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THE USE OF ORGAN CULTURES TO STUDY VESSEL WALL PATHOBIOLOGY

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Abstract

Organ culture of the vessel wall is an useful *in vitro* method to study vascular cell biology. The intact vessel allows for the study of cell-cell and cell-substratum interactions including the structure and function of the vessel wall matrix. Long term organ cultures of porcine aorta show that neointimal formation is due primarily to cell proliferation of pre-existing intimal smooth muscle cells. Neointimal formation in these cultures is more pronounced in the presence of an endothelium that is turning over. In endothelial wound repair studies, the endothelium of the organ culture shows some important differences when compared to tissue culture studies in monolayer culture. Thus, vascular organ cultures can be successfully used to study vessel wall biology in health and disease.

Key Words: Aorta, endothelium, smooth muscle cells, neointima, proteoglycans.

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Introduction

The goal of this review is to describe the use of the organ culture in the study of atherosclerosis. Organ cultures have been used to study endothelial injury and repair, cell proliferation, cell-cell interaction both within the vessel wall and between platelets and/or leukocytes and endothelial cells, and metabolic functions of both endothelial and smooth muscle cells. Aortas from humans (3), rabbits (14, 32, 33, 40), rats (6, 7, 12, 18, 19), pigs (16), and human saphenous vein (2, 39) have been used as have porcine coronary arteries (8), and carotid (12, 30, 31), renal (5, 11) and tail (43) arteries from the rat. Since pigs do develop spontaneous atherosclerosis this model of porcine aortic organ culture is very useful.

The term organ culture implies that the whole organ is present intact. Often some of the adventitial fat is removed from the vessels, however the rest is left intact. The tissue may be cut into rings or into pieces of flat tissue. Explants in which only strips of media are studied are not organ cultures. These explants (4, 20) provide useful data on the function of medial smooth muscle cells but do not have the intact endothelium present.

Neointimal Formation and Smooth Muscle Cell Growth

Our laboratory has developed and used a porcine aortic organ culture system (16) to examine neointimal formation (22, 23). The major reason for using the pig model is because pigs do develop spontaneous atherosclerosis similar to that of humans. An important anatomical feature common to both porcine and human aortas is the presence of smooth muscle cells normally located in the intima, even at an early age (42). In relation to the pathogenesis of intimal thickening, these resident intimal smooth muscle cells may be the single most important difference between the porcine aortic model and vascular organ culture systems from other species (7, 14, 18, 32, 33).

Although many regulators of vascular smooth muscle cell growth are known, including factors derived

from platelets (37), monocytes/macrophages (25, 26, 29, 34) and smooth muscle cells (38, 44), low density lipoproteins (24) and extracellular matrix (17, 45, 46), endothelial cells themselves may play a role in enhancing smooth muscle cell proliferation as suggested by Ross in his "response-to-injury" hypothesis (36). Endothelial cells have been shown to secrete smooth muscle cell growth regulators similar to those produced by platelets and leukocytes (15). This role of the endothelium in regulating smooth muscle cell growth may be critical since they are anatomically the closest cell type to vascular smooth muscle cells. In view of the above information, we used the porcine aortic organ cultures to test the hypothesis that endothelial cells can secrete soluble factors that can regulate neointimal formation.

The Influence of Endothelial Cells on Smooth Muscle Cell Proliferation

Porcine aortic organ cultures were prepared as described previously (16). Briefly, porcine thoracic aortas were obtained from a local slaughterhouse. Following the removal of the adventitial tissues, each aorta was cut longitudinally along the intercostal arteries. The aorta was then pinned down with the endothelium facing up, six 4 x 10 mm rectangular strips of explants were cut from each aorta. Endothelial-denuded organ cultures were prepared by gentle scraping of the lumenal surface with a sterile scalpel blade. This method removed all surface endothelial cells but kept the resident intimal smooth muscle cells within the loose strands of intimal elastic fibrils as shown by transmission and scanning electron microscopy (16). Both nondenuded and denuded organ cultures were incubated with medium 199 containing 5% fetal bovine serum for 7 and 14 days. Control (day 0) and experimental organ cultures were obtained from the same aorta. At the end of each time point, the explants were fixed and processed for paraffin sections. To determine the extent of neointimal formation, the 4-μm paraffin sections were stained with a fluorescent bisbenzimide nuclear dye (Hoechst 33258 from Sigma Chemicals in St. Louis, Missouri). The effect of endothelial cells on neointimal formation was determined by comparing the number of intimal smooth muscle cells in nondenuded organ cultures with that in denuded organ cultures.

