Acute and Long-term Effects of Tissue Culture on Contractile Reactivity in Renal Arteries of the Rat

Jo G.R. De Mey, Martin P. Uitendaal, Harry C.M. Boonen, Marian J.J.F. Vrijdag, Mat J.A.P. Daemen, and Harry A.J. Struyker-Boudier

To evaluate long-term effects of contractile and mitogenic stimuli on the contractile reactivity of arterial smooth muscle, we measured the incorporation of the thymidine analogue 5-bromo-2'-deoxyuridine (BrdUrd) and mechanical responses in arterial segments that had been maintained in tissue culture. The experiments were performed on renal arteries that had been isolated from adult rats, chemically sympathectomized, mechanically denuded from endothelium and mounted under distension. Exposure of arterial segments for up to 3 weeks to culture medium supplemented with fetal calf serum resulted in the following consecutive changes: a strong acute contraction, selective pharmacological changes that included decreased contractile responses to phenylephrine and vasopressin and increased relaxing responses to isoproterenol, increased incorporation of BrdUrd, a progressive fall in contractile responses to all vasoconstrictor stimuli, and an increase in excitability. Serum-free medium resulted in a much smaller acute arterial contraction, induced less incorporation of BrdUrd, accelerated the occurrence of hyperexcitability, but did not affect early pharmacological changes or the subsequent fall in overall arterial contractility with tissue culture. Dialysis of the serum or addition of ketanserin abolished the contractile effect of serum but did not affect the incorporation of BrdUrd or the loss of contractility with tissue culture. Addition of serotonin to serum-free culture medium mimicked the contractile response to serum but not the stimulation of BrdUrd incorporation. These data indicate that tissue culture alters the properties of the arterial wall, that contraction does not underlie the proliferative response of arterial smooth muscle to serum-derived mitogens in vitro, and that stimulation of DNA synthesis does in itself not lead to selective changes in arterial contractility. (Circulation Research 1989;65:1125-1135)

Through changes in size, in number, and in synthetic or contractile activity, arterial smooth muscle cells (ASMCs) play a key role in hypertension and atherosclerosis.1,2 Recently, a number of growth factors were observed to acutely contract isolated aortae.3-5 However, ultrastructural and histochemical analyses of ASMC in culture suggested that ASMCs are transformed from a contractile into a synthetic type when they are stimulated to proliferate.6-7 A similar modulation of phenotype was observed in experimental models of arterial injury in vivo.2,8-10 This injury initially results in profound vasoconstriction and subsequently leads to proliferation of ASMCs.2,10 Prevention of the vasoconstriction by vasodilator treatment reduces the proliferative response to arterial injury.11 Taken together, these observations suggest that there is a sequential relation between contractile and growth responses in the arterial wall. Acute contractile effects could be initially involved in the growth response of the arterial wall to injury and mitogenic factors. During subsequent proliferation of ASMCs the contractility of the arterial wall would be reduced. There are no quantitative data available with respect to these hypotheses. It is also not known whether, in addition to expression of contractile proteins, changes of the phenotype of ASMCs also affect mechanisms of excitation-contraction coupling.

To evaluate the relation between contractile and growth responses in the arterial wall, we maintained isolated arteries in tissue culture, recorded indexes of DNA synthesis and mechanical activity, and
compared acute and long-term effects of absence, presence, and antagonism of serum-derived contractile and mitogenic components. The experiments were performed in arterial segments, which allowed documentation of both quantitative and qualitative changes in arterial function with tissue culture.

**Materials and Methods**

Experiments were performed on segments (1.5–2 mm long) of renal arteries that had been isolated under sterile conditions from 20-week-old male Wistar-Kyoto rats. Except when specifically mentioned, the arterial preparations were chemically sympathectomized and mechanically denuded from endothelium. The segments that had been collected in sterile Hanks' balanced salt solution (HBSS; Gibco, Paisly, UK) were incubated at 37°C for 10 minutes in sterile bicarbonate-free Krebs-Ringer solution (pH 4.00), containing 300 μg/ml 6-hydroxydopamine (Sigma Chemical, St. Louis, Missouri) and rinsed twice in 5 ml HBSS for 10 minutes. The endothelium was removed by passing the shaft of a 20-gauge injection needle (outer diameter 0.9 mm) through the arterial lumen.

