]. A thrombospondin-rich extracellular matrix promotes both the proliferation and migration of VSMC [322] and augments the response to EGF [323].

Glycosaminoglycan heparin is a potent inhibitor of VSMC growth, as demonstrated *in vivo* [324] as well as *in vitro* where,

in addition, confluent cells produce heparan sulfate moieties [325]. Heparin prevents progression through the $G_1$ phase of the cell cycle [326, 327] and decreases responsiveness to EGF [328].

The interaction with endothelial cells is not negligible. Endothelial cells secrete a PDGF-like protein [329] and heparin-like substance [330]. The contribution of these two different factors *in vivo* has not yet been elucidated. It has been reported that confluent endothelial cell-conditioned media have an inhibitory effect on VSMC growth, and in co-culture systems, exponentially-growing endothelial cells stimulate VSMC proliferation [331]. This suggests that endothelial cells at different growth stages produce two distinct growth factors.

*In vitro* **VSMC proliferation in hypertension**

As already mentioned, inappropriate VSMC growth has been observed *in vivo* in pre- and post-hypertensive stages in SHR and other models of hypertension. VSMC obtained from SHR aortae [257, 332-336, 344], or mesenteric arteries [337] proliferate even more in culture. Calf serum-stimulated ornithine decarboxylase activity is greater in SHR and stroke-prone SHR than in WKY [338]. Four weeks of DOCA-salt treatment in Wistar rats can potentiate subsequent smooth muscle cell migration from explants in primary culture and proliferation in up to a second sub-culture [339]. Two weeks of experimental renal hypertension lead to an increase of VSMC proliferation,

even in up to the sixth sub-culture [340], but in the case of hypertension of six weeks duration, the higher proliferation seen in second sub-culture declines and reaches the growth level of control VSMC from normotensive animals. It has been hypothesized that, in healthy animals, there are different VSMC clones, some being capable of more rapid proliferation in response to stimuli. These cells predominate in vessels of injured animals and consequently also in explant cultures and sub-cultures. The limited reduplication capacity of post-mitotic cells [341] is assumed to be the reason for the equal proliferation after six passages. Such a shortly persisting defect in this type of experimental hypertension is characterized by an increase in cell size and a higher percentage of polyploidy in cultivated VSMC [342, 343]. SHR VSMC proliferate constantly more than control cells from WKY, even in longterm culture and their size is similar or slightly smaller [333, 348], corresponding to observations on freshly-dispersed cells [345]. SHR VSMC are hyperresponsive to EGF as compared to cells from either WKY [344, 346] or NIH Black Wistar rats [347] with higher EGF-binding sites [346]. The response to low doses of PDGF alone is similar [336, 344, 347]. However, at high concentrations, this growth factor may stimulate [$^3$H]thymidine incorporation into DNA but, more importantly, it enhances the response to EGF [344]. Increased SHR VSMC growth is associated with an accelerated $Na^+/H^+$ exchange [348] and an elevation of cytoplasmic free calcium [349]. This difference is maintained in longterm culture. Cyclic AMP production, basal

as well as stimulated by isoproterenol, epinephrine or norepinephrine is the same for VSMC from both WKY and SHR strain, in concordance with the fact that both strains have an equal number of high affinity $\beta$-receptors even though WKY VSMC have three times more low affinity $\beta$-receptors than SHR [350]. Calmodulin activator, a highly hydrophobic protein, is present in cultured VSMC from SHR [217], thus conferring greater activity to the calcium/calmodulin system. SHR VSMC elaborate an extracellular matrix in culture that is more stimulatory to the growth of WKY cells than their own kind [351]. SHR cells are also markedly less susceptible to growth inhibition by heparin [352]. VSMC from SHR show an enhanced proliferative response to the mitogenic action of angiotensin II [353] and serotonin [354], as indicated by both [$^3$H]thymidine incorporation and cell number determination. A concomitantly increased formation of [$^3$H]inositol phosphate [353, 354] has been reported, and this effect is blocked by saralazin [355]. Quantitation of protein kinase C by immunoblotting and [$^3$H]phorbol dibutyrate binding reveals no difference between VSMC from SHR and WKY. The sensitivity of protein kinase C to phorbol ester is also the same [353]. VSMC from SHR, however, present a significantly greater number of specific angiotensin II-binding sites [355]. Angiotensin II stimulation *in vitro* increases significantly more cytoplasmic free calcium in SHR than in WKY [356] with *c-fos* and *c-myc* expression [355]. The addition of angiotensin-converting enzyme inhibitor to primary cultures of SHR VSMC has no effect on proliferation or on the

incidence of polyploidy [185]. Vasoconstrictor thromboxane $A_2$ generation is enhanced in SHR VSMC also *in vitro* [212]. Thromboxane synthase inhibitor significantly tempers VSMC doubling time and [$^3$H]thymidine incorporation into DNA in SHR only. A stable analog of thromboxane $A_2$ stimulates VSMC proliferation only in WKY rats. Higher [$^3$H]thymidine incorporation in VSMC from SHR and their stroke-prone SHR derivatives is inhibited by two-day incubation with cholera toxin subunit b [357] with concomitant induction of a contractile phenotype. Since subunit b interacts with gangliosides, its effect on plasma membrane is hypothesized to be responsible for phenotype modulation. Even though inhibition is seen in VSMC cultures from WKY, the cells continue to proliferate at a lower level equal for VSMC from all three strains (WKY, SHR, stroke-prone SHR). Viewing comprehensively, these results suggest a general alteration of VSMC membrane fluidity in genetic hypertension, already observed in erythrocytes [207, 358-360].

**GROWTH FACTORS AND ONCOGENES**

Numerous polypeptide growth factors have been discovered and many of their actions characterized. Because growth factors act primarily in the prereplicative $G_0/G_1$ phase of the cell cycle, their mitogenic effects will be discussed in the next chapter. This section will focus on their isoforms, receptors and chromosomal localization. The chromosomal

mapping of genes provides clinically and pathophysiologically relevant information about loci and their mutual linkage on chromosomes. Breaks at specific loci may result in typical translocations, seen in pathologies such as Burkitt's lymphoma and other hyper- or neoplastic processes [361]. Genes located near the chromosomal breakpoints change their activity possibly as a result of translocation. Oncogenes will be reviewed from the viewpoint of their relation to growth factors.

PDGF is a 30-kD heat-stable cationic polypeptide, first identified in and isolated from platelets [273, 362-364]. Biologically-active PDGF consists of two subunits, A and B, which form disulfide-linked homo- and hetero-dimers. The B-chain of PDGF is structurally and immunologically related to $p^{28sis}$ encoded by the simian sarcoma virus transforming gene, *v-sis* [365, 366]. The cellular homolog of *v-sis*, the protooncogene *c-sis*, actually encodes a polypeptide precursor of the PDGF B-chain [367-369]. It has been mapped in humans on chromosome 22 [370]. There is also considerable (60%) homology between both A and B chains [369]. The PDGF A-chain gene has been localized on chromosome 7 [371]. PDGF purified from platelets contains both PDGF AB and PDGF BB dimers, whereas PDGF AA is produced and secreted by tumor cell lines [371, 372]. All three isoforms are mitogenic, depending on the cell type. Differences in the biological potency of AA, AB and BB forms of PDGF seem to be due to variations in the number and

relative proportions of two PDGF receptors [373, 374]. The type A PDGF receptor binds all three dimeric forms of PDGF whereas the type B PDGF receptor binds PDGF BB with a high affinity and PDGF AB with a low affinity but not PDGF AA [375]. A and B type PDGF receptors are structurally related and organized in a similar fashion [376]. The primary structure of the PDGF receptor is closely related to that of the *v-kit* oncogene product and to receptors for macrophage colony-stimulating factor (CSF-1) [377]. The PDGF receptor is a transmembrane glycoprotein of about 180-kD with a single transmembrane domain. The tyrosine kinase domain is interrupted by a sequence which is unrelated to known tyrosine kinase sequences. The human PDGF receptor gene has been mapped by *in situ* hybridization to chromosome 5 [377]. Activation of the PDGF receptor seems to be mediated by its dimerization, which is closely associated with the activation of intrinsic tyrosine kinase [378].

EGF is a small 6-kD polypeptide originally isolated from a mouse submaxillary gland [379]. It is derived from a high molecular weight precursor, pre-pro-EGF [380], which is related to a region that does not include a sequence for EGF to the extracellular domain of the LDL receptor [381]. Pre-pro-EGF is also related to the protein encoded by the *v-mos* oncogene [382]. Another homology is observed between the EGF precursor and a peptide sequence encoded by the second exon of ANF [383]. There are several similarities between EGF and TGF-$\alpha$ [384]. In

humans, the gene for EGF is localized on chromosome 4 [385]. The plasma membrane receptor for EGF has been purified and cloned. The EGF receptor in human cells is encoded by a gene localized on chromosome 7 [386]. The promoter region of the EGF receptor shows a striking similarity to a promoter region of the human *c-H-ras* protooncogene [387]. The mature EGF receptor is a transmembrane glycoprotein of about 175 kD, consisting of a single polypeptide chain containing at its cytoplasmic region a sequence responsible for tyrosine kinase activity [388]. This tyrosine kinase domain in contrast to the tyrosine kinase domain of the PDGF receptor, consists of an uninterrupted stretch of amino acids. Sequence analysis has revealed a close homology between the transmembrane and cytoplasmic domains of the EGF receptor and *v-erb-B* transforming protein of the avian erythroblastosis virus [389]. Binding experiments have uncovered two distinct affinity sites of the EGF receptor [390]. High affinity sites usually represent a minor percentage (5 to 10%) of the total receptor population and are suggested to be responsible for the mitogenic response. High affinity sites may actually represent receptor dimers of two low ligand affinity monomers. Allosteric oligomerization has been proposed as a mechanism leading to the activation of tyrosine kinase [391].

Fibroblast growth factors (FGFs) were first used to describe polypeptides isolated from the pituitary that stimulated cell division in 3T3 cells or vascular endothelial

cells. Two species were identified: cationic, basic FGF (bFGF) and anionic, acidic FGF (aFGF), with a similar weight of 16 to 18 kD [392, 393]. Both polypeptides have a strong affinity for glycosaminoglycan heparin. It is now recognized that many different growth factors isolated from a variety of tissues and given different names (endothelial cell growth factor, cartilage-derived growth factor) are actually either bFGF or aFGF [394-396]. bFGF and aFGF are structurally related, revealing a 53% sequence homology [397]. Both are highly conserved among species. They have been cloned and mapped in humans on chromosomes 4 and 5 respectively [398, 399]. There are at least three oncogenes related to bFGF and aFGF: *int-2*, *hst*, and FGF-5 [400, 401]. bFGF and aFGF share the same receptor [402, 403]. Low and high affinity binding sites have been identified [404]. The low affinity receptor appears to be associated with heparin-like molecules that may act to facilitate access to high affinity receptors.

TGF-α represents a polypeptide growth factor that is structurally and functionally different from its homonymic TGF-β but is rather homologous in function and structure to EGF [384]. TGF-α binds to the EGF receptor and stimulates its autophosphorylation. TGF-α is a single-chain polypeptide of about 7.4 kD with structural homology to MuSV retrovirus [405]. TGF-α gene has been mapped on human chromosome 2 [361].

TGF-β represents a large family of closely-related

peptides with similar activities (TGF-$\beta_{1 \text{ to } 5}$) [406-409]. There is an even larger superfamily of more diverse factors (inhibin, activin, Mullerian inhibiting substance), based on homology in the C-terminal domain of their precursors [409-411]. TGF-$\beta_1$ is the most widely studied prototype of these factors. It is a disulfide-linked dimer of 2 identical chains of 12.5 kD [412, 413]. TGF-$\beta_1$ was initially described as an activity produced by retrovirally-transformed cells causing anchorage-independent growth when applied to untransformed non-neoplastic cells [414]. It is now well established that TGF-$\beta_1$ is also expressed in many normal cells and tissues [413]. Platelets are the richest source of TGF-$\beta_1$ [270, 415]. The degree of identity between the 5 mature TGF-$\beta$ sequences ranges from 64 to 82%, and, individually, TGF-$\beta$ is even better conserved [409]. TGF-$\beta_1$ is located on human chromosome 19 [416], TGF-$\beta_2$ and TGF-$\beta_3$ on chromosome 1 and 14, respectively [417]. Two glycoprotein receptors (I and II) of 53 and 72-100 kD have been identified as the most widespread high affinity TGF-$\beta_1$ binding sites mediating its signal transduction [418, 419]. TGF-$\beta_1$, unlike many other growth factors, is unable to stimulate tyrosine-specific phosphorylation, indicating that its receptor does not possess the tyrosine kinase domain. The formal identification of TGF-$\beta_1$ receptor cDNA has so far been unsuccessful.

Insulin-like growth factors (IGFs), also called somatomedins, are polypeptides with a marked homology to

insulin [420]. IGF-I (somatomedin C) and IGF-II (somatomedin A) are members of a family of hormones that mediate many of the growth-promoting effects of growth hormone [421, 422]. The physiological properties of IGF-I are different from those from IGF-II. IGF-I is more growth hormone-dependent and more mitogenic than IGF-II which is more insulin-like in its action and is present in the blood at levels three times greater than IGF-I. The determination of primary structure has demonstrated that IGFs are distinct from the insulin molecule. They both consist of a single-chain polypeptide of about 7.5 kD in mature form [420, 423]. IGF-I gene is localized on human chromosome 12, whereas the IGF-II gene resides on human chromosome 11 [424]. The IGF-I receptor, a hetero-tetrameric disulfide-linked complex consisting of two $\alpha$ 130-kD subunits and two $\beta$ 98-kD subunits, also binds insulin with low affinity. It possesses intrinsic tyrosine kinase activity similar to the insulin receptor. In contrast, the IGF-II receptor is a monomer of about 250 kD, does not appear to have any significant affinity for insulin and is devoid of protein kinase activity [425].

The above-mentioned growth factors represent only a segment of agents with mitogenic or mitogenic-like effects. Insulin, besides its metabolic effect, may also stimulate DNA synthesis and cell growth [286, 426]. Nerve growth factor and hematopoietic growth factors are also typical mitogens acting primarily in specific tissues. Moreover, any agent involved

in the regulation of cellular metabolism may have a profound either negative or positive effect on cell growth and proliferation. Considerable advances in our understanding of the structure and effects of these growth factors have been achieved. Their actions include effects unrelated to the control of cell growth as well [283, 427, 428]. One peptide can have both stimulatory and inhibitory activity in a single cell, depending on other signal molecules present [429]. Complex interactions between them are being uncovered. As an example, there is growing evidence that the bimodal mitogenic effects of TGF-$\beta_1$ are mediated through PDGF-AA induction and decreased PDGF receptor A production [290, 430, 431]. Growth factors exert pleiotropic control or modulation at almost every level of embryogenesis, tissue morphogenesis, cell growth and differentiation by autocrine, paracrine and, in some cases, endocrine effects. They are involved in many physiological and pathological situations (wound healing, tumorigenesis, angiogenesis, atherosclerosis). It is now evident that many of these peptides have a much wider spectrum of activity than the biological growth stimulatory/inhibitory effects after which they were originally named. Most of them are better considered as multifunctional agents.

> *"What sense can be made of the multiplicity of actions of so many peptide growth factors? It is apparent that specific peptides are not necessarily limited to a single physiological activity, but rather that growth factors form part of a complex cellular signalling language, in which the individual peptides are the equivalent of characters of an alphabet or code. From this it follows that*

> *informational content resides not in individual peptides, but in the pattern or set of regulatory peptide molecules to which a cell is exposed. The use of combinations selected from a large number of peptide signalling molecules increases the amount of information that can be transmitted. It is not surprising that meaning in this particular cellular language is contextual, because that is generically the case for all languages or codes."*
>
> ***Michael B. Sporn & Anita B. Roberts, 1988*** [428]

While growth factors are extrinsic to the cell, protooncogenes figure prominently as intrinsic factors or genetic regulators able to respond to environmental growth stimuli. However, these extrinsic and intrinsic differences are not so clearcut regarding: 1) the ability of the cell to produce and secrete its own mitogen; and 2) the fact that several oncogenes as mentioned above encode for proteins identical or similar to growth factors or their receptors. Nevertheless, early and late growth-regulated genes may be considered as intrinsic regulatory factors provided they meet criteria other than their simple induction by growth factors [432, 433]. They must be involved in the second messenger signalling pathway or, otherwise, regulate cellular proliferation (e.g., by transactivating other genes).

Among the early growth-regulated genes are several protooncogenes, including *c-fos*, *c-myc* and *c-jun*. *c-fos* and *c-jun* products interact with each other to form a heterodimeric transcription factor complex [434]. The Fos/Jun heterodimer binds with high affinity and specificity to DNA

elements of the consensus sequence identified as AP-1 [435]. The Fos/Jun complex plays an important role in the transactivation of downstream genes and thereby functions as a nuclear mediator coupling external signals to longterm changes in gene expression in the cell [436]. The mechanism of action of *c-myc* protein is not yet clearly understood. Recent findings demonstrate that c-Myc can act via its helix-loop-helix domain as a sequence-specific DNA-binding protein and may play a role as a co-activator of other transcription complexes [228]. Its direct involvement in the regulation of cell proliferation by inducing competence is evident and will be discussed below. The late growth-regulated protooncogenes include *c-myb, c-mos, c-src and c-ras* [433, 437-439]. They are induced in the mid- or late $G_0/G_1$ phase. *c-myb* may act as a transcriptional activator [437]. *c-src* and *c-ras* code for cytoplasmic proteins. The former has tyrosine kinase activity, the latter resembles guanine-binding proteins and has intrinsic GTPase activity [439, 440]. Their precise function is not completely understood and they are thought to play a role in the final transition from the $G_1$ to S phase, in the organization of the DNA-synthesizing machinery and in the transition from S phase to mitosis.

Although not directly regulated by growth factors, cell division cycle (cdc) genes and their products form another group of intrinsic growth regulators, mainly but not exclusively involved in transition from S phase to mitosis.

## CELL CYCLE

The processes that constitute the cell cycle include all biosynthetic mechanisms involved in growth, the provision of energy, development of extending membranes and surface structures, the genesis of new organelles and mechanics of segregation of components at cell division. The advent of the microscope, [$^3$H]thymidine incorporation and flow cytometry has made it possible to understand spatial and temporal processes and to distinguish different stages of the cell cycle. Biochemistry with molecular biology has provided information on underlying mechanisms.

As first shown by Howard and Pelc [441] with plant root cells, the cell cycle may be divided into four phases. This applies also for most but not all eukaryotic cells. After mitosis (M) or cytokinesis, the cell begins its cycle with $G_1$ (first gap), proceeds through the S (DNA synthesis) phase, followed by the $G_2$ phase (second gap), and finally ends its life by dividing into two daughter cells in mitosis (M). Variations in cell cycle times among different cell types and within populations are mainly due to variations in the length of $G_1$, with the duration of S, $G_2$ and M being relatively constant.

Two different approaches have led to a unified view of the cell cycle. The genetic approach to analyzing mutations which arrest the cell cycle at specific points explained

complicated processes as a linear sequence of events. The initiation of each step in the pathway is dependent on completion of the preceding step. This deterministic model, based on the studies of Hartwell [442], assumes that specific genes execute each step. The second cytologic or embryologic approach examines natural points of cell cycle arrest and factors that enable the cell cycle to proceed [443]. It describes a random transition model of oscillation between two states: deterministic (B), consisting of S, $G_2$, M and early $G_1$, and probabilistic (A), the $G_0/G_1$ state. The transition from A to B occurs when one or more randomly fluctuating critical components exceeds the level needed to trigger DNA synthesis. The results of Rao and Johnson [444] support both models. When two interphase cells at different stages of the cell cycle are fused, the more advanced nucleus waits for the completion of events of the retarded nucleus before making any further progress in its cell cycle, thus supporting the deterministic model. The random transition model is supported by observations that fusion of mitotic cells with cells in any other state always induces some form of mitotic response in the interphase nucleus. Two distinct deterministic and probabilistic states are thus suggested. Today's view contains elements from both models, as demonstrated by the studies discussed below.

### $G_0/G_1$ phase

In most cases, there is a temporary gap ($G_1$) between the

completion of mitosis and the onset of DNA synthesis. However, $G_1$ duration may be negligible in early embryos [445] and in a few cultured cells [446]. On the other hand, some *in vivo* cells, such as hepatocytes and neurones, can remain for long periods in the non-proliferating quiescent state ($G_0$). The control of post-embryonic cells *in vivo* and cultured post-mitotic cells occurs during this period. Extracellular factors and nutrients determine whether the cells will continue to cycle. New RNA and protein synthesis are needed [447-449]. Normal animal cells in culture become growth-arrested in the quiescent $G_0$ state when either serum or essential nutrients are withdrawn or when reaching cell to cell contact at confluency. Based on studies with fibroblasts, the $G_0/G_1$ phase has been divided, depending on the effects of limiting factors, into *competence*, *entry*, *progression* and *assembly* subphases, separated by C [450], V [451], and R points [452]. Quiescent BALB/3T3 cells, if not provided in ordered sequence, first with PDGF and subsequently with platelet-poor plasma, do not progress to the S phase [450, 453]. Short PDGF treatment induces a *competence* state, lasting many hours and enabling the cells to respond to platelet-poor plasma components. This first critical point C is situated about 12 hours before DNA synthesis starts, providing that plasma is added [451]. Another *competence* factor, bombesin, is active for other cell types [454]. FGF and hydrocortisone may also substitute for PDGF [455]. Nutrients are not required for the cellular response to PDGF, but amino acids are needed for

the plasma-promoting effect [451]. One of the factors responsible for $G_0/G_1$ progression after C point has been identified as EGF [456]. Its presence is required for approximately 8 hours during the $G_0/G_1$ phase. After priming with this progression factor, fibroblasts traverse the V point located 6 hours prior to DNA synthesis [457]. The subphase between C and V points has recently been named *entry* state [458]. Full progression activity of platelet-poor plasma for BALB/3T3 cells is simulated only by EGF- and somatomedin C-supplemented defined medium [457]. The traverse of the last 6 hours of $G_0/G_1$ requires only IGF-I which can be substituted by hyperphysiologic concentrations of insulin [459, 460]. Cycling non-quiescent cells need only one of these factors to initiate DNA synthesis [461]. The *progression* subphase between V and R is extremely sensitive to cycloheximide, indicating that net protein synthesis is particularly important [462]. After the restriction R point, already-synthesized proteins are assembled into a multiple protein complex, which is the actual machinery for DNA synthesis [452, 463]. This *assembly* subphase is insensitive to cycloheximide [464] and is growth factor-independent. When cycling cells are incubated in medium lacking all growth factors, only those cells within two hours from the S phase can initiate DNA synthesis [461], thus situating the R point two hours before the $G_1$-S boundary.

The transition from the prereplicative $G_0/G_1$ phase to the S phase is regulated by a series of extracellular signals and

the intrinsic ability of the cell to respond to them. After binding to their specific receptors, mitogens stimulate an ordered sequence of biochemical events in the membrane cytosol and nucleus, ultimately leading to DNA synthesis. Different growth factors induce similar basic events and the mechanisms of their differential action in $G_0/G_1$ subphases are not yet elucidated. It may be hypothesized that elicited signalling pathways must cooperate in a subtle manner at different periods, sequentially or in parallel, to converge for the final triggering effect. An immediate consequence of growth factor-binding to surface receptors is the activation of intrinsic tyrosine kinase [465, 466]. Other signals include an increase in monovalent ion fluxes across the plasma membrane [467], a rise in intracellular pH [468, 469], hydrolysis of phosphoinositides [470], augmented cytosolic calcium [471], expression of a group of genes [472-474], elevation of cellular cyclic AMP [475], and internalization and degradation (down-regulation) of the receptor [466, 476]. Internalized growth factors may bind to chromatin rendering the involved DNA regions nuclease resistant [477]. Besides autophosphorylation of the receptor, several potential substrates for tyrosine kinase can be visualized with antibodies to phosphotyrosine [478]. Their importance as mediators of the mitogenic response has not yet been conclusively identified. Stimulation of an amiloride-sensitive $Na^+/H^+$ antiport by growth factors increases intracellular sodium and causes cytoplasmic alkalinization [479]. There is a secondary stimulation of $Na^+/K^+$

pump activity which increases intracellular potassium and restores the electrochemical gradient for sodium. Since stimulation of the proliferative response in quiescent cells depends on maintaining intracellular pH and potassium concentration above critical threshold levels, ionic events may play a permissive role in mitogenesis [467]. The process of phosphoinositide hydrolysis produces diacylglycerol and inositol trisphosphate [470]. The former activates protein kinase C, while the latter mobilizes calcium from the endoplasmic reticulum via specific receptors. An elevation of cytosolic calcium activates calcium-dependent enzymes. The rapid cytosolic increase and membrane hyperpolarization elicited by EGF are not limited to the initial few minutes of stimulation but proceed for longer periods in the form of discrete fluctuations [468, 480]. Calmodulin is a major intracellular calcium-binding protein which mediates most of calcium's effects. Calmodulin needs to be elevated during the entry and progression subphases [481, 482]. Diacylglycerol activates protein kinase C by reducing the calcium requirements of the enzyme and enhances the membrane association [483]. Other agents, such as arachidonic acid, generated from the hydrolysis of phospholipids by phospholipase $A_2$, activate protein kinase C directly [484]. Protein kinase C participates in the mitogenic pathway by regulating other receptor functions, ion channels and gene expression. Protein kinase C phosphorylates the EGF receptors at a specific threonine residue [485], thus decreasing the

apparent affinity of this receptor without changing the total number of sites [486]. Interestingly, PDGF stimulates the phosphorylation of EGF receptors at an identical site via, at least in part, protein kinase C activation [487, 488]. One might speculate that such a mechanism may be implicated in the cell's requirement for prolonged exposure to EGF after PDGF-inducing competence. Activation of protein kinase C leads either directly or indirectly to activation of $Na^+/H$ exchange, which in turn elevates intracellular pH [489]. Thus, protein kinase C may be involved in maintaining the effects of growth factors. A sustained increase in cyclic AMP constitutes a growth-promoting signal for Swiss 3T3 cells. PDGF induces a striking accumulation of cyclic AMP in quiescent 3T3 cells via enhanced synthesis of E type prostaglandins [475]. In contrast, other growth factors do not elevate cyclic AMP levels [490].

