Growth of Rabbit Aortic Smooth-muscle Cells Cultured in Media Containing Diabetic and Hyperlipemic Serum

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SUMMARY

Smooth-muscle cell cultures were grown from thoracic aortas of normal and diabetic rabbits. The effect of diabetic rabbit serum on the growth of these cultures was studied both in the first, rapid-growth phase and the following, more "stationary" phase of growth. Control experiments were carried out on normal sera to which glucose had been added. The concentrations of cholesterol, phospholipid, and triglyceride were the same in both normal and diabetic sera.

Media containing diabetic serum stimulated the growth of cultures significantly in both phases ($2p<0.01$). This occurred in experiments utilizing cells from normal as well as from diabetic rabbits. Control media containing normal serum with added glucose had no such effect. The growth-promoting effects of diabetic serum and of hyperlipemic serum from nondiabetic rabbits were of the same order of magnitude.

Autoradiographic studies showed that the number of ^{3}H-thymidine-labeled cells increased significantly after culture in diabetic serum ($2p<0.005$).

Cells cultured from the very beginning in diabetic serum or normal serum with added glucose were significantly larger than cells grown in control serum ($2p<0.05$ and $2p<0.01$, respectively). Cells grown in hyperlipemic serum were significantly smaller than those grown in normal serum ($2p<0.01$).

These results indicate that diabetic serum contains a factor or factors that stimulate the arterial medial cell to excessive growth. This factor is not glucose, insulin, or lipid. The results may be of relevance for the understanding of human diabetic macroangiopathy. DIABETES *25*:207-15, March, 1976.

Diabetic angiopathy has been considered primarily a pathologic phenomenon of the capillaries and other small blood vessels throughout the body. Epidemiologic, clinical, and autopsy studies have established, however, that abnormalities of the heart and the larger vessels are also very frequent among long-term diabetic patients.[1-3] The nature of this macroangiopathy is not clear today. Many investigators consider it to be identical to atherosclerosis as it occurs in nondiabetic patients, but several facts indicate that at least some components of the large-vessel disease in diabetic patients are specifically diabetic phenomena.

Histologic differences between large coronary vessels from diabetic and nondiabetic patients have been described previously.[4] The linear calcifications of the leg arteries disclosed by x-rays seem to be a characteristic diabetic feature, at least in young and middle-aged persons.[5-7] Large-vessel abnormalities in diabetic patients are related to the duration of the disease, although not so clearly as retinopathy or nephropathy,[3] and some correlation between macro- and microangiopathy has been shown in series of diabetic patients.[2,4,8]

Moreover, epidemiologic studies have indicated that myocardial infarction is two to three times more frequent in diabetic than in nondiabetic patients and that primary mortality is two to three times higher in diabetic patients.[9]

Numerous morphologic studies of human and other primate atherosclerotic arteries—only a few of which are cited here—have revealed that the smooth-muscle cell is the main cell involved in the development of atherosclerotic lesions.[10-13] More complete consideration of this subject may be found in recent reviews.[14,15]

Tissue culture studies of smooth muscle cells have shown that the arterial medial cell will respond with increased proliferation when hyperlipemic serum is added to the media.[16-21]

In the present study we have attempted to elucidate certain aspects of the diabetic macroangiopathy in an in-vitro model first reported by Kao et al.[16] and utilized by Fischer-Dzoga et al.[18,22] for the study of atherosclerosis. We have investigated the growth rate and the morphology of aortic smooth-muscle cells

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from normal and diabetic rabbits, in primary explants incubated in normal, diabetic, and hyperlipemic serum, as well as in normal serum with added glucose.

The results indicate the presence in diabetic serum of a factor that stimulates the growth of aortic smooth-muscle cells.

MATERIALS AND METHODS

Explants. Primary explants of medial smooth-muscle cells from rabbit aortas were obtained with a slight modification of the technic described by Fischer-Dzoga et al.[18] The preparation of the explants was conducted under sterile conditions. Thoracic aortas were collected from either normal or insulin-treated alloxan-diabetic male New Zealand rabbits (2-3 kg.). The aorta was transferred to a Petri dish and immersed in Hank's balanced salt solution at room temperature. Adherent tissue and the adventitia were removed from the aorta, which was then opened longitudinally. The intima was carefully stripped off, leaving the intermediary medial muscle layer. Approximately 100 circular explants 2 mm. in diameter were obtained from each aorta with a skin biopsy punch.