**Tissue Culture**

The tissue culture method that we used for renal artery segments was adapted from those previously described for enzymatically dispersed smooth muscle cells, strips, and rings of arteries. Renal artery rings were mounted on a 20-gauge needle (outer diameter 0.9 mm) and suspended in culture dishes filled with 2.5 ml Dulbecco's minimal Eagle's solution (pH 4.00), containing 300 μg/ml 6-hydroxydopamine (Sigma Chemical, St. Louis, Missouri) and rinsed twice in 5 ml HBSS for 10 minutes. The endothelium was removed by passing the shaft of a 20-gauge injection needle (outer diameter 0.9 mm) through the arterial lumen.

**Recording of Mechanical Activity**

Freshly isolated and tissue cultured arterial segments were mounted horizontally in an organ chamber filled with Krebs-Ringer bicarbonate solution (KRB; millimolar composition: NaCl 118.3, KCl 4.7, CaCl2 2.5, MgSO4·7H2O 1.2, KH2PO4 1.2, NaHCO3 25.0, and glucose 11.1) that was maintained at 37°C and aerated with 95% O2-5% CO2. Resting wall tension and the amplitudes of the contractile response were plotted as a function of diameter. The relationship between resting wall tension (RWT) and diameter (D) could be closely fitted with the exponential: 

$$RWT = RWT_0 \cdot e^{-D}$$

in which RWT0 and St are constants. St was calculated in individual experiments as an index of stiffness. The diameter at which contractile responses to high potassium were maximal will be referred to as the optimal diameter.

In subsequent experiments, the diameter was increased in steps of 100 μm, without intermittent activation, until the optimal diameter for each exper-
FIGURE 1. Tracings of isometric force versus time illustrating responses to high $K^+$ (125 mM) and sodium nitroprusside (NP, 1 $\mu$M) in isolated, sympathectomized, and deendothelialized renal artery segments. A: Freshly isolated arterial segment. B: Preparation maintained in tissue culture for 6 days under distending conditions (see text) in the presence of 20% fetal calf serum. C: Preparations maintained in tissue culture for 6 days under distending conditions in serum-free medium (C1 and C2: preparations isolated from different animals). Arrow and double-shafted arrow illustrate the measurements of spontaneous basal tone and maximal active wall tension, respectively.

Experimental group was reached (1,050 $\mu$m for freshly isolated preparations, 900 $\mu$m for preparations that had been cultured under distending conditions). Further experimentation was performed with all vessels set at their optimal diameter. It included exposure twice to high potassium at a 20-minute interval followed 20 minutes later by administration of 1 $\mu$M sodium nitroprusside (Figure 1). The difference in force between the minimum observed in the presence of sodium-nitroprusside and the maximum observed during the second exposure to high potassium was taken as an index of maximal contractility (Figure 1). This value was converted (force/2 segment length) to maximal active wall tension (MAWT). The difference in wall tension between the minimum observed in the presence of sodium nitroprusside and the average level in drug-free KRB throughout the experiment was taken as an index of spontaneous tone (Figure 1). After removal of the relaxing agent, concentration-response curves were constructed for various pharmacological agents. Between them at least 20 minutes of equilibration in drug-free KRB was allowed. Both the spontaneous basal tone and the further increases in wall tension in response to pharmacological agents were expressed relative to MAWT.

Histology and Immunohistochemistry

The recordings of contractile reactivity were terminated by relaxing the preparations with 10 $\mu$M sodium nitroprusside and exposing them at optimal lumen diameter and at 37° C to periodate-lysine-paraformaldehyde. Thirty minutes later the fixed arterial segments were removed from the
TABLE 1. Effects of Tissue Culture on Mechanical Properties of Arterial Segments

