Isolation of a growth-stimulating agent from human skin fibroblast cultures

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Summary. Cell-free supernatants were harvested from cultures of human skin fibroblasts, were applied on to DEAE-cellulose columns, and the first fraction eluted with phosphate-buffered saline contained the growth-stimulating agent. The eluted fraction was then passed through a series of amicon membranes. After passing through PM-10, the filtrate stimulated growth of bovine vascular endothelial, canine myocardial, and human mammary carcinoma cells.

Cell culture has been used as a productive approach to understanding cellular functions, including experiments by several investigators to study electrical properties of myocardial cells[1-5]. Endothelial cells constitute the inner lining of the blood vascular system. Because of their location at the interface between blood and tissue, they are the chief elements involved in the permeability of blood vessels[6,7]. Abnormalities of endothelial cell structure and function are prominent in the pathology of a number of diseases of blood vessel walls such as thromboangitis[8] and microangiopathy[9].

Several factors that promote the growth of cells in culture have been identified. One of the most potent is the fibroblast growth factor which stimulates the growth of a variety of mesoderm-derived cells[10]. Biopsies of normal human skin when explanted with 10% normal human serum with 100 units/ml penicillin and 100 µg/ml streptomycin proliferate and produce a monolayer of fibroblasts (figure 1). The present studies examined the cell-free spent media harvested from these cultures. A growth stimulating activity was observed with bovine vascular endothelial cells (BVEC), human mammary carcinoma cells (MC) and canine myocardial ventricular cells. Filtration of the spent cell-free media through various molecular weight sieves, a growth stimulating agent with a molecular weight of approximately 10,000 dalton was isolated.

Bovine vascular endothelial cells (BVEC) were initially obtained from Dr Fenelau, were carried in our tissue culture laboratories as described[11]. Human mammary carcinoma cell lines GM, PMA and MW were established in these laboratories[11-13]. Biopsies were obtained from a 72-h infarcted devascularized, interpapillary wedge of approximately 16% of the left ventricle. This was achieved by surgical ligation of the obtuse marginal branches of the circumflex artery supplying this section[14]. These explants were obtained from Dr Roper immediately after biopsy, and cultured in a medium consisting of 40% M 199[15], 45% Earl balanced salt solution and 15% dog serum, with 100 units/ml penicillin and 100 µg/ml streptomycin[16]. Under these conditions, the myocardial explants proliferated yielding monolayers of myocardial cells (figure 2, A and B).

72 h after infarction, biopsies were taken from the infarcted area, border zone and from a normal location. 24 h in culture medium, explants from the infarcted area showed heavy, those from border zone slight and those from normal area lacked in leukocytic-lymphocytic population. These cells were removed when the explants were refed. 72 h later, all cultures containing explants from normal areas, and the majority from the border zone contained bacterial contamination and discarded. The lack of bacterial contamination led to the use of explants from the infarcted area.

The cells from any of the mentioned sources were first washed 3 times with Hanks balanced salt solution (HBSS). The cells were plated at the appropriate density and were allowed to stand for about 3 h in the MEM used for medium before changing to the medium containing the growth factor. Counts of viable and nonviable cells determined by trypan blue exclusion, were taken on triplicate samples after 3 test days. When necessary, corrections were made for plating efficiency (determined to be about 67%) with BVEC. Test on cell lines other than BVEC were carried out in an analogous manner. Serum levels were diminished by ½ or ¼ from the normal amounts that produced optimal in-vitro growth of these cell-lines. Pro-

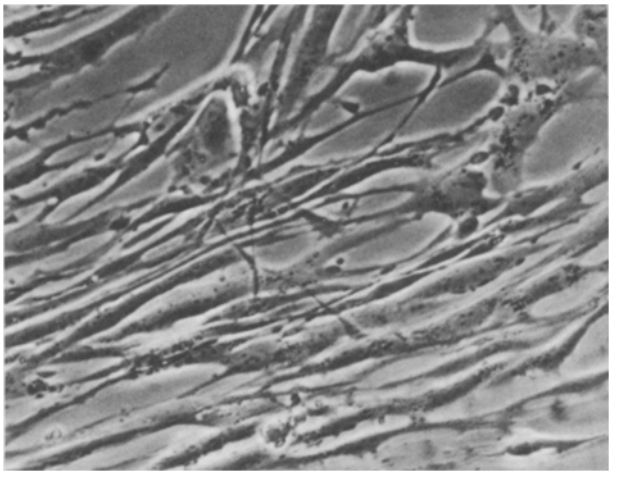


Fig. 1. Normal human skin fibroblasts monolayer grown for 72 h after 3rd transfer as described in text. × 80.