In addition to the events in membrane and cytosol, growth factors rapidly and transiently induce the expression of the cellular oncogenes, *c-fos* and *c-myc*, and other genes whose functions are as yet unknown [472, 473, 491]. The expression of these genes appears even when protein synthesis is inhibited, showing that their transcription does not require production of new proteins. *c-fos* and *c-myc* are suggested to be mediators of competence induction [492-495]. Their translation occurs in mid $G_0/G_1$ and their proteins act as transcriptional activators for later genes [228, 496]. The *c-myc* protein, upon microinjection into nuclei of 3T3 cells, cooperates with

progression factors of platelet-poor plasma in the stimulation of DNA synthesis [494]. Similarly, when *c-myc* is introduced into cells under the control of hydrocortisone-inducible promoter, the addition of hydrocortisone induces DNA synthesis provided progression factors are present [495]. One of the candidate genes regulated by *c-myc* or *c-myb* is cdc2 gene coding for p34 protein kinase whose activity is needed around the R point and later in mitosis [437]. Second messengers stimulated by EGF and IGF-I may interact with early gene products in these effects. Gene transcription, initiated later in $G_0/G_1$, depends on protein synthesis and requires that essential amino acids be brought into the cell. *Ras* gene product is needed during the progression subphase because antibody to *ras* protein blocks transition through $G_0/G_1$ only if microinjected in the later half of the $G_0/G_1$ phase [438]. *Src* protein may be considered a late growth regulatory oncogene, since $pp60^{v\text{-}src}$ has been shown to induce cellular DNA synthesis in the absence of early genes, as if it were acting directly on genes coding for proteins of the DNA synthesizing machinery [439]. Several enzyme activities increase in late $G_0/G_1$, including ornithine decarboxylase [497], dehydrofolate reductase [498, 499], thymidylate synthase [500], thymidine kinase [501], DNA polymerase [502] and $p34^{cdc2}$ kinase [503]. At the end of $G_0/G_1$, during the *assembly* subphase, enzymes move to the nucleus to form a multienzyme replication complex and to catalyse DNA synthesis.

The transition to S phase is controlled not only by positive but also by negative factors. Products of retinoblastoma (Rb) gene provide a block to exit from $G_0/G_1$ and prevent the cell from progressing to S phase. The block can be removed by specific phosphorylation. $p34^{cdc2}$ is hypothesized to be that specific kinase [504, 505]. $G_0/G_1$ block of the cell cycle progression induced by prostaglandins is closely associated with the induction of 68-kD heat shock protein (hsp) [506]. Several authors have reported elevated levels of 70-kD hsp mRNA and protein in late $G_0/G_1$ and S phase and proposed that hsp may play a role in maintaining cell viability [507-509].

**S phase**

The main events during the S phase are the replication of genetic material, that is DNA, and the synthesis of histone and non-histone chromosomal proteins. Chromosomes are very long coiled and supercoiled strings of nucleosomes, each of which is an octamer of histones (two molecules each of histones H2A, H2B, H3 and H4), around which is bound the duplex DNA strand. The entire DNA content of the nucleus must be replicated completely, precisely, and only once in a period of a few hours. This is achieved by initiating bi-directional replication at multiple sites around each chromosome [510, 511]. DNA synthesis in both prokaryotes and eukaryotes requires a number of enzymes and non-enzymatic proteins besides DNA

polymerase. Several enzymes are involved in the synthesis of DNA precursors [497, 499-501]. DNA polymerase α plays a key role in the replication of the eukaryotic genome [512]. Previous reports of induction of DNA polymerase α from $G_1$ to mid S phase of the cell cycle implied that transcription and translation of this gene are closely coupled to the onset of DNA replication [502, 513]. Monoclonal antibodies specific for DNA polymerase α inhibit DNA replication when microinjected into nuclei [514]. The actual event that triggers initiation at an origin of replication is unknown. Within a given cell type, individual genes replicate at defined periods in S phase [515]. Many active genes replicate early in S phase. Protein and RNA synthesis is necessary for initiation and, at a lower level, for continuation of replication [502, 513, 516]. Although the composition of other enzymes directly implicated at the replication fork is not yet fully understood, several activities and protein complexes have been identified. Directly involved in the enzymatic process are: origin-specific binding activity, priming and deoxynucleotide polymerizing activity, helix unwinding, single-stranded DNA binding and DNA ligase activities [517-521].

Recently, a complex termed RF-S has been partially purified by assaying the ability of protein fractions from S phase cells to activate DNA synthesis in extracts from $G_1$ cells [522]. This complex has been found to contain $p34^{cdc2}$ kinase, which is necessary for RF-S activity. Cyclin A

activates $p34^{cdc2}$ and subsequently induces DNA replication. These data suggest a direct role of $p34^{cdc2}$ kinase in activating DNA synthesis, but do not exclude its participation in earlier events. A complex of three polypeptides termed RF-A [519] has been purified and proposed to stabilize the single-stranded region, by its function as a single-stranded DNA-binding protein, for the correct positioning of the polymerase/primase complex. The other essential fraction contains factors required for the formation of a functional pre-synthesis complex at the replication fork. Another complex, RF-C [520], has also been isolated. Reconstituted reactions and hybridization studies with strand-specific, single-stranded DNAs have revealed that in the absence of RF-C, abnormal DNA synthesis occurs preferentially on the lagging strand, and leading strand replication is inefficient. RF-C is therefore necessary for coordinated synthesis of leading and lagging strands at the replication fork.

There is no evidence that specific DNA sequences or proteins are required for the termination of replication. It appears that replication terminates when the two forks meet [510]. DNA topoisomerase II is said to be implicated in separation of the two progeny DNA duplexes [523, 524]. Assembly of these newly-replicated DNA into nucleosomes seems to occur only a short way behind the replication fork. Pulse-chase experiments on both nascent DNA and histones suggest that histones H3 and H4 are deposited first on nascent DNA, then

histones H2A and H2B [525].

In normal eukaryotic cells, DNA is prevented from re-replicating. It has been shown that nuclei replicated *in vitro* are unable to re-replicate in fresh replication extracts until they have passed through mitosis. The only mitotic change which is required is nuclei permeabilization [526]. Based on these studies, a model for the control of DNA replication has been proposed. A hypothetical licencing factor binds to DNA, DNA assembles into the nucleus, licencing factor is inactivated by initiation or passage of the replication fork, and fully replicated DNA cannot re-replicate due to the exclusion of licencing factor until the nuclear envelope is permeabilized during mitosis. Thus, this model can explain why $G_2$ nuclei in $G_1/G_2$ cell hybrids cannot re-replicate until passage through mitosis into the next S phase [444].

**$G_2$ + M phase**

Once chromosomes have been replicated, the cell enters the $G_2$ phase. During this $G_2$ phase, components necessary for the construction and operation of the mitotic apparatus, such as tubulin and other essential spindle proteins, are synthesized [527]. Other regulatory factors reach a critical level, triggering entry into mitosis. There is a continuing need for RNA and protein synthesis. Non-histone proteins are

synthesized during S and $G_2$ [528] and it appears that the formation of these proteins is essential until the $G_2$/M boundary is reached. Several regulatory factors acting at this stage are also already expressed and synthesized during preceding phases, indicating that the normal cell enters this stage at least partially furnished with basic components.

Recently, a new insight into biochemical and genetic regulation at the $G_2$/M boundary was achieved by studies of yeasts and subsequent identification of several homologous regulatory proteins found in human cells. The key protein complex is a maturation-promoting factor (MPF), originally isolated on the basis of the ability of extracts from early frog embryos during mitosis to induce meiotic maturation in immature frog oocytes [529]. Its activity has been detected also in cultured mammalian cells [530]. The early events of mitosis induction by MPF are chromatin condensation and nuclear breakdown preceded by hyperphosphorylation of lamines, the major structural proteins of the nuclear lamina, which gradually becomes depolymarized [526, 531, 532]. The serine/threonine kinase activity of MPF complex is conveyed by a subunit protein, $p34^{cdc2}$ [503]. The kinase activity of $p34^{cdc2}$ is in turn dependent on its phosphorylation state, on tyrosine, serine and threonine residues [533], and is highly regulated by not completely understood series of kinases and phosphatases [498, 534-536]. There is evidence that the activation of MPF/p34 involves a class of proteins around 50

to 65 kd, collectively called cyclins. Cyclins are the other component of MPF besides $p34^{cdc2}$ [537]. Cyclins mRNA and protein levels increase during the $G_2$ phase 4 and 20 times respectively in comparison to the $G_1$ phase, and they are abruptly destroyed at mitosis [538]. A candidate substrate for the MPF/p34/cyclin complex has been identified as $pp60^{c\text{-}src}$ protein, suggesting that $pp60^{c\text{-}src}$ may be the mediator of pleiotropic changes in protein phosphorylation, particularly at the level of the cytoskeleton [539].

The ensuing mitosis assures the equipartition of cell chromosomes at cell division. The four mitotic stages, prophase, metaphase, anaphase and telophase, are in fact arbitrarily defined since mitosis takes place as a continuous process [540, 541]. Each of these steps is contemporaneous with, and in some cases the result of, other cellular processes. During prophase, the duplicate chromosomes condense from a dispersed and metabolic active state to a compact condition suitable for movement. Microtubules and microfilaments of the cytoskeleton disassemble and rearrange, thus destabilizing the S phase organization of the cytoplasm. Mitotic microtubules, the most abundant spindle component and the only one whose presence is clearly essential for chromosome movement, are more labile than their S phase counterparts [527]. At the time of spindle formation, there is an increase of phosphorylation of several proteins [542]. Such post-translational modifications may be important for the

control of mitotic microtubule behavior and disassembly of the nuclear envelope [532]. The onset of metaphase is marked by a breakdown of the nuclear envelope, releasing chromosomes and nucleoplasm to fill the greater part of the cell [531]. The complete spindle is present. The site of spindle microtubule insertion in the centromer region of the chromosome is called kinetochore. The condensed chromosomes take a space-oriented position. One copy of each chromosome (termed chromatid) addresses one end of the cell and begins to align in a plane through the cell midline. Separation of each chromosome into two identical parts (sister chromatids) and movement toward the opposite ends of the cell occur during anaphase. It is followed, in telophase, by the reformation of nuclei and decondensation of the chromosome to reestablish a $G_1$ condition [543]. Nuclear division is generally followed by cell division or cytokinesis.

Brief exposure to serum-free medium in the $G_2$ phase delays the subsequent $G_1$ phase by several hours, demonstrating that $G_2$ events have an indirect effect on the progeny cell cycle, particularly for the fate of cells in subsequent $G_1$ [544]. A tight control of events continues in the new cell cycle. Inhibition of MPF complex arises in early $G_1$ and is thought to be responsible for prevention of entry into mitosis until DNA replication is completed via regulation of chromosome decondensation [543].

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# CHAPTER 2

The presence of VSMC hyperplasia in essential and genetic hypertension *in vivo* is an established phenomenon contributing to the enhanced vascular reactivity. The early rise in blood pressure in genetic models of hypertension makes it difficult to distinguish between primary and secondary events. We used, therefore, *in vitro* system where cells are deprived of *in vivo* high blood pressure and thus, the part of their behavior that persists in longterm culture may better reveal genetic differences. Smooth muscle cells were obtained from SHR and WKY aortae by explant method (Figure 1 and 2). VSMC in culture were characterized by staining with specific anti-smooth muscle myosin antibodies and fluorescence microscopy (Figure 3).

In this chapter, we present data demonstrating that increased proliferation of VSMC from SHR when compared to cells derived from their normotensive controls, WKY, persists in longterm culture and is characterized by higher maximal responsiveness to calf serum, EGF and PDGF and greater specific growth rate, particularly when cells approach confluency. These results suggests that the proliferative defect of SHR VSMC concerns both stimulatory and inhibitory growth pathway.

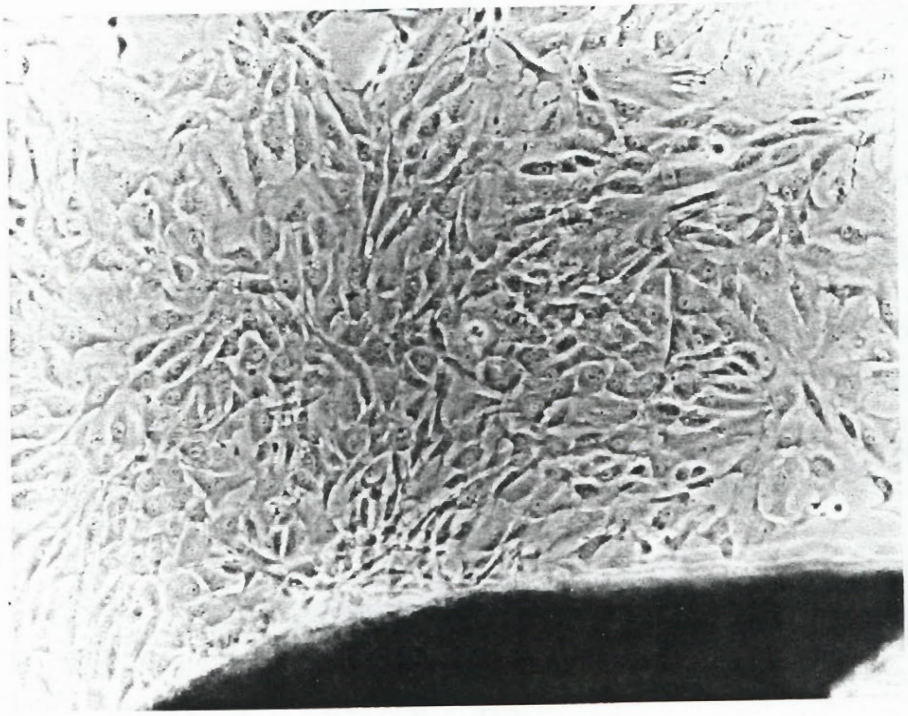

**Figure 1. Outgrowth of smooth muscle cells from an aortic media explant obtained from WKY.** Vascular smooth muscle cells outgrowing from piece of aortic media after 10 days in culture medium. Photographs were taken on an inverted light microscope at 100 x magnification.

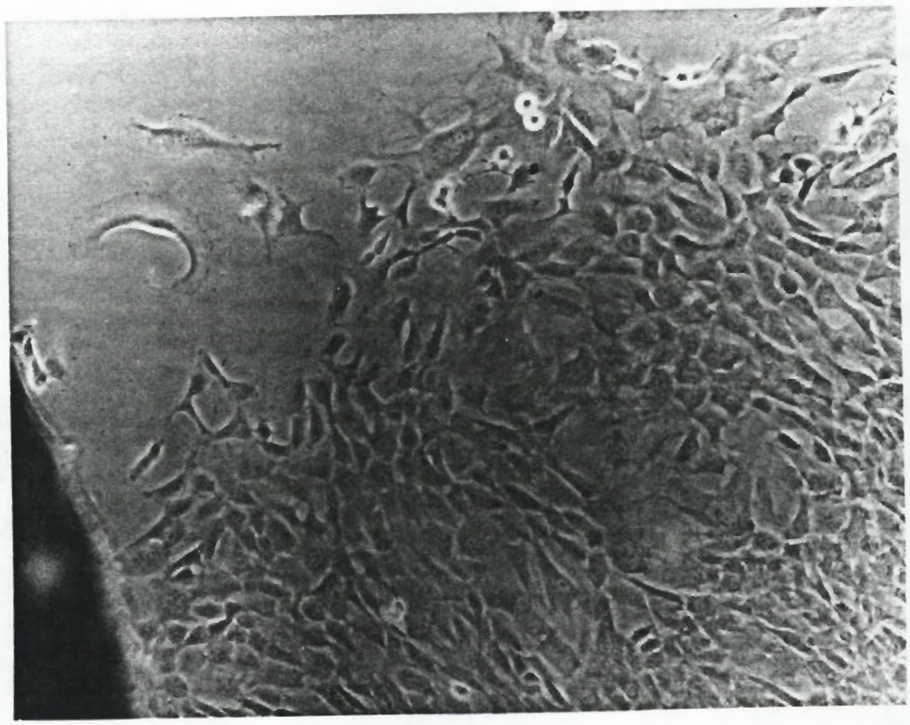

**Figure 2. Outgrowth of smooth muscle cells from an aortic media explant obtained from SHR.** Vascular smooth muscle cells outgrowing from piece of aortic media after 10 days in culture medium. Photographs were taken on an inverted light microscope at 100 x magnification.

WKY

SHR

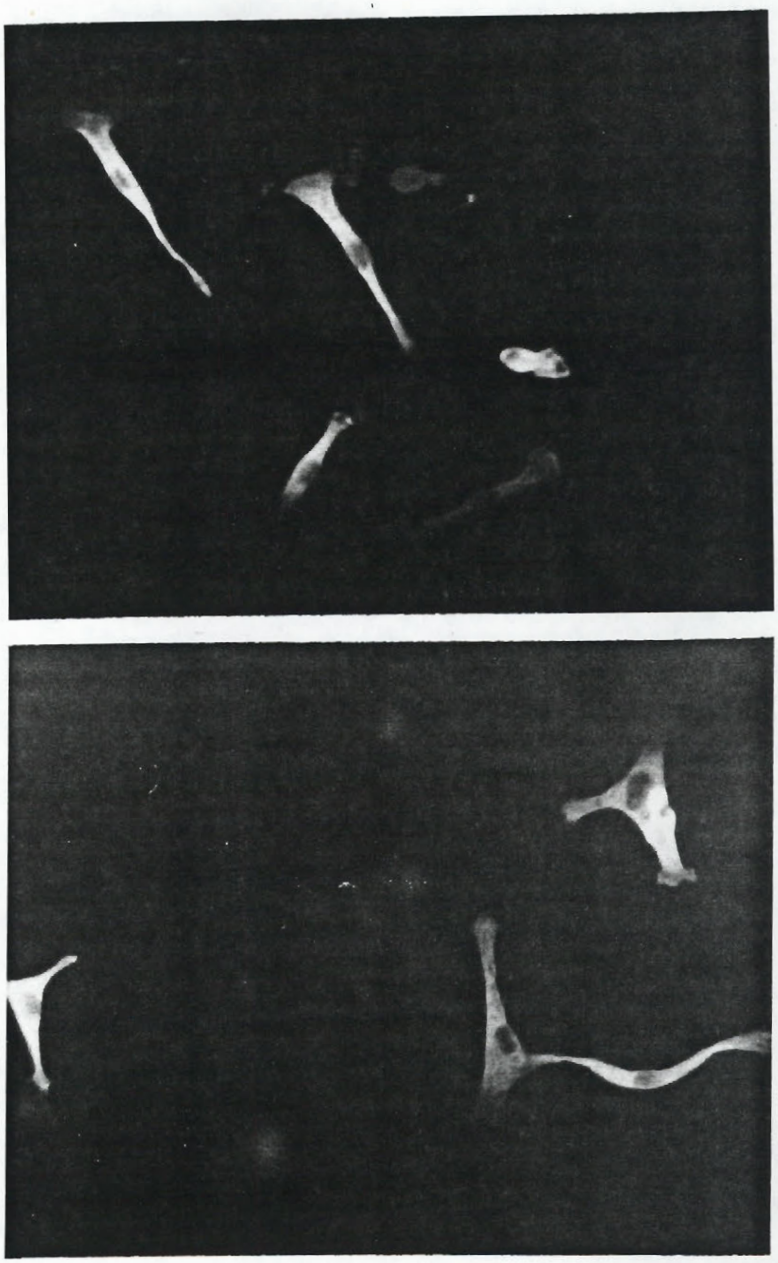

**Figure 3. Immunofluorescence staining of aortic smooth muscle cells from WKY and SHR by specific anti-smooth muscle myosin antibodies.** Cells were sparsely seeded in Lab-Tek Chamber Slides (Nunc Inc., Naperville, IL, USA). After attachment, the cells were rinsed in phosphate buffer saline (PBS), fixed in 2% paraformaldehyde, washed and dehydrated. After washing, they were incubated with rabbit anti-myosin IgG (Biomedical Technologies, Cambridge, MA, USA) in 1:25 dilution in PBS for 60 min, then washed and incubated with labeled secondary antibodies (goat anti-rabbit IgG - fluorescein isothiocyanate, Biomedical Technologies) in 1:25 dilution for 60 min. The slides were then coverslipped and photographs were taken on an inverted microscope, at 200 x magnification, using the mercury lamp as a light source.

# ABNORMALITIES IN GROWTH CHARACTERISTICS OF AORTIC SMOOTH MUSCLE CELLS IN SPONTANEOUSLY HYPERTENSIVE RATS

This work has been published in Hypertension 13: 589-597, 1989

## SUMMARY

Comparative studies have shown that cultured vascular smooth muscle cells from spontaneously hypertensive rats (SHR) proliferate to a higher cell number, grow to a greater density, and have greater specific growth rate, particularly at a higher saturation density, than those of the normotensive Wistar-Kyoto (WKY) control rats. The growth difference was not due to varying cell survival nor to attachment ability after passage. The degree of DNA synthesis was estimated by [$^3$H]thymidine incorporation into newly synthesized DNA. [$^3$H]thymidine uptake increased with escalating concentrations of calf serum and reached a plateau at 5% calf serum in WKY rats, whereas an excessive, continuous rise was observed in SHR with up to a 20% concentration. [$^3$H]thymidine incorporation into newly synthesized DNA was tested after stimulation by platelet-derived growth factor and epidermal growth factor. A significantly higher amount of newly synthesized DNA in vascular smooth muscle cells from SHR was noted when the cells were stimulated by platelet-derived growth factor or epidermal growth factor alone, and their simultaneous addition did not significantly change the 50% effective concentration but heightened the maximal response. These data provide evidence of increased aortic smooth muscle cell proliferation from aortas of SHR after mitogen stimulation and suggest a defect in growth stimulatory-inhibitory control.

## INTRODUCTION

Cardiovascular hyperplasia in hypertension has been described by several authors [1-3] and demonstrated not only in the heart but also in the kidney [4] of newborns representing several models of genetic hypertension [5]. In addition, the heart and kidney of newborn spontaneously hypertensive rats (SHR) present enhanced DNA synthesis *in vivo* [6]. Although hyperplasia may be secondary to high blood pressure [7], it has been demonstrated that vascular smooth muscle cells (VSMC) from SHR proliferate more than VSMC from Wistar-Kyoto (WKY) rats, even in culture [8, 9]. This occurrence indicates an intrinsic genetic abnormality in the control of growth of VSMC from SHR. These studies strongly suggest that cardiovascular hyperplasia may be causally related to essential hypertension. Its involvement as a primary defect in the pathophysiology of hypertension, therefore, has to be ascertained.

The present study was designed to explore the growth characteristics of rat aortic smooth muscle cells in culture since these cells are deprived of *in vivo* high blood pressure and the part of their behavior that persists in culture may better reveal genetic differences. Specifically, we examined 1) proliferation of VSMC and its relation to initial plating density as well as plating efficiency, 2) the specific growth rate of VSMC, 3) the effect of calf serum on DNA synthesis, and 4) the ability of VSMC to enter the S phase after

stimulation by platelet-derived growth factor (PDGF) and epidermal growth factor (EGF).