Each explant was incubated in 3 ml. of growth medium in a 30-ml. plastic flask (Falcon) placed horizontally. The tissue was incubated at 37° C. with 5 per cent CO_2 in 95 per cent air. The growth medium consisted of Basal Medium of Eagle (BME) with Hank's balanced salt solution, supplemented with serum and containing no antibiotics. The medium was changed twice a week. A few days after the explants attached to the bottom wall of the flask, intimal growth appeared from their edges. Growth was observed microscopically at first and then by measurement with the naked eye. The cells formed a monolayer in the periphery and multilayers in the central part of the cultures.

Sera for the growth media. Normal rabbit serum was obtained commercially (Grand Island Biological Co., Grand Island, N.Y.). Diabetic serum was obtained from two groups of male diabetic New Zealand rabbits (1-3½ kg.). The animals were injected intravenously with alloxan (200 mg./kg. body weight) after fasting for 12 hours. The animals were treated with zinc-protamin-insulin (Lilly). Glucosuria, ketonuria, and the 24-hour urine volume were followed daily. All the diabetic rabbits had a fairly constant glucosuria and no ketonuria. They gained weight throughout the experimental period. Urine volume, which varied from 100 to 400 ml. per 24 hours, often corresponded to about 10 per cent of body weight, constituting definite polyuria.

Blood was collected from nonfasting animals by heart puncture under sterile conditions, performed for the first time two weeks after the injection of alloxan. Insulin was withheld 24 hours prior to each bleeding. Rabbits weighing more than 2½ kg. were used not more than three times as blood donors, with a restoration period of at least two weeks between bleedings. Between 15 and 25 ml. of whole blood was taken each time. The serum was collected over a period of four to six weeks from five to seven rabbits and was refrigerated in a sterile bottle until use. Only serum from those rabbits with a blood glucose concentration above 250 mg./100 ml. was included in the serum pool.

Hyperlipemic serum was obtained from nonfasting male nondiabetic New Zealand rabbits (3-4 kg.) that had been fed the atherogenic diet of Liskowski-Mikushin (1 kg. = 956 gm. of Dixie rabbit chow + 40 ml. sunflower oil + 4 gm. cholesterol) for more than one month. Blood was collected by sterile heart puncture.

Chemical methods. Glucose was estimated by means of a glucose oxidase method (Glucostat, Worthington Biochemical Corp., Freehold, N.J.). Cholesterol and triglycerides were determined in an AutoAnalyzer.[23] The phospholipids were measured according to the technics described by Bartlett, Folch, and Lees.[24-26]

Experimental procedure. Two groups of experiments were performed, one focusing on the initial rapid growth period (first five weeks), and the other on the following stationary period.

In the *rapid growth phase experiments*, the cultures were grown in the experimental media from the very beginning. The BME was supplemented with three types of serum in the following concentrations:

1. Ten per cent normal rabbit serum (final glucose concentration = 101 mg./100 ml.).
2. Five per cent diabetic rabbit serum + 5 per cent normal rabbit serum (final glucose concentration = 115 mg./100 ml.).
3. Ten per cent normal rabbit serum with glucose supplementation (final glucose concentration = 127 mg./100 ml.).

Each serum was studied in 11-21 cultures, grown from explants from normal as well as insulin-treated alloxan-diabetic rabbits.

Measurements were performed only on cultures in which initial growth had occurred within the first two weeks. The growth rate of the living cultures was followed by determination of their area after two,

three, and four weeks of incubation. A grid with squares measuring 6.25 mm.^{2} each was placed on the outside of the tissue-culture flask directly over the growing culture. The number of points in the grid falling on the cultures was counted with the grid placed at three different positions. The area of each culture in square mm. was calculated by multiplying the average number of counted points by 6.25.