This study showed that the mean number of intimal smooth muscle cells from nondenuded organ cultures doubled between day 0 and day 7 (Figs. 1a,b, 2) and again doubled between day 7 and day 14 (Figs. 1b,c, 2). We previously reported that the mean number of intimal smooth muscle cells stabilized thereafter (23). The mean number of surface cells of nondenuded organ cultures, which have been identified as endothelial cells by their ability to incorporate DiI-acetylated-LDL (23), did not change significantly during the 2 weeks of incubation (Fig. 2). Both subendothelial cells and medial smooth muscle cells showed positive staining for the smooth muscle actin-specific monoclonal antibody α-SM1 indicating that the neointimal cells were smooth muscle cells (23). Incubation of denuded organ cultures with 5% fetal bovine serum for 7 and 14 days also resulted in an increase in the mean number of intimal smooth muscle cells (Figs. 3a,b,c, 4). But clearly, the increase in intimal smooth muscle cell number following endothelial cell denudation was much less when compared to that seen with nondenuded organ cultures. Thus, our study showed that the presence of endothelial cells was important in the formation of a neointima in our organ culture system.

Endothelial-Derived Soluble Factor

To test whether an endothelial-derived soluble factor was involved, denuded organ cultures were incubated with conditioned medium collected from 4 day (4DCM) and 24 day (24DCM) nondenuded organ cultures. The 4 day time point was chosen because it was found that there was a sudden and dramatic increase in the thymidine index of intimal smooth muscle cells of nondenuded organ cultures between day 4 and day 5 (22). This suggested that if a soluble factor was responsible for the surge of thymidine uptake by these intimal smooth muscle cells, then it would likely be present in the medium at day 4. The 24 day time point was chosen because it represented a period when the number of intimal smooth muscle cells of nondenuded organ cultures had stabilized (23). The cell count from this study showed that when incubated with 4DCM, the mean number of intimal smooth muscle cells doubled between day 0 and day 7 (Figs. 3d,e, 4). Intimal smooth muscle cell number continued to increase steadily after 7 days. The effect of the 24DCM (Figs. 3f,g, 4), on the other hand, generated a neointima that was similar to that obtained when the denuded organ cultures were incubated with 5% fetal bovine serum. Thus, the results from this set of conditioned medium experiments suggested that a soluble factor derived from endothelial cells of nondenuded organ cultures was important in the pathogenesis of neointimal formation.

Vascular Cell Proliferation in Neointimal Formation

The next question we asked was how was the neointima formed? To determine whether there was smooth muscle cell proliferation, nondenuded organ cultures were pulse-labelled with ^{3}H-Thymidine for 24 hours everyday for 14 days. This generated thymidine index profiles for both endothelial cells and intimal smooth muscle cells (Fig. 5). The results showed that the endothelial cell thymidine index reached a maximum at the end of 7 days, then it gradually decreased. Thymidine index of intimal smooth muscle cells followed a similar course to that of the endothelial cells, however, their values were always greater than the endothelial cell thymidine index. It is important to note that although endothelial cells showed thymidine uptake, the mean number of endothelial cells did not significantly change as shown

