

SMOOTH MUSCLE CELL MITOGEN PRODUCTION BY  
ENDOTHELIAL CELLS IN THE LUMENS OF HEALING SYNTHETIC  
VASCULAR PROSTHESES: A POSSIBLE ROLE IN HEALING

Submitted in Partial Fulfillment of the Requirements for  
Graduation with Honors to the Department of Biology  
Carroll College, Helena, Montana

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March 30, 1987

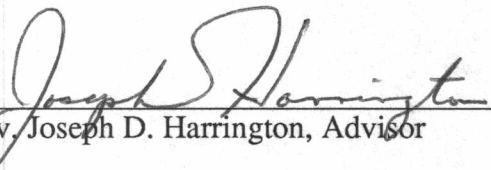
Research performed at the University of Washington,  
Department of Surgery, Atherosclerosis Laboratory, Seattle, Washington,  
under the supervision of Richard Kenagy, Ph. D.

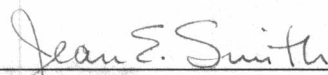



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March 30, 1987

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## ABSTRACT

Media was conditioned by culturing endothelial or medial smooth muscle cells in DME-F12 or by time-course perfusions of segments of aorta, synthetic polytetrafluoroethylene (PTFE) arterial grafts with an intact endothelium, and deendothelialized PTFE grafts. These conditioned media samples were used as culture media for quiescent medial smooth muscle cells. Mitogenesis assays were conducted using radioactive thymidine labeling to test each medium for its ability to stimulate the quiescent smooth muscle cells to enter the S phase from the  $G_1/G_0$  phase. Ratios of the thymidine incorporation of cells cultured in conditioned media to those of the negative control, which lacked mitogens, indicated that healing synthetic grafts with an intact endothelium produced greater levels of mitogens than did undamaged aortas. Attempts, using antibodies to Platelet Derived Growth Factor, a known smooth muscle cell mitogen, to test whether the mitogens produced in the grafts was PDGF indicated that further purification of the antibodies was needed. The possible relationship between mitogen production in arteries and healing synthetic grafts is discussed.

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## INTRODUCTION AND LITERATURE REVIEW

In the last half century great advances have been made using synthetic grafts for vascular reconstruction in the treatment of peripheral arteriosclerosis. These synthetic arterial grafts often fail due to anastomotic narrowing, possibly caused by connective tissue build-up and thrombus.<sup>2</sup> In order to better understand the success and failure of synthetic arterial prostheses, studies are being done about the steps in the healing process and the role growth factors play in it.

The process of healing in synthetic arterial grafts normally involves the accumulation of cells on the luminal surface of the arterial prosthesis. Researchers have demonstrated that endothelium is present on the inner surface of healing grafts with an intima of fibroblasts or smooth muscle cells below it.<sup>2</sup> This accumulation of endothelial cells and smooth muscle cells or fibroblasts occurs differently, depending on the porosity of the prosthesis. Studies show that, in polytetrafluoroethylene (PTFE) grafts with an internodal distance of 30  $\mu\text{m}$ , cellular proliferation, migration, and subsequent covering of the graft lumen associated with healing begins from the anastomosis at both ends of the graft and the adjacent arteries.<sup>2-3</sup> Proliferation continues along the growing edge of the anastomosis, with continued proliferation sometimes occurring at the anastomoses, resulting in occlusion of the vessel and graft failure. In PTFE grafts with an internodal distance of 60  $\mu\text{m}$ , healing occurs from cells derived from an ingrowth of capillaries from tissues outside of the graft.<sup>3</sup> Clowes suggests that capillaries grow through the graft wall from the surrounding granulation tissue. These capillaries provide a source of endothelial cells which proliferate and migrate over the luminal surface of the graft to form a monolayer of endothelium.<sup>3</sup> Further work demonstrated that, cells which stain with anti-smooth muscle cell actin antibody and have the same ultrastructural characteristics as arterial smooth muscle cells, were proliferating beneath











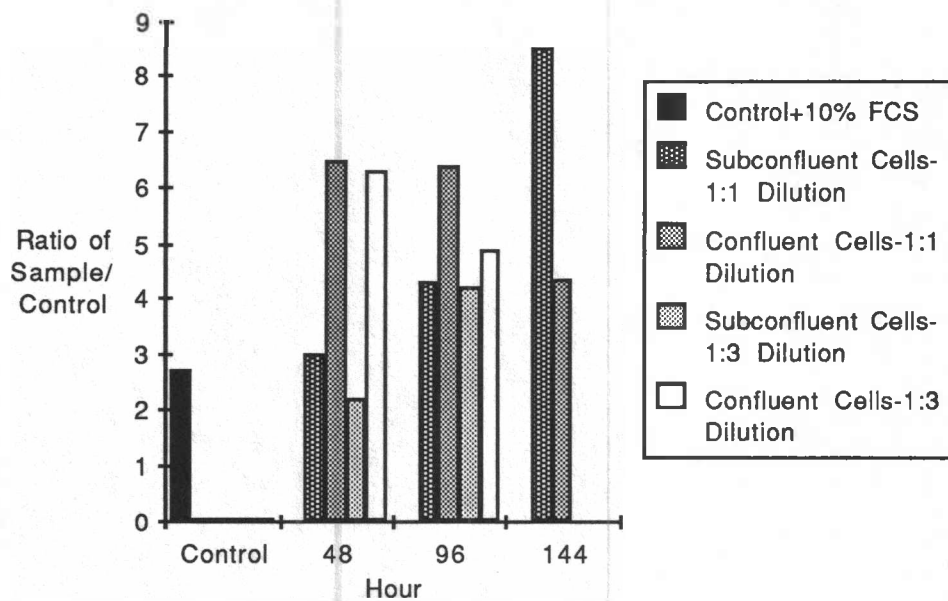












B.

Figure 2. Thymidine Labeling and Ratio of Labeling to Control for Smooth Muscle Cell Conditioned Media. Incorporation of tritiated thymidine, measured as CPM/ $\mu$ g protein, into cultured smooth muscle cells shows that thymidine incorporation into cells cultured in conditioned medium from confluent smooth muscle cells decreases during the 144 hour period. Subconfluent cell conditioned medium shows an ability to cause greater thymidine incorporation into smooth muscle cells as the age of the cells from which the conditioned medium was obtained increases from 48 to 144 hours (A). This corresponds to the degree to which conditioned medium samples stimulate thymidine incorporation and mitogenesis over the control lacking mitogens (B). Included is the value obtained for the control with added mitogens (10% FCS), which is less than the experimentals in all cases except cells cultured in the 1:3 dilution of conditioned medium taken at hour 48 from subconfluent.

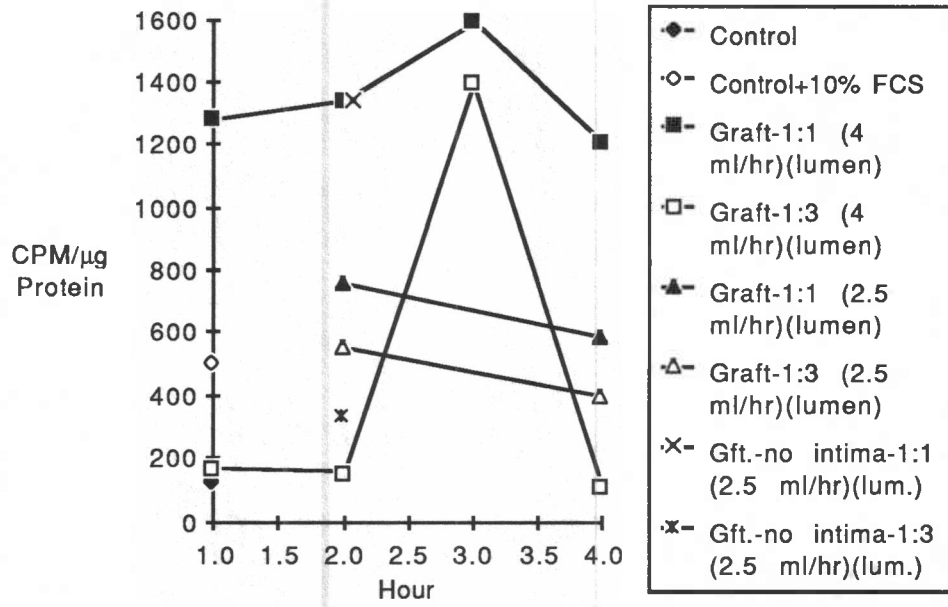
incorporation levels than the control containing mitogens (Fig. 2b.). Since thymidine labeling indicates that cells are synthesizing DNA and have left the  $G_1/G_0$  phase and moved into the S phase, cells which showed higher CPM values after being maintained in a particular conditioned medium indicated the presence of mitogens in the medium.

