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Secretion of a new growth factor, smooth muscle cell derived growth factor, distinct from platelet derived growth factor by cultured rabbit aortic smooth muscle cells

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In attempts to determine the mechanism of proliferation of arterial smooth muscle cells (SMC) in intimal atheromatous lesions, autocrine secretion of growth factors by SMC has recently received much attention. Here we report a new growth factor named smooth muscle cell derived growth factor (SDGF). Cultured rabbit medial SMC secreted SDGF for 1 week during their incubation in serum-free media only after at least 4 passages. SDGF differed from platelet derived growth factor (PDGF) physicochemically, immunologically, and biologically. The properties of SDGF also seemed different from those of other known growth factors that stimulate the proliferation of mesenchymal cells.

Atherosclerosis; Growth factor; PDGF; Autocrine secretion; (Smooth muscle cell)

1. INTRODUCTION

Proliferation of smooth muscle cells (SMC) in the arterial intima is a key event in the pathogenesis of atherosclerosis [1–3]. Some mitogen(s) are essential for the proliferation of SMC. Paracrine secretion of peptide growth factors from platelets (platelet derived growth factor, PDGF) and macrophages or endothelial cells have been reported [1–3], and more recently autocrine secretion of a PDGF-like growth factor by aortic SMC has been demonstrated only in rats [4–8].

We were interested in whether there is an autocrine system in species such as rabbits that are much more susceptible to atherosclerosis than rats. In previous studies we could not detect any mitogenic activity in cultured rabbit aortic medial SMC at the 2nd or 3rd passage. However, we report here that at later passages SMC secreted a mitogenic factor that stimulated their own growth and was distinct from PDGF.

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2. MATERIALS AND METHODS

2.1. *Materials*

Polyclonal antiPDGF antibody and purified PDGF (purity > 95%) were purchased from Collaborative Research Inc. (Lexington, MA) and R & D System Inc. (Minneapolis, MN), respectively.

2.2. *Collection of conditioned medium (CM)*

SMC from rabbit thoracic aortas were cultured as reported [9]. Confluent SMC in T-75 flasks at the 2nd passages were further subcultured by 1:2 split in T-75 flasks. CM was collected as follows. Confluent SMC in T-75 flasks at a certain passage were washed with 10 ml of Dulbecco's modified Eagle's medium (DME) twice and incubated with 10 ml of fresh DME. CM was collected every 2 days with change to 10 ml of fresh DME.

2.3. *DNA synthesis*

Confluent SMC at the 2nd or 3rd passage in 24-well plates (18 mm × 20 mm) were incubated with 1 ml of DME for 24 h, the medium was changed to 1 ml of CM (DME only for the control) containing 1 μCi [^{3}H]thymidine and the cells were incubated for 24 h. The radioactivity of the trichloroacetic acid-insoluble fraction was counted.

2.4. *Cell proliferation*

SMC at passage 2 were seeded onto 24-well plates (10000/well) with 1 ml of DME containing 10% fetal bovine serum and were incubated for 24 h to allow the cells to become

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attached to the wells. Then the cells were washed twice with 1 ml of DME and incubated with DME containing various concentrations of CM for indicated period. Cell numbers were counted in a Coulter counter.

The labeling of PDGF with ^{125}I and ^{125}I-PDGF binding to confluent fibroblast was done as described in [10].

3. RESULTS AND DISCUSSION

Significant mitogenic activity (defined as more than 3 times the control value) was first secreted into conditioned medium at the 4th to 10th passage depending on the primary culture, as shown in fig.1A. One primary culture did not secrete significant amounts of mitogenic activity within 11 passages.

Fig.1B shows typical time courses of secretion of mitogenic activity by confluent SMC from three different primary cultures from passage 4 to 6. SMC secreted the activity ~1 week after the beginning of conditioning (fig.1B).

Pooled conditioned media showed dose-dependent stimulation of DNA synthesis ([^{3}H]thymidine uptake) (fig.2), cell replication (fig.3A) and time-dependent replication (fig.3B). The mitogenic factor stimulated DNA synthesis of not only rabbit aortic SMC but also rat aortic SMC or human skin fibroblasts, although it had most effect on rabbit aortic SMC (table 1). This factor did not stimulate human umbilical vein endothelium.