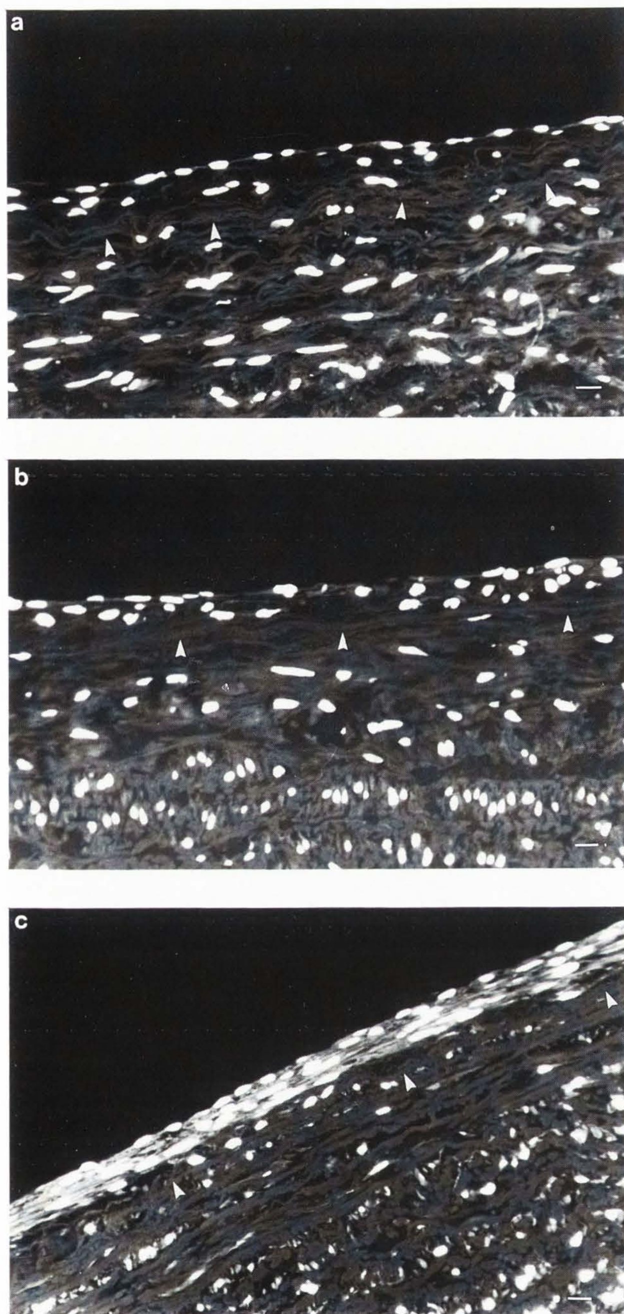


Figure 1 (at left). Fluorescence photomicrographs of nondenuded organ culture paraffin sections stained with the bisbenzimide nuclear dye Hoechst 33258 at day 0 (**a**), day 7 (**b**), and day 14 (**c**). Note the increase in intimal smooth muscle cell number beyond day 0. Arrow heads are directed at the internal elastic lamina. Bar = 10 μm.

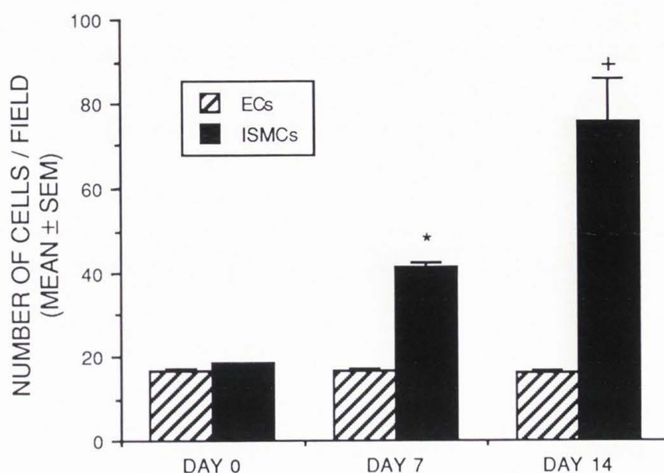


Figure 2. Intimal cell counts from nondenuded organ cultures incubated in 5% fetal bovine serum for up to 14 days. The mean number of endothelial cells (EC) did not change over the 4 week incubation period. The mean number of intimal smooth muscle cells (ISMC) doubled between day 0 and day 7 as well as between day 7 and day 14.
* $p < 0.05$ (comparing number of ISMC between day 0 and day 7)
+ $p < 0.05$ (comparing number of ISMC between day 7 and day 14)
All statistical analyses were performed using a 1-way analysis of variance (ANOVA) test at $p < 0.05$. If the ANOVA result was significant, then a Tukey pairwise comparison test was used to determine exactly which 2 treatment groups were significantly different. N = 6 aortas.

in the cell count study. This indicates that there was loss of endothelial cells in our organ cultures, suggesting perhaps that there was endothelial cell injury and/or the presence of dysfunctional endothelial cells. Endothelial cell loss was evident as a few surface cells showed rounding up with subsequent detachment in nondenuded organ cultures (Fig. 6, arrows). The fact that the endothelial cell turnover rate remained higher than the basal level throughout the incubation period suggested that the endothelium in this model was in a state of injury, however, the nature of this injury remains unclear. On the other hand, the significant increase in intimal smooth muscle cell thymidine incorporation was associated with doublings of intimal smooth muscle cell number during the 2 weeks of incubation. This indicated that smooth muscle cell proliferation played a role in the formation of neointima in our organ culture model. But this does not exclude the possibility of medial smooth muscle cell migration into the intima as part of the mechanism of neointimal formation. If medial smooth muscle cell migration occurred, then one might expect a decrease in the number of medial smooth muscle cells, especially from the area closest to the intima. However, there was no significant change in the mean number of medial smooth muscle cells contained within 5 medial lamellae immediately below the internal elastic lamina (Fig. 7).