Conditioned medium from the animal perfusion model was used in several experiments. The ability of conditioned medium perfused through a synthetic thoraco-abdominal graft at 4.0 ml/hr to stimulate mitogenesis in cultured smooth muscle cells, as indicated by  $^3\text{H}$ -ThD incorporation in CPM/ $\mu$ g protein, increased sharply between the first and third hour of perfusion for both the 1:1 and 1:3 dilutions. This increase was followed by a decrease in

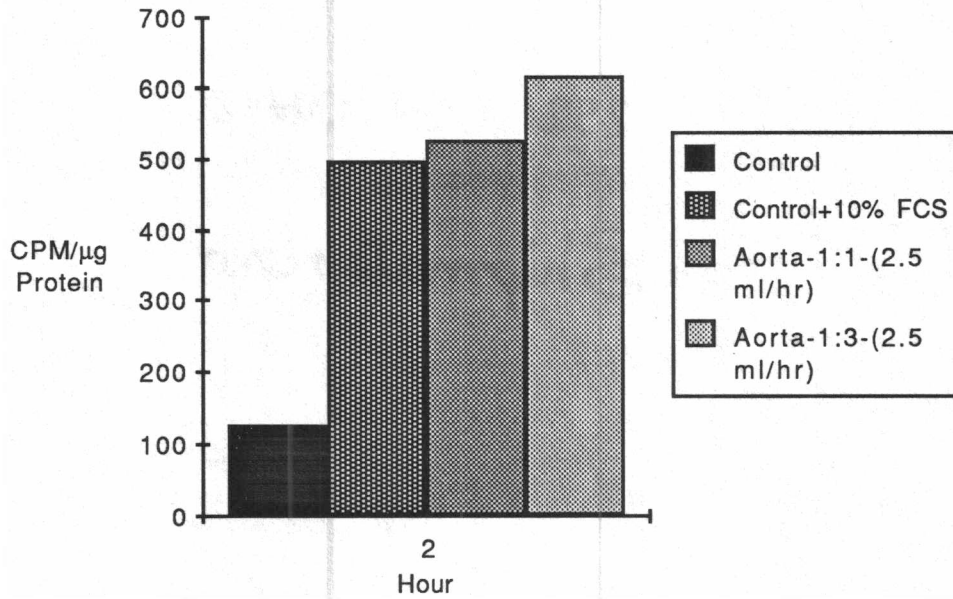
mitogenic ability for both dilutions for conditioned medium removed 4 hours after the beginning of perfusion (Figure 3a). Conditioned medium from grafts perfused at a slower rate of 2.5 ml/hr was removed at only the 2 and 4 hour points, but between these two time periods the mitogenic ability of the medium for medial smooth muscle cells shows an overall decrease for both dilutions as seen for conditioned medium from the graft perfused at 4.0 ml/hr. Conditioned medium for this experiment was also perfused through a deendothelialized thoraco-abdominal at a rate of 2.5 ml/hr at only the 2 hour time spot. While thymidine incorporation for cells cultured in the the 1:1 dilution was greater than that seen for graft conditioned medium from a graft with an intact endothelium at the 2 hour point, the 1:3 dilution was slightly less than the corresponding 1:3 dilution for the normal graft medium. In addition to the grafts, conditioned medium was collected at the 2 hour point from an abdominal aorta with a perfusion rate of 2.5 ml/hr. Smooth muscle cells cultured in both the 1:1 and 1:3 dilutions have thymidine incorporation values measured in CPM/ $\mu$ g protein slightly above those of the control+10% FCS but below the values for the 1:1 dilution of both the normal and deendothelialized grafts (Figure 3c).

As stated previously, medium which had been conditioned by bathing the outsides of the grafts during the perfusion process was also used, although there was no such sample for the aorta. Cells cultured in both the 1:1 and 1:3 dilutions of medium from the outside of the graft with intact endothelium showed thymidine incorporation values above 2500 CPM/ $\mu$ g protein (Figure 3c), greater than any other counts in the experiments; the highest was approximately 1600 CPM/ $\mu$ g protein for the 1:1 dilution of conditioned medium from the lumen of the graft with intact endothelium at the third hour (Figure 3a). Results for the 1:1 dilution of conditioned medium from the deendothelialized graft showed slightly less amounts of thymidine, and the 1:3 dilution was considerably less, with values being near those for the control+10% FCS.

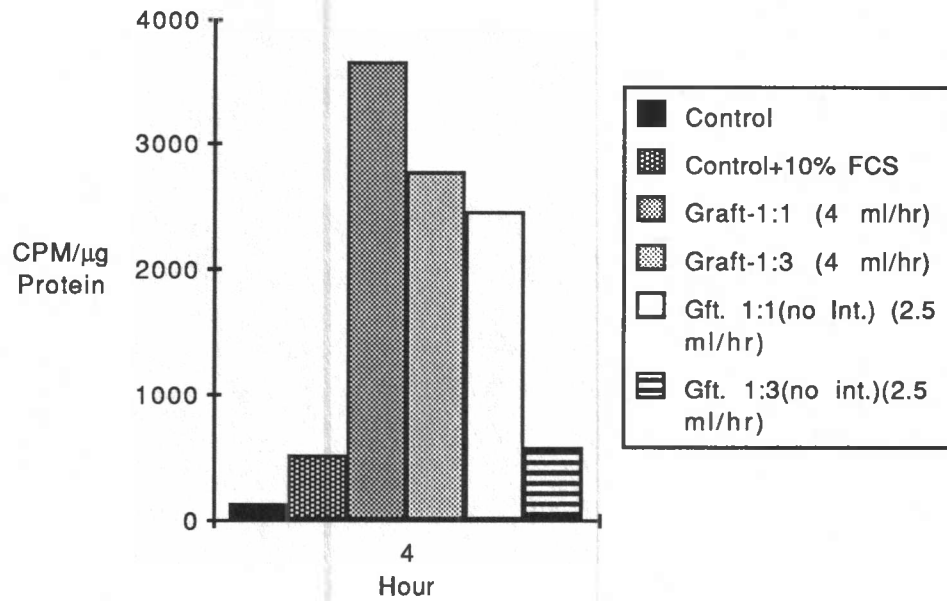
Again, we can look at the ratio of thymidine incorporation of experimental values to



A



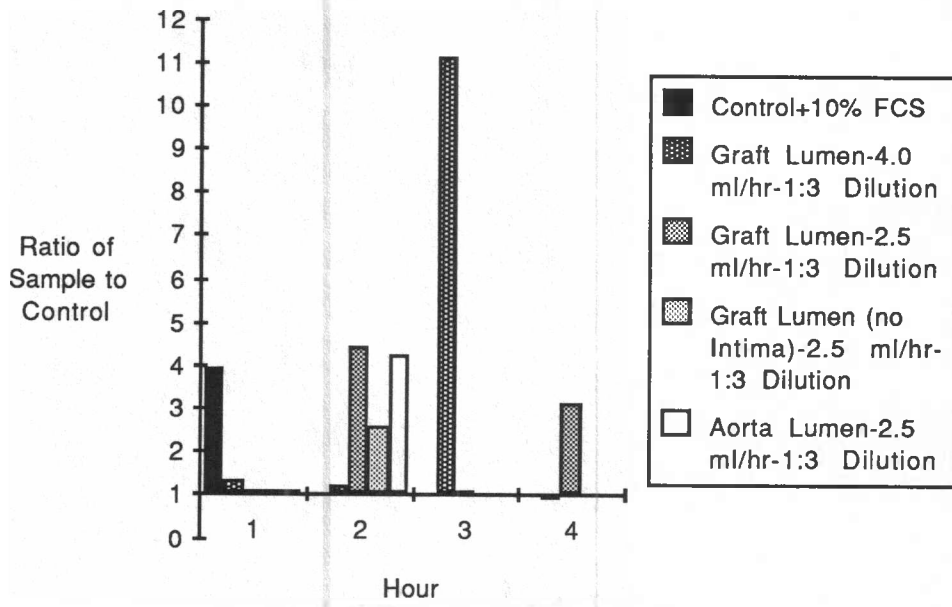
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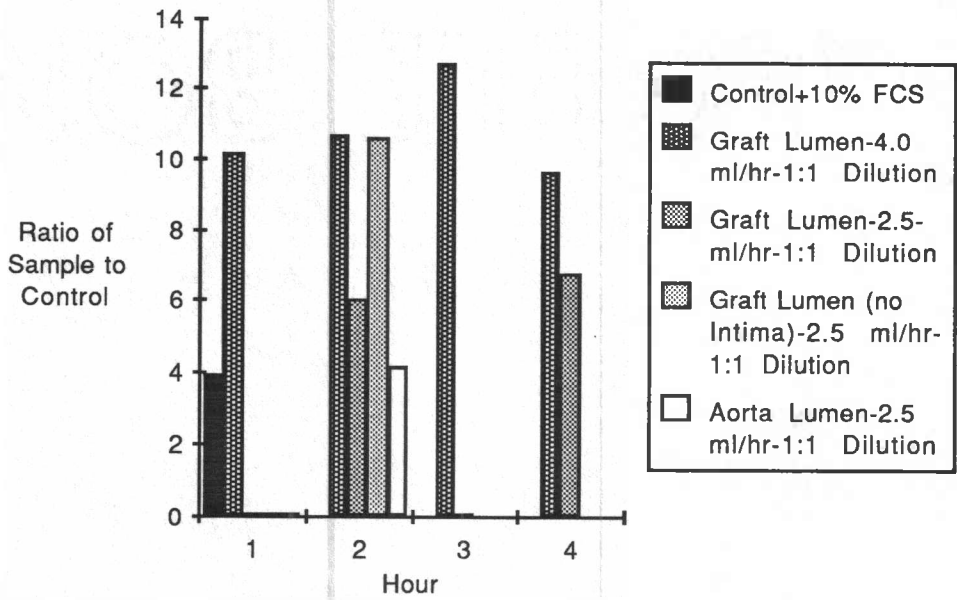
C

