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Smooth Muscle Cell Phenotype and Proliferation

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Summary. **Intimal thickening, commonly observed in atheromatous lesions, mainly consists of smooth muscle cells. The intimal smooth muscle cells have vastly different characteristics from those of medial smooth muscle cells. The major differences are their rapid growth property, their expression of a scavenger pathway and the autocrine system for growth factor in the intimal smooth muscle cells. The existence of an autocrine system is proved by the secretion of smooth muscle cells derived growth factor (SDGF). Intimal smooth muscle cells are considered to be derived from medial smooth muscle cells through migration from the media and proliferation in intima *in vivo*. The change from medial to intimal smooth muscle cells was proved by the co-culture system with endothelial cells *in vitro*. Their rapid growth is observed not only in intimal smooth muscle cells but also in medial smooth muscle cells from the aorta of diabetes mellitus. These findings indicate that smooth muscle cell phenotype change occurs in various stages during the formation of atherosclerosis, and that endothelial cells play an important role in the phenotype change.**

Introduction

One of the most remarkable pathological findings in atheromatous lesions is intimal thickening. The thickened intima is mainly composed of smooth muscle cells. These smooth muscle cells are thought to accumulate first by active migration from the media into the intima, stimulated by migration factors such as platelet derived growth factor and 12-hydroxyeicosatetraenoic acid and then by proliferation in the intima. Many growth factors for proliferation of smooth cells have been reported. The intimal smooth muscle cells have different characteristics from the medial ones, and the phenotype of smooth muscle cells readily changes from a contractile to synthetic type. The smooth muscle cells that accumulate in atherosclerotic lesions are synthetic, not contractile. This change of phenotype, demonstrated in morphological studies, has been reported to be due to response of the smooth muscle cells to an altered environment. Initially the smooth muscle cells have a contractile function, and their cytoplasm is largely filled with thick and thin myofilaments.[1-3] Later, however, the cells synthesize an extracellular matrix and/or divide, and their cytoplasm contains few myofilaments, but large amounts of free ribosomes, rough endoplasmic reticulum and mitochondria.[4,5] Since smooth muscle cells must change from the contractile to the synthetic phenotype[1,6,7] for proliferation, and the pathogenesis of atherosclerosis involves the proliferation of smooth muscle cells in the intima,[8] an understanding of the mechanism controlling this change of the smooth muscle phenotype is important.

Changes in the smooth muscle phenotype can readily be observed in a primary cell culture system.[6,7] Smooth muscle cells with the contractile phenotype cannot be stimulated to divide by platelet derived growth factor, hyperlipemic low density lipoprotein or other serum-derived factors,[6] which are known to be growth factors. However, once the phenotype of the smooth muscle cells has changed to the synthetic type, the smooth muscle cells are responsive to these mitogens and undergo proliferation.[6] Cells that have migrated from explants in the primary culture or have been subcultured at less than confluent seeding densities appear to remain permanently in the synthetic state.[1,7]

Recent studies have shown different responses to atherogenic stimuli of smooth muscle cells depending on the phenotypic state.[9-13] A change from the contractile to the reversible synthetic phenotype of primary cultures of pig and rabbit aortic smooth muscle

cells was reported to be associated with a decrease in their ability to degrade ^{125}I-labeled human low density lipoprotein.[11] Furthermore, we found that smooth muscle cells explanted from intimal lesions formed by cannulation of the rabbit aorta expressed not only the low density lipoprotein receptor but also a scavenger receptor, whereas medial smooth muscle cells expressed only the low density lipoprotein receptor.[14]

As mentioned above, smooth muscle cells show changes in several characteristics, such as proliferation, contraction and lipoprotein metabolism, depending on their environment. We found that intimal smooth muscle cells grew more rapidly than medial smooth muscle cells.[14] However, it is not clear how smooth muscle cells develop their enhanced proliferation in the synthetic state, or what factor(s) is most important for the change in phenotype.