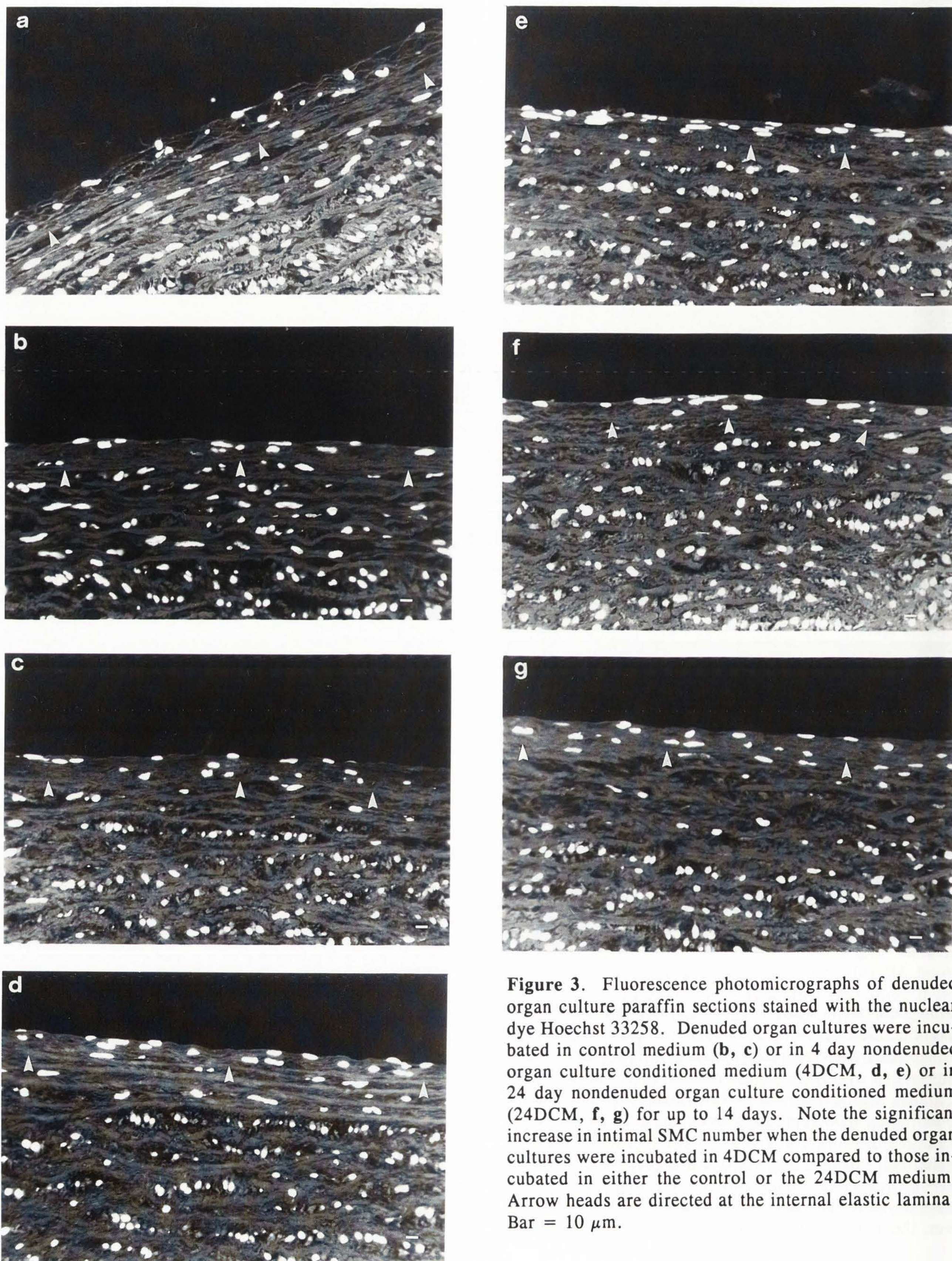
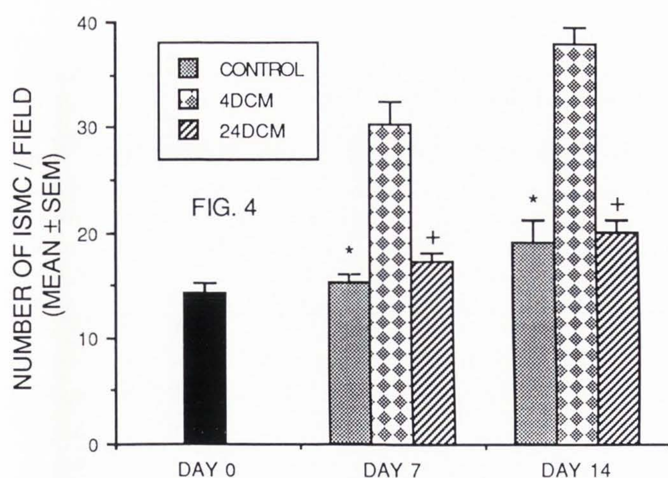