## MATERIAL AND METHODS

### Cell culture

Cultured VSMC were obtained by an explant method [10] from aortas of 10-13-week-old male SHR and WKY rats, as described previously [11, 12]. Both strains were purchased from Charles River Canada (St.Constant, Quebec, Canada). After administration of sodium pentobarbital (0.52 mg/kg) anesthesia, segments of thoracic aortas were aseptically removed by careful dissection. Fat and connective tissue were then discarded, and the aortas were longitudinally opened, pinned onto a wax-coated petri dish with the endothelial side up, and immersed in Dulbecco's Modified Eagle's Medium (DME) (Gibco Labs., Burlington, Ontario, Canada) supplemented with 300 units/ml penicillin and 300 $\mu$g/ml streptomycin (Sigma Chemicals Co., St.Louis, Missouri). Endothelial cells were removed by scraping with a dull scalpel, and segments of the tunica media were teased off the tunica adventitia with fine forceps. These medial segments, which are virtually free of fibroblasts and endothelial cells, were rinsed several times with DME plus 300 units/ml penicillin and 300 $\mu$g/ml streptomycin, cut into small slices (1 $mm^2$), and placed in 25-$cm^2$ tissue culture flasks (Gibco) with a drop of DME plus 100

units/ml penicillin and 100 μg/ml streptomycin plus 15% fetal calf serum (Gibco). The pieces were left for several hours to firmly attach to the bottom of the dishes. The flasks were gassed with 95% air and 5% $CO_2$, loosely stoppered and placed in a 37°C incubator. Fresh medium was gradually added during the first day until a volume of 5 ml was reached, and the dishes were then left undisturbed for at least 7 days. Smooth muscle cells grew from the explants within 10-20 days. At this point, the 15% fetal calf serum was gradually replaced by 10% calf serum (Gibco). When the cells reached confluency in 7-10 days, they exhibited a hill-and-valley pattern typical of smooth muscle cells in culture. They were then passaged by trypsinization with 0.05% trypsin (Gibco) in $Ca^{2+}$- and $Mg^{2+}$-free Dulbecco's phosphate buffered saline and inoculated into 80-$cm^2$ tissue culture flasks at a density of 1 x $10^5$ cells/ml. Under these conditions, the VSMC reacted positively to specific smooth muscle myosin antibodies, as examined by fluorescence microscopy. Morphology of VSMC was verified routinely by light microscopy.

**Cell number determination**

The VSMC were trypsinized, subsequently neutralized, and resuspended in culture medium (DME + 10% calf serum + 100 units/ml penicillin and 100 μg/ml streptomycin). They were then plated in 35-mm Nunclon tissue culture dishes (Gibco) at specified concentrations (1.25 x $10^5$ - 4.0 x $10^5$ cells/plate). After different time intervals, they were trypsinized and

counted in a Coulter counter (Coulter Elec., Inc., Hialeah, Florida).

**Specific growth rate determination**

After trypsinization, VSMC were inoculated at varying concentrations from 4.0 x $10^4$ cells/well in Falcon 24-well cluster dishes (Canlab, Montreal, Quebec, Canada) to 10.0 x $10^5$ cells/plate (Nunclon). At defined time intervals, they were trypsinized and counted. Their specific growth rate was calculated with the formula: $(P_2 - P_1)/P_1(t_2-t_1)$, where $P_1$ and $P_2$ are the cell numbers at respective times $t_1$ and $t_2$.

**Establishment of quiescence**

The trypsinized cells were plated at a concentration of 4.0 x $10^4$ cells/well in culture medium in 24-well cluster dishes. They were allowed to attach for 12 hours, and thereafter the culture medium was changed to DME supplemented with insulin (5 $\mu$g/ml), transferrin (5 $\mu$g/ml), and selenium (5 ng/ml) (ITS) or with plasma-derived serum that had been passed through a Carboxymethyl-Sephadex (Pharmacia, Baie D'Urfé, Quebec, Canada) column to remove PDGF [13]. The cells were incubated in this medium for 48-72 hours to establish quiescence.

**Determination of DNA synthesis**

As described previously [11], the 24-well cluster dishes containing VSMC that had been rendered quiescent were

replenished with fresh DME plus 100 units/ml penicillin and 100 μg/ml streptomycin, and the agents to be tested were added for 24 hours. At the end of this time period, the medium was changed to DME containing 0.5 μCi/ml [$^3$H]thymidine (Dupont, Montreal, Quebec, Canada). After 2 hours, [$^3$H]thymidine incorporation into newly-synthesized DNA was stopped by removal of the labeled medium. Each well was then washed with 1 ml isoosmotic solution (150 mM NaCl) to eliminate excess [$^3$H]thymidine, and the cells were fixed in a 1 ml ethanol:acetic acid (3:1) solution for 10 minutes. The fixative was discarded, and the cells were washed with 1.0 ml $H_2O$. Acid-insoluble material was precipitated by incubation with 1.5 ml cold 0.5 *N* perchloric acid for 15 minutes. The cells were washed further with 1.0 ml cold perchloric acid, and DNA was extracted into 1.5 ml perchloric acid by heating at 80$^o$C for 20 minutes. The perchloric acid containing solubilized DNA was transferred to vials holding 10 ml scintillation fluid (Phase-combining system, Amersham, Oakville, Ontario, Canada). The radioactivity incorporated into newly-synthesized DNA was determined in a liquid scintillation spectrometer.

**Statistical analysis**

The values are given as means ± SEM. The level of significance of differences between the means was evaluated by Student's *t* test for unpaired data, by two-way and three-way analysis of variance (ANOVA) with multiple comparisons

according to the Bonferroni method and by the Wilcoxon nonparametric test as indicated.

## RESULTS

### Vascular smooth muscle cell proliferation in culture

Preliminary experiments evaluated the growth of cultured VSMC derived from normotensive and hypertensive rats by determination of cell number after different periods of time. Cells between passages 8 and 19 were used in these experiments. Table 1 illustrates a typical experiment when the cells were inoculated at a density of $2 \times 10^5$ cells/plate and their number was determined after 4 days in culture medium; the cells from SHR grew to a significantly higher number than those of the normotensive control rats, even after the 19th passage. In this case, the difference between SHR and WKY rats was 41%. After passages 8-13, the difference in the proliferation between VSMC from SHR and WKY rats was 52 + 15%, $n$=13; it did not vary statistically after the 14th-19th passages when the difference was 37 + 7%, n=14. Although variable, the proliferation of cells of SHR from all passages studied was significantly higher than that of normotensive WKY rat cells when compared by using the Wilcoxon nonparametric test ($p < 0.01$, $n$=27).

## TABLE 1

**GROWTH OF VASCULAR SMOOTH MUSCLE CELLS IN RESPONSE TO 10% CALF SERUM AS DETERMINED BY CELL NUMBER**

| | Plating | After 4 days |
|---|---|---|
| Strain | (cells x $10^5$/plate) | |
| WKY | 2.0 | 6.0 ± 0.2 |
| SHR | 2.0 | 8.5 ± 0.3* |

Data are expressed as means ± SEM. WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats.
Cells were used after the 19th passage; n=3;
* $p < 0.01$ as compared with WKY rats by Student's t test.

To ascertain if the increased growth indeed reflected a growth abnormality and not some other properties of cells of SHR, we tested the effect of initial plating density and plating efficiency. To evaluate the influence of initial plating density on cell proliferation, the cells were inoculated at different concentrations and, after 5 days, they were trypsinized and counted. Figure 4 demonstrates that the VSMC from SHR proliferated to a higher cell number ($p < 0.05$ and $p < 0.001$, as compared with WKY rats by two-way ANOVA). Cells inoculated at a high density ($4 \times 10^5$ cells/plate, close to confluence) grew to a maximal density of $6.88 \times 10^5$ cells/plate for SHR, and $4.56 \times 10^5$ cells/plate for WKY rats. A similar final number was obtained when cells were inoculated at a low density of $1.25 \times 10^5$ cells/plate (SHR, $7.68 \times 10^5$ cells/plate; WKY rats, $4.37 \times 10^5$ cells/plate). In the groups of higher cell density at inoculation, we observed more significant differences between the two strains. The relative cell number, expressed in Figure 4 as the ratio of the final cell number to initial plating, decreased with the increasing number of cells plated. This occurrence indicated that cells of both origins were contact inhibited but always at higher densities in SHR. This finding suggested that VSMC from SHR are less sensitive to growth inhibition (see below). Additional experiments, different in time of counting (after 3 and 4 days) and plating number ($1.25 \times 10^5$, $2.0 \times 10^5$, $2.5 \times 10^5$, $4.0 \times 10^5$ and $10.0 \times 10^5$), produced similar results (two-way ANOVA, $p < 0.001$ and $p < 0.02$ respectively). By light

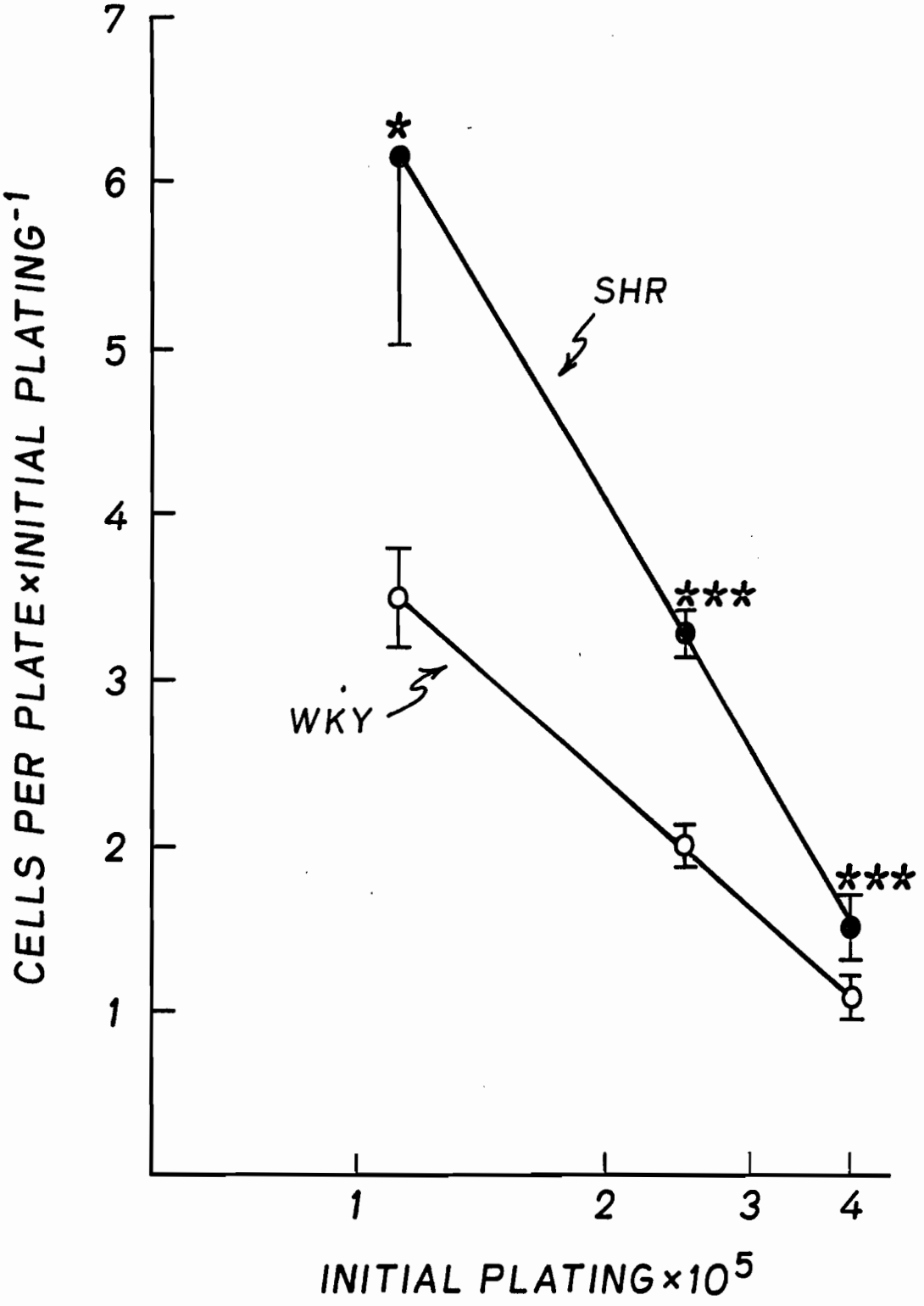


**Figure 4. Plot showing effect of initial plating density on growth of rat aortic smooth muscle cells** (mean ± SEM). SHR, spontaneously hypertensive rats. * $p < 0.05$, *** $p < 0.01$ as compared with Wistar-Kyoto (WKY) rats by two-way analysis of variance.

microscopy, the VSMC from SHR did not present any difference in shape and size relative to WKY cells; at confluence, both exhibited a hill-and-valley growth pattern (hills consist of foci of multilayered cells) typical of cultured smooth muscle cells.

To examine whether the growth difference was due to different cell survival and attachment ability after passage, plating efficiency was also evaluated (Figure 5). Series of cells were seeded at concentrations of $2.0 \times 10^5$ cells/plate, and five plates were trypsinized and counted every second hour during the first 12 hours, then at intervals of 12 and 24 hours after passage. The cells did not present any growth difference during the attachment period. The difference in cell number became significant ($p < 0.02$ by Student's $t$ test, and $p < 0.002$ by two-way ANOVA) only after 4 days in culture.

**Specific growth rate determination**

To examine at which stage of growth the difference appears between the two cell strains, cells after 11th and 12th passages were inoculated at a concentration of $4.0 \times 10^4$ cells/well and counted after 2, 3, 4 and 5 days (Table 2). The specific growth rate was calculated as described in Materials and Methods. At day 2, VSMC from both SHR and WKY rats were in a period of exponential growth, which continued through day 3, approached confluency at day 4, and reached maximum saturation density at day 5 (WKY, $4.1 \times 10^5$

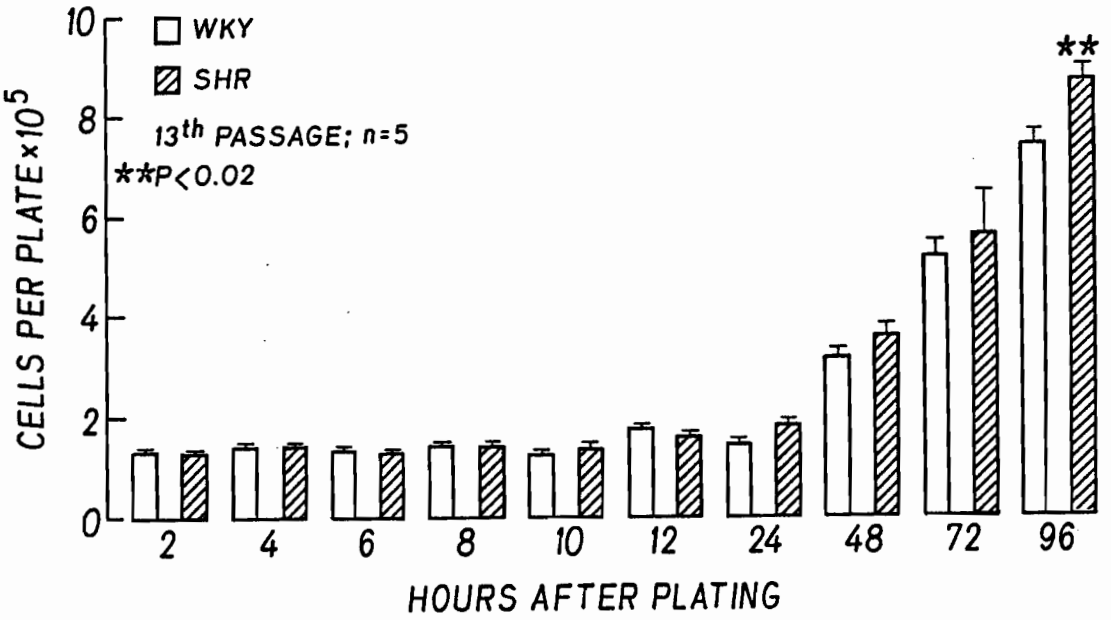


**Figure 5.** Bar graph showing plating efficiency and growth of rat aortic smooth muscle cells (mean ± SEM). SHR, spontaneously hypertensive rats. ** $p < 0.02$ as compared with Wistar-Kyoto (WKY) rats by Student's t test; $p < 0.002$ as compared by two-way analysis of variance.

## TABLE 2

### EFFECT OF TIME ON SPECIFIC GROWTH RATE OF VASCULAR SMOOTH MUSCLE CELLS

| Time (days) | | Specific growth rate (per day) | | |
|---|---|---|---|---|
| $t_1$ | $t_2$ | WKY | SHR | p value |
| SGR computed from inoculation time | | | | |
| 0 | 2 | 0.88 ± 0.06 | 1.03 ± 0.06 | NS |
| 0 | 3 | 1.86 ± 0.08 | 2.13 ± 0.18 | NS |
| 0 | 4 | 1.79 ± 0.16 | 2.52 ± 0.15 | <0.01 |
| 0 | 5 | 1.93 ± 0.11 | 2.56 ± 0.09 | <0.01 |
| SGR computed day by day | | | | |
| 2 | 3 | 1.37 ± 0.11 | 1.46 ± 0.19 | NS |
| 3 | 4 | 0.26 ± 0.08 | 0.54 ± 0.05 | <0.01 |
| 4 | 5 | 0.33 ± 0.05 | 0.27 ± 0.05 | NS |

Data are expressed as mean ± SEM. WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats; SGR, specific growth rate; NS, not significant.
Cells were used after the 11th and 12th passages; data from two independent experiments in triplicate and quadruplicate (n = 7); *p* values were obtained by Student's *t* test; SGR was determined according to the formula $(P_2 - P_1)/P_1(t_2 - t_1)$, where $P_1$ and $P_2$ are cell density at times $t_1$ and $t_2$. Cell density at inoculation was $4.0 \times 10^4$.

cells/well; SHR, 5.7 x $10^5$ cells/well). Through the period of exponential growth, the cells of SHR and WKY rats did not present any difference in specific growth rate. A significantly higher specific growth rate was reached by VSMC from SHR as compared with WKY rats at day 4 and continued at day 5 when the specific growth rate was computed with $t_1$ as the inoculation time (Table 2). When the specific growth rate was computed for each 24-hour period, the significant difference between SHR and WKY rats was confined to a period between days 3 and 4 (Table 2). Since this observation suggests that both cell types are inhibited with increasing density but that this inhibition occurs at a higher density in SHR, the cells were seeded at concentrations of 2.0 x $10^5$, 4.0 x $10^5$ , and 10.0 x $10^5$ cells/plate to directly assess the effect of initial cell density. The highest specific growth rate was noted for both strains when cells were seeded at a low concentration. At this density, these VSMC were growing exponentially; however, there was no significant difference between SHR and WKY rats (Table 3). With inoculation of 4.0 x $10^5$ cells/plate, the low specific growth rate indicates that the cells were already approaching the saturation maximum, and the difference between the VSMC from SHR and WKY rats became significant. The difference between strains became even more significant when cells were inoculated at a high density of 10.0 x $10^5$ cells/plate. At this density, the WKY rat cells were virtually growth arrested, whereas the cells of SHR continued to grow.

TABLE 3

DIFFERENTIAL EFFECT OF CELL DENSITY ON SPECIFIC GROWTH RATE OF VASCULAR SMOOTH MUSCLE CELLS

| Cell density at inoculation (cells $10^5$/plate) | Specific growth rate (per day) | | |
|---|---|---|---|
| | WKY | SHR | % difference (SHR over WKY) |
| 2.0 | 0.73 ± 0.12 | 0.88 ± 0.16 | 121 ± 21 |
| 4.0 | 0.36 ± 0.05 | 0.56 ± 0.02* | 156 ± 14 |
| 10.0 | 0.03 ± 0.01 | 0.19 ± 0.02† | 630 ± 140 |

Data are expressed as mean ± SEM. WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats.
Specific growth rate was determined according to the formula $(P_2 - P_1)/P_1(t_2 - t_1)$, where $P_1$ and $P_2$ are cell density at times $t_1$ (day of inoculation) and $t_2$ (3 days after inoculation).
* $p < 0.02$, † $p < 0.01$ as compared with WKY by Student's $t$ test.

**Effect of calf serum on thymidine incorporation into DNA**

Synchronized WKY rat VSMC from the third passage presented a 7.8-fold increase of [$^3$H]thymidine incorporation after 24 hours of 10% calf serum stimulation whereas VSMC of SHR from the same passage showed a significantly higher (16.5-fold) rise (data not shown). Calf serum also elicited dose-dependent thymidine incorporation into DNA in cultured VSMC. Nonstimulated cells from the 11th passage from both SHR and WKY rats (Figure 6A) synthesized very little new DNA. With rising concentrations of calf serum, DNA synthesis was enhanced in cells of both hypertensive and normotensive origin but reached a plateau in the WKY rat group at a concentration of 5% calf serum, whereas cells from SHR did not, even with 10% and 20% calf serum. Figure 6B illustrates the difference, expressed as "-fold" stimulation. Similar results were recorded with cells from the sixth passage. Maximal DNA synthesis in WKY rat cells was seen at a concentration of 5% calf serum (5.6-fold) whereas cells of SHR continued to synthesize more DNA with 10% and 20% calf serum (17.4-fold and 18.4-fold, respectively) (data not shown). This increased response appeared to be highly reproducible. After stimulation by 10% calf serum, which was the usual concentration in our culture medium, VSMC from SHR after the eighth passage incorporated significantly more [$^3$H]thymidine into DNA than did control cells from WKY rats in 14 separate experiments (Table 4).

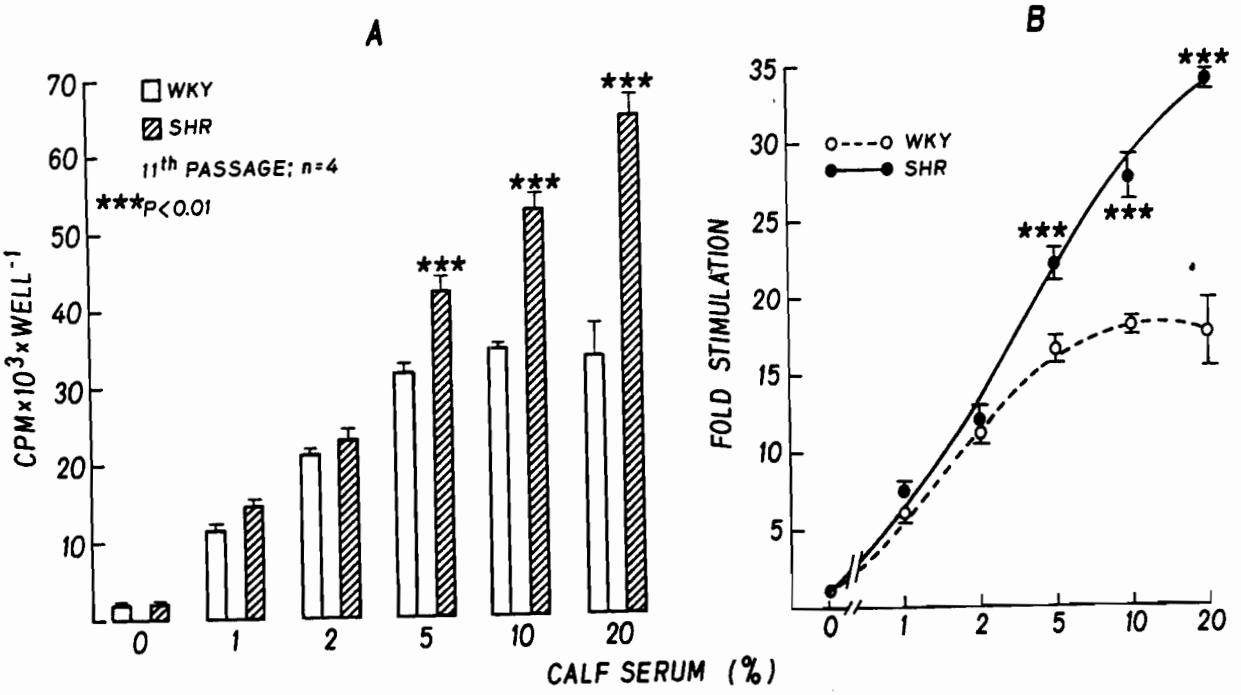


**Figure 6. Graphs showing stimulation of [$^3$H]thymidine incorporation into DNA of rat aortic smooth muscle cells by calf serum** (mean ± SEM). **Panel A**, absolute values (cpm per well). **Panel B**, fold stimulation (ratio of cpm per well at stimulated conditions to cpm per well at baseline). SHR, spontaneously hypertensive rats. *** $p < 0.01$ as compared with Wistar-Kyoto (WKY) rats by Student's t test; $p < 0.001$ as compared by two-way analysis of variance.

## TABLE 4

### GROWTH OF VASCULAR SMOOTH MUSCLE CELLS IN RESPONSE TO 10% CALF SERUM AS DETERMINED BY [$^3$H]THYMIDINE INCORPORATION INTO NEWLY SYNTHESIZED DNA

| Strain | [$^3$H]thymidine incorporation (cpm x $10^3$/well) |
|---|---|
| WKY | 33.5 ± 2.1 |
| SHR | 47.9 ± 2.3 * |

Data are expressed as mean ± SEM. WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats.
Cells were used after the eighth passage; n = 14.
* $p < 0.01$ as compared with WKY by Student's *t* test.