Smooth muscle cell derived growth factor

The "response to injury" hypothesis has been proposed to explain the pathogenesis of atherosclerosis.[8] Platelet derived growth factor is important in the process of the development of this condition. Platelet derived growth factor is secreted from various cells, such as endothelial cells, macrophages and smooth muscle cells in atheromatous lesions, in the arterial wall. However, a recent immunological study on human atheromatous lesions has showed that anti-platelet derived growth factor antibody inhibited only one-third of the total mitogenic activity of the conditioned medium from smooth muscle cells.[15] Therefore, the remaining two-thirds of the total activity were not due to platelet derived growth factor, suggesting the existence of some other growth factor(s).

On the basis of these considerations, we have been interested not only in the change of smooth muscle cells from the contractile to the synthetic phenotype but also in subclasses of synthetic smooth muscle cells. We have also been trying to identify the factor(s) in atheromatous lesions responsible for smooth muscle cell proliferation. As we prepare smooth muscle cells by an explant method, we observe only synthetic smooth muscle cells in our culture system. Still, we have found various phenotypes even among these synthetic smooth muscle cells.

In the present work, smooth muscle cells were explanted from the thoracic aorta of Wistar rats or Japanese white rabbits essentially by the method of Fischer-Dzoga et al.[16] From the second passage, confluent smooth muscle cells in T-75 flasks were subcultured at a 1:2 split ratio in T-75 flasks. Confluent smooth muscle cells in T-75 flasks at the 2nd to 12th passages were washed twice with 10 ml of Dulbecco's modified Eagle's medium and incubated with 10 ml of fresh Dulbecco's modified Eagle's medium. Every 2 days the medium was replaced by 10 ml of fresh Dulbecco's modified Eagle's medium, and the conditioned medium without serum was collected at the 4th to 10th passage. Pooled conditioned medium was used for experiments.

Confluent smooth muscle cells at the 3rd passage in 24-well plates were synchronized to the G_0 stage by serum depletion for 24 h. Then the cells were treated with growth factor(s), and [^{3}H] thymidine incorporation into DNA during incubation for 24 h was measured. Cells were used at the 3rd passage.

Significant mitogenic activity (defined as more than 3 times the control value) was first secreted into the conditioned medium at the 4th to 10th passage, depending on the primary culture, as shown in Fig. 1. This conditioned medium stimulated the proliferation of smooth muscle cells dose-dependently. We named the factor in the conditioned medium with this mitogenic activity the smooth muscle cell derived growth factor."[17,18] Table 1 summarizes the characteristics of smooth muscle cell derived growth factor in comparison with those of other known human growth factors. The results suggest that the smooth muscle

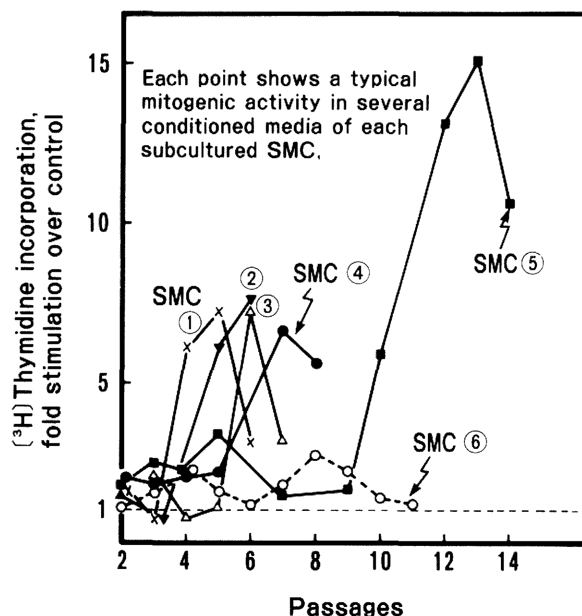

Fig. 1. The effect of a subculture of medial smooth muscle cells on production of mitogenic activity for medial smooth muscle cells. Experiments were repeated 6 times. Each experiment is shown by the number.