<table>
<thead>
<tr>
<th>Days in culture</th>
<th>Endothelium</th>
<th>Distension</th>
<th>Optimal diameter (mm)</th>
<th>Stiffness (mm⁻¹)</th>
<th>Active wall tension (mN/mm)</th>
<th>Spontaneous tone (%)</th>
<th>ED₉₀ K⁺ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>present</td>
<td>NA</td>
<td>1.063±0.028</td>
<td>6.28±0.20</td>
<td>6.03±0.57*</td>
<td>12.8±3.5</td>
<td>29±1</td>
</tr>
<tr>
<td>0</td>
<td>removed</td>
<td>NA</td>
<td>1.031±0.027</td>
<td>5.93±0.43</td>
<td>4.68±0.72</td>
<td>14.1±4.6</td>
<td>28±1</td>
</tr>
<tr>
<td>2</td>
<td>removed</td>
<td>+</td>
<td>0.946±0.027*</td>
<td>5.72±0.52</td>
<td>4.09±0.66</td>
<td>0.0±0.0*</td>
<td>29±1</td>
</tr>
<tr>
<td>6</td>
<td>removed</td>
<td>+</td>
<td>0.904±0.026*</td>
<td>5.47±1.02</td>
<td>3.00±0.40*</td>
<td>16.5±2.3</td>
<td>25±1*</td>
</tr>
<tr>
<td>14</td>
<td>removed</td>
<td>+</td>
<td>0.898±0.059*</td>
<td>7.15±0.79</td>
<td>0.74±0.12*</td>
<td>62.2±6.3*</td>
<td>19±2*</td>
</tr>
<tr>
<td>21</td>
<td>removed</td>
<td>+</td>
<td>~0.900†</td>
<td>5.66±1.60</td>
<td>0.61±0.20*</td>
<td>47.2±8.3*</td>
<td>~20†</td>
</tr>
<tr>
<td>6</td>
<td>present</td>
<td>–</td>
<td>0.803±0.016†</td>
<td>8.06±0.54†</td>
<td>2.49±0.11*</td>
<td>13.2±3.2</td>
<td>23±1*</td>
</tr>
<tr>
<td>6</td>
<td>removed</td>
<td>–</td>
<td>0.816±0.010†</td>
<td>8.17±0.49†</td>
<td>1.26±0.39†</td>
<td>19.7±4.7</td>
<td>25±2</td>
</tr>
</tbody>
</table>

Sympathectomized renal artery segments were maintained in tissue culture for up to 21 days in Dulbecco’s minimal Eagle’s modified medium supplemented with 20% fetal calf serum. From part of the preparations, the endothelium had been removed and part was kept under distension (see text). Data with respect to optimal diameter, stiffness, active wall tension (the difference in wall tension in the presence of 125 mM potassium and 1 mM sodium nitroprusside), spontaneous tone expressed as % of active wall tension, and sensitivity to the contractile effect of potassium (ED₉₀ K⁺ in mM) are shown as mean±SEM. (n=6-18). NA, not applicable.

*The difference from freshly isolated deendothelialized preparations is statistically significant.
†Due to the low level of contractile activity, the optimal diameter and sensitivity to potassium could not be accurately determined.
‡The difference from deendothelialized preparations that had been cultured for 6 days under distending conditions is statistically significant.

For tissues that had been cultured in the presence of BrdUrd, an indirect enzyme-labeled antibody technique with peroxidase-conjugated second step reagents was used to visualize nuclei that had incorporated the thymidine analogue. These sections were counterstained with hematoxylin. To obtain a BrdUrd labeling index for medial cells, the number of nuclear profiles that stained for peroxidase and that were localized in the area enclosed by the internal and external elastic lamina of the section was divided by the total number of medial nuclear profiles. Freshly isolated arteries that had not been exposed to BrdUrd were included in the staining procedures as controls.

**Drugs**

The following pharmacological agents were used: angiotensin II, l-isoproterenol hydrochloride and l-phenylephrine hydrochloride (Sigma), arginine vasopressin (Sandoz, Basle, Switzerland), sodium nitroprusside dihydrate and serotonin creatinine sulphate (Janssen Chimica, Beerse, Belgium), ketanserin tartrate (Janssen Pharmaceutica, Beerse Belgium), and prazosin hydrochloride (Pfizer).