Next we characterized this factor physicochemically and immunologically to determine its relations with other growth factors such as PDGF. Table 2 summarizes the effects of various treatments on the activity of this factor. This factor was stable at 4°C for at least 1 month and at 56°C for 30 min. However, two-thirds of its activity were lost during heat-treatment at 100°C for 10 min, unlike that of PDGF [11]. Freeze-thawing did not reduce its activity. Its activity was increased more than 2-fold by incubation at pH 2.5 at 22°C for 30 min, suggesting either the existence of an acid-labile inhibitor(s) of DNA synthesis in the conditioned medium or the dissociation of polymers of the factor during this treatment. On treatment with 2 mM mercaptoethanol, its activity was almost completely lost suggesting that S–S bonds are important for its mitogenic activity. It was not dialyzable through membranes with a cut-off M_r of

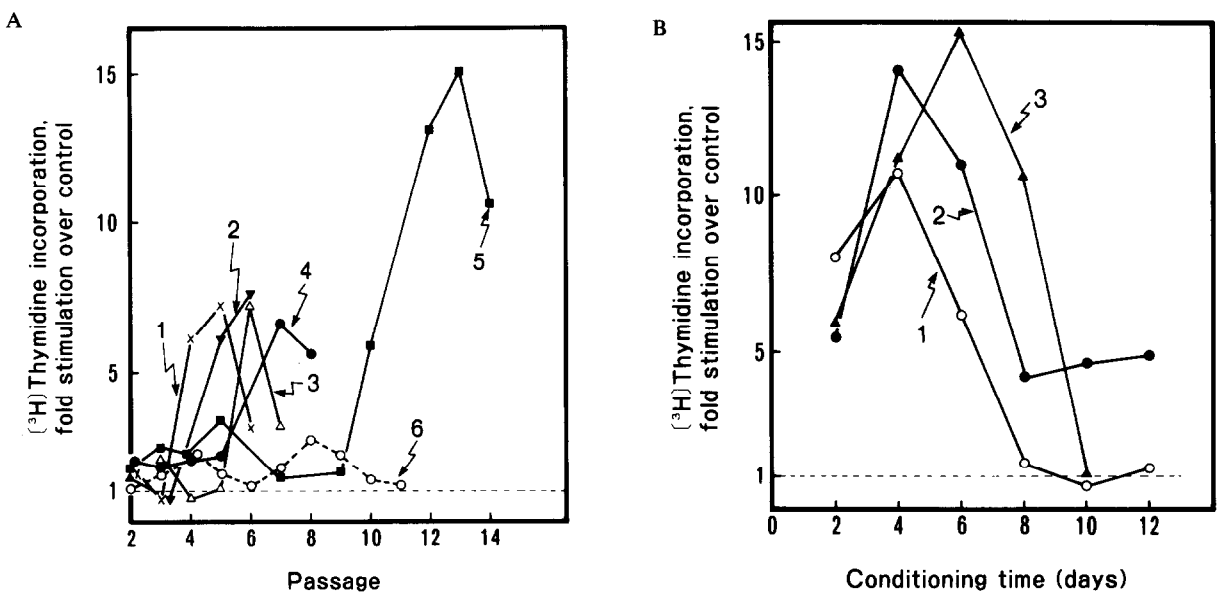

Fig.1. (A) Relationship between passages of cultured SMC and secretion of mitogenic activity. Typical mitogenic activities of several SMC from 6 different primary cultures are shown. Each point shows the highest mitogenic activity for CM from SMC of the same passage. The value for [^{3}H]thymidine incorporation in control cultures was adjusted to 1 and values are for thymidine incorporations as ratios to that of the control. (B) Time course of secretion of mitogenic activity from SMC. Typical patterns of secretion of mitogenic activity by three different cultures (4th–6th passage) are shown.

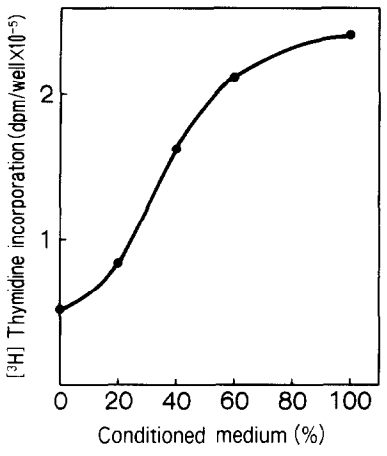

Fig.2. Dose-dependence of effect of pooled CM on DNA synthesis of SMC. CM from SMC at passages 4–12 from about 20 different primary cultures, which had mitogenic activities of more than 3-fold that of the control, were combined and stored at 4°C.

