Organoid culture of cannulated rat resistance arteries: effect of serum factors on vasoactivity and remodeling

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Bakker, Erik N. T. P., Esther T. van der Meulen, Jos A. E. Spaan, and Ed VanBavel. Organoid culture of cannulated rat resistance arteries: effect of serum factors on vasoactivity and remodeling. Am J Physiol Heart Circ Physiol 278: H1233–H1240, 2000.—We developed an organoid culture technique to study the mechanisms involved in arterial remodeling. Resistance arteries were isolated from rat cremaster muscle and mounted in a pressure myograph at 75 mmHg. Vessels were studied during a 4-day culture period in DMEM with either 2% albumin, 10% heat-inactivated FCS (HI-FCS) or 10% dialyzed HI-FCS (12 kDa cut off) added to the perfusate. The albumin group showed a gradual loss of endothelial function and integrity, whereas smooth muscle agonist and myogenic responses were retained. No remodeling was observed. Vessels cultured in the presence of serum showed a progressive constriction. Smooth muscle responses and substance P-induced endothelium-dependent dilation were maintained. An inward remodeling of 17 ± 4% in the HI-FCS group and 26 ± 3% in the dialyzed HI-FCS group was found, while media cross-sectional areas were unchanged. These data show that pressurized resistance arteries can be maintained in culture for several days and undergo eutrophic remodeling in vitro in the presence of high molecular weight serum factors.

vascular smooth muscle; pressure myograph; growth factors; endothelium; myogenic response

PERIPHERAL RESISTANCE and local tissue perfusion depend on both the structure and contractile activation of the resistance vasculature. Consequently, pathological alterations in tone and structure may lead to chronic ischemia as well as hypertension. An increase in the ratio between wall thickness and lumen diameter is found in human essential hypertension and various animal models of hypertension (14). This alteration can be the result of growth and/or the rearrangement of existing materials. The latter is referred to as eutrophic remodeling (21). The mechanisms controlling resistance vessel caliber are only partly understood. Thus the causality of relations between blood pressure, vascular tone, and vessel wall remodeling and hypertrophy remain the subject of ongoing discussion. Over the years the in vitro study of blood vessels (26–28) has proven to be a very useful tool in the unraveling of mechanisms involved in acute control of tone. It could therefore be envisioned that the study of vessels in organoid culture over longer periods of time similarly will improve our understanding of chronic control of diameter, reactivity and wall thickness. Some studies on vessels in organoid culture have been published. With the use of isometrically mounted renal arteries, DeMey et al. (10) showed that tissue culture itself impairs constrictor responses to some agonists, whereas serum was found to induce constriction and increase DNA synthesis. Lindqvist et al. (19) reported that unloaded rings of rat tail artery show an impaired contractility after a 4-day incubation period with FCS. Mechanical loading conditions may well be of major importance when studying in vitro cultured segments. The technique that most closely mimics the in vivo situation in this respect is the cannulated vessel setup. This approach does not in itself impose mechanical deformation of the vessel wall, which could interfere with the orientation of its structural elements and influence structural adaptation. A further advantage of the cannulated vessel setup is that pressure and flow can be controlled independently.

Bardy et al. (3) developed an organ culture system in which segments of rabbit aorta are pressurized and perfused for several days. These authors studied the effects of pressure and flow on DNA synthesis, matrix proteins, and smooth muscle marker proteins in rabbit aorta (3, 4, 7). Yet, no studies have been reported on cannulated resistance vessels in culture. Therefore, we aimed to develop an organoid culture system in which functional as well as structural adaptations of resistance arteries to mechanical parameters can be studied. For this purpose, we used cannulated resistance arteries from rat cremaster muscle. We report here on changes in smooth muscle and endothelial function, depending on composition of the incubation medium. Most importantly, we found that resistance arteries are capable of eutrophic remodeling in vitro. This structural change may be mediated by growth factors present in serum and relate to their contractile properties.