**Statistics**

All data are shown as mean±SEM, with n indicating the number of preparations. Statistical significance of differences was evaluated using analysis of variance followed by Bonferroni’s test. A value of p<0.05 was accepted to denote statis-

---

**FIGURE 2. Effects of tissue culture on responses to pharmacological vasoconstrictor agents in isolated, sympathectomized, and deendothelialized renal arteries.** Freshly isolated segments (0 days) and preparations that had been maintained in the presence of 20% fetal calf serum in tissue culture for 2 or 6 days in the presence or absence (*) of distension, were mounted for recording of isometric force development at optimal diameter. Maximal contractile responses to angiotensin II (AT II, 1 µM, [ ]), arginine vasopressin (AVP, 30 nM, [ ]), phenylephrine (PHE, 10 µM, [ ]), and serotonin (5HT, 10 µM, [ ]) were expressed as a fraction of the contractile response to 125 mM potassium (for absolute values, see Table 1) and are shown as mean±SEM (n=6).
FIGURE 3. Effects of tissue culture on responses of isolated sympathectomized and deendothelialized renal arteries to isoproterenol (left) and sodium nitroprusside (right). Arterial segments were maintained in tissue culture under distending conditions for 2 (○—○) or 6 (△—△) days in medium supplemented with 20% fetal calf serum. Cumulative concentration response curves were consecutively constructed to isoproterenol and sodium nitroprusside after the preparations had been mounted for recording of isometric force development and had been made to contract with 30 mM potassium. For comparison, data obtained in freshly isolated preparations (●—●) were also included. Data were expressed as fraction of the response to 30 mM potassium in the absence of relaxing agent and are shown as mean±SEM. (n=6).

Results

Freshly Isolated Preparations

Freshly isolated renal arteries displayed little spontaneous tone (Figure 1, Table 1), but contracted in response to a variety of stimuli (Figure 2). Maximal responses to arginine vasopressin (AVP, 30 nM), phenylephrine (PHE, 10 μM), and serotonin (5HT, 10 μM) were comparable to those to high K+ (125 mM, Figure 2). Angiotensin II (Ang II, 1 μM) on the other hand caused a contractile response that was significantly smaller than that to 125 mM K+ (Figure 2). Freshly isolated preparations that had been made to contract with 30 mM K+ failed to relax in response to isoproterenol (10 nM to 1 μM) but relaxed in response to sodium nitroprusside (10 nM to 1 μM, Figure 3).

Exposure of freshly isolated renal arteries to culture medium caused a small contractile response (Figure 4, top left). When the medium was supplemented with fetal calf serum, this resulted in further increases in contractile force. Half-maximal responses were obtained with 2-5% serum (not shown). In the presence of 20% serum, the amplitude of the contractile response was at least as large as that to 125 mM K+ (Figure 2). Freshly isolated preparations that had been made to contract with 30 mM K+ failed to relax in response to isoproterenol (10 nM to 1 μM) but relaxed in response to sodium nitroprusside (10 nM to 1 μM, Figure 3).

Tissue Culture: Effects of Time

In the first series of culture experiments, renal artery segments were suspended on a distending support (outer diameter 0.9 mm) in medium supplemented with serum for up to 3 weeks. Judging from immunohistochemical staining for von Willebrand factor, the endothelium was effectively removed by inserting the support in the arterial lumen at the start of the culture and remained absent throughout the 3-week period. Throughout the entire period, the overall organization of the arterial wall into media and adventitia was maintained. After 6 days of culture in the continuous presence of BrdUrd, 80.5±5.9% (n=6) of the nuclei in the media had incorporated the thymidine analogue (Figure 4, top right) indicating marked DNA synthesis in arterial smooth muscle cells under these conditions.

Table 1 summarizes the effects of proceeding tissue culture on mechanical properties of arterial segments. The optimal lumen diameter of the preparations rapidly decreased to match the diameter of the support they were cultured on. The stiffness of the preparations was not affected throughout the culture period; contractile responses on the other hand changed markedly during culture. After short-term culture, the preparations displayed little spontaneous tone. After 2 and 3 weeks, marked spontaneous tone was observed. Contractile responses to 125 mM K+ were not affected after 2 days and were reduced to 65%, 20%, and 15% after 6, 14, and 21 days, respectively (Table 1, Figure 5,6). Sensitivity to the contractile effect of K+ was similar in renal arteries that had been freshly isolated or cultured for 2 days (Table 1, Figure 6). However, arteries cultured for 6, 14, and 21 days were hypersensitive to the contractile effect of K+ (Table 1, Figure 6).