Figure 3. Thymidine Labeling for Perfusion Experiment 1-Aortas and Graft Lumens and Outsides. Incorporation of  $^3\text{H-TdH}$  into medial smooth muscle cells cultured in various conditioned media is indicated in CPM/ $\mu\text{g}$  Protein. A negative control containing no mitogens and a positive control with 10% FCS were run. Values for cells cultured in 1:1 and 1:3 dilutions of conditioned media from thoraco-abdominal PTFE grafts with intact intimas perfused at 4.0 (collected at 1-hour intervals) and 2.5 ml/hr (collected at 2-hour intervals) and from a deendothelialized thoraco-abdominal PTFE graft perfused at 2.5 ml/hr (collected at the 2-hour point only) are shown in (A). Values for the abdominal aorta perfusion (2.5 ml/hr) (collected at the 2-hour point) are shown in (B), and (C) shows values for conditioned medium from the outsides of each vessel except the aorta.

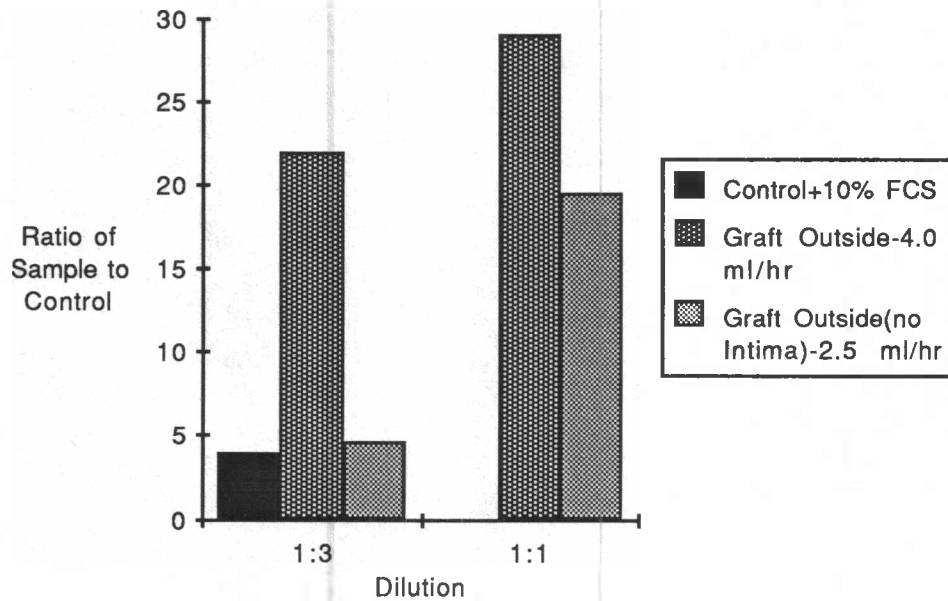
those of the control lacking mitogens. The control with mitogens (10% FCS) caused a fourfold amount of thymidine incorporation over the negative control and therefore shows a fourfold mitogenic effect on smooth muscle cells in culture. 1:3 dilutions of conditioned medium from grafts and the abdominal aorta perfused at 2.5 ml/hr, and the positive control all showed similar ratios to the negative control. The deendothelialized graft perfusate was less than this, with a ratio of only 2.5 times that of the control; and ratios for cells cultured in medium from the normal graft perfused at 4.0 ml/hr are only 1.5 that of the control for the first two hours, rise to eleven-fold over the control for the third hour, and drop to a ratio below 1.0 for the fourth hour, indicating less mitogenesis capabilities than the negative control (Figure 4a). Ratios for the dilution of the same media showed that the aorta still has



A



B



C

Figure 4. Thymidine Labeling Ratios to Control for Perfusion Experiment 1-Lumens and Outsides. Ratios are shown here of the amount of thymidine incorporation by cells cultured in the conditioned media from Fig. 3. to cells cultured in the negative control with the 1:3 dilutions from the lumen of each sample shown in (A), the 1:1 dilutions from the lumens in (B), and both dilutions for the outsides of each vessel in (C).

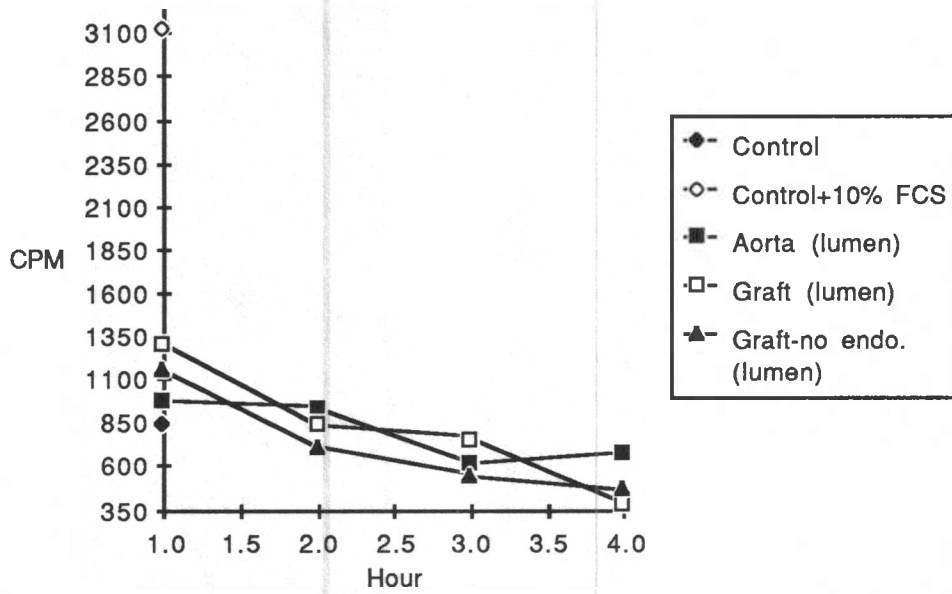
approximately a fourfold mitogenic ability over the negative control, but ratios for other samples are greater. Perfusate from the normal graft perfused at 4.0 ml/hr now show ratios of ten-fold over the control for the first two collections with a rise and fall similar to those in the 1:3 dilution for hours 3 and 4. Conditioned medium from the normal graft perfused at 2.5 ml/hr has ratios of approximately six-fold over the control for both the second and fourth hour collections, while that from the deendothelialized graft perfused at the same rate was ten-fold (Figure 4b). Collections of conditioned medium at the fourth hour from the outside of the deendothelialized graft perfused at 2.5 ml/hr had ratios to the control that were greater than that of the positive control but less than that of the conditioned medium from grafts with intact endothelium perfused at 4.0 ml/hr, the latter of which at a dilution of 1:1 caused cultured smooth muscle cells to incorporate thymidine approximately 29 times that of the negative control (Figure 4c).

Using synthetic grafts and arteries taken from a different baboon, experiments

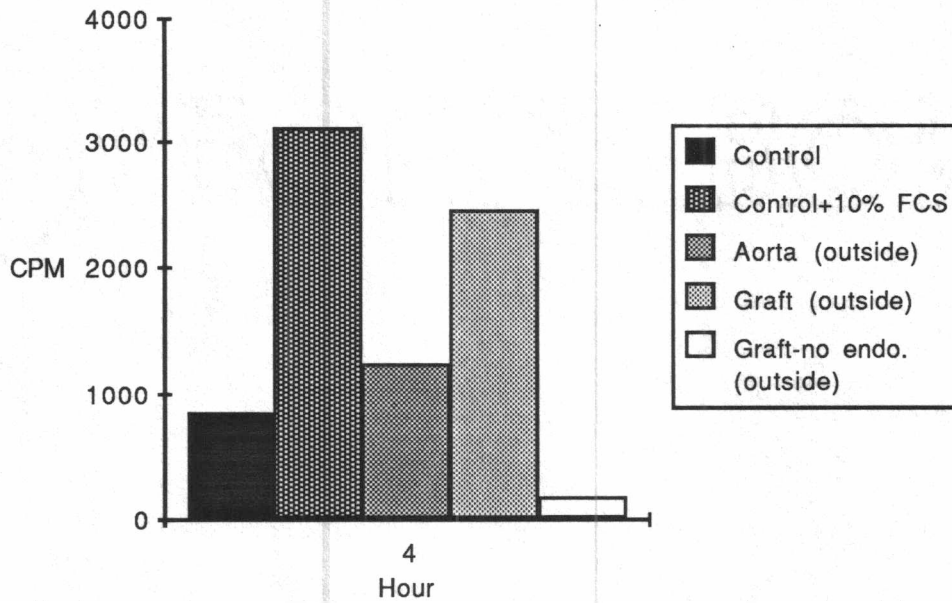
similar to the previous set were conducted. Conditioned medium for use on cultured smooth muscle cells was obtained from time-course perfusions at a rate of 4.0 ml/hr of a section of the abdominal aorta, and a normal and deendothelialized section of a thoraco-abdominal PTFE graft, but only 1:1 dilutions of the conditioned medium were used as culture medium for the smooth muscle cells. Due to difficulties with the spectrophotometer, no protein assays were done, so incorporation of radioactive thymidine was measured as CPM rather than CPM/ $\mu$ g protein.