METHODS

Male Wistar rats (250–350 g) were decapitated after sedation with 100% CO₂, in accordance with institutional guidelines. Cremaster muscles were excised and placed in cold physiological saline solution composed of (in mM) 119...
NaCl, 4.7 KCl, 1.18 KH$_2$PO$_4$, 1.17 MgSO$_4$, 25 NaHCO$_3$, 1.6 CaCl$_2$, 0.026 EDTA, 5.5 glucose, and 1.0 HEPES. A 6- to 8-mm-long segment of the first order arteriole was dissected and cut into two pieces. One piece was fixed for 30 min in a mixture of 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer for histology or electron microscopy. The other segment was mounted in a pressure myograph under sterile conditions. The segment was stretched to its in situ length and kept at this length during the experiment. The vessel was superfused with DMEM containing penicillin (100 IU/ml) and streptomycin (0.1 mg/ml) that was recirculated from a 50-ml reservoir and refreshed daily. The solution was equilibrated with 19% O$_2$, 76% N$_2$, and 5% CO$_2$, pH 7.4.

The vessel was superfused with DMEM containing penicillin, and streptomycin (0.1 mg/ml) and kept at this length during the experiment. The other segment was mounted in a pressure myograph and cut into two pieces. One piece was fixed for 30 min in a mixture of 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer for histology or electron microscopy.

Electron Microscopy. Tissues were sectioned using a cryotome at −20°C. Sections of 8 µm thickness were stained with either toluidine blue or picrosirius red, dehydrated in a graded series of alcohol and embedded in mounting medium (Entellan, Merck). Media cross-sectional areas were determined with Image-Pro Plus software from three sections of each preparation and averaged.

Fig. 1. Spontaneous tone of vessels cultured in the presence of albumin, heat-inactivated FCS (HI-FCS), or glycine. In the albumin group, FCS was obtained from Life Technologies. FCS was obtained from bovine serum. Statistical analysis. Data are expressed as means ± SE where n is the number of arterial segments studied. A repeated-measurement ANOVA followed by a post hoc Dunnett t-test was performed to determine the significance of differences between days. A paired t-test was used for all other comparisons. Differences were considered significant at P < 0.05.

RESULTS

Spontaneous tone. A total number of 18 vessels was included in the present study. Passive diameters (at 75 mmHg) were similar for all three groups: 181 ± 6 µm for albumin (n = 6), 178 ± 8 µm for HI-FCS (n = 6), and 183 ± 5 µm for dialyzed HI-FCS (n = 6). All vessels developed spontaneous tone during the equilibration period, resulting in similar active diameters (at 75 mmHg): 113 ± 8, 110 ± 9, and 117 ± 5 µm for albumin, HI-FCS, and dialyzed FCS, respectively. As shown in Fig. 1, the segments in the albumin group partially lost tone on day 2, regained tone on the third day and again partially lost tone on the fourth day. Typically, rhythmic vasomotion appeared on the second or third day and continued throughout the experiment. Vessels that were cultured with HI-FCS showed a progressive increase in spontaneous tone. Typically, a normal level of spontaneous tone was observed on day 1, which then gradually increased. This constriction was affected by neither ketanserin (1 µM; n = 3), an inhibitor of serotonin receptors, nor by losartan, an inhibitor of ANG II receptors (10 µM; n = 3), when added to the vessel at the end of the experiment (data not shown). Also shown in Fig. 1, a similar development of contraction.
dilation was observed with dialyzed serum, suggesting that small contractile factors present in serum were not involved. Only washout of serum at the end of the experiment with fresh DMEM resulted in a significant dilation of 35 ± 5 µm (n = 3). In both serum groups, vasomotion was less apparent but observed occasionally during the second or third day.

Responses to agonists. As shown in Fig. 2, in the albumin group responses to substance P gradually declined and were essentially lost at day 4. In contrast, responses increased in the HI-FCS group. Responses were significantly increased on day 3 compared with day 1. Similarly, with dialyzed HI-FCS responses were significantly increased on days 2 and 3.

As shown in Fig. 3, responses to the endothelium-dependent dilator ACh gradually declined in all three groups. In the albumin group, dilatory responses appeared to decline even more rapidly as observed with substance P. Responses were significantly decreased at days 3 and 4 and actually converted to a small constriction on day 4. Neither HI-FCS nor HI-dialyzed FCS prevented loss of dilatory responses to ACh during culture.