Compared with contractile responses to K+, tissue culture affected responses to some, but not all,
pharmacological stimuli more drastically. Figure 2 summarizes relative maximal amplitudes of contractile responses to agonists following increasing time in culture. After 2 days, that is, at a point in time when sensitivity and maximal responses to $K^+$ were not affected, responses to Ang II, AVP, and PHE were drastically reduced while those to 5HT were not affected. After 6 days, when responses to $K^+$ were reduced by 35%, relative maximal responses to AVP remained low, those to PHE had partially recovered, and those to 5HT were still not affected (Figure 2). While tissue culture resulted in increased sensitivity to $K^+$, it did not affect that to 5HT and drastically decreased that to PHE (Figure 6). Tissue culture did not affect relaxing responses of precontracted arteries to sodium nitroprusside. But unlike freshly isolated preparations, segments that had been cultured for 2 or 6 days responded to isoproterenol with concentration-dependent relaxations (Figure 3).

**Tissue Culture: Effects of Media Composition**

To identify those changes with culture that were due to cellular proliferation, we compared responses of vessels that had been maintained in the presence and absence of serum, a defined source of growth factors. The index was significantly lower after exposure to different conditions than after exposure to serum alone. Yet some 20% of the medial cells incorporated BrdUrd in their nuclei following immunohistochemistry. Data are shown as fractional responses (1 equals 100% of the acute contractile effect of the culture media and the spontaneous tone following culture, 1 mN/m tension following culture and 100% of the medial cells for the BrdUrd labeling fraction) and are shown as mean ± SEM (n=6). Culture medium composition: I, Dulbecco's minimal Eagle's modified medium (DMEM); II, serotonin; III, DMEM plus 20% dialyzed fetal calf serum; IV, DMEM plus 20% fetal calf serum; V, D. calf serum and 1 μM ketanserin.

**Figure 4.** Acute and chronic effects of tissue culture media on isolated, sympathectomized, and deel arteries. Top left: Freshly isolated arterial segments were mounted for recording of isometric force following exposure to culture medium; the resulting acute contractile response is shown. Top right and bottom were maintained for 6 days in culture medium under distension in the continuous presence of bromo-2'-deoxyuridine (BrdUrd). They were subsequently mounted for recording of isometric force and spontaneous basal tone (bottom right) were measured as illustrated in Figure 5. Subsequently fixed, and the percentage of medial cells that had incorporated BrdUrd in their nuclei following immunohistochemistry. Data are shown as fractional responses (1 equals 100% of the acute contractile effect of the culture media and the spontaneous tone following culture, 1 mN/m tension following culture and 100% of the medial cells for the BrdUrd labeling fraction) and are shown as mean ± SEM (n=6). Culture medium composition: I, Dulbecco's minimal Eagle's modified medium (DMEM); II, serotonin; III, DMEM plus 20% dialyzed fetal calf serum; IV, DMEM plus 20% fetal calf serum; V, D. calf serum and 1 μM ketanserin.
In the third series of experiments, we attempted to gain more insight into the contributions of chronic exposure to serum-derived mitogenic and contractile stimuli to some of the changes observed following tissue culture. Renal artery segments were therefore exposed for 6 days to different culture media. They consisted of Dulbecco’s minimal Eagle’s modified medium with the following additions: none, 10 μM 5HT, 20% dialyzed serum, 20% undialyzed serum, or 20% undialyzed serum plus 1 μM ketanserin. The results are summarized in Figure 4. Serum caused marked nuclear incorporation of BrdUrd (Figure 4, top right). This was considerably less following serum-free conditions with or without 5HT. The stimulation of DNA synthesis by serum was not affected by dialysis or by the presence of ketanserin. Contractile responses to K⁺ were similarly reduced following tissue culture in either of the five media used (Figure 4, bottom left), suggesting that the long-term absence or presence of mitogenic or contractile stimuli or the inhibition of their effects did not influence changes in contractility with time. Spontaneous tone was lowest in vessels cultured in the absence or presence of ketanserin, intermediate in vessels that had been kept in the presence of dialyzed serum or in serum-free medium with 5HT, and was highest in those maintained in serum-free medium alone (Figure 4, bottom right). The pattern of the spontaneous basal tone was highly variable ranging from low frequency rhythmic contractions to a tonically maintained activity (Figure 1).