In the *stationary growth phase experiments,* the cultures were grown in HBME containing 10 per cent normal rabbit serum until the stationary growth phase was reached (five weeks). At this point the flasks were divided into five experimental groups and one control group, each consisting of 10 cultures. The 60 cultures were selected and paired so that each of the 10 cultures in the control group corresponded in size to one of the 10 cultures in each experimental group. The control group was kept in the same medium used in the preceding rapid-growth phase, HBME with 10 per cent normal rabbit serum (final glucose concentration = 101 mg./100 ml.). The five experimental groups were grown in HBME with the following additions:

1. Five per cent diabetic rabbit serum + 5 per cent normal rabbit serum (final glucose concentration = 115 mg./100 ml.).
2. Five per cent diabetic rabbit serum + 5 per cent hyperlipemic rabbit serum (final glucose concentration = 112 mg./100 ml.).
3. Five per cent hyperlipemic rabbit serum + 5 per cent normal rabbit serum (final glucose concentration = 101 mg./100 ml.).
4. Five per cent normal rabbit serum with addition of glucose + 5 per cent hyperlipemic rabbit serum (final glucose concentration = 112 mg./100 ml.).
5. Ten per cent normal rabbit serum with glucose supplementation (final glucose concentration = 127 mg./100 ml.).

Point counting (as descibed above) was used to determine the area of the cultures in the stationary growth phase after four, eight, and 12 days of exposure to the various media.

Cell proliferation also was measured by the percentage of cells that incorporated ^{3}H-thymidine as identified by autoradiography. Four days after beginning the exposure of the cultures to the various sera, the growth media were enriched with 0.2 μCi./ml. ^{3}H-thymidine (New England Nuclear, Boston, Mass.). The three-day labeling period was followed by a growth period in nonradioactive media until the cultures were fixed in situ with formalin at day 12.

The bottom of each plastic flask was cut out and the formalin-fixed cultures rinsed in water. The slides were dipped briefly three times in nuclear track emulsion NTB2 (Kodak) at 45° C., then placed upright for 30 minutes at 28° C. in an atmosphere of 80 per cent humidity before being transferred to light-tight boxes containing a drying agent and refrigerated for about two weeks. Dektol was used for developing at 17° C. for two minutes. The slides were then rinsed briefly in water, fixed for three minutes, and finally rinsed in water for 30 minutes. The slides were stained with hematoxylin-eosin. The number of ^{3}H-thymidine-labeled and nonlabeled cells within a grid was counted in two or more cultures prepared for autoradiography from each group. The counting was performed on the peripheral monolayer of the cultures at a magnification of 200 X. A total of 200-450 nuclei was counted from each group.

Cell size was determined after completion of the experimental phase. The growth media were removed and the cultures washed in phosphate buffer. The cells were fixed in Bouin's solution or formalin. The bottom of each plastic flask was cut out and handled as a regular histologic preparation. Gomori's aldehyde-fuchsin method was applied to the Bouin-fixed tissues. The cells fixed in formalin were stained with hematoxylin for quantitative measurements and oil red O for lipid.

The size of the smooth-muscle cells was estimated by point-counting the peripheral monolayer in two or more cultures from each group. At the working magnification each square in the grid covered 2,500 μ^2.

The measuring procedure was the following: the cytoplasmic area within the grid was calculated by counting the number of points falling on the cells. The average figure obtained in three different positions of the grid was multiplied by 2,500. The number of nuclei was also determined within the frame of the grid and mean size of the cells calculated.

Statistical methods. Paired comparison was performed by means of Wilcoxon's signed-rank test.[27] Proportions between labeled and nonlabeled cells were evaluated statistically with a χ^2 test.[27] Wilcoxon's test was applied for comparison of nonpaired samples.[28] A 2-p value of less than 0.05 was used as the limit of significance.