Serotonin induced similar contractile responses on day 1 in all three groups. As shown in Fig. 4, responses in the albumin group increased progressively during the culture period. Responses were significantly increased on days 3 and 4 compared with day 1. A comparable increase in contractile responses was observed in the dialyzed HI-FCS group, but not in the HI-FCS group. In the latter group responses were unchanged over the culture period.

Active pressure-diameter relations. As shown in Fig. 5, all segments displayed a myogenic pressure-diameter relationship on day 1. Thus vessels distended in the lower pressure range and constricted at higher pressures. In the albumin group, the pressure-diameter relation was not significantly altered on day 4. In both serum groups, a downward shift of the pressure-diameter relation was observed. As shown in Fig. 5B, the increased constriction was reflected over the whole pressure range, indicating that myogenic reactivity was preserved. Diameters were significantly smaller at each pressure level for day 4 versus day 1.

Passive pressure-diameter relations. Passive pressure-diameter relations were obtained at the start and at the end of each experiment. As shown in Fig. 6A, the relationship was unchanged after a 4-day culture period in the albumin group. However, vessels that were cultured in the presence of either HI-FCS or HI-dialyzed FCS showed a marked decrease in inner diameters over the whole pressure range (Fig. 6B). For instance, at 75 mmHg diameters were decreased by 17 ± 4% in the HI-FCS group and by 26 ± 3% in the dialyzed HI-FCS group.

Histology. Media cross-sectional areas were determined in freshly isolated vessel segments and compared with their counterparts that underwent the experimental protocol. Cross sections showed a clearly
discernable media surrounded by fibrous tissue consisting mainly of collagen, as indicated by picrosirius red staining. As shown in Fig. 7, media cross-sectional areas of control and cultured segments were not statistically different in all groups.

Electron microscopy. Freshly isolated arterial segments showed a layer of endothelial cells covering the luminal side of the vessel (Fig. 8A). As expected, the endothelial cells were separated from the smooth muscle cells by a lamina elastica interna. The media consisted of two layers of smooth muscle cells orientated circumferentially. The adventitia contained collagen fibers that were mainly orientated longitudinally and fibroblasts. Vessels that were cultured in the presence of albumin showed nearly complete absence of endothelial cells at day 4. This process appeared to involve a detachment of endothelial cells from the luminal surface, as shown in Fig. 8B. Here, a vessel is shown at the second day of culture in the presence of albumin. Endothelial cells are still present but only partly connected to the underlying tissue. In vessels that were cultured with serum, endothelial cells were present at day 4, as illustrated in Fig. 8C. In general, the cells appeared to be somewhat less elongated compared with control, and occasionally small parts of the endothelial cells stretched out into the vessel lumen. The internal elastic lamina was still present and the orientation of the smooth muscle cells was unchanged in both the albumin and serum groups. Smooth muscle cells did not show signs of mitosis.

DISCUSSION

The main findings of the present study are that 1) basal tone, myogenic reactivity and contractile responses remain present in vessels cultured in the absence or presence of serum. In the presence of serum, vessels 2) developed a progressive constriction, 3) showed a marked inward remodeling without a change
in media cross-sectional area, and 4) maintained endothelium-dependent dilation.

Model characteristics. Our ultimate goal is to have an in vitro technique for the study of long-term control of resistance artery caliber and function. Although on previous occasions vessels were cultured without mechanical load (19), on needles (10), or in a wire myograph (23), our experiments were done under physiological pressure. In this respect, the cannulated vessel setup more closely mimics the in vivo situation while offering the advantages of independent control of the chemical and physical environment. It should be mentioned, however, that the current technique is limited to vessels of $50–100 \, \mu m$ in diameter because smaller vessels are too difficult to isolate and cannulate. The pressure utilized in the present study (75 mmHg) was based on in vivo pressure measurements in the cremaster muscle, reported by Meininger et al. (20). Roughly, pressure drops from 95 mmHg at the proximal end to 65 mmHg at the distal end in first order arterioles such as used in the present study, indicating that these vessels are true resistance vessels.