Figure 3. Fluorescence photomicrographs of denuded organ culture paraffin sections stained with the nuclear dye Hoechst 33258. Denuded organ cultures were incubated in control medium (**b, c**) or in 4 day nondenuded organ culture conditioned medium (4DCM, **d, e**) or in 24 day nondenuded organ culture conditioned medium (24DCM, **f, g**) for up to 14 days. Note the significant increase in intimal SMC number when the denuded organ cultures were incubated in 4DCM compared to those incubated in either the control or the 24DCM medium. Arrow heads are directed at the internal elastic lamina. Bar = 10 μm.



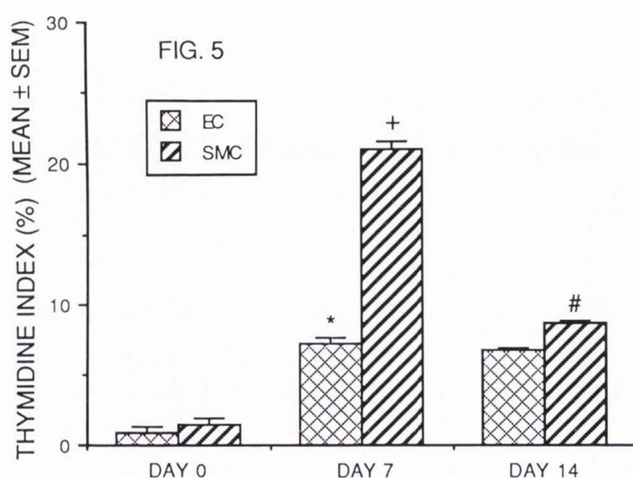


Figure 4 (at left). Intimal SMC (ISMC) counts of denuded organ cultures incubated for up to 14 days in 5% fetal bovine serum (CONTROL) or in 4 day- (4DCM) or 24 day- (24DCM) nondenuded organ culture conditioned medium. The number of ISMC was significantly greater with the 4DCM treatment compared to the effects of control or 24DCM which had similar effects.
* $p<0.05$ (comparing number of ISMC with control and with 4DCM treatment at the indicated time points).
+ $p<0.05$ (comparing number of ISMC with 4DCM and with 24DCM treatment at the indicated time points).
All statistical analyses were performed using a 1-way analysis of variance (ANOVA) test at $p<0.05$. If the ANOVA result was significant, then a Tukey pairwise comparison test was used to determine exactly which 2 treatment groups were significantly different. N = 6 aortas.

Figure 5 (at right). Mean thymidine index of endothelial cells (EC) and intimal smooth muscle cells (SMC) of non-denuded organ cultures incubated in 5% fetal bovine serum for up to 14 days. EC thymidine index peaked following 7 days of incubation and declined thereafter. The SMC thymidine index was greater than that of EC but showed a similar course over the 2 weeks of incubation.
* $p<0.05$ (comparing mean EC thymidine index at day 0 and at day 7).
+ $p<0.05$ (comparing mean SMC thymidine index at day 0 and at day 7).
# $p<0.05$ (comparing mean SMC thymidine index at day 7 and at day 14).
All statistical analyses were performed using a 1-way analysis of variance (ANOVA) test at $p<0.05$. If the ANOVA result was significant, then a Tukey pairwise comparison test was used to determine exactly which 2 treatment groups were significantly different. N = 28 aortas.

But still, one could argue that medial smooth muscle cells could have migrated into the intima but the migrated cells could have been repopulated by proliferation from adjacent non-migrated medial smooth muscle cells. This possibility was excluded because the thymidine index of medial smooth muscle cells was very low throughout the incubation period (Fig. 8). A peak was reached by day 21 (23), well after the time that the most prominent increase in intimal smooth muscle cell number had occurred. Therefore, our data suggested that medial smooth muscle cell migration played only a minor role in the formation of the neointima in our organ culture system.