RESULTS

The concentrations of cholesterol, triglycerides, phospholipids, and glucose in the pools of diabetic, hyperlipemic, and normal serum are shown in table 1. The glucose concentration in the second pool of serum

TABLE 1

Lipid and glucose values of the normal (control), diabetic, and hyperlipemic sera

Sera	Exp.	Cholesterol	Triglycerides	Phospholipids	Glucose
Control serum	1.	93 mg.%	80 mg.%	187 mg.%	122 mg./100 ml.
	2.	86 mg.%	86 mg.%	160 mg.%	120 mg./100 ml.
Diabetic serum	1.	50 mg.%	— n.d.	93 mg.%	317 mg./100 ml.
	2.	64 mg.%	177 mg.%	152 mg.%	425 mg./100 ml.
Hyperlipemic serum	1.	1,167 mg.%	43 mg.%	525 mg.%	62 mg./100 ml.
	2.	1,154 mg.%	84 mg.%	569 mg.%	72 mg./100 ml.

from diabetic rabbits is somewhat higher than that of the first. The concentration of lipids in the diabetic serum is at about the same level as in the normal serum.

Rapid-growth-phase experiments. After four weeks the outgrowth of the smooth-muscle cells from the explants from normal rabbits was significantly larger in the cultures exposed to the diabetic serum than in cultures grown in normal serum or in normal serum with added glucose. There was no significant difference between the growth-promoting effect of normal serum with and normal serum without added glucose (figure 1, table 2).

The outgrowth of the smooth-muscle cells from explants from diabetic rabbits was significantly greater in diabetic serum than in normal serum with or without added glucose (figure 2, table 3). The growth rates differed in the two experiments using normal serum with added glucose: in one of them the growth rate was the same as in the control experiments with normal serum, but in the other it was lower (table 3, experiments 1 and 2).

Stationary-growth-phase experiments. Figure 3 shows the areas of the cultures grown in the various experimental media as a percentage of the size of those grown in the normal control medium.

On the average, control medium with added glucose caused a depression of growth, but this result was not statistically significant. In contrast, all other experimental media led to a considerable increase in growth, amounting to 60-80 per cent on day 12 (2-p values between 0.01 and 0.05), although there was no difference between the effect of the various types of experimental media (figure 3).

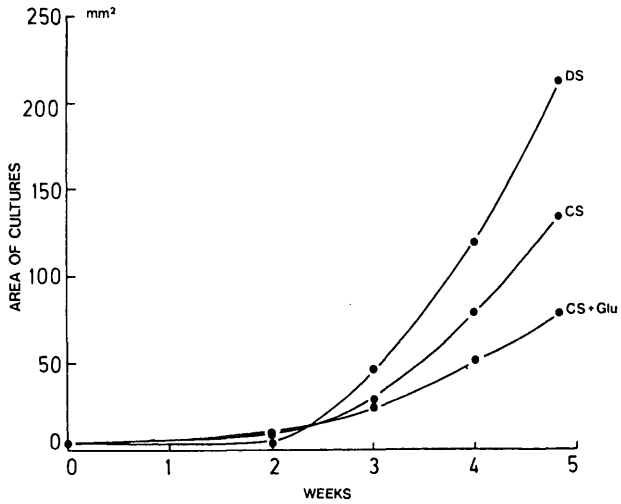

FIG. 1. The growth rate of primary cultures of normal rabbit aortic smooth-muscle cells in the rapid-growth phase. Experiment 1. DS=diabetic serum, CS=control serum, CS+GLU=control serum + glucose.

The diabetic serum, as well as the hyperlipemic serum with and without added glucose, induced a significant increase in the proportion of ^{3}H-thymidine-labeled cells in the peripheral monolayer of

TABLE 2

Effect of diabetic rabbit serum on area of *normal* rabbit aortic SMC cultures (rapid-growth phase)

Sera	Exp.	Number of cultures	Mean area of cultures	Range of culture areas	Statistical comparison 2-p values CS	CS + GLU
Control serum	1.	23	114.4 mm.²	79.1-185.5 mm.²		
(CS)	2.	18	132.3 mm.²	52.0-233.0 mm.²		
Control serum	1.	21	91.2 mm.²	43.7-141.5 mm.²	> 0.05	
+ glucose (CS + GLU)	2.	11	109.8 mm.²	58.4-150.0 mm.²	> 0.05	
Diabetic serum	1.	15	192.0 mm.²	104.2-261.0 mm.²	< 0.01	< 0.01
(DS)	2.	17	167.4 mm.²	92.3-245.5 mm.²	<0.05	< 0.01