**Effect of platelet-derived growth factor and epidermal growth factor on thymidine incorporation into DNA**

To evaluate whether this increased growth response to calf serum was due to established growth factors, we tested the effects of PDGF and EGF in synchronized VSMC. The results of PDGF stimulation on [$^3$H]thymidine incorporation into newly-synthesized DNA are shown in Figure 7. VSMC from SHR were more liable to respond to PDGF and to commit themselves in the synthetic S phase than those from WKY rats at doses from 0.03 $\mu$M to 0.3 $\mu$M.

When DNA synthesis was tested in response to EGF (Figure 8), a stimulatory effect was evident at concentrations from 0.017 $\mu$M to 0.17 $\mu$M. The plateau reached in cells from both groups was significantly higher in SHR. However, the 50% effective concentration ($EC_{50}$) did not differ significantly, as determined by dose-response analysis [14]; the only statistically significant difference was the maximal response ($R_{max}$) (Table 5).

Finally, the simultaneous effect of PDGF and EGF on [$^3$H]thymidine incorporation into DNA was tested. In these experiments, cells were stimulated by 0.006 and 0.06 $\mu$M of PDGF and with increasing concentrations of EGF from 0.017 to 17 $\mu$M (Figure 9). At all dose levels of these growth factors, VSMC from SHR showed a greater response than those from WKY rats. The difference between cells from the two strains

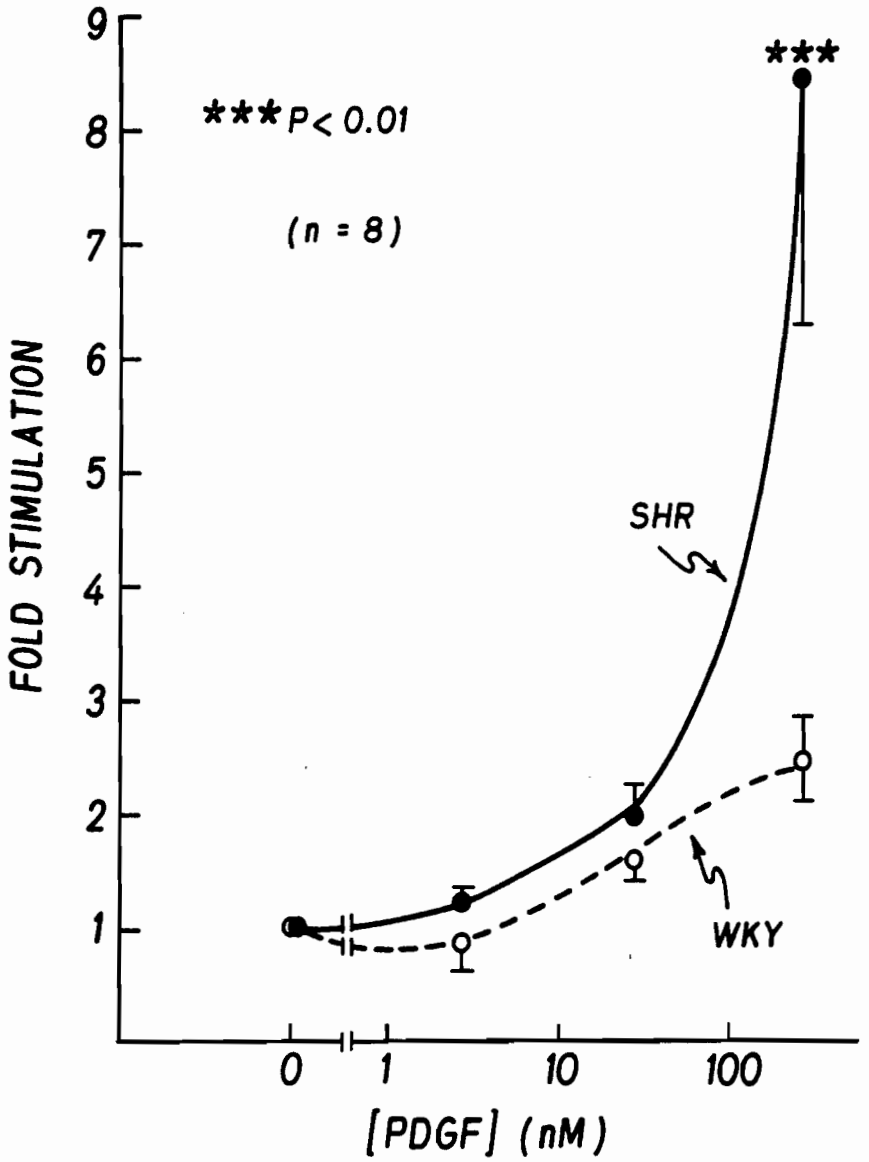


**Figure 7.** Graph showing stimulation of [$^3$H]thymidine incorporation into DNA of rat aortic smooth muscle cells by platelet-derived growth factor (PDGF) (mean ± SEM). SHR, spontaneously hypertensive rats. *** $p < 0.01$ as compared with Wistar-Kyoto (WKY) rats by Student's t test; $p < 0.001$ as compared by two-way analysis of variance.

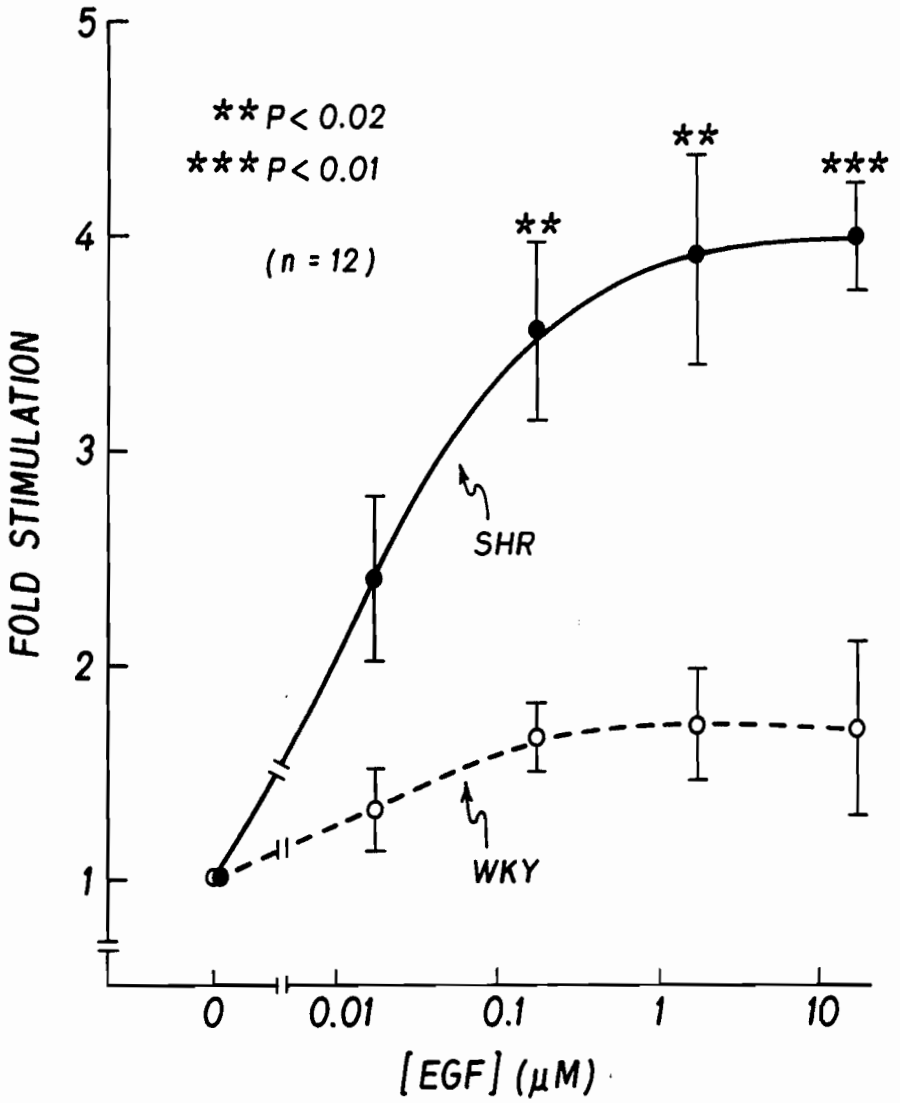


**Figure 8.** Graph showing stimulation of [$^3$H]thymidine incorporation into DNA of rat aortic smooth muscle cells by epidermal growth factor (EGF) (mean ± SEM). SHR, spontaneously hypertensive rats; ** $p < 0.02$, *** $p < 0.01$ as compared with Wistar-Kyoto (WKY) rats by Student's t test; $p < 0.001$ as compared by two-way analysis of variance.

TABLE 5

GROWTH OF VASCULAR SMOOTH MUSCLE CELLS IN RESPONSE TO PLATELET-DERIVED GROWTH FACTOR AND EPIDERMAL GROWTH FACTOR AS DETERMINED BY DOSE-RESPONSE ANALYSIS OF [$^3$H]THYMIDINE INCORPORATION INTO NEWLY SYNTHESIZED DNA

| | $EC_{50}$ | | $R_{max}$ | |
|---|---|---|---|---|
| PDGF | ($\mu$M EGF) | | | |
| (nM) | WKY | SHR | WKY | SHR |
| 0 | 0.02 ± 1.58 | 0.02 ± 0.54 | 1.70 ± 0.01 | 4.00 ± 0.01* |
| 6 | 0.18 ± 0.09 | 0.06 ± 0.02 | 3.96 ± 0.08 | 6.19 ± 0.93* |
| 60 | 0.02 ± 0.12 | 0.02 ± 0.002 | 3.50 ± 0.53 | 12.50 ± 2.50† |

Data are expressed as mean ± SEM. PDGF, platelet-derived growth factor; EGF, epidermal growth factor; $R_{max}$, maximal response by fold stimulation of thymidine incorporation; WKY, Wistar-Rats; SHR, spontaneously hypertensive rats.
* $p < 0.01$, † $p < 0.05$ as compared with WKY by Student's *t* test.

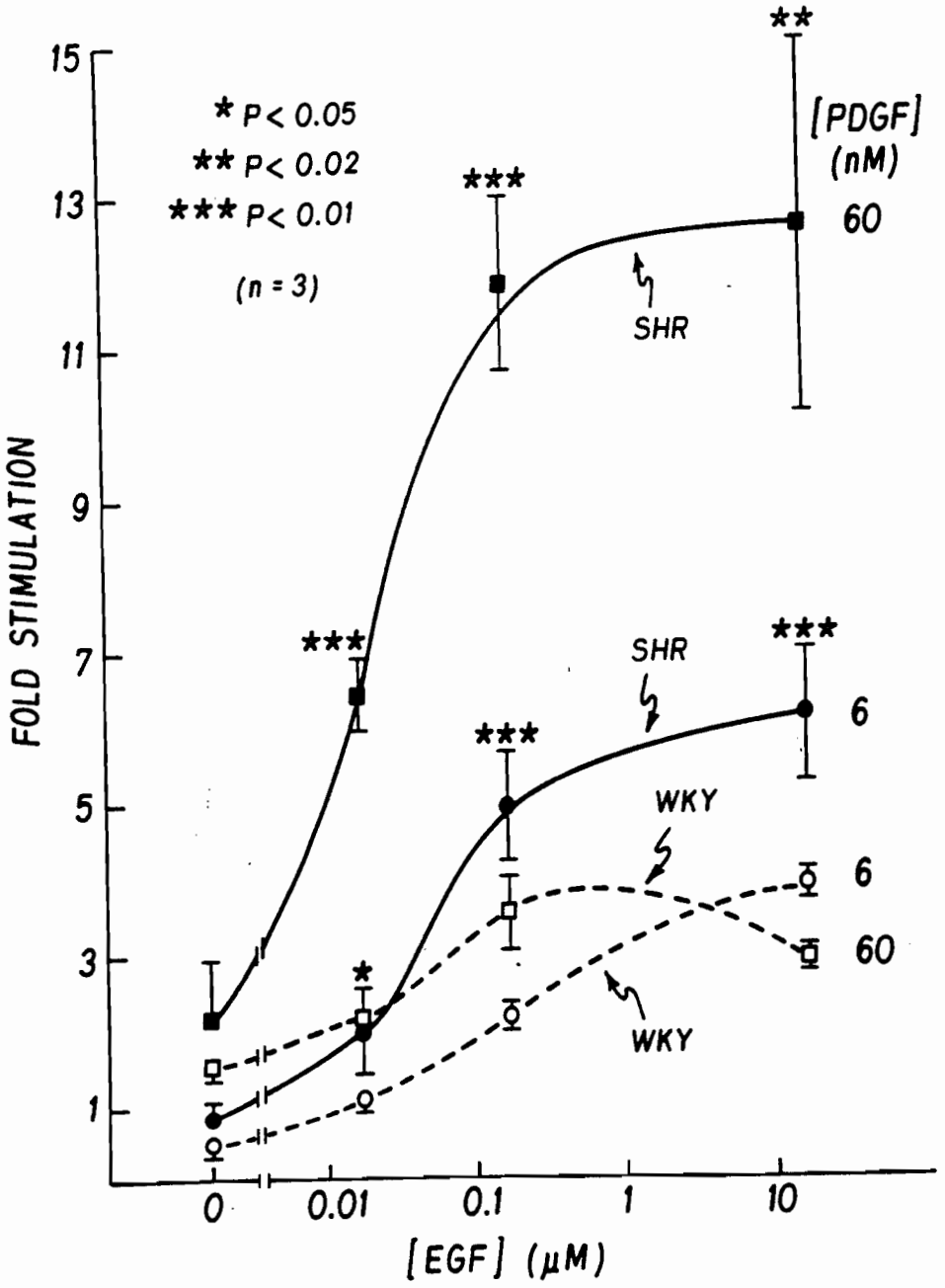


**Figure 9. Graph showing stimulation of [$^3$H]thymidine incorporation into DNA of rat aortic smooth muscle cells by platelet-derived growth factor (PDGF) and epidermal growth factor (EGF)** (mean ± SEM). SHR, spontaneously hypertensive rats. * $p < 0.05$, ** $p < 0.02$, *** $p < 0.01$ as compared with Wistar-Kyoto (WKY) rats by Student's t test. Similar results were obtained by three-way analysis of variance.

was most evident at 0.06 $\mu$M of PDGF in the presence of increasing doses of EGF; [$^3$H]thymidine incorporation plateaued at 1.7 $\mu$M EGF with both PDGF concentrations. As seen in Figure 9 and demonstrated in Table 5, the addition of PDGF did not change the $EC_{50}$, but increased the $R_{max}$ of EGF.

## DISCUSSION

The proliferation of VSMC has been considered a consequence of the vascular injury induced by hypertension. However, Folkow [15] has proposed that a portion of elevated peripheral vascular resistance in hypertension results from structural alterations in the vessel wall mass. A 5% change in the average internal radius of resistance vessels can produce a 25% increase in peripheral vascular resistance. The notion that cardiovascular hyperplasia may be causally related to spontaneous hypertension is supported by studies [4-6] demonstrating that cardiac and renal hyperplasia associated with genetic hypertension is present in newborns of four different spontaneously hypertensive models but absent in offsprings of parents with renal and experimental hypertension. However, the pathophysiological role of smooth muscle cell hyperplasia in hypertension still remains controversial since there are reports of only hypertrophy [16] or increased DNA content without mitosis due to cell polyploidy [17]. Nevertheless, using a three-dimensional

dissector, Mulvany et al [2] and Baandrup et al [18] have provided evidence of hyperplasia in mesenteric resistance vessels of SHR. Studies on newborn SHR and WKY rats with a different DNA content in hearts and kidneys [4-6] have not definitively proven that the increase of DNA synthesis is a primary event in hypertension since newborn SHR already present slightly elevated blood pressure [19], and even this elevation may have had pathophysiological consequences.

The investigations of Yamori et al [8] and Clegg et al [9], as well as our own findings of enhanced proliferation of cultured VSMC from SHR, support the possibility that hyperplasia of VSMC is a genetic abnormality in hypertension since the cells are deprived of high blood pressure and continue to grow independently faster, even after 19 passages. Although our results show that VSMC from SHR proliferate faster approaching confluency and reach higher density, they have to be attached to the culture dish to proliferate, and they express an absolute requirement for growth factors quantitatively similar to cells from WKY rats. However, evaluation of the specific growth rate suggests that the inhibitory effect of contact on cell growth is abnormal in cells from SHR. During exponential growth, there appeared to be no difference between the two cell strains. At the time of decline of the specific growth rate, when the cells approach the confluence, the slope of decline is less steep in SHR and results in continuous growth at densities at which cells from

WKY rats are already growth arrested.

The transition from the prereplicative $G_0/G_1$ phase to the synthetic S phase of the cell cycle is a critical, regulated step of cell proliferation. At this point, normal cells require growth factors, such as competence factor PDGF [20, 21], and progression factor EGF [22, 23], which are components of serum. PDGF is a cationic, 30-kD glycoprotein composed of two peptide chains with the B chain encoded by the protooncogene *c-sis* [24-26]. EGF is a single chain, anionic 6045-d polypeptide, whose receptor probably represents the product of the *c-erb*-B protooncogene [22, 27]. Both growth factors induce an ordered sequence of biochemical events with a rise in $Ca^{2+}$-CaM, $pH_i$, and tyrosine phosphorylation of specific substrates, which occur as crucial events in the pathway leading ultimately to DNA replication. Although expressed through independent signals, both PDGF and EGF regulate *c-fos* and *c-myc* mRNA levels, which may finally trigger DNA synthesis [28]. In addition to their mitogenic and other functions, both have striking effects on the control of vascular function [29-31].

EGF and PDGF, under normal circumstances, act in concert with other stimulatory or inhibitory agents and growth factors, such as heparin or transforming growth factors (TGF). TGF$\alpha$ share sequence homology to EGF and bind also to its receptor with indistinguishable biological activities *in vitro* [32]. TGF$\beta$, on the other hand, have a different primary

sequence [33], bind to a unique receptor distinct from the EGF receptor [34], and exert both stimulatory and inhibitory effects on cell proliferation [35-37]. They inhibit the serum or PDGF-mediated proliferation of VSMC at subconfluent densities but augment the growth of VSMC at high cell density [38]. TGF$\beta$ have been also reported to inhibit EGF induction of transin mRNA [39], whose expression had been shown to be elevated by oncogenic transformation and stimulation of growth factors [40]. Smooth muscle cell growth can be also inhibited *in vitro* [41] as well as *in vivo* [42] by heparinlike molecules. Heparin reduces the number of EGF receptors on VSMC in a manner that closely parallels its antiproliferative effect [43].

By determining cell number, the present study demonstrated an increased proliferation of VSMC in response to calf serum and excluded simple polyploidy as the only difference in cells derived from SHR [17]. The experiments reported here suggest abnormal decline of the growth rate with approaching confluence. The growth factors and mechanisms responsible for the contact inhibition or slowing of the specific growth rate at high density are not all known. The selective role of stimulatory and inhibitory growth factors will have to be further evaluated at this growth-declining phase, which appears to be abnormally late in SHR. This abnormality of growth inhibition may be due to an imbalance between the expression of the EGF receptor or its effectiveness and to the different expression or effectiveness

of TGF$\beta$ and other growth stimulatory-inhibitory factors.

In addition, we observed that newly-synthesized DNA, after stimulating quiescent smooth muscle cells by both PDGF and EGF, is significantly higher in VSMC from SHR. This suggests that cells derived from SHR are more liable to enter the synthetic S phase after mitogen stimulation. The two strains differ in their maximum responsiveness, particularly when approaching confluence. The regulatory effects of cell density on the selective reduction of the high-affinity receptors for EGF and TGF$\beta$ have been reported [44]. The simultaneous stimulation by PDGF and EGF revealed that PDGF heightened the maximal response but did not alter the $EC_{50}$ of EGF; thus, the additive effect of these factors was illustrated. The different responses to circulating growth factors seen in our study may occur at different sites in the biochemical pathway leading to DNA synthesis. They may be caused by alterations of growth factor receptors, a modulated regulation of transmembrane signalling (G-protein, phosphatidylinositol phosphodiesterase, tyrosin kinase), an abnormal release of intracellular messengers (inositol trisphosphate, calcium), a different sensitivity of subsequent steps (diacylglycerol release, protein kinase C activation, tyrosine phosphorylation), or a modified synthesis of protooncogenes (*c-fos, c-myc*) believed to control the early steps to DNA synthesis. Indeed, many cellular defects in SHR and other models of essential hypertension have been

described, namely, altered cell membrane ion transport [45-47], changed cyclic nucleotides [48] and adenylate cyclase system [49], increased inositol trisphosphate [50, 51] and cytosolic $Ca^{2+}$ levels [52], and higher calmodulin activity [53]. Genetic abnormalities of any of these steps may be, in turn, regulated by yet unknown genes. Our pilot study in fact indicates an altered expression of *c-myc* and *c-fos* in response to growth factors [54]. The present investigation has clearly demonstrated abnormalities of growth of cultured smooth muscle cells from SHR in response to growth factors. These abnormalities of growth suggest an abnormal balance between stimulatory and inhibitory control mechanisms. The genetic linkage of this phenomenon to the expression of hypertension and its pathogenetic and molecular mechanisms deserves further evaluation.

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# CHAPTER 3

In Chapter 2, we demonstrated, besides the greater specific growth rate at saturation densities that VSMC from SHR when compared to their control cells from WKY, incorporated more [$^3$H]thymidine into newly synthesized DNA after mitogenic stimulation. This observation suggested that VSMC from SHR may be more liable to enter the synthetic S phase of the cell cycle. In the following Chapter, we present the initial characterization of kinetics of cultured VSMC in parallel with the evaluation of their size and morphology, using flow cytometry technique and light microscopy.

# INTRINSIC FACTORS INVOLVED IN VASCULAR SMOOTH MUSCLE CELL PROLIFERATION IN HYPERTENSION

This work has been accepted for publication in Clinical and Investigative Medicine, 1991

## SUMMARY

The exaggerated response to growth factors of vascular smooth muscle cells from spontaneously hypertensive rats when compared to cells from normotensive control Wistar-Kyoto rats persists in culture, indicating an intrinsic/genetic defect. The time course of [$^3$H]thymidine incorporation shows that synchronized vascular smooth muscle cells from sponataneously hypertensive rats start to synthesize new DNA earlier after mitogenic stimulation than cells from normotensive rats. Flow cytometry demonstrates that in cell population growing in 10% calf serum for three days there is a higher proportion of cells from spontaneously hypertensive rats in the S phase of the cell cycle. The same proportions in the $G_2$+M phase of growing as well as synchronized cells from normotensive and hypertensive rats indicate no difference in polyploidy. Forward light scatter analysis reveals no difference in cell size. These results suggest that the kinetics of vascular smooth muscle cells from normotensive and spontaneously hypertensive rats are different. Since the defect seems to be in the prereplicative phase of the cell cycle susceptible to regulation by extrinsic factors, we studied the effect of the calmodulin inhibitor, W-7, on DNA synthesis. Pilot experiments indicate that the effect of calmodulin inhibitor W-7 on growth is the same for both strains, and furthermore suggests the involvement of a previously-reported calmodulin activator in hypertension.

## INTRODUCTION

Increased peripheral vascular resistance is one of the significant pathogenic mechanisms of essential hypertension. Partly the result of enhanced vascular reactivity, the augmented vascular resistance is also due to structural alterations in vessel wall mass, as proposed by Folkow [1]. The notion that cardiovascular hyperplasia may be causally related to spontaneous hypertension is supported by studies demonstrating that cardiac and renal hyperplasia associated with genetic hypertension is present in newborns of four different spontaneously hypertensive models but absent in offspring of parents with renal and experimental hypertension [2]. An increased number of laminae is observed in aortae from spontaneously hypertensive rats (SHR), even in the fetal stage [3]. The pathophysiologic role of vascular smooth muscle cell (VSMC) hyperplasia in hypertension remains controversial since there are reports of hypertrophy or increased DNA content without mitosis due to cell polyploidy [4]. However, the histological studies of Mulvany and others have provided evidence of *in vivo* hyperplasia in mesenteric resistance vessels of SHR [5, 6]. Aortic and mesenteric smooth muscle cells from SHR proliferate more than VSMC from normotensive control Wistar-Kyoto (WKY) rats even in culture [7-12], indicating an abnormal phenotype independent of elevated blood pressure *in vivo*. We and others have demonstrated that VSMC from SHR present an exaggerated maximal response to calf

serum, epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) stimulation [9, 10]. The current studies are centered on ***in vitro*** characterization of this intrinsic defect.

Several abnormalities of cell cycle-related events have been noted in vessels and cultured VSMC from SHR; among them is an altered $Ca^{2+}$/calmodulin system [13-16]. Its role in the exaggerated growth potential of VSMC from SHR is not yet clearly understood. We have, therefore, additionally evaluated the modulatory effect of calmodulin, one of the essential cell cycle regulators.