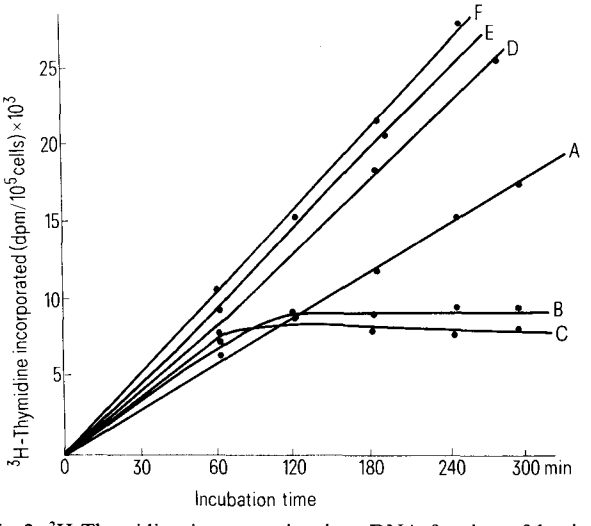
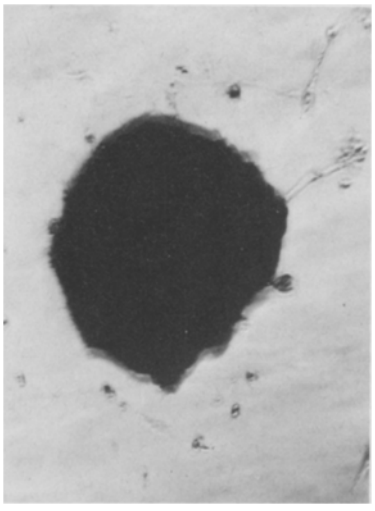


Fig. 2. ^{3}H-Thymidine incorporation into DNA fraction of bovine vascular endothelial cells: 5 ml of 10^7 per ml of the viable cells were incubated in Eagle's-MEM supplemented with (A) 10% fetal calf serum, (B and C) with 5 and 10 µg per ml of bovine serum albumin, and (E, E, and F) with 5, 10 and 20 µg per ml of the growth-stimulating agent(s), respectively. At time intervals aliquots of 10^5 cells were removed and pulsed for 60 min with ^{3}H-thymidine. The incorporation of the label into DNA was determined as described under methods[18].



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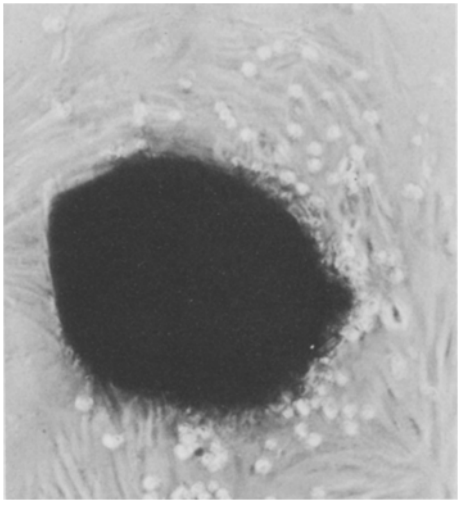

b

Fig. 3. Canine myocardial ventricular explants 72 h after adherence to glass. *A* Explants in Eagle's-MEM supplemented with fetal calf serum. *B* In Eagle's-MEM supplemented with 5 µg/ml of human skin fibroblast growth stimulating agent(s).

tein concentration of the growth stimulating agents was determined using the method of Lowry et at.[17] with bovine serum albumin as a standard.