**Tissue Culture: Effects of Distension and Removal of the Endothelium**

In the final series of experiments, we evaluated whether, unlike contractile and mitogenic factors, distension and removal of endothelium contributed to the alterations of the properties of the arterial wall with tissue culture. Sympathectomized renal artery segments were therefore either left intact or denuded from endothelium and suspended on a nondistending support (outer diameter, 0.25 mm) in medium plus serum for 6 days.

The optimal lumen diameter was significantly smaller and the stiffness significantly larger in preparations that had been kept undistended in tissue culture than in freshly isolated arteries or preparations cultured under distending conditions (Table 1). This was observed irrespective from removal of endothelium. Vessels cultured in the absence and presence of distension displayed similar spontaneous tone (Table 1). Also in this respect, removal of the endothelium had no significant effect. In denuded distended and in nondenuded nondistended cultured segments, contractile responses to 125 mM K⁺ were comparable, that is, 50–60% of that in freshly isolated arteries. In vessels that had been denuded from endothelium and kept undistended, maximal responses to K⁺ averaged only 25% of control (Table 1).

The degree of distension also affected pharmacological properties following tissue culture. Responses to Ang II and PHE were reduced to a larger extent in undistended culture vessels than in distended (Figure 2). Again in undistended vessels responses to 5HT were not selectively affected by tissue culture (Figure 2).

**Discussion**

Mechanical, pharmacological, and growth responses could be recorded in arterial segments following tissue culture. Classical culture conditions induced acute contraction, altered pharmacological properties, stimulated DNA synthesis, decreased contractility, and increased excitability in arterial smooth muscle. The acute contractile effects did not contribute to the stimulation of DNA synthesis.
Culture of enzymatically dispersed ASMCs is widely used to evaluate cellular mechanisms involved in responses to proliferative stimuli (for review, see Chamley-Campbell et al). Ultrastructural, immunohistochemical, and biochemical analyses of arterial smooth muscle cells in culture suggested that they are transformed from a contractile into a synthetic phenotype in response to proliferative stimuli. Histochemical analyses of the thickened intima following arterial injury in vivo suggested a similar loss of contractile and cytoskeletal proteins in arterial smooth muscle cells that proliferate in vivo, although a number of purified and synthetic growth factors were recently observed to acutely contract isolated aortae. These observations could suggest the following sequence of events: Growth factors acutely contract arterial smooth muscle. The mechanical response itself, its underlying second messengers, or accompanying metabolic consequences could force arterial smooth muscle cells from a differentiated state into a less-contractile proliferative state and then through the cell cycle.

To obtain direct and quantitative evidence with respect to this hypothesis and reasoning that contractile activity not only depends on contractile and cytoskeletal proteins but also on mechanisms of excitation-contraction coupling, we recorded contractile properties in arterial segments that had been maintained in tissue culture. The culture procedure was adapted from those previously described for arterial cells, explants, and rings. Our adaptation consisted of the use of muscular renal artery segments that had been intentionally sympathectomized and denuded from endothelium and that were maintained in vitro under some level of distension. When disconnected from their cell bodies, nerve endings are unlikely to be preserved for longer periods of time. We therefore preferred to destroy them at the start of the culture. The endothelium is a possible source of endogenous vasoactive agents and can modulate proliferative responses of underlying smooth muscle cells. We therefore preferred to remove it at the start of most of the cultures. Mounting of the segments on a distending support prevented active diameter changes during culture and allowed for the maintenance in vitro of some level of distension. Together with endothelial removal and exogenous growth factors, this distension could promote proliferation of arterial smooth muscle cells. The rigid support in the lumen of the arterial preparations however prevented structural changes in intima. Besides this, the arterial culture shared properties with in...
vivo experimental models of arterial injury such as removal of endothelium, medial stretch, exposure of arterial smooth muscle cells to serum-derived contractile and mitogenic factors, and stimulation of intra-arterial DNA synthesis. The use of arterial segments rather than dispersed cells allowed for quantitative and qualitative assessment of contractile reactivity and for the recording of growth responses in arterial smooth muscle cells at normal density in their natural micro-environment.