cell derived growth factor is distinct from the factors reported previously, such as platelet derived growth factor, fibroblast growth factor, epidermal growth factor and somatomedin C. Unlike medial smooth muscle cells, intimal smooth muscle cells secreted a substantial amount of mitogenic activity into the conditioned medium at early passages, such as the 2nd and 3rd passages (data not shown). This activity was not inhibited by a polyclonal antibody to platelet derived growth factor. Therefore, the accelerated growth property of intimal smooth muscle cells may be due to the autocrine secretion of the smooth muscle cell derived growth factor. Intimal smooth muscle cells, which grow more rapidly than medial smooth muscle cells, produced more smooth muscle cell derived growth factor than the latter.

These results suggest that the enhanced proliferation of intimal smooth muscle cells might be due to their high production of smooth muscle cell derived growth factor. Furthermore, these results indicate that synthetic smooth muscle cells have different phenotypes. For clarification of the mechanism of change from smooth muscle cell derived growth factor-non-producing smooth muscle cells to smooth muscle cell derived growth factor-producing intimal smooth muscle cells, cell-to-cell interaction in the arterial wall was considered.

The role of the endothelial cell on smooth muscle cell phenotype change

First, the effect of endothelial cells on the growth of smooth muscle cells was examined. Endothelial cells were co-cultured with smooth muscle cells in millicells. After co-culture with endothelial cells and then removal of the endothelial cells, medial smooth muscle cells were found to grow faster than control smooth muscle cells, as shown in Fig. 2. This enhanced growth persisted during two subsequent subcultures, but was not apparent during the third subculture. These results suggest that some factor(s) derived from endothelial cells changes the character of the medial smooth muscle cells and that the change persists for two passages.

Medial smooth muscle cells co-cultured with endothelial cells secreted more smooth muscle cell derived growth factor than control smooth muscle cells at the corresponding time of culture (Fig. 3). After the co-culture of macrophages instead of endothelial cells with smooth muscle cells in the same way, macrophage-conditioned smooth muscle cells were also found to produce smooth muscle cell derived growth factor. Platelet derived growth factor is considered to be produced by both endothelial cells and macro-

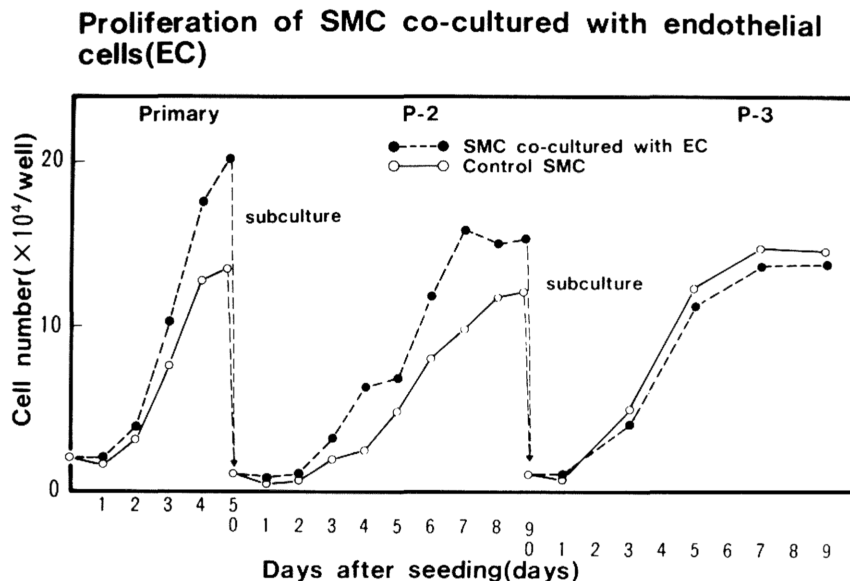

Fig. 2. The effect of endothelial cells on smooth muscle cell growth. Medial smooth muscle cells were cultured with endothelial cells. After incubation, the endothelial cells were removed, the smooth muscle cells were cultured further, and cell growth was examined. "Primary" means the first passage after removal of endothelial cells.