Unlike the previous set of experiments, none of the conditioned media samples showed a greater ability for mitogenesis for smooth muscle cells than the positive control+10% FCS. Cells cultured in conditioned medium taken from the first hour collection from the lumen of the graft with an intact intima had higher CPM values, and thus more mitogenic ability, than the other samples (Figure 5a). A steady decrease in this ability is seen for subsequent collections of this medium with the sharp increase at hour 3 observed in the previous experiment being absent. Medium taken from the lumen of the deendothelialized graft also continuously decreased throughout the four-hour collection period and caused lower levels of thymidine incorporation in smooth muscle cells than that seen for the medium from the graft with intact endothelium, except at hour four where the two CPM values have fallen below those of the negative control and are approximately equal. Perfusate from the aorta has a mitogenic ability less than either graft sample and slightly above that of the negative control for the first two collections, which decreases to slightly below that of the control for the second two collections (Figure 5a). Conditioned media taken from the outside of each vessel after four hours also show a lesser capability to cause thymidine incorporation than the control+10% FCS (Figure 5b). Medium from the graft with intact endothelium appears more mitogenic than media from either the aorta or the deendothelialized graft, which is the least mitogenic.

Ratios of the mitogenic ability of the samples to the negative control show that the

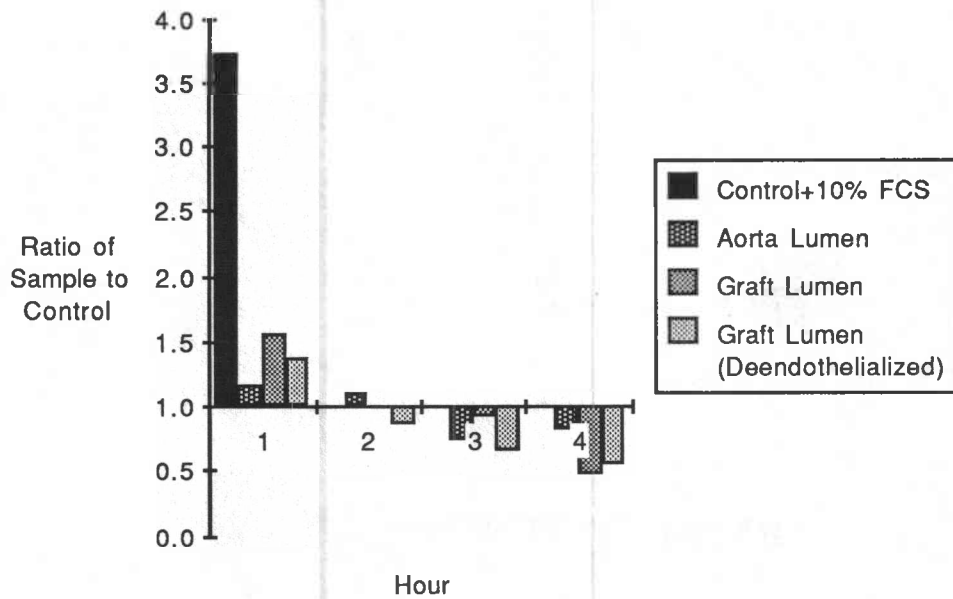


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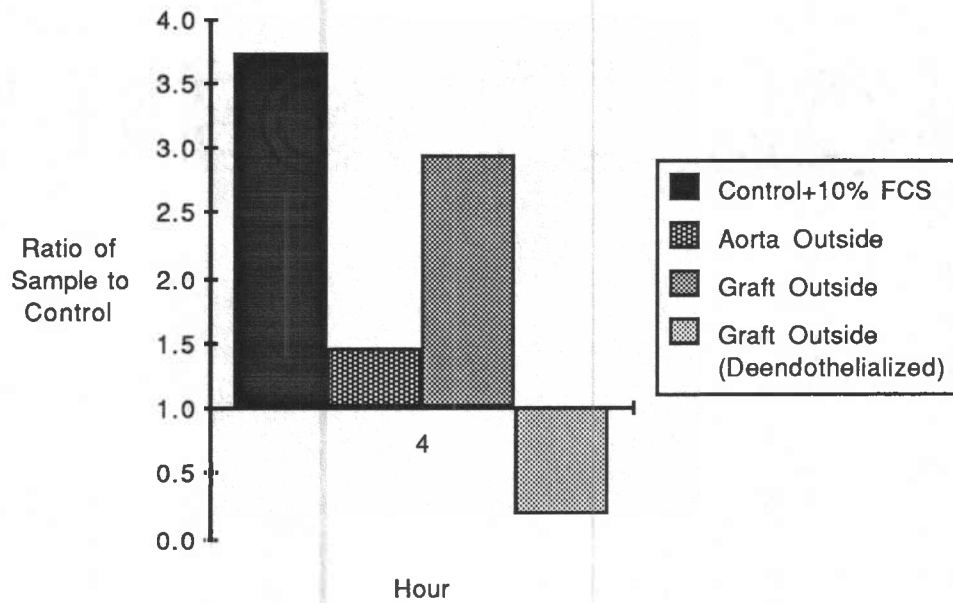


B

Figure 5. Thymidine Labeling for Perfusion Experiment 2-Lumens and Outsides. A second set of conditioned medium experiments using time-course perfusions relates the mitogenic ability of media from a segment of abdominal aorta, a thoraco-abdominal PTFE graft with an intact endothelium, and a deendothelialized graft for cultured smooth muscle cells by counting the CPM from  $^3\text{H-TdH}$  incorporation. Values for media obtained from the lumens of these vessels are indicated in (A), while those for the outsides are in (B).



A



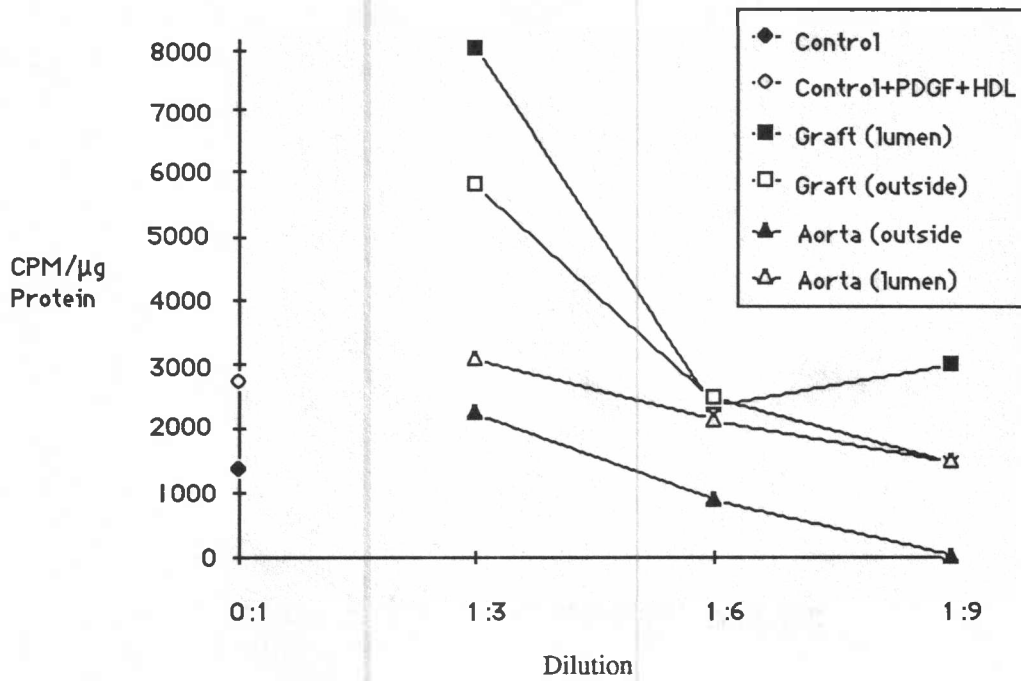
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Figure 6. Thymidine Labeling Ratios for Perfusion Experiment 2-Lumens and Outsides. Using values obtained for thymidine incorporation caused by conditioned medium from vessels shown in Figure 5a and b., the mitogenic ability, expressed as a ratio of the CPM of experimental samples to the negative control, is graphed for conditioned medium from the lumens (A) and outsides (B) of each vessel.