The technique of organoid culture of cannulated vessels has been applied to rabbit aortic segments (3, 4, 7). These studies focused on DNA synthesis, matrix proteins, and smooth muscle phenotype, and did not include observations on contractile function and endothelium-dependent dilation. Also, the process of remodeling has not been studied before in either large or small cannulated segments. Large and small arteries differ in many physiological and pharmacological aspects (16, 22), notably the presence of basal tone, myogenic reactivity, and the sensitivity of the endothelium to agonists. Therefore, observations of small arteries could contribute to our understanding of tissue perfusion and blood pressure. The present model allows studies on vessels believed to be representative for at least part of the resistance artery vasculature. A further, more practical advantage of small vessels is that the internal diameter can be readily observed and measured by video techniques.

We first attempted to culture vessels in a serum-free medium, as serum is an undefined source of many substances, including growth factors. However, our results obtained with the albumin-cultured segments indicate that serum is required to maintain endothelial function. This result is in agreement with findings in cultured endothelial cells, where serum deprivation is known to induce apoptosis (15). Serum was added to the perfusate, and not to the superfusate, to stay as close to the in vivo situation as possible. It remains to be established whether lower concentrations of serum are sufficient to preserve endothelial function. Our findings on the effect of serum seem at variance with results obtained by Yamawaki et al. (31). These authors found that endothelial function of mechanically unloaded rabbit mesenteric arteries was preserved after incubation in serum-free medium for 7 days. Rather, serum was found to attenuate substance P-induced relaxation. Part of this discrepancy may be related to treatment of the serum, because we heat-inactivated serum to avoid complement-induced endothelial dysfunction (9, 18). Clearly, optimal culture conditions need to be established for different preparations.

Fig. 7. Media cross-sectional areas of vessels cultured in the presence of albumin, HI-FCS, or dialyzed HI-FCS ($n=6$ vessels each). Cross-sectional areas were unchanged after a 4-day culture period in all groups.

Fig. 8. A: electron microscopic image of a freshly isolated, pressurized cremaster muscle arteriole. Flat, elongated endothelial cells cover the lumen. Lamina elastica interna separates the endothelium from two layers of smooth muscle cells. Adventitia consists of collagen fibers and fibroblasts. Bar, 1 \mu m. B: image of a vessel cultured in presence of 2% albumin on day 2. Note this vessel was not pressurized during fixation. Same magnification as in A. C: image of a vessel cultured in the presence of 10% HI-FCS at day 4. Same magnification as in A.
A small pressure gradient was applied to generate a flow sufficient to refresh the luminal fluid. Thus the arteries were kept under low-flow conditions, with shear stresses < 1 dyn/cm². Since flow may be an important parameter in remodeling, our results may depend on this condition. The low-flow condition may also explain some of the morphological changes of the endothelial cells, such as the less elongated appearance that was observed.

A further concern in this study is that isolation of arterioles implicates a loss of innervation. The absence of functional nerve endings could alter both the contractility and remodeling processes. It has been shown that perivascular nerves are involved in media hypertrophy after infusion of ANG II in rats (24). However, our data show that perivascular nerves are not required for eutrophic remodeling, while normal contractility also was preserved.

Responses to agonists. All vessels in the present study gradually lost their responsiveness to ACh. Since the serum-incubated vessels showed actually an increased responsiveness to another endothelium-dependent dilator, substance P, we conclude that the loss of the ACh response is not related to a general deterioration of endothelial function. More likely, a loss of muscarinic receptors may account for the observed results, in agreement with observations on cultured endothelial cells (25). Alternatively, downstream signaling pathways (2) might have been affected. In this respect, endothelial nitric oxide synthase levels have been shown to be affected by the presence of serum during culture of vascular segments (31). In the serum-free group, both ACh and substance P-induced responses were lost, in agreement with the electron microscopic images showing dissociating endothelial cells on day 2 and the absence of endothelial cells on day 4.

To test contractile responsiveness, serotonin was chosen since DeMey et al. (10) demonstrated that responses of wire-mounted segments to this agonist are preserved during culture, as opposed to phenylephrine and vasopressin responses. Serotonin receptors are present on smooth muscle and endothelium, which mediate constriction and dilation respectively (29). The increased contractile response to serotonin in the albumin group is in accordance with the loss of a counteracting effect of the endothelium. However, it is unclear why contractile responses in the dialyzed FCS group similarly increase, despite the presence of endothelial cells. Taken together, the agonist-induced responses in this study show that smooth muscle cells remain reactive for at least 4 days without serum in our setup, although serum is required for endothelial cell viability.