Discussion

There are several reasons for using vascular organ cultures to study physiological and pathological conditions of the vessel wall. Unlike cell cultures, organ cultures provide a more natural microenvironment for cell-cell and cell-matrix interaction. Organ cultures also lack the complexity of *in vivo* studies where confounding factors sometimes make results difficult to interpret. In addition, once the questions have been answered using organ cultures, *in vivo* conditions can always be introduced back into the organ culture system in a step-wise manner. An example of this is the work done on the reorientation of the centrosome in migrating endothelial cells at a wound edge (35). Rogers reported that following endothelial wounding using porcine aortic organ cultures, the centrosome of endothelial cells from the first row along the wound reoriented toward the wound edge before cell migration. This finding supports *in vitro* endothelial cell wounding studies (13, 47), except that the results obtained with cultured cells occurred much faster. This is likely due to differences in cell-matrix interactions in the two systems. Having established this wound model in the organ cultures, flow and ultimately blood elements could then be added to the organ culture system to mimic more closely the *in vivo* conditions.

In the present study, our porcine aortic organ cultures were used to examine the regulation of intimal hyperplasia which is a characteristic feature leading to advanced fibrofatty plaques. Aortic organ cultures from

other species (6, 12, 14, 18, 32, 33, 40) and explants from different regions of the vascular tree (5, 12) have also been used to study the same problem. Previous studies using co-culture and organ cultures have shown that arterial smooth muscle cell growth can be inhibited (5, 9, 11), or not affected (12, 41) by the endothelium. Using porcine aortic organ cultures, we showed that smooth muscle cell growth can be regulated by the endothelium. The fact that the increase in intimal smooth muscle cell number was associated with a period of intense endothelial cell turnover and that stabilization of this intimal thickening occurred as the proliferation of endothelial cells decreased suggested that endothelial cell injury and/or death resulted in the release of smooth muscle cell mitogens into the medium, stimulating, directly or indirectly, the proliferation of the subjacent resident intimal smooth muscle cells. In addition, it was recently suggested that cells undergoing replication can release basic-fibroblast growth factor (bFGF) (10), a growth factor that is not normally secreted (1, 21). This is consistent with our finding that the decrease in endothelial cell turnover was associated with a decrease in intimal smooth muscle cell proliferation. Also, it has recently been shown *in vivo* that bFGF may be important in the pathogenesis of intimal hyperplasia induced by balloon catheter injury of the intima-media (27), and that addition of antibody to bFGF could reduce the extent of intimal smooth muscle cell proliferation (28). We have not yet identified the putative soluble factor(s) that regulate smooth muscle cell proliferation in our organ culture system.

Endothelialized organ cultures from other species that contain no resident intimal smooth muscle cells did not result in appreciable intimal thickenings even when incubated with medium containing up to 30% fetal bovine serum (7, 14, 18, 30, 32, 33). However, organ cultures of human aortas (3), which consist of a few layers of intimal smooth muscle cells covered by a patchy layer of endothelium, resulted in a significant increase in the number of intimal smooth muscle cells following incubation in serum-supplemented medium. In a recent study, Newby's group (2, 39) found that endothelialized, but not denuded, explants of human saphenous veins resulted in significant neointimal formation following incubation with 30% fetal bovine serum for up to 2 weeks. Although the intima is ill-defined in saphenous veins, there are smooth muscle cells located closely apposed to the endothelium. The authors then reported that approximately 50% of the neointimal cells following 2 weeks of incubation were the result of cell proliferation. In view of these results, it appears that the presence of resident intimal smooth muscle cells is critical for neointimal formation in organ culture models where there is no apparent medial injury (14). However, the presence of resident intimal smooth muscle cells makes it difficult to precisely define the role of medial smooth muscle cell migration in neointimal formation.

Another area that needs to be examined in this organ culture system is the regulation of the extracellular matrix by the endothelial and smooth muscle cells. This is important since it is well known that various matrix components can regulate the growth and differentiation (17, 45, 46) of vascular smooth muscle cells. Using organ cultures of rat carotid arteries, Merrilees and Scott (30, 31) reported that an increase in inner medial glycosaminoglycan (GAG) synthesis was associated with explants lined with mitotically active endothelial cells. Also, denuded organ cultures resulted in decreased GAG synthesis in the same region (30, 31). They further characterized, using ^{3}H-glucosamine and ^{35}S incorporation indices, that hyaluronate accounted for 54% of the total amount of label in the intima and 43% of the label in the inner media while sulfated GAGs were predominantly distributed in outer layers of the arterial wall.