## MATERIAL AND METHODS

### Cell culture

Cultured VSMC were obtained by an explant method [17] from aortae of 10- to 13-week-old male SHR and WKY rats, as described previously [9, 18]. Smooth muscle cells grew from the explants within 10 to 20 days. At confluency, they exhibited a "hill-and-valley" formation typical of smooth muscle cells in culture. They were passaged by trypsinization with 0.05% trypsin (Gibco, Burlington, Ontario, Canada) in $Ca^{2+}$, $Mg^{2+}$-free Dulbecco's phospate buffer saline (PBS) and used between the 7th and 20th passages. The subcultured cells were maintained in Dulbecco's modified Eagle's medium (DME) (Gibco)

supplemented with 10% calf serum, 100 units/ml penicillin and 100 lg/ml streptomycin, which was replaced every 3rd day. VSMC reacted positively to smooth muscle specific myosin antibodies (Biomedical Technologies Inc., Sloughton, MA, USA), as examined by fluorescence microscopy. Their morphology was verified routinely by light microscopy. Cultures were photographed on a Diaphot Nikon inverted microscope (Nikon Canada Inc., Montreal), using a phase contrast objective. When indicated, the cells were made quiescent after 16 to 20 hours of attachment by replacing the culture medium with defined serum-free medium (DME containing 2.5 lg/ml insulin, 2.5 lg/ml transferrin and 2.5 ng/ml selenium) for 72 hours.

**Determination of DNA synthesis by thymidine incorporation**

Quiescent cells, plated in 24-well cluster dishes at a density of 4 x $10^4$ cells/well, were stimulated by calf serum in DME. The amount of newly-synthesized DNA was evaluated by $^3$H-thymidine incorporation, as described previously [18]. Briefly, the stimulating medium was removed, and DME containing 0.5 $\mu$Ci/ml $^3$H-thymidine was added for 2 hours. Each well was washed with 1 ml of 150 mM NaCl, and the cells were fixed in 1 ml ethanol:acetic acid (3:1) for 10 minutes, then washed with 1 ml $H_2O$. Acid insoluble material was precipitated by 15 minutes of incubation with 1.5 ml cold 0.5 N perchloric acid, washed further with 1 ml perchloric acid, and DNA was extracted into 1.5 ml perchloric acid by heating at 80$^o$C for 20 minutes. The radioactivity incorporated into

newly-synthesized DNA was determined in a liquid scintillation spectrometer. The same method was used in studies evaluating the effects of the calmodulin inhibitor, W-7 [N-(6-aminohexyl)-5-chloro-1-naphtalenesulfonamide] (Sigma), on DNA synthesis.

**Analysis of cellular DNA content by flow cytometry**

Cells were inoculated in 80-$cm^2$ flasks at a concentration of 5 x $10^5$ cells/flask. Cells made quiescent or growing in 10% calf serum were harvested by trypsinization, then resuspended in Dulbecco's PBS containing 320 $\mu$M $Ca^{2+}$ and 60 $\mu$M $Mg^{2+}$ ($PBS^+$), fixed in 50% ethanol and kept at 4°C. Immediately before flow cytometry analysis, they were washed in Dulbecco's $PBS^+$ and resuspended in 2 ml $PBS^+$. DNA was stained by propidium iodide (20 $\mu$g/ml), a specific dye for nucleic acids (Sigma, St.Louis, MO, USA). To prevent propidium iodide binding to RNA, 10 $\mu$g/ml of ribonuclease A (Sigma) were added to each sample. After 30 minutes of incubation at 37°C, the cells were analysed in a FACScan flow cytometer (BDIS, San Jose, California) with an argon laser adjusted to emit 15 mW at 488-nm wavelengths. From each cell sample, 5 to 10 x $10^3$ events were accumulated per histogram. The proportion of cells in cell cycle phases was determined from each histogram, using the model described by Baisch et al. [19]. VSMC size was estimated simultaneously from the same cell samples by analysis of forward light scatter.

### Statistical analysis

The values are given as means ± SEM. The level of significance of differences between the means was evaluated by Student's t test for unpaired data, by two-way ANOVA and by the Wilcoxon non-parametric test, as indicated.

## RESULTS

### Time course of thymidine incorporation into DNA

Quiescent, synchronized cells were stimulated by 20% calf serum in 30 hours time course. Quiescent VSMC from both WKY and SHR incorporated virtually no $^{3}$H-thymidine into DNA for the first 8 hours after the addition of calf serum (Figure 10). Although from then on both types of cells presented a time-dependent increase of newly-synthesized DNA in response to mitogenic stimulation, SHR cells incorporated significantly more $^{3}$H-thymidine than control cells after 12 hours of stimulation ($p<0.0005$ by ANOVA), and reached a significantly higher plateau at 24 and 30 hours ($p<0.0005$ by ANOVA). These results indicate that synchronized SHR VSMC, after mitogenic stimulation, start to synthesize new DNA earlier than WKY cells.

### Characteristics of quiescent and growing cells

VSMC were inoculated in culture medium containing 10% calf serum. After attachment, the medium was replaced by

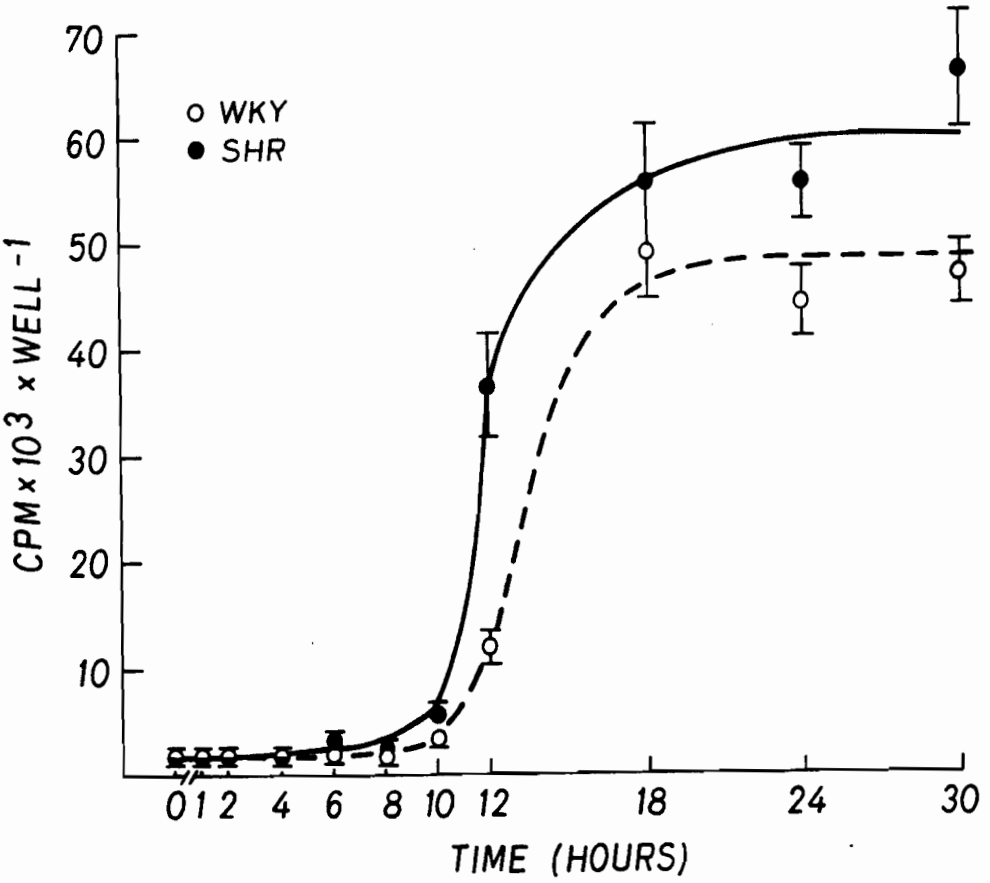


**Figure 10. Time course of $^3$H-thymidine incorporation into DNA of rat aortic smooth muscle cells following calf serum stimulation.** Quiescent cells were stimulated by 20% calf serum for the periods indicated, then pulsed for 2 hours by $^3$H-thymidine, and the radioactivity incorporated was measured in precipitated, acid-insoluble material. The values represent the means ± SEM of at least three experiments, each performed in quadruplicate.
WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats. $p<0.0001$, difference between the two strains as evaluated by two-way ANOVA.

defined serum-free medium or 10% calf serum-containing medium. Three days later, the cells were trypsinized and fixed, and the proportion of cells in different cell cycle phases was assessed with a FACScan flow cytometer. Table 6 and Figure 11A show the cell cycle phase proportions of WKY and SHR VSMC growing in culture medium containing 10% calf serum or defined serum-free medium for 72 hours. In serum-free medium, the proportion of WKY and SHR cells in the $G_0/G_1$ phase was significantly higher ($p<0.0005$, Student's t test), mostly on account of cells in the S phase whose proportion was reduced about three times when compared to cells growing in 10% calf serum. Quiescent VSMC from SHR presented the same $G_0/G_1$ proportions as WKY cells. In 10% calf serum, there was a small but significantly lower ($p<0.01$, Wilcoxon test) proportion of SHR VSMC in the $G_0/G_1$ phase, accompanied by a higher percentage ($p<0.01$) of S-phase cells, when compared to WKY. This reflects that, in the presence of 10% calf serum, VSMC from SHR proliferate at a higher rate. No difference between WKY and SHR was detected in the $G_2$+M phase, either in quiescent VSMC in serum-free medium or in VSMC growing in 10% calf serum, indicating the same proportion of tetraploid cells.

In a pilot experiment, quiescent VSMC were further stimulated by 10% calf serum for 12 and 24 hours (Figure 11A). Analysis of cell cycle phases showed that at 12 hours a higher proportion of SHR cells was in the S phase than cells

## TABLE 6

### PERCENTAGE OF QUIESCENT AND GROWING VASCULAR SMOOTH MUSCLE CELLS IN CELL CYCLE PHASES AS DETERMINED BY FLOW CYTOMETRY

| Strain | Cell cycle phase | Culture condition | |
|---|---|---|---|
| | | Growing (n=11) | Quiescent (n=13) |
| WKY | $G_0$-$G_1$ | 66 ± 3 | 82 ± 1‡ |
| | S | 20 ± 3 | 6 ± 1‡ |
| | $G_2$-M | 14 ± 1 | 12 ± 1 |
| SHR | $G_0$-$G_1$ | 61 ± 3* | 81 ± 1‡ |
| | S | 24 ± 3* | 8 ± 1‡ |
| | $G_2$-M | 15 ± 1 | 11 ± 1† |

* $p < 0.01$ by the Wilcoxon test comparing SHR vs. WKY.
‡ $p < 0.0005$, † $p < 0.01$ by the Student's t test comparing quiescent vs. growing cells.
WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats.
Culture conditions:
Growing, cells for 3 days in 10% calf serum. Quiescent, cells for 3 days in defined serum-free medium.

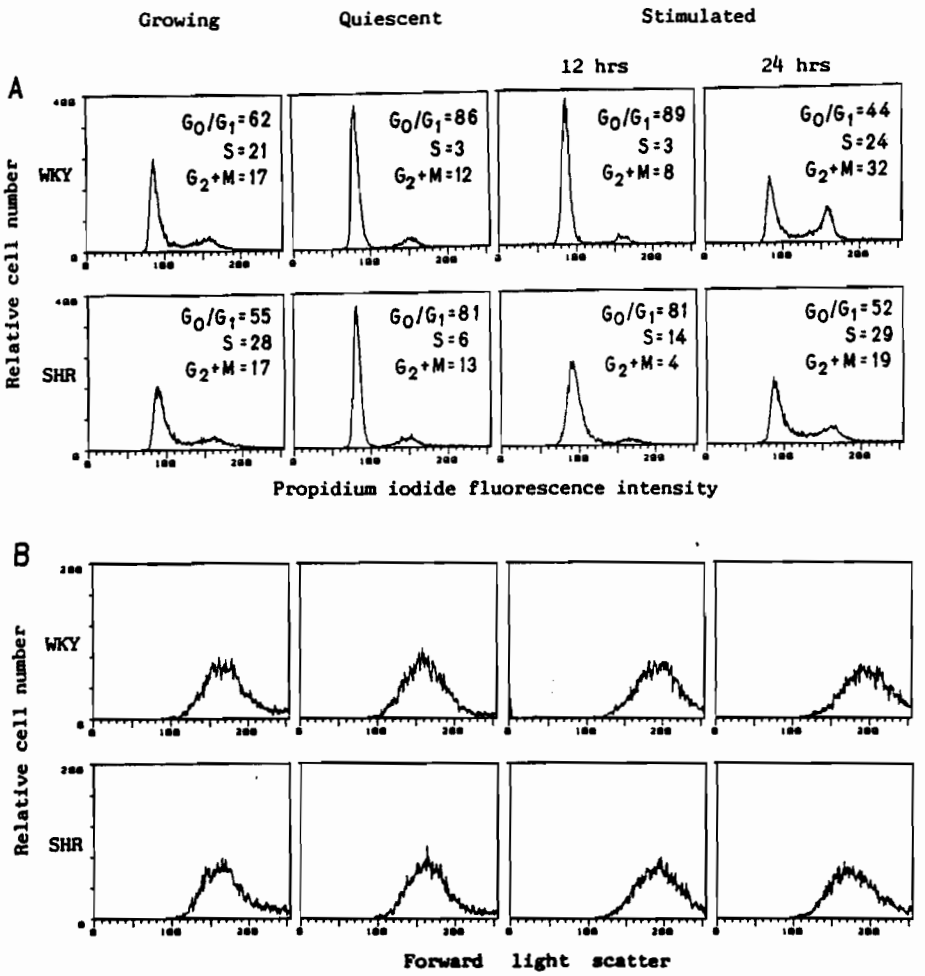


**Figure 11. Computer-drawn histograms of cultured WKY and SHR aortic smooth muscle cells, as determined by flow cytometry.** Cells under indicated conditions were harvested, treated with RNAse, stained with propidium iodide, and analysed by FACScan. The percentage of cells in cell cycle phases was assessed according to the Baisch model [19]. Propidium iodide fluorescence corresponds to the amount of DNA bound. Forward light scatter is proportional to relative cell size. Typical experiment, total events per histogram: $5 \times 10^3$.
Growing, cells for 3 days in 10% calf serum.
Quiescent, cells for 3 days in defined serum-free medium.
Stimulated, quiescent cells stimulated by 10% calf serum for the periods indicated.
WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats.

from WKY, supporting the data on $^{3}$H-thymidine incorporation. At 24 hours, WKY cells started to enter the $G_2$+M phase, while a proportion of VSMC from SHR had already left the $G_2$+M phase and reentered the new cell cycle. The earlier presence of SHR cells in a new cell cycle was substantiated by the fact that after 30 hours of stimulation, VSMC from SHR accumulated a higher amount of $^{3}$H-thymidine in DNA (Figure 10). These data suggest that the cell cycle kinetics of VSMC from WKY and SHR are different. As analysed by forward light scatter. Cell size demonstrated no difference between VSMC from WKY and SHR, either in growing, quiescent or stimulated cell populations (Figure 11B).

The morphologic appearance of VSMC in distinct growth stages of culture is depicted in Figure 12, as recorded by phase contrast microscopy. The shape and size of VSMC from both WKY and SHR were similar in all growth states. After 3 days in serum-free defined medium, quiescent cells revealed no mitosis and their number was clearly lower when compared to cells growing for the same period in 10% calf serum. In growing populations, we can notice a greater number of VSMC from SHR. They reached confluency and started to form multilayered "hills", which was not yet the case for VSMC from WKY. Quiescent cells stimulated by 10% calf serum for 24 hours displayed a marked increase in mitosis in WKY and SHR when compared to quiescent, non-stimulated cells. Even by microscopic observation, there was a noticeably greater number

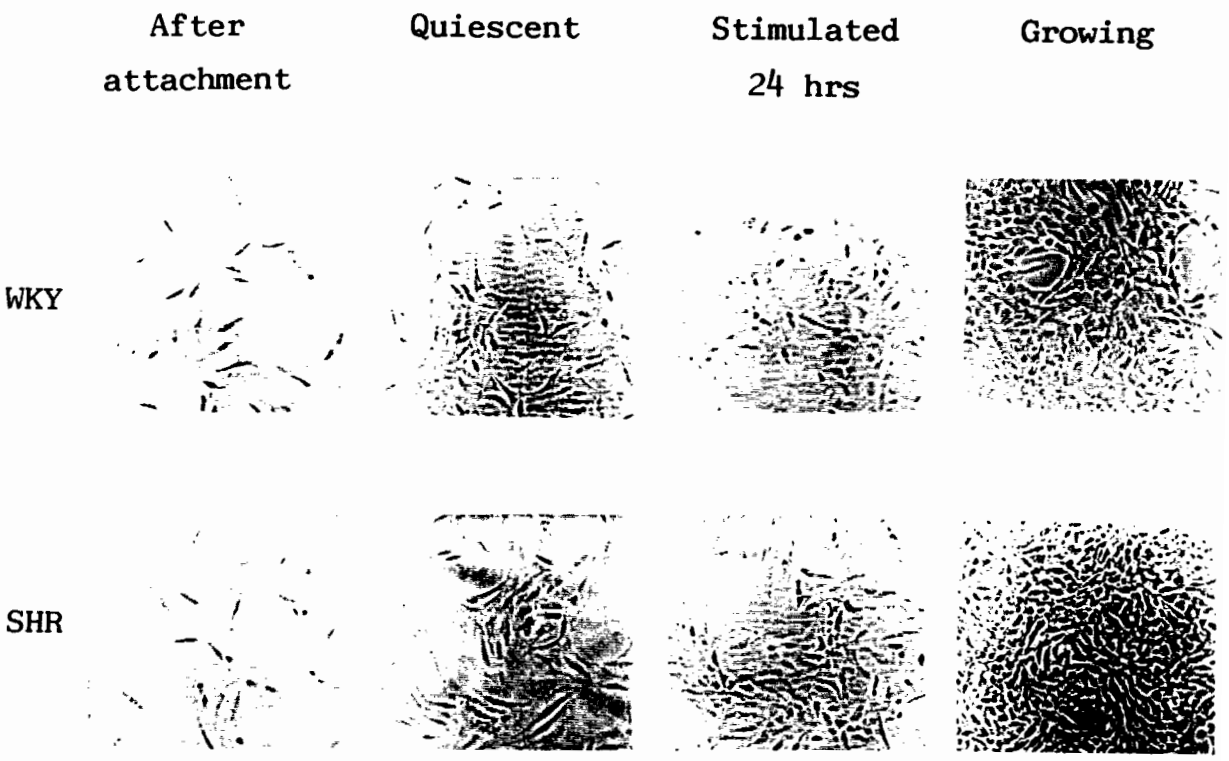


**Figure 12. Morphologic appearance of aortic smooth muscle cells at distinct growth stages in cell culture.** Photographs were taken on an inverted light microscope at 100 x magnification.
After attachment, cells 16 hours after inoculation in 10% calf serum.
Quiescent, cells for 3 days in defined serum-free medium.
Stimulated, quiescent cells stimulated by 10% calf serum for the period indicated.
Growing, cells for 3 days in 10% calf serum.
WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats.

of mitosis in VSMC from SHR than in WKY.

**Effect of W-7 on DNA synthesis**

W-7 inhibited 10% calf serum stimulated DNA synthesis in a dose-dependent manner in VSMC from both WKY and SHR (Figure 13). Maximal inhibition was reached at a 150 $\mu$M W-7 concentration in cells of both origins. At 100 $\mu$M concentration of W-7, the suppression of DNA synthesis was significantly ($p<0.02$, Student's t test) more pronounced in WKY (89 $\pm$ 2%) than in SHR (72 $\pm$ 5%). However, the 50% of inhibition dose ($ID_{50}$) for WKY and SHR cells, as determined by 4-parameters logistic analysis [20], was 66 $\pm$ 8 $\mu$M and 78 $\pm$ 7 $\mu$M respectively, not significantly different between strains. This indicates that the calmodulin sensitivity of VSMC from WKY and SHR to inhibition is similar and suggests that calmodulin alone is not responsible for the more rapid and greater DNA synthesis in VSMC from SHR.

## DISCUSSION

The increased growth potential of VSMC potentially represents one of the crucial anomalies in hypertension. With $^{3}$H-thymidine incorporation into DNA and flow cytometric analysis of DNA content we demonstrated that cultured VSMC from SHR proliferate at higher rate than VSMC from WKY because of earlier DNA synthesis after calf serum stimulation. No

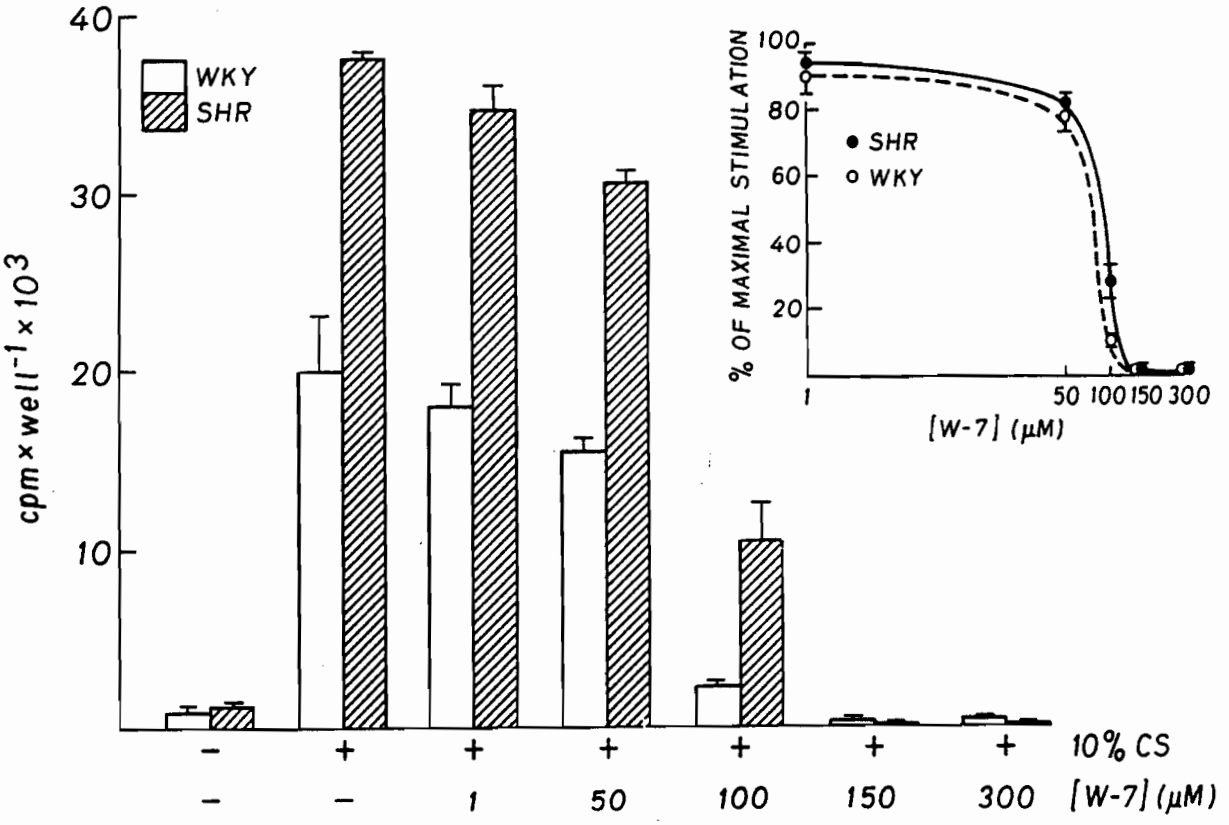


**Figure 13. Inhibition of calf serum-stimulated DNA synthesis by the calmodulin inhibitor W-7 in rat aortic smooth muscle cells.** Quiescent cells were stimulated by 10% calf serum with the addition of escalating concentrations of W-7. After 24 hours, they were pulsed with $^{3}$H-thymidine, and the radioactivity incorporated was measured in precipitated, acid-insoluble material. The values represent the means ± SEM of two experiments, each performed in quadruplicate.
WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats.

difference was seen in cell size and polyploidy between WKY and SHR, corresponding to observations in freshly-isolated VSMC from these rats [21]. The transition from the prereplicative $G_0/G_1$ phase to the synthetic S phase of the cell cycle is regulated by extracellular signals and the intrinsic ability of the cell to respond to them. At this point, normal cells require extrinsic growth factors, such as competence factor PDGF and progression factor EGF, which are contained in platelets and are the most abundant mitogens of serum. VSMC from SHR respond in exaggerated manner to PDGF and EGF [9, 10]. Higher growth-promoting activity, found in platelets from hypertensive diabetic patients [22], suggests a pathophysiological role of these extrinsic growth factors in diseases characterized by increased VSMC proliferation.