This effect was not affected by α1-adrenoceptor blockade but was abolished by the 5-HT2-receptor antagonist ketanserin. The acute contractile effect could also be prevented by dialysis of the serum. This suggests that the acute contractile effect of serum was entirely due to 5HT and that no contractile effect of high molecular weight growth factors could be detected. It is of interest in this respect that unlike rat aorta, rat renal arteries and rat and human resistance arteries fail to contract in response to platelet-derived growth factor (Bassett et al. and J.G.R. De Mey and P. Schiffer, unpublished observations), a candidate mitogen in fetal calf serum.

To judge from nuclear incorporation of BrdUrd, 80% of the medial cells synthesized DNA during the initial 6 days of arterial culture in the presence of serum. The extent of DNA synthesis observed in the absence of serum (20% during 6 days) is high compared with the slow turnover of ASMCs in vivo in adult rats. The relative stimulation in serum-free conditions in vitro could be due to endogenous mitogens produced in response to injury and to the removal of inhibitory influences from the endothelium. The additional proliferative effect of serum was not affected by dialysis or ketanserin and could not be mimicked by serotonin. This indicates that stimulation of DNA synthesis was due to one or more high molecular-weight serum components and could be dissociated from the acute contractile effect. Thus, strong mechanical activity such as that induced by 5HT, stimulation and the underlying second messengers like diacylglycerol, inositol triphosphate, and calcium did not stimulate DNA synthesis by themselves or contribute to the stimulation induced by serum-derived mitogens. These observations in arterial segments contrast with earlier findings in cell cultures of aortic smooth muscle cells.

Arterial tissue culture resulted in a progressive drop of contractile responses to depolarization. This could be compatible with the above-mentioned modulation of phenotype of ASMCs under proliferating conditions. The decline in contractility with tissue culture was observed irrespective of the extent to which the different culture media that were used stimulated intra-arterial DNA synthesis. Tissue contractility thus faded in culture independent of cellular DNA synthesis. It could be of interest in this respect that phenotypic changes in the expression of contractile proteins in ASMC culture not only depend on cellular turnover but also on cellular density. The high cellular density in the arterial media thus might have prevented selective phenotypic changes in arterial segments under proliferating conditions in vitro. Whether arterial segments were exposed to culture conditions that induced mild or strong contraction did not affect their subsequent maximal responses to high potassium. Loss of contractile reactivity with tissue culture thus occurred independently of chronic exposure to either mitogenic or contractile stimuli. A variety of other phenomena could contribute to the observed nonselective fall in contractility with time. They include changes in orientation of smooth muscle cells, changes in intracellular and extracellular force transmitting structures, and overall tissue decay. We have not obtained evidence in favor or against any of these suggestions. But neither cellular proliferation nor adaptation to pronounced contractions seems to play an overriding role.

With arterial tissue culture, contractile reactivity not only changed quantitatively but also qualitatively. While freshly isolated arteries displayed little spontaneous tone, basal activity was prominent in segments that had been cultured for longer periods of time. The occurrence of spontaneous activity was markedly accelerated in serum-free conditions but neither the absence, presence, or antagonism of serotonin affected it. Thus, this change with culture cannot be attributed to mitogenic or contractile influences. The mechanism of spontaneous activity remains unclear. Two observations could be of interest. Small increases in extracellular potassium concentration, which in freshly isolated blood vessels relax contractile responses through activation of electrogenic sodium transport, caused further increases in tension in spontaneously active cultured arteries (see Figure 6). In addition, spontaneous tone became more prominent as the recording of reactivity proceeded, that is, the more contractile stimuli had been applied (see Figure 6). This suggests that the spontaneous tone following tissue culture could be due to reduced activity of mechanisms, such as electrogenic sodium transport, that are involved in relaxation of arterial smooth muscle. A similar mechanism could underlie increased sensitivity to the contractile effect of potassium.