Spontaneous tone. Basal tone (1) and myogenic responses (28) are fundamental properties of resistance vessels and of key importance for the control of organ perfusion. We therefore investigated whether both phenomena are maintained under culture conditions. In the presence of albumin alone, basal tone was slightly variable over the days, but was maintained up to day 4 (Fig. 1). Likewise, myogenic regulation was preserved (Fig. 5). Endothelial cells were absent by day 4. Thus in addition to the lack of effect of acute removal of the endothelium on myogenic responses (12), these data point out that the absence of the endothelium for a longer period does not affect this resistance artery property.

Vessels cultured in the presence of serum showed a progressive increase in constriction (Fig. 1). As was the case in the albumin group, myogenic regulation was maintained at day 4, albeit acting on a much deeper level of tone (Fig. 5B).

Serum may contain small contractile factors like serotonin, which may induce constriction during organ culture (10). However, the contractile effect of serum was not prevented by serum dialysis, and inhibition of serotonin receptors with ketanserin was ineffective. Schifers et al. (23) demonstrated that ANG II induces a slowly developing tonic increase in wall tension after a latency of 12 h in cultured renal arteries. However, involvement of ANG II can be prevented by serum dialysis, as the local renin-ANG system of the cremaster muscle depends on exogenous angiotensinogen (30). In addition, an inhibitor of ANG II receptors, when added to the vessel at the end of the experiment, had no effect on tone in our experiments. Possibly, the progressive increase in constriction may involve large molecular growth factors present in serum. In addition to their mitogenic effects, many growth factors have been shown to be potent vasoconstrictors. Platelet-derived growth factor was one of the first to be identified as a constrictor substance (6). Epidermal growth factor induces constriction in the DOCA-salt rat model, but not in control rats (13), and transforming growth factor-β constricts rat cremaster arterioles (17). In general, growth factor-induced constriction is associated with a slow onset of contraction and is tonic in nature (5). Thus these characteristics would fit the progressive increase in spontaneous tone observed in our experiments.

Remodeling. We found a marked reduction in the passive inner diameter of arteries cultured in the presence of serum. In particular, a downward shift of the passive pressure-diameter relation was observed over the tested pressure range between 25 and 125 mmHg. No change in media cross-sectional area was found, indicating that a rearrangement of structural elements had occurred during the experiment. Such structural adaptation is referred to as eutrophic remodeling (21). The passive diameter of arteries at sufficiently high pressures is determined by the length, amount and orientation of collagen fibers (11). Therefore, these data point to a relatively rapid reorganization of the collagen fibers in the wall.

Remodeling was found both in HI-FCS and dialyzed HI-FCS, but not in serum-free cultured segments. The concomitant occurrence of constriction and remodeling raises the possibility that these effects are linked.
There are basically two possibilities for such a linkage. First, both processes might share intracellular signaling pathways, and an agonist in serum could induce both effects. Recently, basic fibroblast growth factor (bFGF) has been implicated in remodeling in a mouse model. Treatment with an anti-bFGF antibody inhibited inward remodeling induced by ligation (8), whereas bFGF-deficient mice exhibit impaired smooth muscle contractility (32). Thus bFGF is implicated in both constriction and remodeling. The present coincidence of deep constriction and remodeling may therefore be attributed to the presence of bFGF or other growth factors in the serum. A second possibility, of potentially more importance, is that in the long run, passive characteristics follow the active diameter, irrespective of the mode of contraction. It is conceivable that a continuous rearrangement of extracellular matrix fibers occurs in the wall. When formed in a deeply constricted vessel, this would limit dilation and result in inward remodeling. It is, however, clear that this hypothesis requires a better understanding of matrix organization and turnover.

In summary, we have developed an organoid culture technique for resistance arteries in which contractile responses and endothelial function are maintained for 4 days. This period is sufficient to observe substantial remodeling. This technique should provide a valuable tool for the study of tone and structure regulation under physiological conditions in vessels of a size relevant for control of organ perfusion and peripheral resistance.

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REFERENCES

7. Birukov KG, Bardy N, Lehoux S, Merval R, Shirinsky VP, and Tedgui A. Intraluminal pressure is essential for the mainte-
27. VanBavel E, Mooij T, Giezeman MJ, and Spaan J A. Cannula-