After binding to their specific receptors, growth factors induce an ordered sequence of biochemical events in the membrane, cytosol and nucleus, ultimately leading to DNA synthesis. The $Ca^{2+}$/calmodulin system is one of the crucial components of this pathway. PDGF and EGF markedly stimulate $Ca^{2+}$ release from intracellular stores via inositoltrisphosphate, generated together with diacylglycerol by activated phospholipase C [23, 24]. Calmodulin is an ubiquitous intracellular $Ca^{2+}$-binding protein, mediating many of the $Ca^{2+}$-regulated events in the eucaryotic cell. Elevation of calmodulin levels at the $G_1/S$ boundary is required both for the entry of quiescent cells into cell cycle

[25] and for progression through both $G_1$ and S in exponentially growing cells [26]. Moreover, a constitutive increase of intracellular calmodulin levels via the expression of a transfected calmodulin gene leads to a shortening of cell cycle length due to a reduced duration of the $G_1$ phase [27]. To ascertain the involvement of the calmodulin system, described as defective in genetic hypertension [13, 15, 16, 24, 28, 29], we evaluated the effect of calmodulin inhibitor, W-7, on DNA synthesis. The results of the present study show that W-7 inhibits VSMC from both WKY and SHR in a similar manner, suggesting that other parts of the calmodulin system rather than calmodulin itself may be involved in the abnormal proliferation of SHR cells. A calmodulin activator [15] has been demonstrated in SHR and other models of essential hypertension, potentially altering calmodulin-mediated responses. The latter may include the mitogenic response and transition from the $G_0/G_1$ to the S phase of the cell cycle. A W-7 concentration of about 100 $\mu$M decreases DNA synthesis in SHR cells to levels comparable to that in WKY cells without the drug, and at this concentration the degree of inhibition of DNA synthesis was significantly more pronounced in WKY than SHR. Further exploration of the "normalizing" aspect of this anticalmodulin agent is needed.

In addition to the events in membrane and cytosol, growth factors induce the expression of the cellular oncogenes *c-fos* and *c-myc* [30]. We have observed that, after mitogenic

stimulation, the expression of *c-fos* is more elevated in VSMC from SHR than in cells from WKY, but only at high cell densities [31] (and see Chapter 5). Specific growth rate differences between VSMC from SHR and WKY are most evident when cells approach confluency, suggesting a defect also in contact inhibition [9]. Transforming growth factor $\beta_1$ (TGF$\beta_1$) is well known for its bimodal effect on cell growth [32] and complex interactions with PDGF and EGF [33]. TGF$\beta_1$ inhibits VSMC growth at low cell densities and enhances it at high cell densities [34, 35]. TGF$\beta_1$ is, therefore, a good candidate to be involved in the differential growth of VSMC from SHR and WKY at high cell densities and in defective contact inhibition in SHR. Our preliminary results indicate that TGF$\beta_1$ expression precedes and parallels the density-dependent increased growth rate and less steep decline of contact inhibition [36] (and see Chapter 5).

The intrinsic factors leading to enhanced proliferation, the exaggerated response to growth factors, and the defective contact inhibition of VSMC in hypertension are still not fully understood. The persistance of this growth defect in culture emphasizes its primary character in genetic hypertension. Support for this hypethesis comes also from evidence of enhanced *in vivo* DNA synthesis in the heart, kidney and aorta already in newborn SHR compared to normotensive controls [37]. The pathophysiologic role of VSMC proliferation *in vivo* in genetic hypertension is not fully understood. In both genetic

and secondary hypertension, structural changes in the vessel wall are predominant pathologic features together with a higher contractile response of that vascular mass. Genetically hypertensive rats appear to have both hyperplastic and hypertrophic components contributing to vascular changes in the established phase of the disease [38]. Hypertrophy accompanied by polyploidy is observed in induced hypertension and seems to be dominant in the adult aorta, while hyperplastic changes are more evident in the resistant vasculature [39, 40]. In long-term cultures, however, it is hyperproliferation which is the predominant phenotype in both aortic [9] and mesenteric [12] VSMC from SHR. Moreover, long-term treatment of essentially hypertensive patients as well as SHR with antihypertensive drugs does not eliminate all the structural changes, suggesting further that high blood pressure is not the sole determining factor [41-44]. Thus, a tendency towards hyperplasia is present in both aortic and resistance vessels of SHR. Hypertrophy, observed in adulthood, seems to be a consequence of elevated blood pressure, while hyperplasia may represent a genetic defect. The pathogenetic linkage between VSMC hyperproliferation and hypertension remains to be determined.

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# CHAPTER 4

In Chapter 3, we demonstrated that VSMC from SHR start to synthesize new DNA earlier than their control cells. In Chapter 4, using two different flow cytometry approaches, new in studies of VSMC proliferation, we quantitated progression from $G_0/G_1$ to the S phase of the cell cycle as well as cell cycle transition kinetics. Hypothesis about the localization of the defect in $G_0/G_1$ subphases is proposed.

# ACCELERATED ENTRY OF AORTIC SMOOTH MUSCLE CELLS FROM SPONTANEOUSLY HYPERTENSIVE RATS INTO THE S PHASE OF THE CELL CYCLE

This work has been submitted to
Journal of Clinical Investigation

## SUMMARY

The present study was designed to characterize the growth kinetics of the exaggerated proliferative response to mitogens of vascular smooth muscle cells from spontaneously hypertensive rats compared to cells from normotensive Wistar-Kyoto controls. Cellular DNA content, analyzed by flow cytometry, demonstrated a 4-hr accelerated entry into the S phase of the cell cycle of vascular smooth muscle cells from spontaneously hypertensive rats: the significant (4.5-fold) increase in the percentage of cells in the S phase occurred after 12 hrs of calf serum stimulation. A 3.9-fold increase of cells in the S phase was seen in the normotensive controls only after 16 hrs. Transit through the cell cycle was quantitated by flow cytometry using the Hoechst 33 342 - bromodeoxyuridine substitution technique. Vascular smooth muscle cells from hypertensive rats went through the cell cycle 4 hrs ahead of cells from normotensive Wistar-Kyoto rats. This accelerated transit was mostly due to a faster rate of entry into the S phase from spontaneously hypertensive rats cells. Persistence of this new intermediate phenotype in cell culture suggests its primary pathogenetic role in spontaneous hypertension.

## INTRODUCTION

Increased peripheral resistance in hypertension is the result of an augmented mass of the vessel wall together with higher contractile activity of that vascular mass. Narrowing of the vessel lumen due to arterial wall thickening is a common feature in hypertension. Vascular smooth muscle cell (VSMC) hyperplasia in the spontaneously hypertensive rat (SHR) model of essential hypertension has been demonstrated in resistance vessels by histological studies [1, 2]. Aortic and mesenteric smooth muscle cells from SHR proliferate more rapidly than cells derived from normotensive Wistar-Kyoto (WKY) control rats [3-9] *in vitro*, supporting the possibility of an abnormal intermediate phenotype which is not a consequence of elevated blood pressure. Structural changes in the mass of resistance arteries may contribute to their increased responsiveness to vasoactive agents in hypertension *in vivo* [10]. Hyperresponsiveness to vasoactive agents has also been observed in freshly isolated [11] and subcultured VSMC from SHR [12].

The early rise in blood pressure in genetic models of hypertension [13] makes it difficult to distinguish between primary and secondary events occurring in adultood in these models. We have been able to demonstrate increased proliferation in heart, kidney and aorta already in SHR neonates *in vivo* [14]. These results are sustained by greater

numbers of laminae in SHR aortae, even in the fetal stage [15]. The notion that cardiovascular hyperplasia may be causally related to spontaneous hypertension is supported by studies demonstrating that cardiac and renal hyperplasia associated with genetic hypertension is present in newborns of four different spontaneously hypertensive models but absent in offspring of parents with renal and experimental hypertension [16]. We and others have previously shown that the greater proliferation of SHR VSMC, persisting under culture conditions, is not due to varying cell survival or attachment ability after passage, and that these cells manifest an exaggerated maximal response to calf serum, epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) [6, 7]. VSMC from SHR grow to a greater density and their specific growth rate is higher than that of WKY, particularly when they approach confluency [6]. Characterization of VSMC growth kinetics is therefore essential to an understanding of these abnormalities. The present study evaluated, by flow cytometry, the cell cycle transition kinetics of cultured VSMC from SHR and their normotensive WKY controls.

## MATERIAL AND METHODS

### Cell culture

Cultured VSMC were obtained by an explant method [17] from aortae of 10- to 13-week-old male SHR and WKY rats, as

described previously [6, 18]. In preparation for the experiments, the cells were made quiescent after 16 to 20 hrs of attachment by replacing the culture medium with defined serum-free medium (DME containing 2.5 μg/ml insulin, 2.5 μg/ml transferrin and 2.5 ng/ml selenium) or 0.2% calf serum in DME for 72 hrs.

**Cellular DNA content analysis by flow cytometry**

Cells were inoculated in 80-$cm^2$ flasks at a concentration of 5 x $10^5$ cells/flask and made quiescent, as described above. They were stimulated with 10% calf serum and arrested by trypsinization at various times from 0 to 24 hrs. Cells resuspended in Dulbecco's PBS containing 320 μM $Ca^{2+}$ and 60 μM $Mg^{2+}$ ($PBS^+$) were fixed in 50% ethanol and kept at 4°C. Immediately before cell cycle analysis, they were washed in Dulbecco's $PBS^+$ and resuspended in 2 ml $PBS^+$. DNA was stained with propidium iodide (20 μg/ml), a specific dye for double stranded nucleic acid (Sigma, St.Louis, MO, USA). To prevent RNA staining by propidium iodide, 10 μg/ml of ribonuclease A (Sigma) was added to each sample. After 30 min of incubation at 37°C, the cells were analysed in a FACScan flow cytometer (BDIS, San Jose, California) with an argon laser adjusted to emit 15 mW at 488-nm wavelengths. From each cell sample, 10 x $10^3$ events were accumulated for each histogram. The proportion of cells in cell cycle phases was determined from each histogram, using the model described by Baisch et al. [19]. VSMC size was estimated simultaneously from the same cell

samples by analysis of forward light scatter.

**Flow cytometric analysis of bromodeoxyuridine (BrdU) substitution**

Cells were inoculated in 80-$cm^2$ flasks at a concentration of 7.5 x $10^5$ cells/flask and made quiescent as above. Cell suspension samples were prepared and processed for cell cycle analysis as described [20]. Briefly, the cells were stimulated by DME containing 10% calf serum to which BrdU (Boehringer Mannheim Corp., Dorval, Quebec, Canada) was added at a concentration of 10 μg/ml. To overcome BrdU's cytotoxic effect due to inhibition of ribonucleotide reductase [21], deoxycytidine (Boehringer Mannheim Corp.) was added in equimolar concentrations (8 μg/ml) to BrdU. Cell suspensions were obtained by trypsinization at various time periods after calf serum stimulation and the addition of BrdU and deoxycytidine. The cells were then resuspended in $PBS^+$, fixed in 50% ethanol and kept at $4^oC$. After washing in $PBS^+$, they were stained with 1 μg/ml of the adenine/thymidine-specific binding dye, Hoechst 33 342 (Molecular Probes, Inc., Eugene, OR, USA), in the presence of 0.05% NP-40. Fluorescence analysis was performed on a FACStar (BDIS) with an argon laser adjusted to emit 50 mW at 365-nm wavelengths. For each Hoechst fluorescence histogram, 5 x $10^3$ cells were accumulated. In two control experiments, half of each sample was stained by propidium iodide and analyzed by FACScan as described above.

**Statistical analysis**

Values are given as means ± SEM. The level of significance of differences between the means was evaluated by Student's t test for unpaired data and by two-way and multivariate ANOVA [22].

## RESULTS AND DISCUSSION

**Analysis of cellular DNA content**

Quiescent cells were stimulated at time 0 with 10% calf serum. After different periods, they were trypsinized, fixed and stained with the DNA-binding dye propidium iodide, and processed by FACScan for cellular DNA analysis. The intensity of fluorescence emitted is directly proportional to DNA content. The time course of analysis is presented in Figure 14 (typical experiment) and Table 7 (data from 5 independent experiments). Quiescent cells were mostly seen in the $G_0/G_1$ compartment. After 12 hrs of stimulation, the proportion of VSMC from SHR in the S phase increased significantly (4.5-fold, ANOVA p=0.0003, 8 hrs vs. 12 hrs). In the case of VSMC from WKY, this (3.9-fold) increase of S phase cells occurred 16 hrs after stimulation (ANOVA p=1.2249, 8 hrs vs. 12 hrs, and p = 0.0003, 12 hrs vs. 16 hrs), indicating that the transition from the $G_0/G_1$ to the S phase of the cell cycle was accelerated by 4 hrs in SHR VSMC. The proportion of VSMC in the S phase reached a maximum at 16 hrs in SHR and at 20

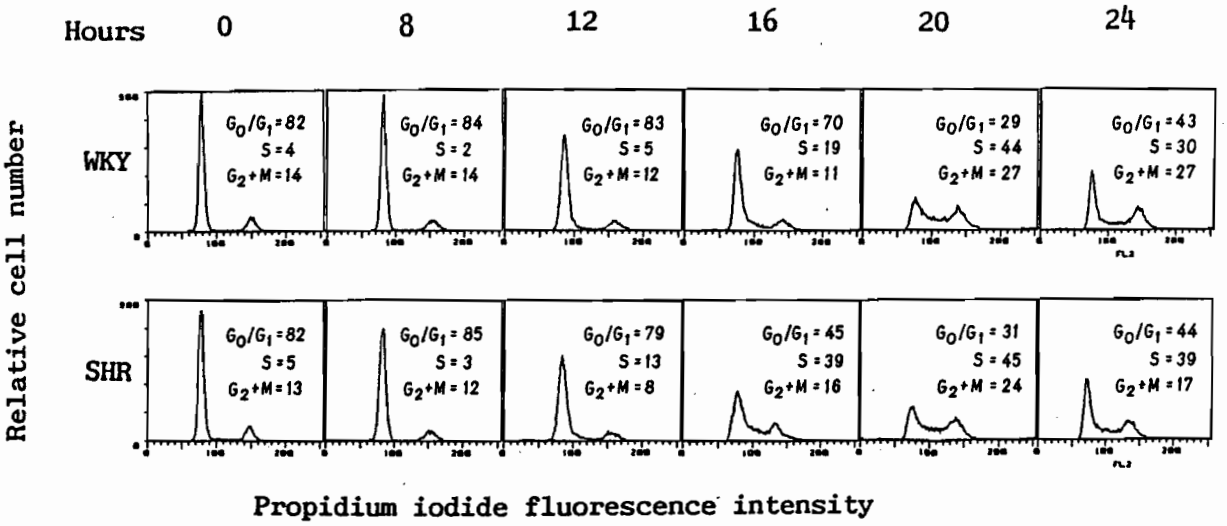


**Figure 14. Cell cycle analysis of aortic smooth muscle cells from WKY and SHR.** Cells were made quiescent by serum starvation and then stimulated with 10% calf serum. They were harvested at various times (between 0 and 24 hrs), treated with RNAse, stained with propidium iodide and analyzed on a FACScan flow cytometer. A total of $10 \times 10^3$ cells were accumulated for each DNA histogram. The proportion of cells in each phase of the cell cycle was determined using the Baisch mathematical model.

## TABLE 7

## ESTIMATED PROPORTIONS OF SYNCHRONIZED WKY AND SHR VASCULAR SMOOTH MUSCLE CELLS DURING TIME COURSE FOLLOWING CALF SERUM STIMULATION

| PHASES | STRAIN | HOURS | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 8 | 12 | 16 | 20 | 24 |
| $G_0/G_1$ | WKY | 83 ± 1 | 81 ± 2 | 81 ± 2 | 58 ± 4 | 34 ± 3 | 48 ± 2 |
| | SHR | 80 ± 1 | 81 ± 2 | 75 ± 3 | 45 ± 2 | 39 ± 4 | 46 ± 2 |
| S | WKY | 4 ± 1 | 5 ± 2 | 8 ± 2 | 31 ± 4 | 40 ± 2 | 29 ± 2 |
| | SHR | 7 ± 1 | 4 ± 1 | 18 ± 3 | 37 ± 2 | 38 ± 3 | 35 ± 2 |
| $G_2$-M | WKY | 13 ± 1 | 15 ± 1 | 11 ± 1 | 12 ± 1 | 27 ± 2 | 23 ± 3 |
| | SHR | 13 ± 1 | 15 ± 1 | 7 ± 1 | 18 ± 1 | 23 ± 3 | 19 ± 1 |

The data are expressed as means ± SEM from 5 independent experiments.
WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats.
$p < 0.007$, difference between the two strains as evaluated by multivariate analysis of variance.

hrs in WKY, and was paralleled by a decreased proportion of cells in the $G_0/G_1$ phase (Table 7). After 24 hrs of stimulation, the cell cycle distribution of VSMC was similar to that of exponentially growing populations. Analysis of forward light scatter demonstrated no difference in cell size between WKY and SHR VSMC (data not shown), confirming previous observations on freshly-isolated [11] as well as cultured [23] VSMC from these rats.

**Analysis of cell cycle phases by BrdU substitution**

Quiescent cells were stimulated with 10% calf serum and the thymidine analogue BrdU was added at the same time. The cells were then trypsinized, fixed in time course as above and stained with Hoechst 33 342. The BrdU incorporated in newly-synthesized DNA in place of thymidine quantitatively quenches Hoechst 33 342 fluorescence. Cells, when they progress through the S phase, incorporate BrdU and will thus not increase their fluorescence intensity. After mitosis, the amount of Hoechst fluorescence in daughter cells (designated as $G_1'$) is approximately 50% of $G_0/G_1$ cells which have not incorporated BrdU. $G_1'$ cells therefore appear in a new peak of quenched fluorescence. This permitted us to follow the decrease of $G_0/G_1$ cells and the progression through the cell cycle, as recorded by the appearance and increase of daughter cells in the $G_1$ peak that have passed through the S and $G_2$ + M phases.

After stimulation with 10% calf serum and the addition of BrdU, the S compartment, to the right of the major $G_0/G_1$ peak, remains empty (Figure 15). From 8 hrs on, we observed the quenched fluorescence of $G_1'$ cells to the left of the $G_0/G_1$ peak with higher proportions in the case of SHR cells; WKY cells followed the SHR pattern with approximately a 4-hr lag period (Figures 15 and 16). The proportion of $G_0/G_1$ cells continued to decrease until 32 hrs. The transition kinetics of VSMC from WKY and SHR in 3 independent experiments are summarized in Figure 16. Since each cell in $G_0/G_1$ gives rise to 2 daughter cells after mitotic division, the numbers of $G_1'$ cells were divided by 2 to obtain the real transition kinetics [24]. The distribution of $G_0/G_1$ minus $G_1'/2$ difference was plotted against time after stimulation and BrdU addition. A linear (SHR, r=0.992, WKY, r=0.994) relationship appeared between 12 and 28 hrs. At earlier times (between 0 and 8 hrs), the apparent increase in the proportion of $G_0/G_1$ cells corresponded to entry into this peak of original $G_2$ + M cells that had undergone mitosis without BrdU incorporation. The intercepts of the curves with line 0 corresponded to the time when half of the cells which were initially in the $G_0/G_1$ phase had progressed through the cell cycle and had divided. The curves were parallel, showing no measurable difference in transition kinetics. The shift of the SHR VSMC curve to the left is explained by 4-hr accelerated $G_0/G_1$-S phase transition.

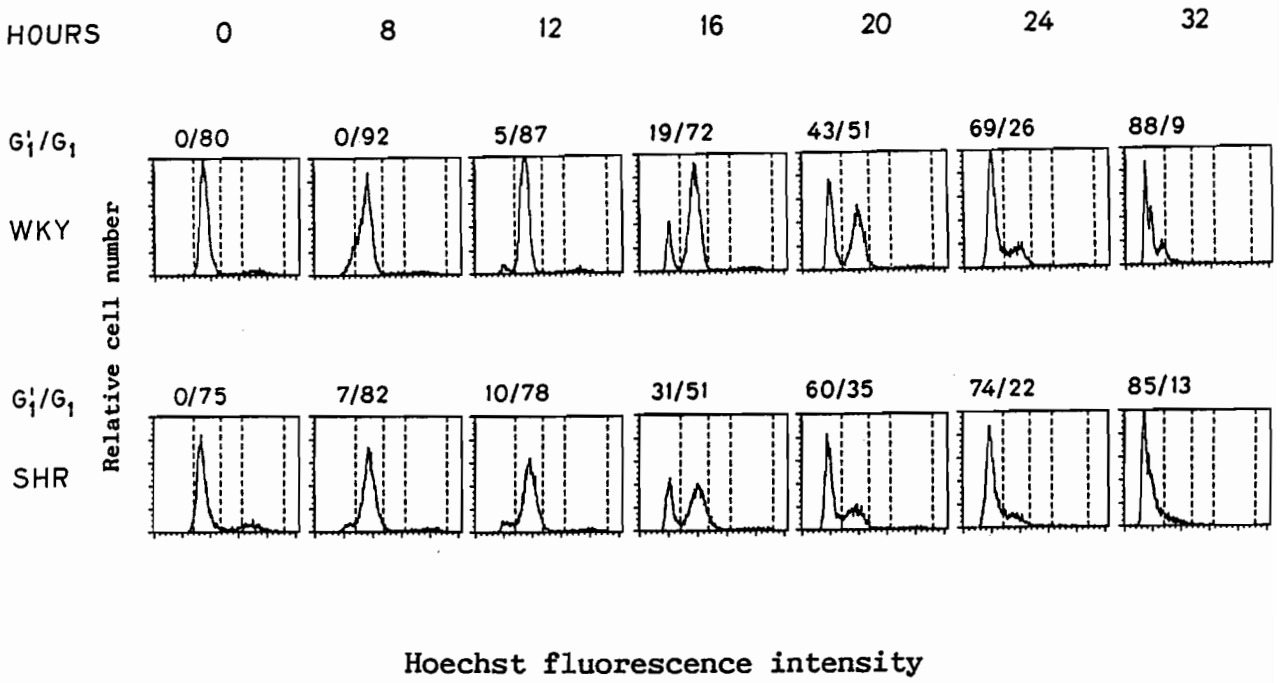


**Figure 15. Cell cycle analysis of WKY and SHR aortic smooth muscle cells using the Hoechst 33 342 - BrdU technique.** Cells were made quiescent by serum starvation and then stimulated with 10% calf serum in the presence of BrdU. They were harvested at various times (between 0 and 32 hrs), stained with Hoechst 33 342 and analysed on a FACStar PLUS. $G_1$ peak refers to cells that had been in the $G_0/G_1$ phase at the time of BrdU addition and have not transited through S and $G_2$ + M, whereas the $G_1'$ peak refers to those cells that transited through the S and $G_2$ + M phases. A total of 5 to 10 x $10^3$ cells were accumulated for each histogram.