Future studies could examine the effects of flow, leukocytes, platelets, and serum components such as low density lipoprotein on the regulation of processes which lead to atherosclerotic plaque formation. Also, agents that might inhibit intimal hyperplasia can be introduced to this organ culture model to study their effects on neointimal formation, stabilization, and regression.

Acknowledgements

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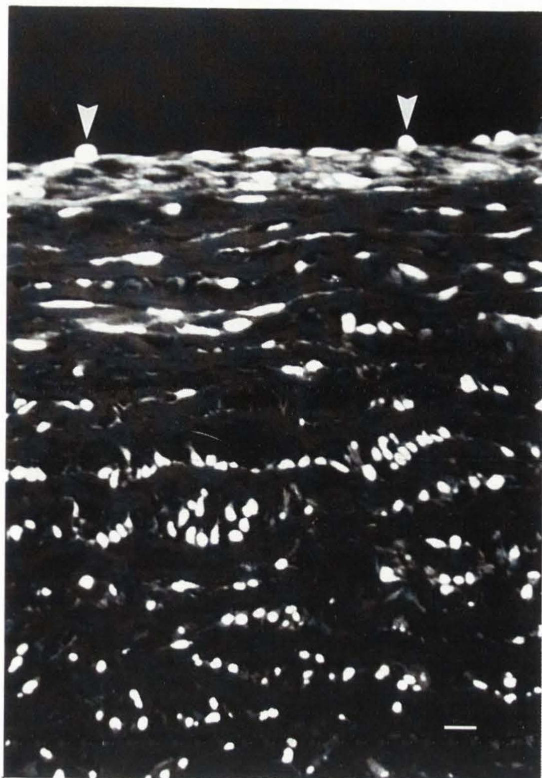

Figure 6. Fluorescence photomicrograph of a section of nondenuded organ culture at day 14 stained with the nuclear dye Hoechst 33258 at day 14. Arrow heads point to the nuclei of 2 rounded surface cells about to detach from the explant. Bar = 10 μm.

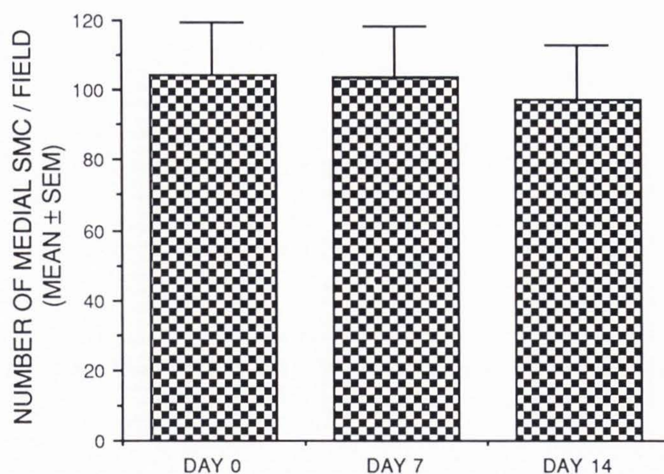

Figure 7. Medial smooth muscle cell counts from nondenuded organ cultures incubated in 5% fetal bovine serum for up to 14 days. The mean number of medial SMC located within the top five medial lamellae below the internal elastic lamina did not significantly change over the 2 week incubation period. N = 6 aortas.

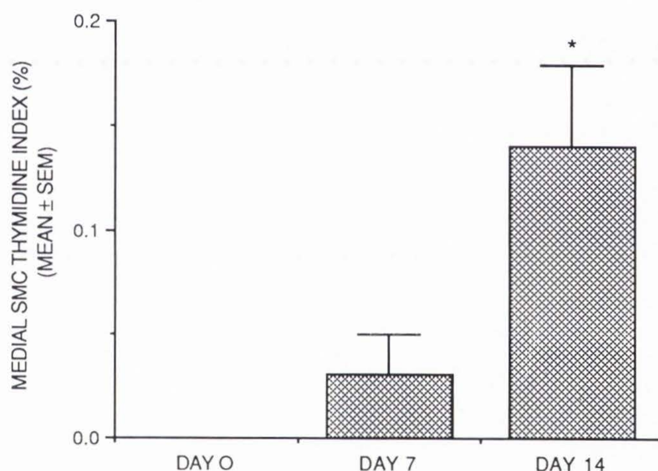

Figure 8. Mean thymidine index of medial smooth muscle cells located within the top five medial lamellae below the internal elastic lamina. The mean medial SMC thymidine index at days 7 and 14 were low and showed an increase late in the incubation period.
* $p < 0.05$ (comparing mean medial SMC thymidine index between day 7 and day 14)