6000. In fact this dialysis slightly increased its activity, suggesting the presence of a low molecular inhibitor(s) in the conditioned medium. Its activity was almost completely lost on treatment with trypsin. The latter two results suggest that the mitogenic factor is a polypeptide. Chromatography on Sephadex G-75 gel showed that the factor had a molecular mass of ~8700 Da, which is different from those of reported growth factors, including PDGF and PDGF-like particles [11–14]. We named this factor SDGF. Details of its purification will be reported elsewhere.

Secretion of PDGF-like substances from SMC of rats has been reported [4–7]. Therefore, we tested whether this factor was immunologically similar to PDGF-like substances. As shown in fig.4A, polyclonal anti-PDGF antibody at 20 μg/ml caused ~80% inhibition of enhancement of DNA synthesis by unlabeled purified PDGF (at 5 ng/ml). However, it did not affect DNA synthesis enhanced by 100% conditioned medium, indicating that SDGF does not cross-react immunologically with PDGF. To confirm this conclusion, we tested the competitive inhibition by conditioned medium of ^{125}I-PDGF binding to human skin fibroblasts. The binding of 2 ng/ml of ^{125}I-PDGF to human skin fibroblasts was dose-dependently inhibited by unlabeled PDGF but was not inhibited by conditioned medium (fig.4B).

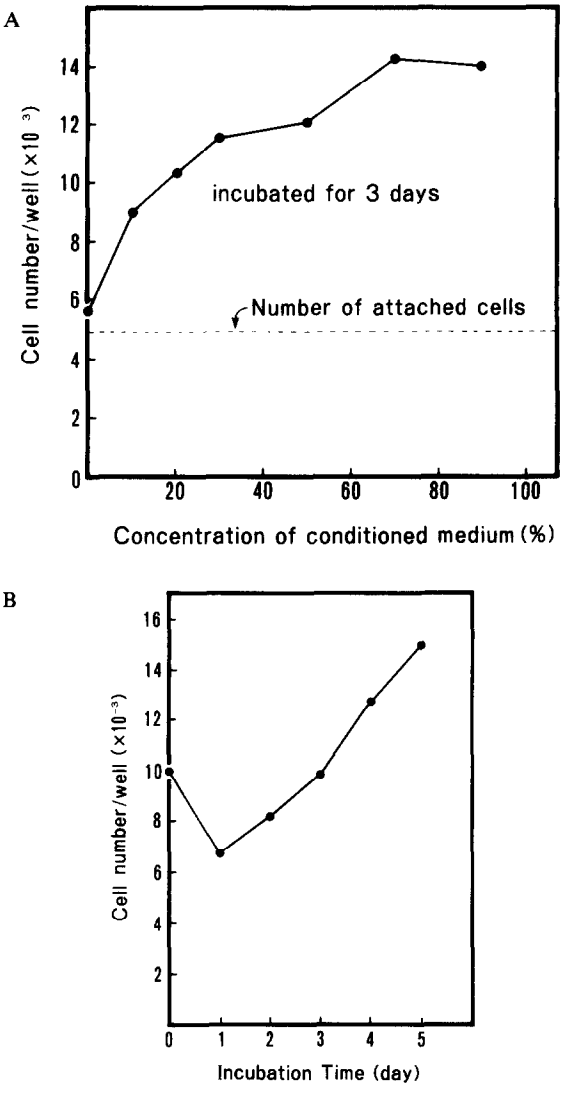

Fig.3. (A) Dose-dependence of effect of pooled CM on replication of SMC. (B) Effects of pooled CM on replication of SMC as a function of the incubation time. Cells were incubated with 30% pooled CM until day 5.

Thus the SDGF in the conditioned medium probably did not bind to the PDGF receptor.

The above results show that SDGF differs from PDGF physicochemically and immunologically. For the following reasons SDGF also seems to differ from other well-characterized growth factors such as epidermal growth factor (EGF), somatomedin-C, and fibroblast growth factor(s) (FGF), which like SDGF stimulated growth of

Table 1

Target cell specificity of conditioned medium (CM)

Target cells	DNA synthesis of CM (50%)	
	n-fold stimulation over control	Unit[a]
Rabbit SMC	6.5	2.60
Rat SMC	2.1	0.36
Human skin fibroblasts	1.7	0.68
Human umbilical cord vein endothelium	0.8	–