Arterial responses to serotonin were not selectively affected by tissue culture irrespective of the chronic absence, presence, or antagonism of the indolamine during culture. Sensitivity for serotonin was not altered. Maximal responses progressively decreased to the same extent as those to high potassium. In contrast, responses to Ang II, PHE, and AVP were drastically reduced within 2 days of culture, that is, at a point in time when neither sensitivity nor maximal responsiveness to either K+ or 5HT were significantly affected. In addition, while freshly isolated arteries failed to respond to isoproterenol, significant relaxing responses to the


**Acknowledgments**

We wish to thank Dr. B. Schutte (Department of Internal Medicine, University of Limburg) for supplying anti-BrdUrd antibodies, E. Geurts and M. Hogenboom for preparing the manuscript, and M. Heuts for assisting in part of the reactivity measurements.

**References**

1. Folkow B: Physiological aspects of primary hypertension. *Physiol Rev* 1982;62:547-554

**β-adrenergic agonist were consistently observed in arteries that had been cultured for as short as 2 days. Also this change displayed selectivity since relaxing responses to sodium nitroprusside were not affected by arterial tissue culture. Since serum-free conditions resulted in marked spontaneous contractile activity, the relation of these pharmacological changes to proliferative responses could not be addressed in the present study. It could be noteworthy though that the adrenergic changes in sympathectomized arteries in tissue culture are opposite to those that are observed following in vivo denervation of arteries of adult animals (for review, see Reference 35). They are in the direction of the pattern that is characteristic for arteries of juvenile animals (for review, see Reference 35), the arterial smooth muscle cells of which proliferate at a rate that is several times higher than in arteries of adult animals. This, together with observations of Slotkin and coworkers concerning the postnatal development of adrenergic mechanisms in the heart and kidneys, could suggest differences in adrenergic control of proliferating and quiescent tissues including arterial smooth muscle.

Although proliferating conditions markedly stimulated intra-arterial DNA synthesis in the present study, they did not selectively affect mechanical properties of isolated arteries, and the possibility that they altered pharmacological properties remains speculative. In contrast, the absence of distension and endothelium led to selective changes. The vessels that had remained undistended in culture became narrower and stiffer and responded more to vaso-pressor and less to α-adrenergic stimulation than arteries that had been maintained under distending conditions. In vessels that had been denuded from endothelium, the fall in maximal responsiveness to high K⁺ was more marked when they had been left undistended than when some external stress was maintained on the tissue. Observations in blood vessels from hypertensive animals clearly indicated the possibility of quantitative and qualitative alterations in arterial function as secondary adaptation to elevated transmural pressure.**

In summary, contractile and growth responses could be recorded in arterial segments following tissue culture. Classic tissue culture conditions acutely caused strong contractions, chronically affected mechanical and pharmacological properties of arterial smooth muscle, and stimulated DNA synthesis in the arterial wall. Stimulation of cellular DNA synthesis, but neither the acute nor the chronic alterations in contractility, could be attributed to mitogenic factors. Extensive contractile activity did not in itself induce proliferation of arterial smooth muscle cells or participate in the effects of serum-derived mitogens. Extensive stimulation of DNA synthesis on the other hand did not in itself reduce tissue contractility.
30. Doyle VM, Creba JA, Ruegg UT, Hoyer D: Serotonin increases the production of inositol phosphates and mobilises calcium via the 5HT2 receptor in A7R5 smooth muscle cells. *Naunyn Schmiedebergs Arch Pharmacol* 1986;333:98–103

**KEY WORDS**  • arterial smooth muscle  • DNA synthesis  • mechanical activity
Acute and long-term effects of tissue culture on contractile reactivity in renal arteries of the rat.

J G De Mey, M P Uitendaal, H C Boonen, M J Vrijdag, M J Daemen and H A Struyker-Boudier

_Circ Res._ 1989;65:1125-1135
doi: 10.1161/01.RES.65.4.1125

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1989 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/65/4/1125

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org/subscriptions/