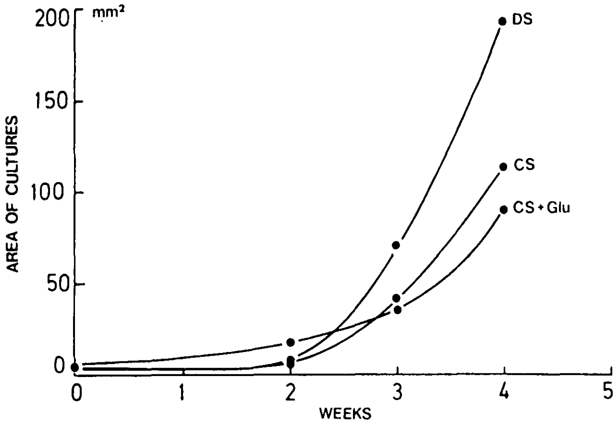

FIG. 2. The growth rate of primary cultures of alloxan-diabetic rabbit aortic smooth-muscle cells in the rapid-growth phase. Experiment 1. DS=diabetic serum, CS=control serum, CS+GLU=control serum + glucose.

was no statistically significant difference between the mean size of cells grown in normal serum and diabetic serum (table 7). Cells propagated in hyperlipemic serum were significantly smaller than cells grown in normal serum (2p<0.01). Cells grown in media supplemented with both diabetic and hyperlipemic serum were significantly smaller than those grown in diabetic serum (2p<0.01). In glucose-enriched normal serum the cells were significantly larger than those grown only in normal serum (2p<0.05) (figure 5), but in glucose-enriched hyperlipemic serum they were the same size (table 7).

DISCUSSION

The in-vitro studies of the rapid-growth phase demonstrated that outgrowth of smooth muscle cells from normal and diabetic aortic explants is significantly larger in the presence of diabetic serum than in normal serum. However, the proliferation is appar-

TABLE 3

Effect of diabetic rabbit serum on area of *diabetic* rabbit aortic SMC cultures (rapid-growth phase)

Sera	Exp.	Number of cultures	Mean area of cultures	Range of culture areas	Statistical comparison 2-p values CS	CS + GLU
Control serum	1.	17	132.4 mm.²	50.0-182.2 mm.²		
(CS)	2.	18	122.3 mm.²	57.8-202.1 mm.²		
Control serum	1.	20	80.4 mm.²	31.2-125.0 mm.²	< 0.01	
+ glucose (CS + GLU)	2.	16	133.0 mm.²	57.5-206.0 mm.²	> 0.1	
Diabetic serum	1.	17	180.7 mm.²	31.2-266.7 mm.²	< 0.01	< 0.01
(DS)	2.	19	165.6 mm.²	104.1-297.8 mm.²	= 0.01	< 0.05

the cultures, in comparison to the effect of normal serum (table 4). Furthermore, diabetic serum in combination with hyperlipemic serum increased the proportion of labeled cells significantly more than either the diabetic or hyperlipemic serum alone (2p<0.005).

Measurements of cell size. For the determination of cell size two different fixatives were used, Bouin's solution and formalin. It appears that cell size is much smaller in the preparations fixed in Bouin's solution. In the rapid-growth experiments, normal smooth-muscle cells propagated in the diabetic serum or in normal serum with added glucose were significantly larger than those grown in normal serum (figure 4, table 5). In fact, the normal serum with added glucose produced significantly larger cells than did the diabetic serum (figure 4, table 5). In the second experiment with diabetic serum the average value showed the same trend, but the differences were not statistically significant. The diabetic smooth-muscle cells were also enlarged after exposure to diabetic serum or normal serum with added glucose (table 6).