phages, so these results suggest that platelet derived growth factor induced the phenotypic change of smooth muscle cells. This possibility is supported by the fact that the platelet derived growth factor-B chain gene is the same as the c-sis oncogene. For investigation of this possibility, medial smooth muscle cells were cultured with platelet derived growth factor for 4 days. On further culture in the absence of platelet derived growth factor, they were found to proliferate more rapidly than control smooth muscle cells cultured without platelet derived growth factor, and this increased growth was shown to be blocked by an anti-platelet derived growth factor. These results suggest that platelet derived growth factor, the factor observed *in vitro* on cell-to-cell interaction as described above, is one of the factors stimulating smooth muscle cell derived growth factor production.

The above results can be summarized as follows: 1) Medial smooth muscle cells are in the contractile state *in vivo,* but come to be in the synthetic state when cultured. However, they start to secrete smooth muscle cell derived growth factor only after prolonged culture. Their secretion of smooth muscle cell derived growth factor is probably due to their continuous stimulation by serum and platelet derived growth factor produced by arterial cells. 2) Intimal smooth muscle cells are in a synthetic state *in vivo* and secrete smooth muscle cell derived growth factor. They secrete smooth muscle cell derived growth factor from an early stage of culture. 3) Endothelial cells or macrophages enhance changes in the characters of medial smooth muscle cells toward those of intimal smooth muscle cells with respect to growth and growth factor secretion. These findings suggest that smooth muscle cells migrate from the media to the intima and are in part modulated by platelets, endothelial cells, or macrophages to become so-called

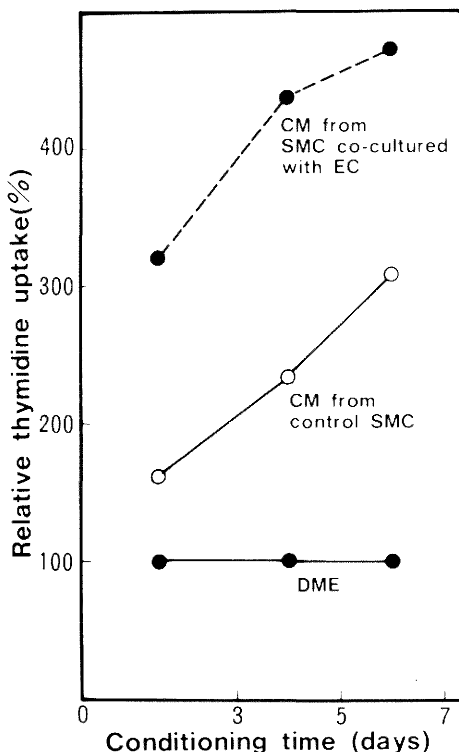

Fig. 3. The effect of a conditioned medium on smooth muscle cell proliferation. Conditioned mediums were collected from smooth muscle cell cultures with or without endothelial cells.