All statistical analyses were performed using a 1-way analysis of variance (ANOVA) test at $p < 0.05$. If the ANOVA result was significant, then a Tukey pairwise comparison test was used to determine exactly which 2 treatment groups were significantly different. N = 28 aortas.

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Discussion with Reviewers

M. Richardson: Would the authors speculate as to the nature of the endothelial injury? Is there any evidence that the metabolic activity of the endothelial cells altered during the period of organ culture?
Authors: We do not know the nature of the endothelial injury. However, the injury may be related to the removal of the tissue and its placement *in vitro* resulting in changes in cell shape and gap formation between adjacent cells. Metabolic activity of endothelial cells was not examined.

M. Richardson: Was there any correlation between the endothelial cell proliferation rate and the thickness of the neointima?
Authors: The increased thickness of the neointima occurred following the period of greatest endothelial cell turnover.

A. Newby: The authors appear to have excluded all the surface cells from their analysis of intimal smooth muscle cells. It would be helpful to clarify this point. In cultures of human saphenous veins, we have found that some intimal surface cells are smooth muscle cells.
Authors: Some surface cells of the nondenuded organ cultures may be smooth muscle cells, especially after two weeks of incubation. Our data on DiI-acetylated-LDL staining shows that on occasions, the surface cells may be intimal smooth muscle cells since an occasional area showed lack of surface staining.

A. Newby: The control of medial cell proliferation may deserve more prominence. Presumably the authors believe that the pre-existing intimal cells in their cultures ultimately derive from the media. Their point that migration from the media may be a rate-limiting step is well taken, and born out by unpublished data we have in both organ culture and animal models of angioplasty. Nevertheless, there has to be some migration to get the whole process started. The observation that medial cell proliferation is stimulated by injury is a consistent finding in organ cultures and may be of relevance to atherogenesis where injury might be caused by oxidized LDL or by macrophages.
Authors: We have no evidence to indicate that the pre-existing intimal smooth muscle cells resulted from migration of medial cells. Pigs are found to have aortic intimal smooth muscle cells at an early age. The role of medial cell migration in the formation of the neointima in our organ culture system remains unclear. In *in vivo* balloon injury models, one sees prominent medial migration into the intima. We also showed that medial smooth muscle cell proliferation was very low and occurred much later than the proliferative activity in the intima. This is likely due to the lack of mechanical trauma to the media in this model.

A. Newby: It may be helpful to include some comments on cell viability and how it may be maintained in organ culture.
Authors: Since endothelial cells showed uptake of acetylated-LDL throughout the four weeks of incubation, we conclude that they were indeed viable. Also, results from the nuclear staining showed no changes such as pyknosis or other nuclear abnormalities, although occasional medial smooth muscle cell necrosis has been observed, this is likely due to the lack of nutrient supply to such a thick piece of vessel. In other organ cultures similar to ours, total tissue ATP (adenosine triphosphate)/ ADP (adenosine diphosphate) concentration was not altered (see ref. 39).

R. Saban: Under the heading **Vascular Cell Proliferation in Neointimal Formation**, the authors state: "This indicates that there was loss of endothelial cells ... suggesting perhaps that there were endothelial cell injury and/or the presence of dysfunctional endothelial cells" and that "the endothelium in this model was in a state of injury." Therefore, it is important to describe the incubation conditions such as temperature, pH, composition of medium 199 and its source.
Authors: The organ cultures were incubated with medium 199 with Earl's salt (Gibco Laboratories, Grand Island, NY). The cultures were then incubated at 37 °C in a humidified chamber equilibrated with 5% (v/v) CO_2 in air at pH 7.4. These are standard conditions used for our aortic endothelial and smooth muscle cell cultures.

A. Newby: I did not find inclusion of so many similar micrographs helpful. In demonstrating the difference between endothelium denuded and intact preparation, only the morphometric data can stand up to objective analysis.
Authors: The plates of the different time points add an important dimension to the paper and show the readers how the morphometric data was performed.