In the stationary-growth-phase experiments, there

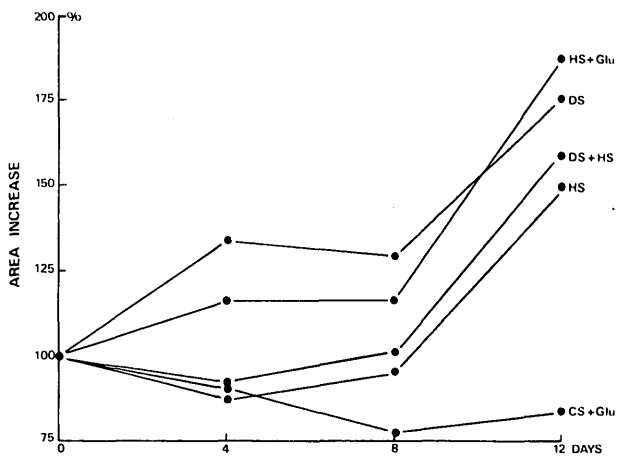

FIG. 3. The growth rate of primary cultures of normal rabbit aortic smooth-muscle cells in the stationary-growth phase. The growth of cultures in normal serum has been set to 100 per cent. Experiment 1. DS=diabetic serum, CS=control serum, HS=hyperlipemic serum, CS+GLU=control serum + glucose.

TABLE 4

Effect of diabetic rabbit serum on the incorporation of ^{3}H-thymidine into rabbit aortic SMC cultures

Sera	Control serum (CS)	Diabetic serum (DS)	Diabetic + hyperlipemic (DS + HS)	Control + hyperlipemic (HS)	Control serum + glucose + hyperlipemic (HS + GLU)
Total number of counted cells	456	281	370	215	375
Percentage of labeled cells	3.73%	11.03%	22.70%	9.30%	42.13%
χ^2-test	CS/DS	DS/DS + HS	CS/DS + HS	CS/HS	CS/HS + GLU
2-p Values	< 0.005	< 0.005	<0.005	<0.025	<0.005

ently not due to the glucose content of the diabetic serum, since the addition of glucose to normal serum did not enhance growth.

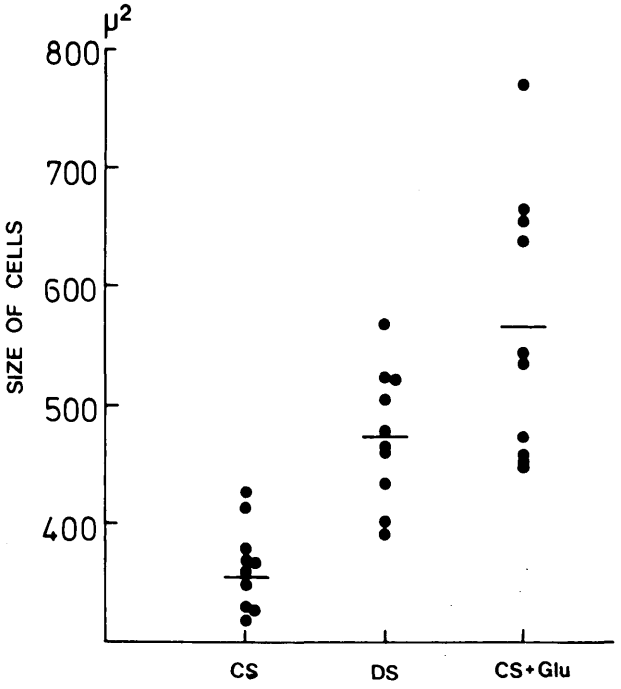

FIG. 4. The size of normal arterial medial cells in the rapid-growth phase. Experiment 1. DS=diabetic serum, CS=control serum, CS+GLU=control serum + glucose.

It has previously been demonstrated that the growth rate of the arterial medial cells can be accelerated by addition of hyperlipemic serum to the growth medium.[16,17,19,21] However, since there were virtually no differences between the cholesterol, triglyceride, and phospholipid contents of the diabetic serum and the normal serum, the stimulation of growth observed in these experiments cannot be ascribed to the amount of lipids. Recently several abnormalities of the apolipoprotein pattern of diabetic rats were described by Bar-On et al.[29] Although their animals were hyperlipoproteinemic and had been fed a high-sucrose diet, their findings make it clear that the subunit proteins should be examined in future experiments with aortic cultures grown in media containing serum from diabetic animals.