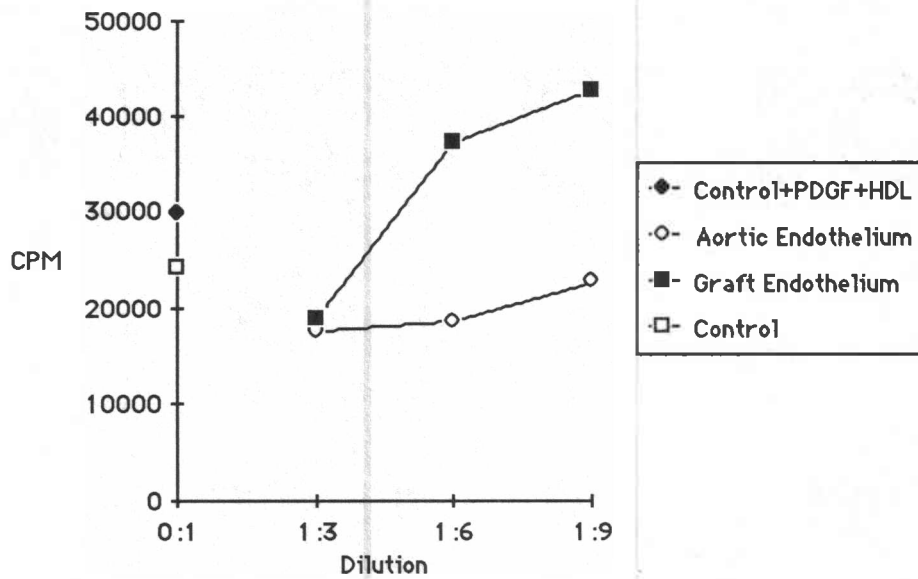
positive control is approximately three times as mitogenic as the negative control. In support of what has already been stated, conditioned medium from the first hour of the graft with intact endothelium has the next highest ratio of approximately 1.5, which decreases at subsequent time spots to a ratio of less than 1.0, indicating a mitogenic ability less than the negative control (Figure 6a). Conditioned medium from the deendothelialized graft and aorta show a similar decrease in ability for mitogenesis, with ratios dropping from values above to below 1.0. Observations for conditioned media from the outside of the vessels, however, indicate that while the graft with the intact endothelium still has the greatest mitogenic ability, approximately 3.0 times that of the negative control, only conditioned medium from the outside of the deendothelialized graft has a mitogenic ability that falls below that of the negative control (Figure 6b).

As in the last two sets of experiments, conditioned medium was removed from perfusions of a segment of abdominal aorta and, in this case, an aorto-iliac PTFE graft. The perfusion was not a time-course perfusion, and after five hours, perfusate was removed from the lumens and from the outside of the vessels. Endothelial cells were stripped from the intima of some segments of both vessels, cultured, and used to produce conditioned medium. Again, mitogenic ability is expressed as CPM/ $\mu$ g protein due to thymidine incorporation. A decrease in mitogenic ability is observed for conditioned media obtained from the animal model perfusions as the dilution of conditioned media is increased from 1:3 to 1:6 to 1:9 (Figure 7a). Conditioned media removed from the cultured endothelial cells, however, showed the opposite trend; mitogenic ability, expressed as CPM, increased as the dilutions were increased from 1:3 to 1:9 (Figure 7b).

Ratios of the amount of thymidine incorporation into smooth muscle cells cultured in conditioned media to that of controls lacking mitogens indicated that mitogenic ability of the 1:3 dilution of medium from the graft lumen is the highest of any sample, being almost six-fold over the negative control; the control with PDGF and HDL (high density lipoprotein)



A

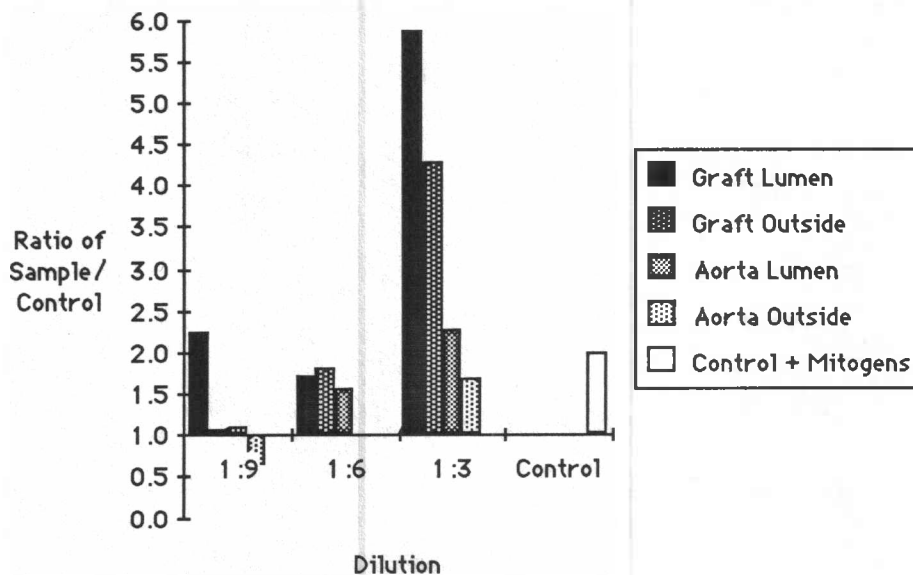


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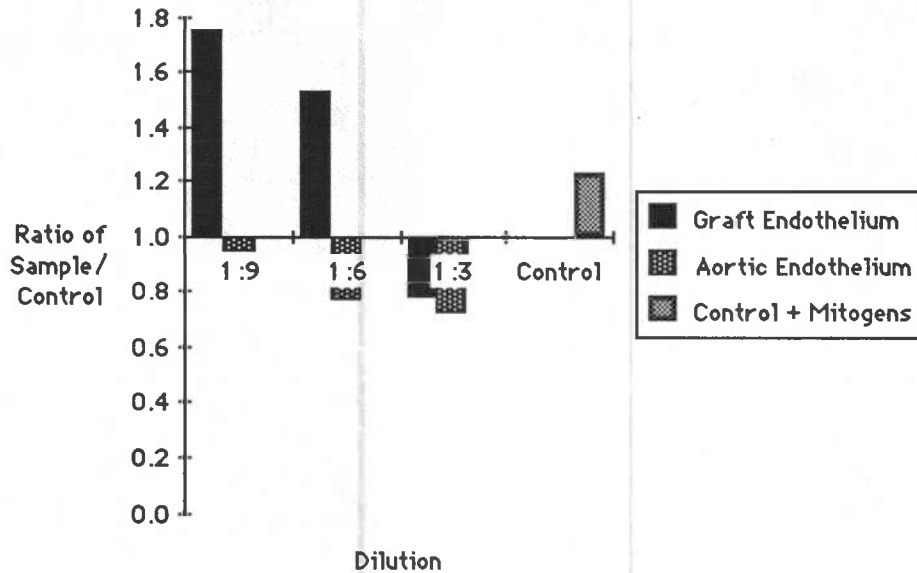
Figure 7. Thymidine Labeling for Perfusion Experiment 3 and Cultured Endothelium Conditioned Media. Mitogenic abilities of 1:3, 1:6, and 1:9 dilutions of conditioned media from five-hour perfusions of segments of an aorto-iliac PTFE graft and an abdominal aorta are shown in (A) for both the lumens and outsides. (B) shows the mitogenic ability of the same dilutions of conditioned medium taken from cultured endothelium from segments of the same vessels.

added was only two times as mitogenic as the negative control (Figure 8a). For both the 1:3 and 1:9 dilutions, conditioned medium from the lumen and outside of the graft show the greatest mitogenic ratio to the control, while the aorta lumen and outside were the least. For the 1:6 dilution the samples were all fairly close, and there was no 1:3 dilution of conditioned medium from the outside of the aorta due to contamination. Conditioned media from cultured endothelium showed that graft endothelium was more mitogenic than aortic endothelium for all three dilutions, but that both media had a mitogenic capability which was only a fraction of that of the negative control (Figure 8b). This was true for all three dilutions of medium obtained from the aortic endothelium.