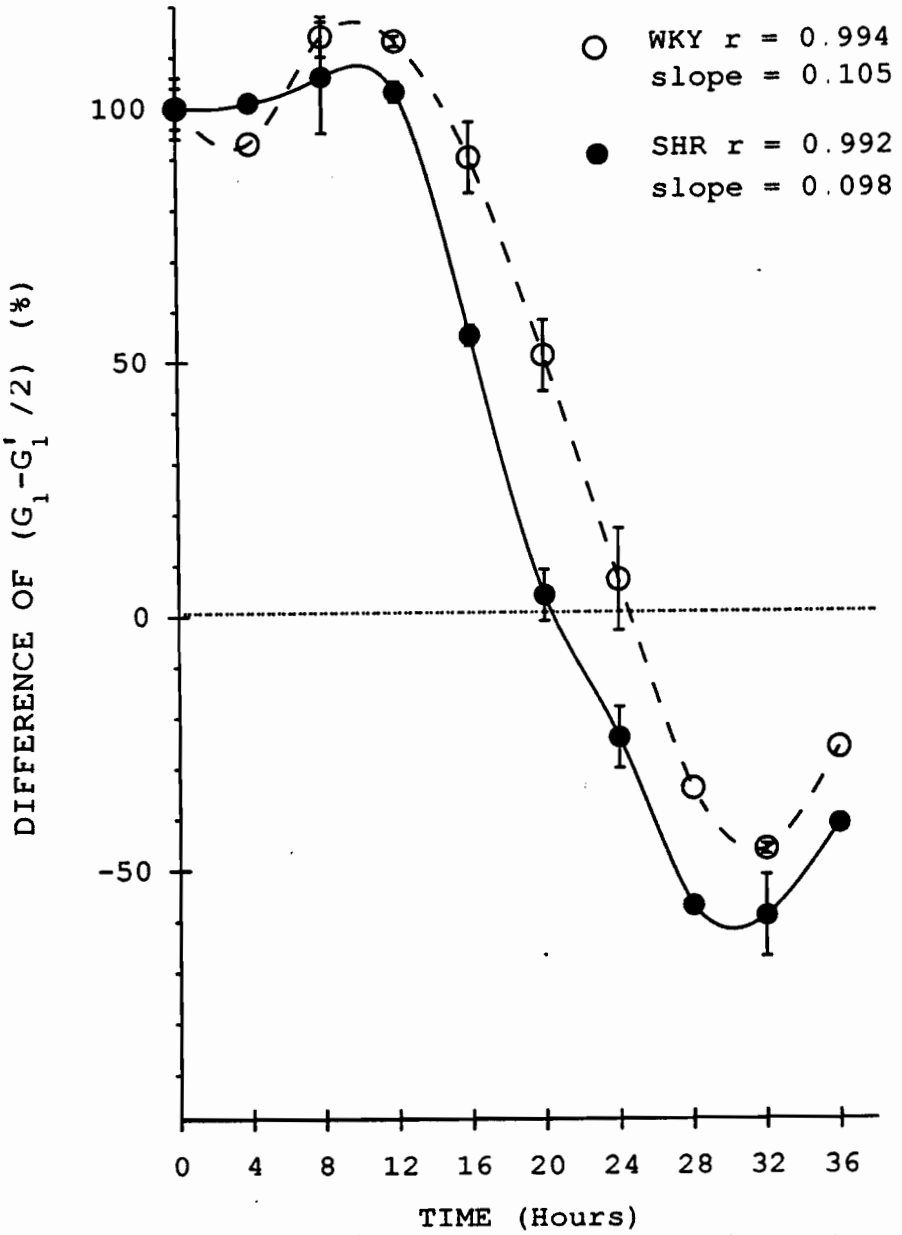


**Figure 16. Determination of the transition kinetics of WKY and SHR aortic smooth muscle cells.** The proportions of cells in $G_1$ and $G_1'$ compartments were obtained from histograms similar to those shown in Fig. 2. The proportions of cells in the $G_1'$ peak were divided by a factor of 2 since each cell gives rise to two daughter cells after mitotic division. Differences of ($G_1$ minus $G_1'/2$) were plotted against time after calf serum stimulation and BrdU addition. Means $\pm$ SEM from 3 independent experiments. Initial values are normalized to 100. Line 0 represents equal proportions of $G_1$ and $G_1'/2$ cells. The intercepts of the curves with line 0 correspond to the time when half of the cells have progressed through the cell cycle and have divided.

Transition from the prereplicative $G_0/G_1$ phase into the synthetic S phase of the cell cycle is regulated by extracellular signals and the intrinsic ability of the cell to respond to them. $G_0/G_1$ events leading ultimately to DNA synthesis comprise a series of sequential signals in a cause-effect relation, including cis and transacting regulators. Depending on the effects of limiting factors, the prereplicative phase has been divided into *competence, entry, progression* and *assembly* subphases, which are separated by C [25], V [26] and R [27] points. After the restriction R point, transit through *assembly* to the S phase is serum-independent [28]. The results of our previous and present experiments led us to formulate a hypothesis about localization of the defect in the cell cycle. The expression of one of the immediate-early genes, proto-oncogene *c-fos*, was investigated in our laboratory [29] (and see Chapter 5). At a density comparable to that used in the present studies, calf serum stimulation induced the same kinetics and levels of *c-fos* mRNA in quiescent VSMC from both WKY and SHR. This suggests that up to competence C point there is no difference between WKY and SHR cells, since competent cells immediately produce mRNAs, including *c-fos*, which appears in a few minutes, and *c-myc*, which appears later.

EGF is the most important progression factor acting downstream of competence [30]. VSMC from SHR were demonstrated [6, 7] to present a greater response to EGF than WKY cells. The

response to low concentrations of PDGF, the competence-inducing factor, has been reported to be the same in VSMC from SHR [7, 31]. EGF induces *c-fos* but is required to be present for at least 8 hrs to express its progression effect [30]. The rapid cytosolic $Ca^{2+}$ increase and membrane hyperpolarization elicited by EGF are not limited to the initial few minutes of stimulation but proceed for longer time periods in the form of discrete fluctuations. Calmodulin, a major intracellular $Ca^{2+}$-binding protein, has to be elevated during the *entry* and *progression* subphases [32, 33]. Although calmodulin levels were found in our laboratory to be the same for both WKY and SHR cells, a calmodulin activator was demonstrated in SHR VSMC, thus conferring greater activity to the $Ca^{2+}$-calmodulin system and possibly influencing entry into the S phase [34]. It seems conceivable therefore that the major defect of SHR VSMC resulting in accelerated entry into the S phase is situated between the competence and restriction points. The potential consequence of progression through the cell cycle with 4 hrs accelerated entry into the S phase in SHR VSMC is illustrated in Figure 17.

The prereplicative events necessary for the eventual onset of the S phase can begin during the previous cycle [35]. An additional defect is noted in the faster disappearance of $G_2$-M cells at 12 hrs of stimulation in SHR (Table 7). We cannot therefore exclude a minor defect which would indirectly influence $G_1$-S transition.

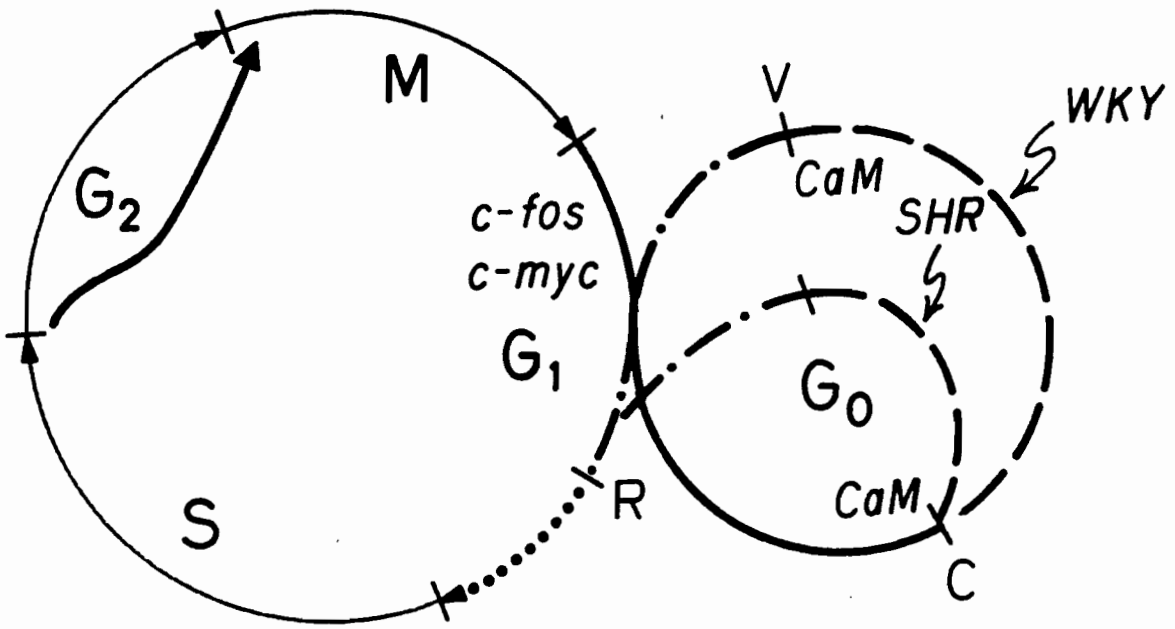


**Figure 17. Schematic illustration of accelerated entry of aortic smooth muscle cells from SHR into the S phase of the cell cycle.** The conventional cell cycle ($G_1$, S, $G_2$, M phases) is modified to indicate $G_0/G_1$ (bold line) activities preceeding entry into the S phase. They may be divided into *competence* ( ———— , end of M to C point), *entry* (— — — , C to V point), *progression* (—·——·— , V to R point) and *assembly* (····· , R to beginning of S) subphases. The defect of SHR cells seems to be situated between the C and R points. An additional minor alteration is evident in the $G_2$ phase.

Several other abnormalities of cell cycle-related events have been noted in vessels and cultured VSMC from SHR and other models of essential hypertension. The internal pH in resistance vessels of SHR is more alkaline [36]. The exaggerated growth of cultured SHR VSMC is accompanied by a heightened $Na^+/H^+$ exchange [37] and elevated cytoplasmic free $Ca^{2+}$ and intracellular $Na^+$ [38, 39]. Altered phosphoinositide turnover has been noted in the SHR aorta [40] and erythrocytes [41]. Their cause-effect relation to increased proliferation of SHR VSMC is not established. Any of these steps may be regulated by yet unknown genes.

In conclusion, accelerated entry into the S phase of the cell cycle is an intrinsic abnormality of VSMC from SHR and in addition to a well established higher contractile response to vasoconstrictors [42], presents a new intermediate phenotype in genetic hypertension. Although the pathogenetic linkage with hypertension remains to be determined, its persistence under culture conditions suggests a primary defect independent of blood pressure.

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# CHAPTER 5

In Chapter 2, we demonstrated that VSMC from SHR in comparison to the cells derived from normotensive WKY rats present a greater specific growth rate when approaching confluency or when seeded at high cell densities, suggesting an altered contact inhibition. It was therefore of relevance to understand and characterize further this altered contact inhibition. As a first step, we have studied the expression and effect of TGF-$\beta_1$, a factor with bimodal actions on proliferation of vascular smooth muscle and other cell types. Both endogenous expression and the effect of exogenously added TGF-$\beta_1$ were examined with respect to time and different VSMC densities. *c-fos* expression served as a marker of mitogenic response at different cell densities.

# TGF-$\beta_1$ EXPRESSION AND EFFECT IN AORTIC SMOOTH MUSCLE CELLS FROM SPONTANEOUSLY HYPERTENSIVE RATS

This work is in press
in Hypertension, Vol. 17, June 1991

## SUMMARY

Previous studies demonstrated that in addition to an increased response to growth factors, cultured vascular smooth muscle cells derived from spontaneously hypertensive rats (SHR) grow to a greater density when compared to cells from normotensive Wistar-Kyoto rats (WKY). Transforming growth factor $\beta_1$ (TGF-$\beta_1$) has a bimodal effect on vascular smooth muscle cells growth, depending on cell density. The present study investigated the relationship between cell density and expression of the proto-oncogene *c-fos* and TGF-$\beta_1$ in cells from WKY and SHR. The results demonstrate an increased accumulation of *c-fos* mRNA in calf serum-stimulated cells derived from SHR but only at a high cell density. The expression of TGF-$\beta_1$ mRNA was enhanced in growing cells from SHR at every density studied, as early as 24 hours after inoculation, with a further increase at later times. The effect of exogenous TGF-$\beta_1$ on new DNA synthesis was evaluated by [$^3$H]thymidine incorporation. At a low cell density, TGF-$\beta_1$ had no effect on DNA synthesis in vascular smooth muscle cells from either WKY or SHR. At a high cell density, there was a significant increase of DNA synthesis in response to TGF-$\beta_1$ in cells from SHR without any effect in WKY. In conclusion, contact inhibition of vascular smooth muscle cells from SHR at a higher cell density is accompanied by an earlier expression of the marker gene *c-fos* and preceeded by an exaggerated expression of TGF-$\beta_1$. Considered together with the stimulating

effect of exogenous TGF-$\beta_1$ at a high cell density, the results suggest an abnormal feedback control (autocrine stimulation) of this growth factor and its involvement in altered contact inhibition of vascular smooth muscle cells from SHR.

## INTRODUCTION

Abnormal vascular smooth muscle cell (VSMC) growth has been suggested to be one of the significant contributors to increased peripheral vascular resistance in hypertension [1, 2]. Several studies, including ours, have demonstrated greater VSMC proliferation in spontaneously hypertensive rats (SHR) when compared to normotensive Wistar-Kyoto controls (WKY) in response to growth stimuli, such as calf serum, epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) [3-6]. The expression of this intermediate phenotype appears to be stable up to the 20th passage. An additional intermediate phenotype of this abnormal growth is an increased specific growth rate, which is most apparent when cells approach confluency [6]. It has been suggested that the production of an extracellular matrix may be essential for the expression of enhanced VSMC proliferation in SHR [7]. Transforming growth factor $\beta_1$ (TGF-$\beta_1$) has been shown to stimulate the expression and synthesis of several extracellular matrix proteins [8, 9]. It also possesses multiple and complex regulatory functions that influence cell growth [10]. TGF-$\beta_1$ is present in

significant amounts in platelets [11, 12] and is synthesized by a variety of cells in culture [13]. This factor acts as a bifunctional modulator of VSMC growth since it inhibits serum- or PDGF-mediated proliferation at a low cell density and potentiates it at a high cell density [14, 15]. PDGF and EGF elevate the expression of TGF-$\beta_1$ which can also induce its own message [16, 17]. TGF-$\beta_1$ mRNA has recently been demonstrated to increase in aortae of experimental DOCA/salt hypertensive rats [18]. These findings support the *in vivo* implication of synthesis and release of growth factors from VSMC and their autocrine/paracrine role. However, the involvement of TGF-$\beta_1$ in essential hypertension has not yet been studied. The present investigation focused on the growth of cultured VSMC from SHR and WKY at different cell densities in relation to contact inhibition, the expression of the proto-oncogene *c-fos* and TGF-$\beta_1$ as well as the effect of this growth factor added exogenously.

## MATERIAL AND METHODS

### Cell culture

Cultured VSMC were obtained by an explant method from aortae of 10- to 13-week-old male SHR and WKY rats (Charles River Canada, St. Constant, Quebec, Canada or Taconic, Germantown, N.Y., USA), and their phenotypes in sub-culture were characterized as described previously [6, 19-21] (see Chapters 2 and 3). Briefly, cells from both origins stained

positively for specific anti-smooth muscle myosin antibodies, presented at confluency a "hill-and-valley" formation, typical for smooth muscle cells in culture and were of similar size. The cells were used for experiments between 6th and 20th passages. When indicated, the cells were made quiescent after 16 to 20 hrs of attachment by replacing the culture medium [Dulbecco's Modified Eagle's medium (DME), 10% calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin] with defined serum-free medium (DME containing 2.5 μg/ml insulin, 2.5 μg/ml transferrin and 2.5 ng/ml selenium) or DME + 0.2% calf serum for 72 hrs.

**RNA extraction and Northern blot analysis**

Total cellular RNA was isolated from VSMC by the acid guanidium thiocyanate-phenol-chloroform method [22]. Ten μg of total RNA were denatured by heating (65°C for 10 min) in a buffer of 20 mM 3-[N-morpholinopropanelsulfonic] acid, pH 7.0, 50% formamide, 16% formaldehyde, and size-fractionated by electrophoresis on 1% agarose and 1.8% formaldehyde gels in 20 mM 3-[N-morpholinopropanelsulfonic] acid, 5 mM sodium acetate and 1 mM EDTA, pH 7.0 at 100 V for 4 hrs. The gels were stained with ethidium bromide, photographed and transferred to nylon filters (Genescreen, New England Nuclear, Boston, MA) in 3.0 M NaCl and 300 mM sodium citrate. The blots were prehybridized at 42°C for 3-4 hrs in a buffer containing 50% formamide, 750 mM NaCl, 250 mM $NaPO_4$, 5 mM EDTA, 0.5% SDS, 5x Denhart's solution (0.1% Ficoll, 0.1% polyvinylpyrollidone,

0.1% BSA) and 200 $\mu$g/ml sheared and denatured salmon sperm DNA. The blots were hybridized overnight at 42°C in 50% formamide, 1x Dernhardt's solution, 750 mM NaCl, 250 mM $NaPO_4$, 5 mM EDTA, 0.5% SDS, 10% dextran and 200 $\mu$g/ml sheared and denatured salmon sperm DNA with $^{32}$P-labelled cDNA probes of *v-fos* or TGF-$\beta_1$. The *v-fos* probe consisted of the 5.75-kb Hind III fragment of pFBJ-2 plasmid [23]. The TGF-$\beta_1$ probe consisted of the 1.6-kb EcoR1 fragment of pMurB-2 plasmid [24]. cDNAs were radiolabelled by the random priming technique [25]. After hybridization, the filters were washed with 50% formamide, 500 mM NaCl, 25 mM $NaPO_4$, 1 mM EDTA, 0.5% SDS at 42°C for 30 min and with 75 mM NaCl, 5 mM $NaPO_4$, 1 mM EDTA, 0.1% SDS at 65°C for 1 hr. They were exposed to Kodak X-RPM1 film with 2 intensifying screens at -80°C. The developed films were scanned with a densitometer (Bio-Rad Labs., Mississauga, Ontario, Canada); areas under each peak, evaluated as optical density (OD) multiplied by peak's width, were used as quantitative estimates of the amount of mRNA accumulation. The samples obtained from WKY and SHR VSMC were prepared and run on the same gel simultaneously in identical conditions.

**Thymidine incorporation**

[$^3$H]thymidine incorporation into newly-synthesized DNA was performed as described previously [6] (see Chapter 2). Briefly, quiescent VSMC in 24-well cluster dishes were treated for 24 hrs with TGF-$\beta_1$ (from porcine platelets of at least 95% purity - R&D Systems, Inc., Minneapolis, MN, USA) in

DME + 0.2% calf serum. The medium was then removed, and DME containing 0.5 $\mu$Ci/ml [$^3$H]thymidine (Dupont, Montreal, Quebec, Canada) was added for 2 hrs. Each well was washed with 150 mM NaCl, fixed in ethanol:acetic acid (3:1) for 10 min, and washed with $H_2O$. Acid insoluble material was precipitated by 15 min of incubation with cold 0.5 N perchloric acid, washed, and DNA was extracted into 1.5 ml perchloric acid by heating at 80$^o$C for 20 min. The radioactivity incorporated into newly-synthesized DNA was determined in a liquid scintillation spectrometer.

**Statistical analysis**

The values are given as means ± SD. The level of significance of difference between means was evaluated by Student's t test for unpaired data and by 3-way ANOVA.

## RESULTS AND DISCUSSION

Since our previously reported results have demonstrated an increased response to growth factors [6] and an abnormality in $G_1$-S phase transition of the cell cycle, it seemed warranted to evaluate the expression of the proto-oncogenes implicated in the regulation of the $G_1$ phase (Figure 18). Cells were inoculated at $4x10^3$ cells/cm$^2$ and $17x10^3$ cells/cm$^2$ and left in 10% calf serum for 20 and 72 hrs respectively. The former cells remained at low cell density whereas the

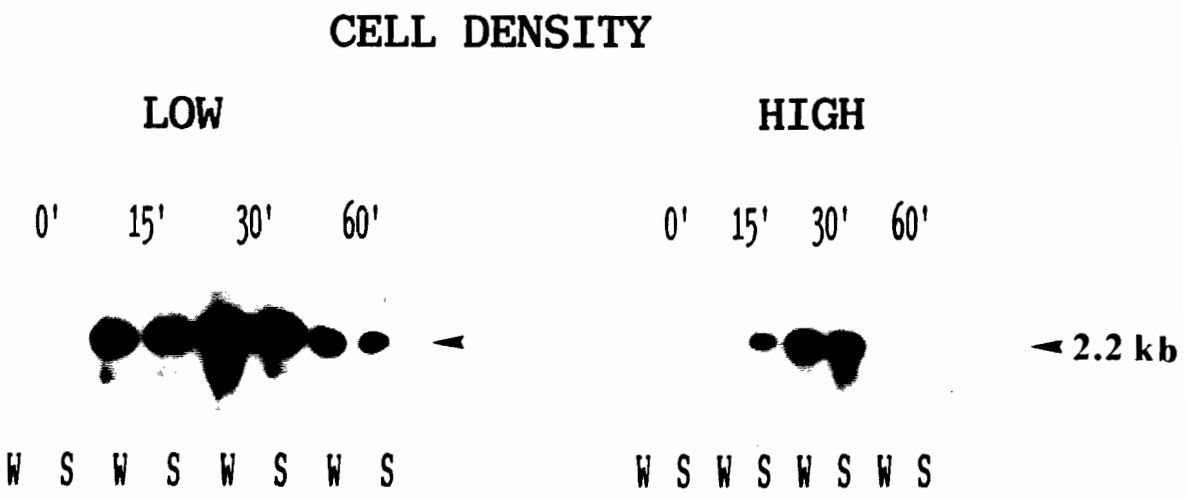


**Figure 18.** ***c-fos*** **expression in vascular smooth muscle cells from WKY and SHR in response to calf serum stimulation.** *Low cell density*: Cells were inoculated in DME + 10% calf serum at 4 x $10^3$ cells/$cm^2$ in 200-$cm^2$ flasks. After 20 hrs of attachment, the medium was changed for defined serum-free medium for 72 hrs. *High cell density*: Cells were inoculated at 17 x $10^3$cells/$cm^2$ and left in DME + 10% calf serum for 3 days. The medium was then changed for defined serum-free medium for an additional 72 hrs. Quiescent cells were stimulated in both cases by 10% calf serum, harvested in time course (0, 15, 30 and 60 min), and RNA was extracted. Ten $\mu$g of total RNA were applied in each well. Gels were stained with ethidium bromide for the control of quantification of RNA samples. Membranes were hybridized with a probe of *v-fos* labelled with $^{32}$P by random priming. W, Wistar-Kyoto rats, S, spontaneously-hypertensive rats.

latter reached high cell density of 70 to 100 x $10^3$ cells/$cm^2$ as can be seen in experiment presented in Figure 19 (upper panel). In both cases, after 20 or 72 hrs in 10% calf serum, cells were made quiescent by 72 hrs incubation in defined serum-free medium. Figure 18 follows the *c-fos* mRNA accumulation in response to 10% calf serum in quiescent VSMC from WKY and SHR at low and high inoculation densities. *c-fos* was rapidly induced from 15 min after mitogenic stimulation, with a maximum at 30 min and a decline after 1 hr. No significant difference between WKY and SHR was seen at a low cell density. When the mRNA was quantified by densitometry in five different experiments, maximum expression was always observed at 30 min, and was 9.8 $\pm$ 2.6 and 9.9 $\pm$ 2.2 (OD x mm) vs 5.3 $\pm$ 2.5 and 4.9 $\pm$ 2.6 (OD x mm) at 15 min for WKY and SHR respectively. At a high cell density, the *c-fos* expression at 15 min of calf serum stimulation, was 8.4-fold higher in SHR when compared to WKY, as evaluated by densitometry in two independent experiments. A lesser 1.4-fold difference persisted at 30 min after the addition of growth stimuli. This increased expression of *c-fos* at a high cell density in VSMC from SHR seems to be a marker of a reduced contact inhibiton and hyperresponsiveness to mitogens.

To further investigate the effect of cell density on rat VSMC proliferation, cells were inoculated at low (1.8 x $10^3$), medium (40 x $10^3$) and high (100 x $10^3$ cells/$cm^2$) densities, and their proliferation was followed in 10% calf serum by

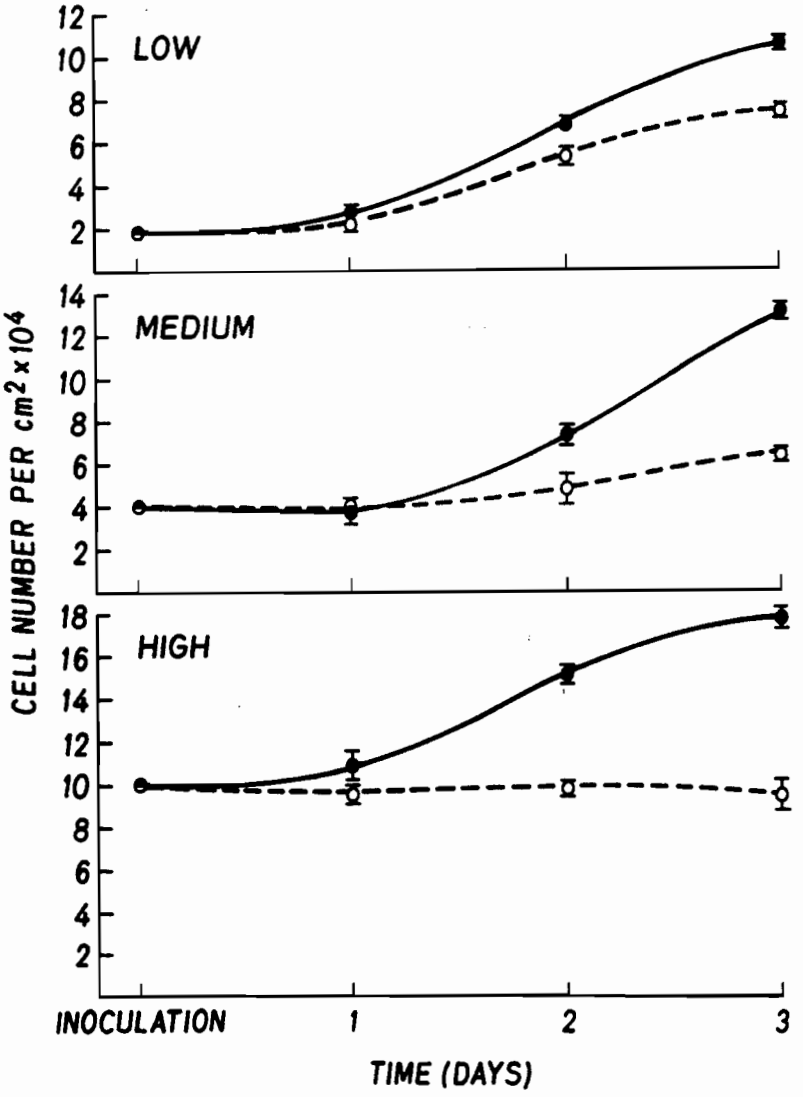


**Figure 19. Growth of vascular smooth muscle cells from WKY and SHR at three different cell densities.** Cells were inoculated in DME + 10% calf serum at densities of 18 x $10^3$ (low), 40 x $10^3$ (medium) and 100 x $10^3$ (high) cells/$cm^2$ in 25-$cm^2$ flasks. They were trypsinized 24, 48 and 72 hrs later, and the cell number was determined in a Coulter counter. The data are expressed as means ± SD from two experiments performed in triplicate. Empty circles, (- - -) Wistar-Kyoto rats; solid circles, (———) spontaneously hypertensive rats.

counting the cells over 3 days (Figure 19). WKY and SHR cells proliferated with significantly different kinetics ($p < 0.001$, strain comparison by 3-way ANOVA). Proliferative kinetics of VSMC from both strains was significantly changed by cell density of inoculation ($p < 0.001$, effect of cell density by 3-way ANOVA). As seen in Figure 19, the difference between WKY and SHR cell number (reached at 3 days) increased from low to high density. At high seeding density, there was a full growth arrest in cells of WKY origin while proliferation continued over the following 3 days in cells derived from SHR. We suggest that this lack of contact inhibition at a high density represents an additional intermediate phenotype of VSMC in SHR.