[a] 1 unit = mitogenic activity of 1% fetal bovine serum

Rat aortic SMC were from a 3-month-old male Wistar rat. Human skin fibroblasts were from the foreskin of a 35-yr-old man. Human umbilical cord vein endothelium was cultured as described in [18]

mesenchymal cells: the molecular mass of EGF [15,16] (6045 Da) is different from the tentative molecular mass of SDGF (8700 Da). EGF is heat-stable and acid-labile, whereas SDGF is heat-labile and acid-stable. Unlike SDGF, EGF alone did not stimulate DNA synthesis in cultured rabbit aortic SMC (not shown). No somatomedin C was detected by radioimmunoassay in the pooled conditioned medium. The reported molecular mass of FGF [17] is more than 10 kDa, which is different

Table 2

Effects of various treatments on the mitogenic activity of conditioned media

Treatment	Mitogenic activity (%)
None	100
Stored at 4°C for 1 month	112
Heated at 56°C for 30 min	99
Heated at 100°C for 10 min	35
Freeze-thawed	95
pH 2.5 at 22°C for 30 min	231
Dialyzed in membranes of 3500 cut-off	153
6000 cut-off	149
2 mM mercaptoethanol at 22°C for 30 min	6
0.01% trypsin at 37°C for 30 min	6
0.1% trypsin at 37°C for 30 min	2

Mitogenic activity was assayed by measuring [^{3}H]thymidine incorporation into DNA as described in the legend to fig.1A. 'None' means DNA synthesis in the presence of 100% pooled CM without any previous treatment of the latter

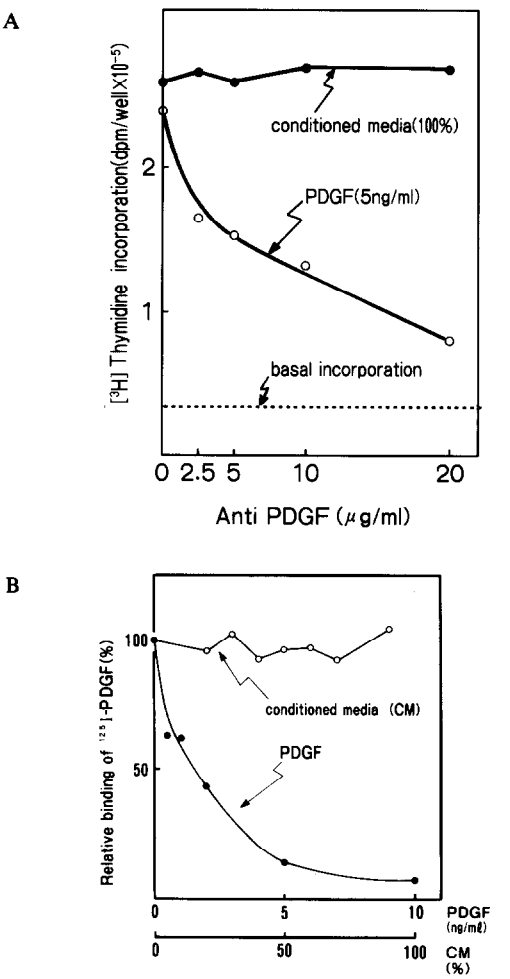

Fig.4. (A) Effects of anti PDGF antibody on DNA synthesis of SMC stimulated by pooled CM or PDGF. AntiPDGF antibody was added to the culture at the same time as pooled CM (100%) or PDGF (5 ng/ml). The dotted line shows the radioactivity in the absence of any growth factor. (B) Effects of pooled CM and unlabeled PDGF on the binding of ^{125}I-PDGF to human fibroblasts (4th passage).

from the tentative molecular mass of SDGF (8700). FGF is acid-labile whereas SDGF is acid-stable. FGF stimulates DNA synthesis of human umbilical cord endothelium, unlike SDGF (table 2). These indirect lines of evidence suggest that SDGF is a new growth factor, not previously reported.

The pathogenic significance of SDGF is unknown. Possibly SDGF is secreted in vivo by intimal SMC which migrate from the aortic media

and replicate many times. Successive passages of the SMC in culture could mimic this in vivo process in atheromatous lesions. In preliminary experiments we found that cultured intimal SMC from atheromatous lesions of rabbit aortas induced by the balloon-cathether method secreted a mitogenic factor(s) at the 2nd and 3rd passages, when cultured medial SMC still did not secrete SDGF. Thus SDGF secreted in an autocrine manner may be important for proliferation of the SMC in atheromatous lesions. We are now studying intimal SMC.

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