In an attempt to identify the mitogens present in media conditioned by aorta and PTFE graft segments and endothelial cells cultured from them, unused refrozen conditioned medium from the previous experiment was thawed and diluted to 1:3. Smooth muscle cells were cultured in either the conditioned medium dilution alone, conditioned medium with anti-PDGF, or conditioned medium with non-immune serum to test for the presence of PDGF. Thymidine incorporation measured in CPM is greater for cells cultured in the 1:3 dilution of conditioned medium from the lumen of the aorto-iliac graft + anti-PDGF than for



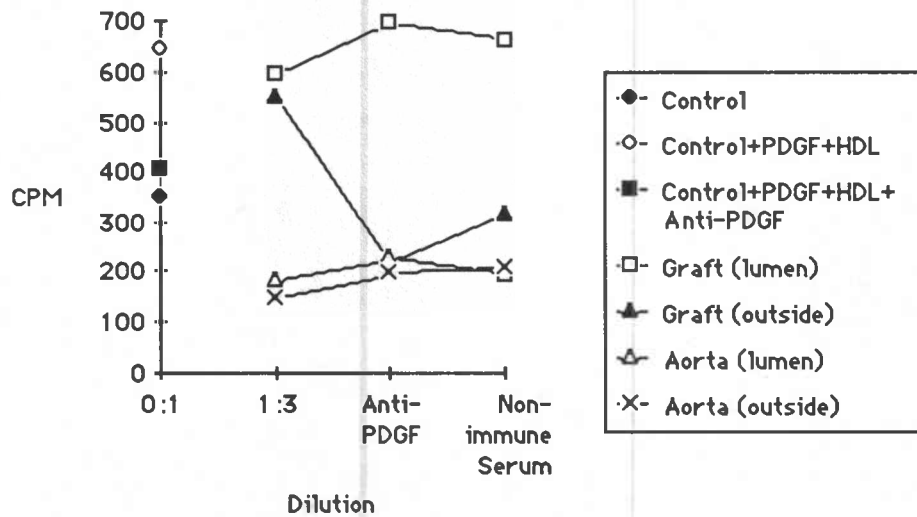
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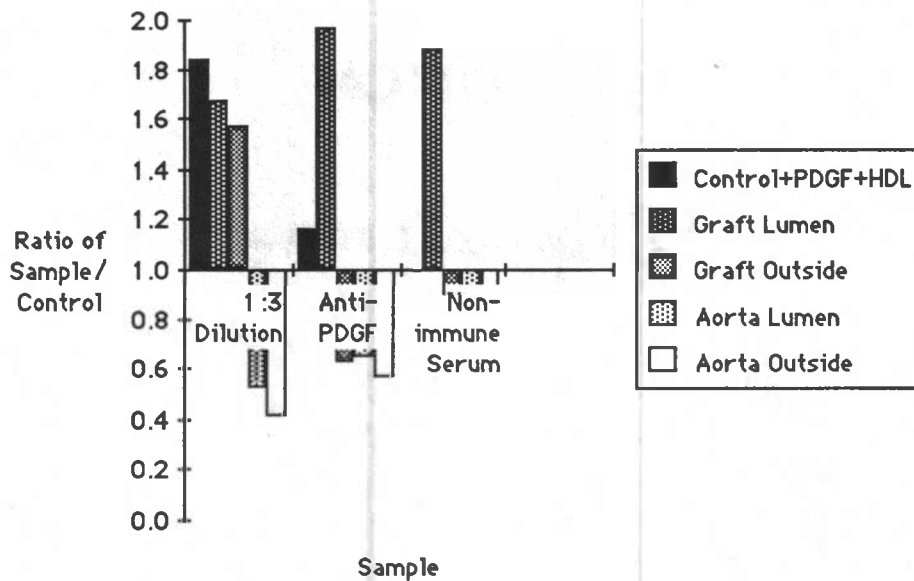
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Figure 8. Thymidine Labeling Ratios to Control for Perfusion Experiment 3 and Cultured Endothelium Conditioned Media. Ratios of the amount of thymidine incorporation into smooth muscle cells caused by dilutions of conditioned medium to the control indicate the degree of mitogenesis over the negative control. Values are shown for the conditioned medium samples in Fig. 7a. in (A) and for samples from Figure 7b. in (B).

either the 1:3 dilution alone or with the non-immune serum (Figure 9a). However, mitogenesis caused by conditioned medium from the outside of the graft is less than that for the dilution alone or the dilution with non-immune serum. Thymidine incorporation in cells cultured in conditioned medium from both the lumen and outside of the abdominal aorta exhibited a slight increase with the addition of anti-PDGF, but was lower than values for conditioned medium from both the lumen and outside of the graft. Again, the degree of mitogenesis can be expressed by relating this data to the ratio of thymidine incorporation by cells cultured in conditioned medium to that of the negative control. Inconsistencies concerning the effect of adding anti-PDGF or non-immune serum to cultured smooth cells are evident in the results. With the exception of cells cultured in conditioned medium from the lumen of the graft with added anti-PDGF or non-immune serum, all ratios were less than that of the positive control containing mitogens (PDGF and HDL), which is approximately 1.8 fold over the negative control (Figure 9b). Mitogenic ability for the positive control drops to only 1.2 fold over the negative control with the addition of anti-PDGF, but for the

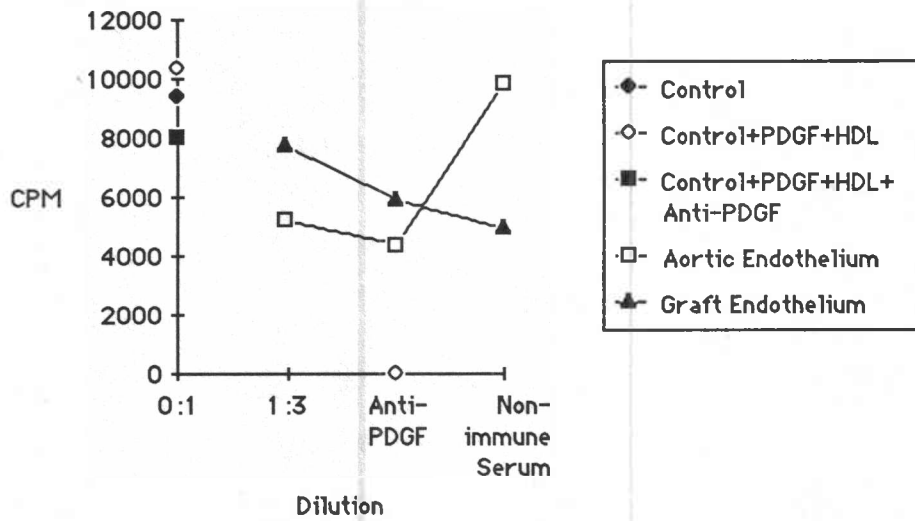


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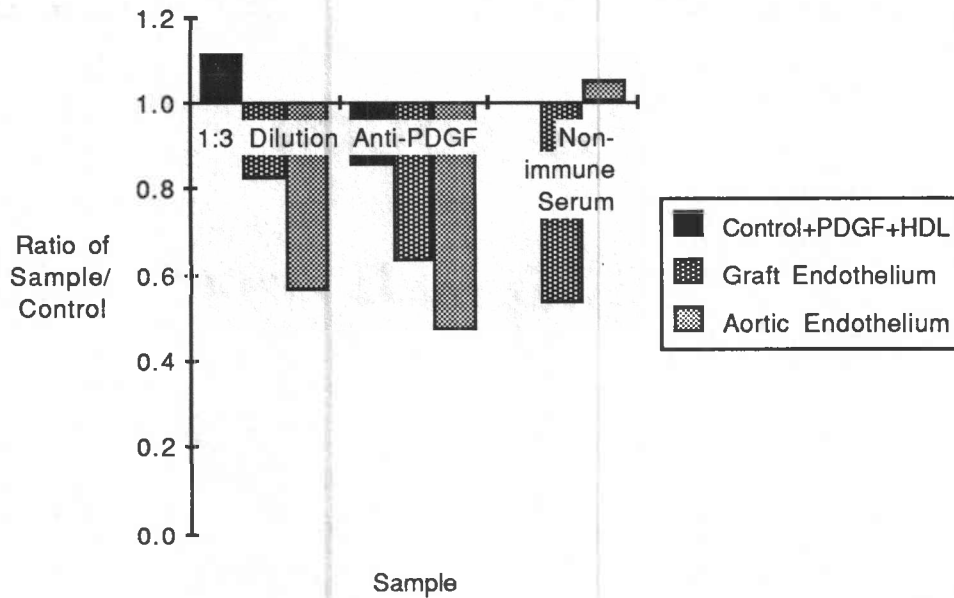


B

Figure 9. Thymidine Labeling and Ratios to Control for Mitogen Qualification in Perfusion Experiment 3. Thymidine incorporation into smooth muscle cells cultured in 1:3 dilutions of conditioned medium alone, with anti-PDGF, or with non-immune serum added, is shown for conditioned medium obtained from the lumen and outside of perfusions of a segment of abdominal aorta and an aorto-iliac graft in (A). The ratio of these values to the negative control indicates the degree of mitogenesis of the sample over or under the control (B).