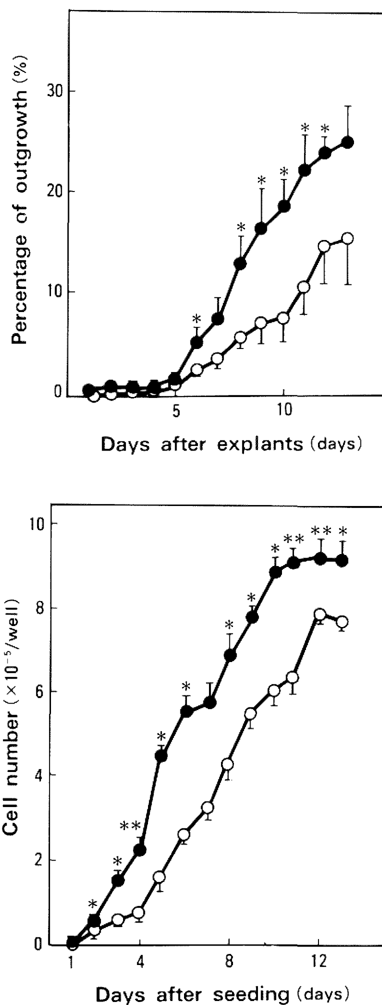

Fig. 4. Outgrowth and proliferation of smooth muscle cells from diabetic and control rat aorta.
(A) outgrowth (B) proliferation

Table 1. Comparison of growth factors

	PDGF	EGF	IGF-I	FGF	IL-1	SDGF
Smooth muscle cell proliferation	↑ (alone) ↑ (with EGF)	↗ (alone) ↑ (with PDGF)	↗ (alone) ↑ (with PDGF)	↗ (alone) ↑ (with EGF)	——	↑
Endothelial cell proliferation	——	——	——	——	↑	——
Collaboration with SDGF	+	+	+	+	−	
Molecular weight (KD)	27-33, 14, 18	6.0	7.6	16	12-20	8.7
Heat-stability (100℃, 10')	+	+	+	−	−	−
Acid-stability	+	−		−	+	
PI	9.8-10.2	4.5	8.2	4.7 (acidic)	5 (α)	4.2
				9.5 (basic)	7-8 (β)	5.9
RIA for IGF-I			+			−
Inhibition by anti-PDGF	+					−
Competition with ^{125}I-PDGF	+					−

SDGF, smooth muscle cell derived growth factor: PDGF, platelet derived growth factor: EGF, epidermal growth factor: FGF, fibroblast growth factor.

intimal smooth muscle cells. Atherosclerotic plaques must be formed by these pathological cells.

Smooth muscle cell phenotype in the medial layer

Does the synthetic phenotype of smooth muscle cells appear only in the intimal layer of atherosclerotic lesions? Atherosclerosis is considered to be caused by many risk factors. One of these risk factors, diabetes mellitus, causes quite advanced atherosclerotic lesions in young subjects. These clinical findings suggest that diabetes mellitus patients possess a different type of smooth muscle cells. Therefore, the phenotype of diabetes mellitus-smooth muscle cells was examined. In rats with diabetes induced by streptozotocin, the rate of outgrowth from aortic specimens and the proliferation of smooth muscle cells in subcultures were both enhanced (Fig. 4). These diabetic animals did not show atherosclerotic lesions, but did show intimal thickening, and the accelerated growth of their smooth muscle cells was thickening, and the accelerated growth of their smooth muscle cells was maintained for several passages in culture. As mentioned above, in earlier passages the smooth muscle cells from the intimal layer secreted more smooth muscle cell derived growth factor than smooth muscle cells from the medial layer. However, the secretion of smooth muscle cell derived growth factor by diabetes mellitus-smooth muscle cells was similar to that by control smooth muscle cells. Therefore, the enhanced proliferation of diabetes mellitus-smooth muscle cells is not dependent on the smooth muscle cell derived growth factor, and diabetes mellitus-smooth muscle cells have a different phenotype from intimal smooth muscle cells in non-diabetic animals.

Another possible factor inducing the phenotypic change is platelet derived growth factor, because

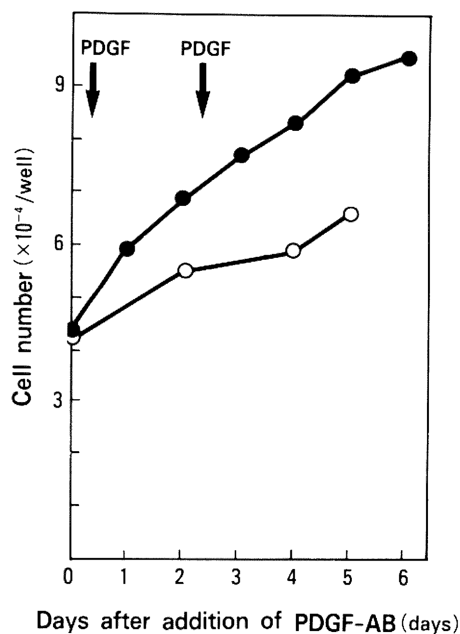

Fig. 5. The effect of platelet derived growth factor on smooth muscle cell proliferation from diabetic and control rat aorta.