^{3}H-thymidine incorporation into DNA was determined at 30-min intervals during incubation of 10^5 viable cells/ml of Eagle's MEM in presence and absence of the growth factor, as described by Delecluse et al.[18].

All cell lines tested for response to the growth stimulating agent, were plated in the standard growth medium from stock cultures into wells of Falcon Micro Test II plates at subconfluent densities ranging from 10,000 to 40,000 cells/well. Following cell attachment after 6–24 h, medium was changed with the addition of the growth stimulating agent (5–500 µg/ml) to the test cultures. The cultures were refed every 72 h with growth factor containing medium. At time intervals the wells were photographed, and cell counts and viability were determined, the latter by trypan blue dye exclusion. Control cultures were treated identically except for the omission of the growth agent.

Results and discussion. Cell-free supernatans harvested from 72-h cultures of normal human skin fibroblasts were fractionated on DEAE-cellulose, and the 1st fraction eluted with phosphate-buffered saline contained the major growth stimulating activity. This latter fraction then successively

Isolation and partial purification of the human fibroblast growth stimulating factor. Cell-free supernatants were harvested from confluent cultures of human skin fibroblasts and examined for growth stimulating activity after various stages of purification

Purification step	Bovine vascular endothelial cells			Human mammary carcinoma cells		
	N	Control (%)	P	N	Control (%)	P
Supernatant	5	142.5± 7	0.05	8	138.7± 7	0.01
DEAE-cellulose fraction I	4	187.2± 8	0.01	6	217.8± 9	0.01
Filtrate from amicon membrane						
XM-100	3	225.8± 9	0.01	6	289.1±11	0.005
XM-50	3	277.3±10	0.01	6	347.2±14	0.005
PM-30	3	363.3±19	0.005	6	398.3±24	0.005
UM-20	3	378.4±21	0.005	6	463.7±28	0.001
PM-10	3	405.6±25	0.005	6	496.1±32	0.001

P, probability computed as described by Dixon and Massey[19]. N, number of experiments. 5 days after plating, bovine vascular endothelial cells or mammary carcinoma cells were incubated for 24 h in medium containing 5 µg/ml of the growth stimulating factor assessed as protein[16]. The results are expressed as cpm ^{3}H-thymidine incorporated during the terminal h per µg DNA as percentage of control. Typical control values taken as 100% incorporation were 250, and 450 cpm per µg DNA for bovine vascular endothelial and mammary carcinoma cells, respectively. The results represent the mean ± SD.

Table 2. Viability of cells in Eagle's MEM supplemented with the growth stimulating factor. Cells were plated as described in "Materials and methods" with or without the addition of the growth stimulating factor. Viability were measured by trypan blue dye exclusion. Viability is expressed as the percentage of viability in presence of the growth stimulating factor compared to that of controls

Cell line	Control*	Percent of viability at the following concentrations of the growth stimulating factor			
		5 µg/ml	100 µg/ml	200 µg/ml	400 µg/ml
Bovine vascular endothelial	100	395.1±20	570.3±32	1575.4±50	3758.3±65
Canine myocardial cells	100	174.3± 9	227.6±10	735.6±25	2275.9±39
Mammary carcinoma					
GM	100	487.5±32	595.3±21	1476.9±47	3285.8±47
PMA	100	517.2±37	765.3±42	1985.9±53	4165.9±68
MW	100	468.9±29	515.3±35	1673.8±52	3685.9±66

Typical control values taken as 100% incorporation were 250, 150 and 450 cpm for bovine vascular endothelial, canine myocardial and mammary carcinoma cells, respectively. The above results represent the mean ± SD of 5 separate experiments.