A



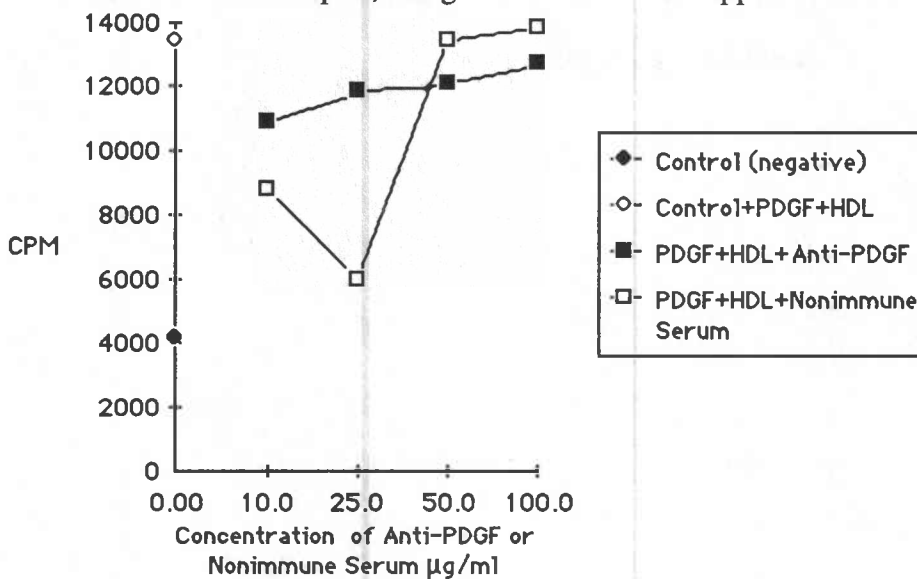
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Figure 10. Thymidine Labeling and Ratios to Control for Mitogen Qualification in Cultured Endothelial Cells Conditioned Media. Thymidine incorporation into smooth muscle cells cultured in 1:3 dilutions of conditioned medium alone, with anti-PDGF, or with non-immune serum added, is shown for conditioned medium obtained from cultured endothelial cells from the lumen of perfusions of a segment of abdominal aorta and an aorto-iliac graft in (A). The ratio of these values to the negative control indicates the degree of mitogenesis of the sample over or under the control (B).

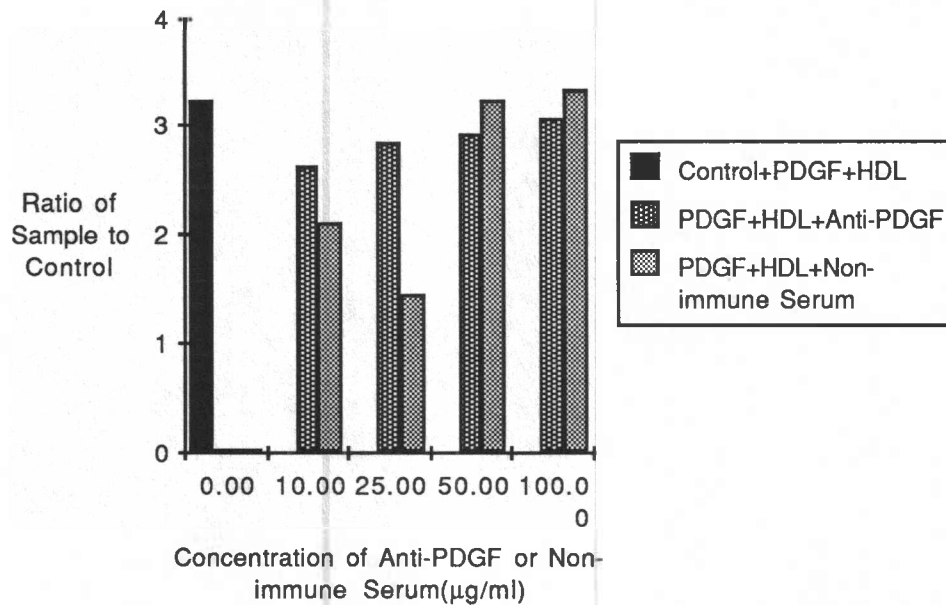
graft, luminal, conditioned medium, it increases from approximately 1.7 fold to almost 2 fold over the negative control with the addition of anti-PDGF. Other conditioned media samples exhibit mitogenic abilities which are a fraction of that seen for the negative control, with the exception of the 1:3 dilution of conditioned medium from the outside of the graft which is approximately 1.6 times as mitogenic as the negative control.

Tests using conditioned medium from the cultured aortic and graft endothelial cells were also inconsistent. Thymidine incorporation into smooth muscle cells cultured in conditioned medium from cultured graft endothelial cells decreases for the 1:3 dilution alone, the 1:3 dilution with anti-PDGF, and the dilution with non-immune serum respectively (Figure 10a). Values for cells in medium from aortic endothelial cells decreases with the addition of anti-PDGF, but increases sharply when non-immune serum is present in the medium dilution. In addition, with the exception of conditioned medium from the aortic endothelial cells with non-immune serum added, conditioned media CPM ratios to the negative control indicate that their mitogenic ability is only a fraction of that of the control (Figure 10b).

Inconsistencies in the results for the qualification of PDGF as the mitogen present in conditioned medium samples, along with the fact that it appeared that the anti-PDGF used



A



B

Figure 11. Thymidine Labeling and Ratios to Control for Mitogen Qualification in the Anti-PDGF Dose Response. (A) shows thymidine incorporation into smooth muscle cells cultured in medium containing PDGF and HDL alone, or with these mitogens and anti-PDGF or non-immune serum added at increasing concentrations. The ratio of these values to the negative control indicates the degree of mitogenesis of the sample over or under the control (B).

was capable of acting as a mitogen itself, prompted dose response tests for both the anti-PDGF and non-immune serum. Smooth muscle cells were cultured in medium with PDGF and HDL as mitogens and in increasing concentrations of either anti-PDGF or non-immune serum. Again, there were inconsistencies. As the concentration of anti-PDGF present in the medium increases, the amount of thymidine incorporation also increases (Figure 11a). With increasing concentrations of non-immune serum present in the medium, the amount of thymidine incorporation also increases, but at a concentration of 25.0 µg/ml there is a sharp drop in this value. In addition, ratios of the CPM values for each concentration to the negative control indicated that medium containing anti-PDGF at any concentration is almost as mitogenic as the control with PDGF, HDL, and no antibodies to PDGF (Figure 11b). Furthermore, the 10.0 and 25.0 µg/ml concentrations of non-immune

Table 1. Data Values for Perfusion Experiment 3. Actual values and standard variations are shown for samples from Figure 7a in the column designated as Thymidine incorporation as CPM (counts per minute). The actual values for the ratio of each of these samples to the negative control as graphed in Figure 8a is shown also, with variation calculated as the standard propagation of error.

Sample	Dilution	Thymidine Incorporation (CPM)	Ratio to Negative Control
Control		1361 ± 746	
Control + PDGF+HDL		2717 ± 474	2.00 ± 1.15
Graft			
Lumen	1:3	7984 ± 277	5.87 ± 3.22
	1:6	2362 ± 2773	1.73 ± 2.24
	1:9	3029 ± 190	2.23 ± 1.23
Outside	1:3	5853 ± 72	4.30 ± 2.36
	1:6	2482 ± 73	1.82 ± 1.00
	1:9	1447 ± 4	4.30 ± 2.36
Aorta			
Lumen	1:3	3083 ± 199	2.27 ± 1.25
	1:6	2136 ± 225	1.57 ± 0.88
	1:9	1479 ± 189	1.09 ± 0.61
Outside	1:3	2305 ± 3033	1.69 ± 2.40
	1:6	Contaminated	
	1:9	863 ± 169	0.63 ± 0.36

serum exhibit less mitogenic ability over the negative control than did the corresponding concentrations of anti-PDGF.

The variability of the results makes interpreting and drawing conclusions from them rather difficult. The fact that in most experiments only duplicate samples, rather than a large

number, were run makes statistical calculations inaccurate. Few of the experiments involved the use of conditioned medium from the same source; and if it was from the same source, an abdominal aorta for instance, it was not from the same animal and most often was not perfused for the same amount of time. The main reason we conducted procedures in this way, instead of establishing a solid base of specific data, was to get a general overview of the production of mitogens capable of stimulating growth in smooth muscle cells by graft and aortic endothelium. As demonstrated in Table 1, the standard deviations between the samples were often greater than the averages themselves. While the actual data is shown for only one experiment, other data exhibited similar variability. Likewise, the ratio of thymidine incorporation of the sample averages to the control average often has an error, calculated as the standard propagation of error, which is a substantial fraction or greater than the ratio itself. In many cases, variability in the control is large enough to make the standard propagation of error for the ratios large, even if variability between actual samples was small. Because of this, data values often overlap; and differences in mitogenic ability of two conditioned media, as shown by graphs, were not concrete but may show general trends.

## DISCUSSION AND CONCLUSIONS

### **Role of Mitogen Production by Endothelial Cells**

Mitogenesis tests indicate that cells, presumably endothelial, in the lumen of both abdominal aortas and synthetic 60  $\mu\text{m}$  PTFE grafts produce mitogens capable of stimulating growth in smooth muscle cells. It appears that graft endothelium produces a greater amount of mitogens than aortic endothelium. In most cases the amount of mitogens present in conditioned medium taken from these vessels decreases as the medium samples are removed at later time periods in the perfusions. Even so, mitogen production in the grafts remains at higher levels than seen in aortas.