platelet derived growth factor was the main growth factor in the serum used in the culture system. Therefore, the effect of platelet derived growth factor on the proliferation of diabetic smooth muscle cells was examined. Results showed that platelet derived growth factor enhanced the proliferation of diabetic smooth muscle cells over that of the control (Fig. 5).

Platelet derived growth factor binds to three kinds of specific receptors, $\alpha\alpha$, $\alpha\beta$, and $\beta\beta$, on the cell surface. The change in the response to platelet derived growth factor might be closely related with the enhanced expression of a platelet derived growth factor-β-receptor(s). Binding studies showed that the binding of platelet derived growth factor to diabetes

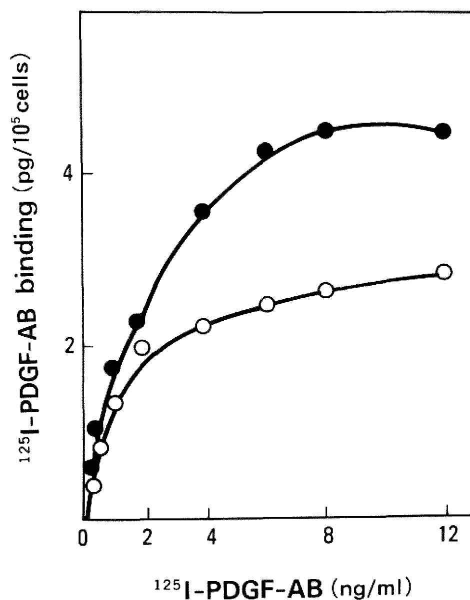

Fig. 6. Platelet derived growth factor binding to smooth muscle cells from diabetic and control rat aorta.

Exp. 1	Exp. 2	Exp. 3
C D	C D	C D

Fig. 7. Platelet derived growth factor-β-receptor mRNA expression in cultured smooth muscle cells from diabetic and control rats. C: Control, D: Diabetes mellitus, RNA: 5 μg

mellitus-smooth muscle cells was greater than that to control smooth muscle cells (Fig. 6), and Scatchard analysis showed that the enhancement was not caused by an increase in the affinity, but rather an increase in the number of the receptors.

Therefore, platelet derived growth factor-β type receptor mRNA expression was studied. Expression of the mRNA was found to be increased in cultured aortic smooth muscle cells from a diabetes mellitus compared with that in control smooth muscle cells (Fig. 7), and also in the medial layer of the aorta of diabetes mellitus rats. These results indicate that the enhanced proliferation of diabetes mellitus-smooth muscle cells is caused by the enhanced synthesis of platelet derived growth factor-β-receptor, and that the phenotype of smooth muscle cells in the diabetic aorta is different from that in the control aorta. These diabetes mellitus-smooth muscle cells may have an increased ability to form atherosclerotic lesions, and especially intimal thickening, in response to atherogenic stimuli.

Concluding remarks

These findings may be summarized as follows: The phenotype of smooth muscle cells in the arterial wall changes depending on circumstances, either in the process of migration of smooth muscle cells from the medial layer to the intimal layer, or in the medial layer itself.

These phenomena were actually observed, but the mechanism of the change is still unknown. Another problem, not mentioned in this paper, is whether the cells that migrate from the media to intima are cloned. There are reports that cells carrying a gene from atheromatous lesions had the features of intimal cells, but contradictory results have also been reported.[19]

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