To search for potential regulators of the pathogenesis of this phenotype, we evaluated the expression and effect of TGF-$\beta_1$. Cells were inoculated at the same densities as in experiments depicted in Figure 19. TGF-$\beta_1$ mRNA accumulation was evaluated in cells growing in DME + 10% calf serum for 24, 48 and 72 hrs after inoculation in three independent experiments. As seen in Figure 20, TGF-$\beta_1$ mRNA was detectable in VSMC from both WKY and SHR 24 hours after seeding. While TGF-$\beta_1$ expression decreased in WKY with increasing cell density, VSMC from SHR expressed TGF-$\beta_1$ regardless of their density. The differential expression of TGF-$\beta_1$ between WKY and SHR was therefore most evident at medium and high cell densities. Increased TGF-$\beta_1$ mRNA accumulation at 24 hrs after

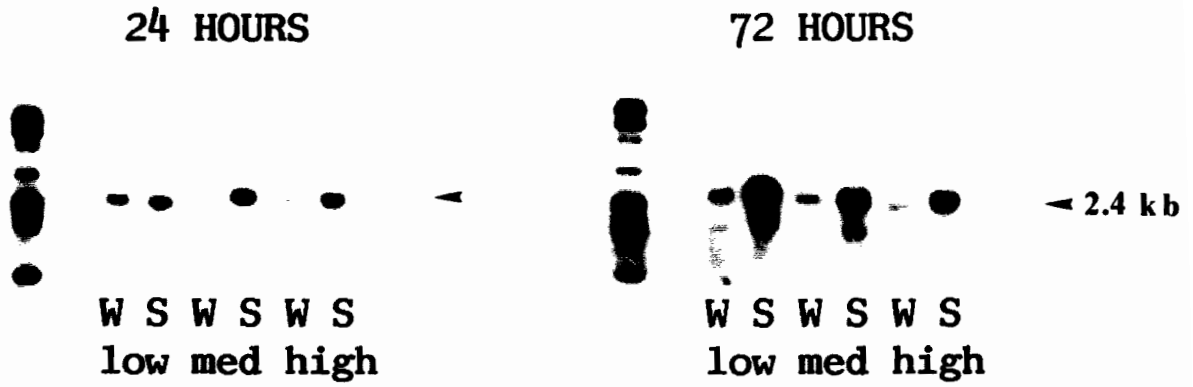


**Figure 20. TGF-$\beta_1$ expression in growing vascular smooth muscle cells from WKY and SHR at different cell densities.** Cells were inoculated in DME + 10% calf serum at densities of 18 x $10^3$ (low), 40 x $10^3$ (medium) and 100 x $10^3$ (high) cells/$cm^2$ in 200-$cm^2$ flasks. After 24 and 72 hrs, they were harvested and RNA extracted. Ten $\mu$g of total RNA were applied in each well. Gels were stained with ethidium bromide for the control of quantification of RNA samples. Membranes were hybridized with a TGF-$\beta_1$ probe labelled with $^{32}P$ by random priming. W, Wistar-Kyoto rats; S, spontaneously hypertensive rats.

inoculation in SHR as compared to WKY was observed in all experiments performed. TGF-$\beta_1$ expression in both strains at 48 hrs (data not shown) and 72 hrs was higher than at 24 hrs after seeding. At 72 hrs, TGF-$\beta_1$ mRNA declined in both WKY and SHR with increasing cell density. Nevertheless, the difference between WKY and SHR persisted and was actually most evident again at high densities, suggesting a potential role of TGF-$\beta_1$ in the altered contact inhibition in SHR. The presence and secretion of TGF-$\beta_1$ protein are currently being investigated in our laboratory. Since TGF-$\beta_1$ enhances its own expression, it is conceivable that this positive autocrine/paracrine effect is exaggerated in VSMC from SHR.

The DNA synthesis in response to exogenous TGF-$\beta_1$ was studied at low and high cell densities. VSMC were synchronized for 3 days in DME + 0.2% calf serum, then treated with increasing concentrations of TGF-$\beta_1$ in DME + 0.2% calf serum for 24 hrs (Figure 21). In these experiments, control VSMC from WKY and SHR responded to 10% calf serum stimulation with significant differences at both low (WKY, 20,500 $\pm$ 1,800 cpm/well; SHR, 30,100 $\pm$ 1,300 cpm/well, $p < 0.001$ as compared WKY vs SHR by Student's t test for unpaired data) and high densities (WKY, 79,400 $\pm$ 10,000 cpm/well; SHR, 96,000 $\pm$ 3,300 cpm/well, $p < 0.02$ as compared WKY vs SHR by Student's t test for unpaired data), as previously shown [6] (see Chapter 2). The dose-response to exogenous TGF-$\beta_1$ is illustrated in Figure 21. SHR cells presented higher basal [$^3$H]thymidine incorporation

at both cell densities, not seen in our previous studies [6] (see Chapters 2 and 3). It was most probably due to cell synchronization in DME + 0.2% calf serum in contrast to our previous use of DME + plasma-derived serum or defined serum-free medium. Since TGF-$\beta_1$ has been shown to need a minimal presence of PDGF for its effect [14], 0.2% calf serum was used here, and the basal levels of [$^3$H]thymidine incorporation reflected a described hyperresponsiveness of VSMC from SHR to growth factors. The data revealed no dose effect of TGF-$\beta_1$ at a low density in VSMC from both WKY and SHR. At a high cell density, there was no effect of TGF-$\beta_1$ in VSMC from WKY. In contrast, in cells of SHR origin, [$^3$H]thymidine incorporation increased significantly with escalating concentrations of TGF-$\beta_1$ ($p < 0.001$ by 3-way ANOVA).

Additional experiments examined the density-dependent rise in [$^3$H]thymidine incorporation stimulated by TGF-$\beta_1$ in SHR. The effect of increasing cell density on the response to 10 ng/ml of TGF-$\beta_1$ is depicted in Figure 22. At a low density, there was no response to TGF-$\beta_1$ in either WKY or SHR. An optimal response was observed at a medium cell density with also a slight effect of TGF-$\beta_1$ in WKY cells and a greatest difference between SHR and WKY. The difference persisted at a high density where TGF-$\beta_1$ again had no effect on WKY cells. Given the fact that cells in these experiments were synchronized prior to TGF-$\beta_1$ addition, we did not observe the inhibitory effect of TGF-$\beta_1$ at low cell densities either in

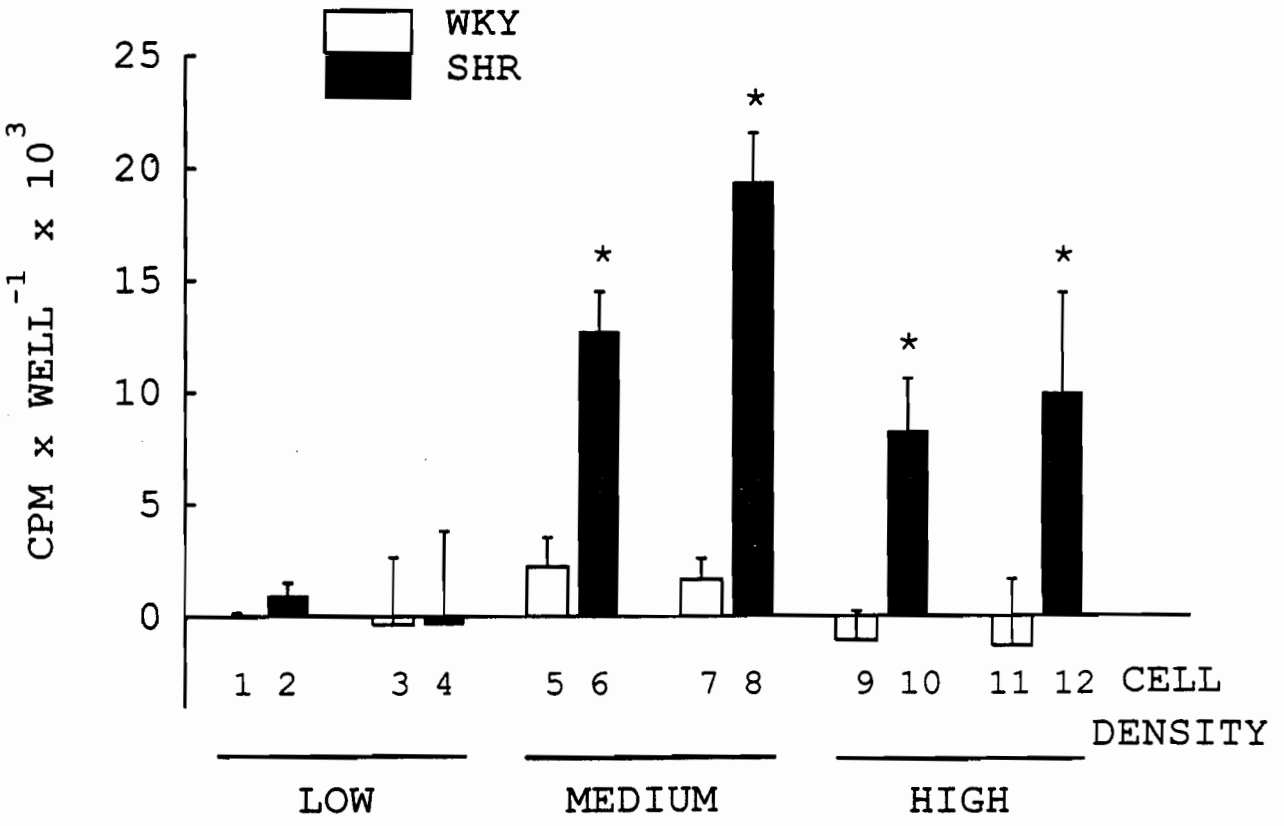


**Figure 22. Effect of cell density on TGF-$\beta_1$ stimulated [$^3$H]thymidine incorporation into DNA of vascular smooth muscle cells from WKY and SHR.** Cells were inoculated in 24-well cluster dishes at low, medium or high densities in DME + 10% calf serum. After 20 hrs of attachment, the medium was replaced by DME + 0.2% calf serum for 72 hrs. The number of quiescent cells was determined in control wells (in $10^3$ cells/cm$^2$): **1**, 13 $\pm$ 2; **2**, 13 $\pm$ 2; **3**, 30 $\pm$ 4; **4**, 29 $\pm$ 3; **5**, 58 $\pm$ 3; **6**, 57 $\pm$ 7; **7**, 44 $\pm$ 5; **8**, 60 $\pm$ 2; **9**, 121 $\pm$ 8; **10**, 128 $\pm$ 25; **11**, 239 $\pm$ 9; **12**, 305 $\pm$ 15; means $\pm$ SD; n=4. Quiescent cells were treated with 10 ng/ml TGF-$\beta_1$ in DME + 0,2% calf serum for 24 hrs. The cells were then pulsed by [$^3$H]thymidine for 2 hrs, and the radioactivity incorporated was measured in precipitated acid insoluble material. The net effect of TGF-$\beta_1$ was calculated as cpm/well of 10 ng TGF-$\beta_1$ stimulated cells minus cpm/well of non-stimulated, quiescent cells. The data are expressed as means $\pm$ SD, n=4, *p<0.001 by Student's t test comparing WKY vs SHR. WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats.

WKY or SHR, as has been demonstrated in growing VSMC [15, 26].

The growth-promoting effect of TGF-$\beta_1$ was proposed to be responsible for the establishment and maintenance of the multilayered "hill" formation in confluent VSMC *in vitro* [14]. The formation of "hill-and-valley" occurred earlier in VSMC from SHR. An abnormal expression of TGF-$\beta_1$ mRNA in cells of SHR origin suggests that this growth factor may be secreted in higher amounts and modulate the differential proliferative characteristics of SHR cells in an autocrine/paracrine manner. It has been shown that TGF-$\beta_1$ positively regulates its own expression [16]. The data presented here demonstrate an abnormal expression and response to TGF-$\beta_1$ and suggest that the autoinduction of TGF-$\beta_1$ is particularly enhanced in growing VSMC from SHR. Such a defect in feedback regulation of an endogenous growth factor may be involved in the expression of abnormal growth phenotypes in SHR, resulting in greater VSMC proliferation and increased peripheral vascular resistance. Nevertheless, the pathogenetic involvement of the increased expression and effect of TGF-$\beta_1$ in primary hypertension requires genetic studies of segregation of this phenotype with high blood pressure. TGF-$\beta_1$ also induces the expression of PDGF-A mRNA in VSMC and increases the secretion of PDGF-like protein [27]. Whether or not TGF-$\beta_1$ exerts its growth stimulatory effect directly or indirectly via PDGF-like molecules is not yet clear. Investigation with neutralizing

antibody and antisense RNA of these growth factors are underway in our laboratory.

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# CHAPTER 6

# GENERAL DISCUSSION

# AND

# CLAIMS TO ORIGINALITY

## GENERAL DISCUSSION

Comparison of hypertensive and normotensive strains for polymorphisms in a given trait is a first step in evaluating both its pathophysiologic role in and pathogenetic linkage with hypertension. The characterized abnormality may then be used in genetic studies to see whether or not it segregates with blood pressure either in F2 and backcross generations or in recombinant inbred strains.

Few reports of increased proliferation of cultured VSMC from SHR had been published before 1988. These studies, with the exception of those of Clegg et al. [1] had been performed only with cells in early passages [2, 3]. Since enhanced proliferation may shortly persist in culture, even when induced *in vivo* by secondary hypertension [4, 5], it was considered important to investigate proliferative behavior in longterm cultures. We demonstrated that VSMC from SHR proliferated more than cells from their normotensive controls, WKY rats, even after 20 passages. The greater proliferation was not due to different survival or attachment ability after seeding, as shown by similar plating efficiency. Even though VSMC from SHR grew to a greater density, as observed in parallel by others [6], they had to be attached to the culture dish to proliferate and they expressed an absolute requirement for growth factors, qualitatively similar to cells from WKY. It ensues from the fact that both cell types made quiescent by

incubation in either PDGF deprived serum or defined medium containing insulin, transferrin and selenium, similarly incorporated very little [$^3$H]thymidine. Calf serum elicited a dose-dependent increase of [$^3$H]thymidine incorporation in VSMC from both strains. However, WKY cells reached a plateau at 5% calf serum stimulation whereas a continuous rise was observed in SHR with a concentration of up to 20%.

The critical step of cell proliferation is the transition from the pre-replicative $G_0/G_1$ phase to the synthetic S phase of the cell cycle. This step is regulated by extracellular signals and the intrinsic ability of cells to respond to them. Normal cells require at least two factors in this step: PDGF and EGF [7]. Both are components of whole serum [8, 9]. PDGF induces a competence state, enabling the cells to respond to EGF and transit across the competence (C) point [10, 11]. EGF drives the cell through the progression and entry subphases [12]. We have demonstrated that quiescent VSMC from SHR present an exaggerated maximal response to both factors. EGF and PDGF alone were weakly mitogenic for cells from normotensive animals. PDGF stimulated excessive [$^3$H]thymidine incorporation in VSMC from SHR but only at high doses (300 nM = 10 ng PDGF/ml). However, at lower doses (6 and 60 nM), it potentiated the effect of EGF. Similar results have been published simultaneously by other investigators [3, 13]. Besides being potent mitogens, PDGF and EGF have been shown to be powerful vasoconstrictors [14, 15]. Folkow [16, 17] has

demonstrated that increased hyperresponsiveness of hypertensive vessels to vasoactive agents may be solely explained on the basis of greater vascular mass, the responsiveness of each unit of that mass being the same in hypertensive and normotensive animals. This paradigm, however, has not yet been definitively proven since single freshly-isolated VSMC from SHR presented an exaggerated sensitivity to noradrenaline and cell size was similar to that of WKY [18]. Nevertheless, PDGF and EGF may regulate vessel responsiveness by also stimulating VSMC growth, thus leading to stiffening of the vessel wall. Several growth-related events have indeed been described to be abnormal in vessels, cultured VSMC and other cell types in SHR [19-29].

In the vessel wall *in vivo*, VSMC are found in a so-called contractile phenotype distinguished by a very low rate of proliferation [30, 31]. A similar situation is evident in culture at confluency provided the cells reach a critical saturation density in less than two weeks. VSMC under our conditions reached confluency in one week and displayed a "hill-and-valley" formation. Specific growth rate declined with increasing cell density in VSMC from both strains but less rapidly in SHR. The difference between WKY and SHR was accentuated as cells approached confluency. VSMC from SHR always reached greater saturation density, suggesting that there may also be a defect in contact inhibition. Thus, the hightened proliferation of VSMC from SHR seemed to present two

defects: hyperresponsiveness to mitogens and altered contact inhibition. The following investigation focused on their further characterization. The time course of [$^3$H]thymidine incorporation showed that quiescent SHR cells start to synthesize new DNA earlier after calf serum stimulation than WKY cells. Analysis of cellular DNA content, using flow cytometry on propidium iodide-stained cells, demonstrated a 4-hour accelerated entry into the S phase. No difference in cell size was observed by forward light scatter. Flow cytometry using the Hoechst 33 342 - bromodeoxyuridine substitution technique demonstrated that SHR transited through the cell cycle 4 hours ahead of WKY cells. This was due to accelerated entry into the S phase since transition kinetics were comparable for VSMC from both strains. With other results from our laboratory, some of them presented in Chapters 2 through 5, we are now able to formulate a hypothesis about the localization of this defect in the cell cycle. At comparable low cell densities, the response to low concentrations of PDGF and the *c-fos* expression, one of the mediators of competence, were the same for WKY and SHR cells, suggesting that up to C point, there is no difference between the two strains. The significantly greater response to EGF, a progression factor, suggests that, indeed, the defect is situated downstream of C point. After R point, the cells are fully committed to progression to the S phase, and the assembly subphase is growth factor-independent. Comparable calmodulin levels in WKY and SHR VSMC [32] and calmodulin

sensitivity to inhibition, shown here to be similar, do not exclude the involvement of the calcium/calmodulin system in that defect. Calmodulin activator, reported by our laboratory in genetic hypertension [32, 33], may be a potential candidate mediating an accelerated exit from the progression and entry subphases, since calmodulin is required in these stages of the cell cycle [34, 35].

In addition to the accelerated entry into the S phase, we characterized defective contact inhibition. When seeded at a high cell density, VSMC from WKY were contact inhibited, but cells from SHR continued to proliferate. They responded more to calf serum as evidenced by an earlier expression of a the marker gene, *c-fos*. The underlying mechanism of hyperresponsiveness to calf serum seems to be different from hyperresponsiveness at a low cell density. At a low cell density, the expression of *c-fos* was similar in VSMC from both strains.

The cellular mechanisms involved in contact inhibition are poorly understood. Heparin, one of the components of extracellular matrix, is inhibitory to VSMC, but VSMC from SHR are less sensitive to its suppressive effect [36]. One of the growth factors influencing the composition of the extracellular matrix, TGF-$\beta_1$, has been found to inhibit serum- or PDGF-stimulated VSMC proliferation at a low cell density and to enhance it at a high cell density [37, 38]. Such

regulators might be potentially involved in the pathogenesis of altered contact inhibition in SHR.

In growing VSMC, TGF-$\beta_1$ mRNA presented higher accumulation in the case of SHR at all cell densities as early as 24 hours after inoculation with further increase at later times. Interestingly, at a low cell density, quiescent VSMC from either WKY or SHR did not respond to treatment with exogenous TGF-$\beta_1$. At a high cell density, dose-dependent [$^3$H]thymidine incorporation was seen only in SHR. Since TGF-$\beta_1$ has been shown to autoregulate its own expression, an abnormal feedback control (autocrine/paracrine stimulation) may be involved in the altered contact inhibition of SHR cells.

Two intermediate phenotypes are described in this thesis: 1. accelerated entry into the S phase of the cell cycle in response to mitogenic stimulation, including calf serum, EGF and PDGF; 2. altered contact inhibition accompanied by the expression of *c-fos* at a high cell density, potentially resulting from an abnormal expression and responsiveness to TGF-$\beta_1$. Altered autocrine/paracrine stimulation may be involved in both defects. The two phenotypes should be studied for their pathogenetic linkage to high blood pressure, employing cultured VSMC obtained from recombinant inbred strains. Such rat strains are now available in our laboratory [39]. The two phenotypes may be regulated by different genes.

It is conceivable that these intermediate phenotypes may not segregate together in the same recombinant inbred strain. Their relative quantitative or qualitative contributions have to be determined.

There is increasing evidence that both human essential hypertension and animal genetic hypertension represent a heterogenous disease. The recent observation of salt-sensitive and salt-resistant substrains of the SHR model of hypertension, depending on their breeding origins, lies in that direction [40]. Thus, increased proliferation may also be involved primarily and pathogenetically only in some SHR substrains.

Besides being a primary factor, VSMC proliferation may be pathophysiologically crucial as a "fixative" factor responsible for the maintenance and development of primary as well as secondary hypertension. It was suggested that intermittent pressure elevations could act as a "triggering" mechanism [41-43], provided they are frequent and/or substantial enough to raise the integrated pressure [43]. Structural adaptation of the cardiovascular system, including narrowing of the wall lumen, would take place. Blood pressure would rise subsequently to maintain adequate perfusion and would present a further stimulus for hypertrophy and proliferation. Such a "vicious circle" would cause a gradual increase of blood pressure.

Yet a third aspect may be imminently important: structural cardiovascular changes as a risk factor for morbidity and mortality in hypertension [44]. Left ventricular hypertrophy represents the highest risk for cardiovascular morbid events, independently of age, male sex and blood pressure [45-47]. The association between left ventricular hypertrophy and sudden death is more striking than the association between high blood pressure and sudden death [48]. Left ventricular hypertrophy is not consistently improved with blood pressure control, similarly to vascular hypertrophy/hyperplasia [49-51]. An understanding of VSMC growth abnormality in hypertension therefore remains crucial. Higher VSMC proliferation as a primary defect pathogenetically linked to hypertension or its consequences deserves further investigation.

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## CLAIMS TO ORIGINALITY

**A)** We demonstrated for the first time that the increased proliferation of VSMC from SHR as compared to WKY persists in longterm culture (3rd-20th passages), and

> **1:** is not due to the different plating efficiency;
>
> **2:** is not due to the direct involvement of calmodulin itself;
>
> **3:** is characterized by a greater responsiveness to mitogens, including calf serum, EGF and PDGF, suggesting a defect in growth stimulatory pathway;
>
> **4:** is characterized by a higher specific growth rate at saturation densities, and cell proliferation at a density at which WKY cells are already arrested, indicating an altered contact inhibition.

**B)** The defect of the growth stimulatory pathway was investigated by flow cytometry analysis of the cell cycle kinetics, the technique used for the first time in studies of VSMC proliferation. By that means, we demonstrated

> **1:** 4-hour accelerated entry of VSMC from SHR into the S phase of the cell cycle as compared to WKY;
>
> **2:** similar transition kinetics of VSMC from both WKY and SHR.

C) We demonstrated that the altered contact inhibition of VSMC from SHR is characterized by

1: an earlier expression of *c-fos* protooncogene at a high cell density;

2: an exaggerated expression of TGF-$\beta_1$ preceding the altered contact inhibition;

3: an abnormal stimulatory response to exogenous TGF-$\beta_1$ at a high cell density.

These results suggest the involvement of autocrine/paracrine regulation of TGF-$\beta_1$ in altered contact inhibition.