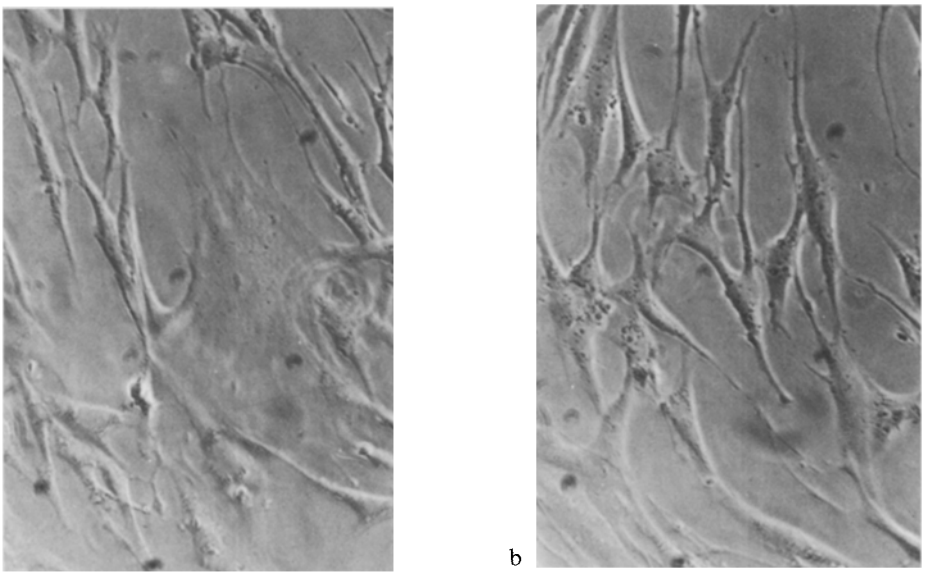

Fig. 4. Human mammary carcinoma cells on their 156th transfer. 72 h after subculture into *A* Eagle's-MEM supplemented with fetal calf serum (50%), and *B* Eagle's-MEM supplemented with 5 µg/ml of human skin fibroblast growth-stimulating agent(s).

passed through a series of amicon membranes. The filtrates from XM-100, XM-50, PM-30, UM-20 and PM-10 contained the growth stimulating activity. The data summarized in table 1 indicate that successive filtration through the amicon membranes produced progressive increase in the growth stimulating activity of both bovine vascular endothelial and human mammary carcinoma cells. When examined on polyacrylamide gel electrophoresis, the filtrate from PM-10 yielded 1 major and 1 faint protein band (unpublished observations). The amicon membranes retained large amounts of proteins which had no effect on cellular growth. It is suggested that the described procedure, concentrated and increased the specific activity of the growth stimulating agent(s).

When bovine vascular endothelial cells (BVEC) were incubated in Eagle's MEM supplemented with 10% heat inactivated fetal calf serum, and at time intervals pulsed for 60 min with ^{3}H-thymidine, curve A in figure 2 showed progressive incorporation of the label into DNA fraction of BVEC. If fetal calf serum was replaced with 5 or 50 µg/ml of bovine serum albumin (BSA), curves B and C (figure 2) showed a progressive increase during the 1st h, then a constant incorporation of ^{3}H-thymidine into DNA fraction of BVEC. Replacing fetal calf serum with 5, 10 or 20 µg/ml of the human fibroblast growth-stimulating agent(s) produced progressive increase in the incorporation of ^{3}H-thymidine into the DNA fraction of BVEC. It is suggested that the growth stimulating agent(s) promoted and sustained the growth of BVEC.

70 h after they adhered to glass, canine myocardial ventricular explants in Eagle's MEM supplemented with 10% fetal calf serum produced very few cells (figure 3, A), whereas in Eagle's-MEM supplemented with 5 µg/ml of the human fibroblast growth-stimulating agent(s), the explants produced heavy mixed population of round and fibroblast-like cells (figure 3, B).

The data summarized in table 2 indicated that the growth-stimulating agent(s) effect on bovine vascular endothelial, canine myocardial, or mammary carcinoma cells is concentration dependent.

The described experiments were focussed on the effects of growth-stimulating substance(s) on bovine vascular endothelial and canine myocardial cell cultures. It is suggested that the agent(s) contribute to revascularization of myocardial infarcted areas. The capacity to stimulate blood vessel growth may be also a fundamental property of neoplastic cell populations as compared with their normal tissues of origin. The significance of neovascularization for tumor growth and the biological mechanisms involved in this process is of current interests[20,21]. The described growth-stimulating agent(s) isolated from cultures of human skin fibroblasts, also stimulated growth of human mammary carcinoma cells (figure 4) and may contribute to in-vivo vascularization of the tumor.

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