This may explain observations made by Clowes, et. al. that endothelial cells are necessary for smooth muscle proliferation in healing grafts. Undisturbed arteries, in this case the abdominal aorta, have a layer of endothelium with an underlying intima of smooth muscle cells maintained in a quiescent state, which would imply that the endothelial layer in uninjured arteries does not produce mitogens. In healing grafts, where smooth muscle cells must proliferate to a confluent monolayer, it appears that the endothelial layer may function to produce mitogens for growth promotion in the underlying smooth muscle cells. Since graft perfusate does appear to be more mitogenic than aorta perfusate, the data, though highly variable, suggests that this may be the reason for the presence of an endothelial layer before smooth muscle cell proliferation in healing 60  $\mu\text{m}$  PTFE arterial grafts.

One complication with the results is that mitogen production above that of the negative controls is observed for the outsides of the aorta, normal graft, and deendothelialized graft. In the case of the grafts, one possible explanation for the presence of mitogens in medium conditioned on the outside is that mitogens may leak through the pores on the surface of the unhealed portions of the graft. The high levels seen there could be due to the fact that

conditioned medium was removed from the outside only once, after 4 or 5 hours, when the experiment was concluded, while medium from the lumen was perfused through the vessel constantly and removed at 1 or 2 hour intervals. Therefore, only a fraction of the mitogens produced in the lumen are retrieved in each collection, while medium from the outside contains all of the mitogens from the entire time period. Removing medium from the outside of the graft at the same time as the inside would provide better data for comparisons.

Results from experiments using conditioned medium taken from cultured medial smooth muscle cells, several intervals after their original plating, may indicate several things. First, smooth muscle cells plated originally confluent produce more mitogens than subconfluent cells or medium with serum containing mitogens for the first 48 hours. This supports past research suggesting the possibility that smooth muscle cells may produce PDGF<sup>3</sup>. This ability decreases with time in confluent cells, while it increases above that of positive controls at later time periods for subconfluent cells. A possible explanation for this is that confluent cells undergo contact inhibition and gradually lose their ability to produce mitogens and experience growth arrest, while subconfluent cells, having plenty of free space to grow into, increase mitogen production. Although we did not pursue this, collecting medium from subconfluent cells for a longer time period may reveal that as the cells become confluent, their mitogen-producing ability decreases, like that of cells plated at confluency.

This ability of subconfluent smooth muscle cells to produce their own mitogens *in vitro* may play an important role in their proliferation and growth during the healing process of synthetic PTFE arterial grafts. Healing 60  $\mu\text{m}$  grafts display migration and proliferation of endothelial cells, and later smooth muscle cells, from capillary ingrowth from tissues outside of the graft.<sup>3</sup> The smooth muscle cells which migrate into the developing lumen of healing grafts begin in a subconfluent state but may be stimulated to proliferate by mitogens produced not only by the graft endothelium but also by the smooth muscle cells themselves. When smooth muscle cells reach confluency in the intima of the fully healed graft, one

possibility could be that they decrease mitogen production to levels equal to that seen in normal aortas and become quiescent as seen *in vitro*. Mitogen production by subconfluent smooth muscle cells in culture may also help explain the fact that mitogen production seen in deendothelialized grafts is sometimes greater than that in aortas. Even with the endothelium stripped away and its mitogens no longer present, the subconfluent smooth muscle cells of the healing graft could produce their own mitogens. This however, conflicts with evidence that the endothelial layer is necessary for smooth muscle proliferation. An alternative possibility explaining the higher level of mitogens present in deendothelialized grafts over aortas is that mitogens produced by the removed endothelium had been adsorbed to smooth muscle cells, or more likely, the graft material itself; however, our work did not focus on this possibility, and further studies must be done to verify it.

Another phenomenon that must be explained is the fact the certain conditioned media demonstrate a mitogenic ability less than that of the negative control. Results from experiments using conditioned medium from cultured endothelial cells taken from aorta and graft segments exhibit this. While the endothelial cells have a fairly constant mitogenic ability at 1:3, 1:6, and 1:9 dilutions slightly less than the control, mitogenic ability of graft endothelium increases from approximately 0.8 times that of the control at the 1:3 dilution to 1.75 fold over the control at the 1:9 dilution. This increased mitogenesis with decreasing dilutions of conditioned medium suggests that these cultured graft endothelial cells may produce an inhibitor. While this cannot be stated with assurance without further research, endothelial cells in culture may produce an inhibitor for smooth muscle cells which must have a critical concentration to achieve inhibition. The concentration is present at a 1:3 dilution of conditioned medium, but as the dilution increases to 1:9, the concentration necessary for inhibition is no longer present. At this point, mitogens which may also be present in the medium would be able to stimulate cellular proliferation. This has not, however, been observed in the perfusion experiments, and further work must be done to

discover the possibility of the production of an inhibitor, as well as mitogens, in grafts and arteries if we are to understand their mechanism of healing.

#### **Qualification of Mitogens**

Variations in the results for experiments attempting to qualify the specific mitogen produced by graft and aortic endothelial cells limit our conclusions. Antibodies to PDGF were used because PDGF is a potent mitogen for smooth muscle cells. If PDGF was in fact the mitogen being produced by graft and aorta endothelium, the addition of anti-PDGF to the conditioned medium before adding it to test cells should theoretically eliminate or greatly reduce the mitogenic ability of the medium. Non-immune serum was used to insure that nothing in the medium lacking antibodies to PDGF, but later used to suspend the anti-PDGF, was capable of inhibiting or stimulating growth of smooth muscle cells.

The results suggest that both the non-immune serum and anti-PDGF are impure. Neither should be mitogenic, and the anti-PDGF should decrease smooth muscle cell proliferation if PDGF is present. However, in some cases, a 1:3 dilution of conditioned medium with one of the two added, exhibits more mitogenic ability than the 1:3 dilution alone. At other times, smooth muscle cells show less growth when the non-immune serum is present than if anti-PDGF is present.

Inconsistencies in results using conditioned medium led to dose response experiments with PDGF in serum free medium and increasing concentrations of either non-immune serum or anti-PDGF. Again, the results indicate problems with the antibodies and non-immune serum. Smooth muscle cell proliferation should decrease as the concentration of anti-PDGF increases, since PDGF was the mitogen used. However, as the concentration of anti-PDGF increases, so does smooth muscle cell proliferation, indicating that either the antibody or the medium containing it is mitogenic and prevents accurate identification of PDGF. Likewise,

the non-immune serum is mitogenic for smooth muscle cells, especially as its concentration rises. Therefore, before we can attempt to identify PDGF as the mitogen produced by graft or artery endothelial cells, more work must be done to purify the antibody used to eliminate mitogens.

#### **Continuing Research and Conclusions**

Several things can be done to improve the results of future experiments involving mitogenesis assays using conditioned media samples from arteries and synthetic grafts. The first thing is to use identical protocols in many experiments to accumulate a large enough data base to do adequate statistical analysis for use in establishing patterns in mitogen production. As already stated, better purification methods for antibodies to PDGF are needed in order to identify the mitogen in conditioned media. Also, often times the media was thawed and refrozen several times, altering the integrity of polypeptide growth factors. Avoidance of repeated temperature changes may help provide more consistent results. Finally, segments of grafts and arteries taken from baboons were of varying lengths. This means that the difference in total cell count of the vessel could alter the results. More cells present in one vessel could indicate a greater total mitogen production than in another segment with fewer cells, even if the cells of both segments have the same rate of production. Either segments of the same length must be used, or a correction factor correcting for the difference in total cell number should be used. Knowing the diameter and length of the vessel, a total surface area could be calculated. A cell count of a given area of the vessel, along with its surface area, could be used to calculate a total cell count. Then, results could be standardized by calculating the amount of CPM from thymidine incorporation per cell. While these steps probably will not solve all of the problems of variation, they may provide more a accurate way of comparing data.

Research concerning the role of growth factors produced by the endothelium of healing synthetic grafts continues at the University of Washington, Atherosclerosis lab, and supports the hypothesis that endothelium is necessary for the production of growth factors to stimulate smooth muscle cell proliferation. Increasing unpublished evidence supports this and points to PDGF as the mitogen. In addition, data indicating the production of an inhibitor by graft and arterial endothelium is becoming stronger, making understanding of the role of growth factors in synthetic arterial graft healing more complicated.

As already stated, the variability of the results from this research make definite conclusions impossible. However, some trends can be observed. The endothelium of healing synthetic PTFE grafts does produce mitogens for smooth muscle cells at a greater level than for normal aortas. This supports the findings that endothelial growth into the graft is necessary before smooth muscle cells appear. Further research may reveal the exact nature of the growth factors (probably PDGF) involved and a better understanding of the healing process. Knowing the exact role of growth factors in smooth muscle cell proliferation in synthetic grafts, we may be able have better control over the healing process. Furthermore, continued production of smooth muscle cell mitogens may be responsible for their overproliferation in and subsequent failure of synthetic arterial prostheses. Knowing the role of growth factors produced in the graft lumen, we may also be able to prevent or decrease the amount of arterial graft failure.

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