The difference between the growth-promoting effects of diabetic serum and commercial normal serum could be due to the diet of the animals from which the sera were obtained. However, other studies carried out in this laboratory have demonstrated that the growth of cultures is the same using the commercial normal serum or normal serum obtained from rabbits fed the same diet as the diabetic rabbits.[30]

The factor or factors in the diabetic serum that stimulate the growth of smooth-muscle cells in vitro have not been identified, although alloxan and insulin can be excluded. In the present studies diabetic serum

TABLE 5

Effect of diabetic rabbit serum on size of *normal* rabbit aortic SMC (rapid-growth phase)

Sera	Exp.*	Mean size of cells	Range of cell sizes	Statistical comparison CS	CS + GLU
				2-p values	
Control serum	1.	364.0 μ^2	317.7-426.7 μ^2		
(CS)	2.	1,322.5 μ^2	1,007.0-1,591.4 μ^2		
Control serum +	1.	565.3 μ^2	451.8-771.6 μ^2	< 0.01	
glucose (CS + GLU)	2.	1,822.0 μ^2	1,010.7-3,821.6 μ^2	< 0.01	
Diabetic serum	1.	474.4 μ^2	390.0-568.7 μ^2	<0.01	= 0.01
(DS)	2.	1,635.7 μ^2	1,114.4-2,261.9 μ^2	<0.05	> 0.1

*The cells in experiment 1 were fixed in Bouin's solution, while the cells in experiment 2 were fixed in formalin.

TABLE 6

Effect of diabetic rabbit serum on size of *diabetic* rabbit aortic SMC (rapid-growth phase)

Sera	Exp.*	Mean size of cells	Range of cell sizes	Statistical comparison 2-p values CS	CS + GLU
Control serum (CS)	1.	589.9 μ^2	467.7-769.7 μ^2		
	2.	1,209.6 μ^2	918.2-1,777.0 μ^2		
Control serum + glucose (CS + GLU)	2.	1,516.2 μ^2	1,087.1-2,267.4 μ^2	< 0.05	
Diabetic serum (DS)	1.	824.1 μ^2	555.3-1,293.7 μ^2	< 0.01	
	2.	1,556.4 μ^2	1,039.2-2,029.9 μ^2	< 0.02	> 0.1

*The cells in experiment 1 were fixed in Bouin's solution, while the cells in experiment 2 were fixed in formalin.

TABLE 7

Effect of diabetic rabbit serum on size of *normal* rabbit aortic SMC (stationary-growth phase)

Sera	Exp.*	Mean size of cells	Range of cell sizes	Statistical comparison 2-p values CS	DS	HS
Control serum (CS)	1.	1,184.9 μ^2	531.4-1,932.5 μ^2			
	2.	3,085.0 μ^2	1,988.4-4,925.6 μ^2			
Diabetic serum (DS)	1.	1,422.5 μ^2	1,064.0-2,085.0 μ^2	> 0.1		
	2.	3,097.7 μ^2	2,292.0-4,241.1 μ^2	> 0.1		
Hyperlipemic serum (HS)	1.	706.4 μ^2	467.8-1,008.7 μ^2	<0.01		
	2.	1,919.8 μ^2	1,493.1-2,528.4 μ^2	<0.01		
Diabetic + hyperlipemic serum (DS + HS)	1.	1,048.0 μ^2	817.3-1,272.0 μ^2		< 0.01	< 0.01
	2.	2,408.0 μ^2	1,683.0-3,109.0 μ^2		< 0.01	< 0.01
Hyperlipemic serum + glucose (HS + GLU)	1.	1,363.6 μ^2	1,136.0-1,687.0 μ^2			< 0.01
	2.	2,262.0 μ^2	1,968.0-2,779.0 μ^2			< 0.01

*The cells in experiment 1 were fixed in Bouin's solution, while the cells in experiment 2 were fixed in formalin.

was collected from rabbits with insulin deficiency established with alloxan, and to avoid any acute effects of alloxan the blood was collected for the first time two weeks after alloxanization. As a further precaution, insulin was not injected for 24 hours prior to the blood sampling, and only blood from rabbits with a blood glucose concentration above 250 mg./100 ml. was used. Thus, it is highly unlikely that the injected insulin could be responsible for the difference in growth between the cultures in normal serum and in diabetic serum.

Diabetic serum had a clear-cut effect on the growth of cultures in both the rapid and the stationary growth phases. However, there was no difference between the growth response of aortic explants from diabetic and normal rabbits, indicating that the diabetic serum must be the important factor stimulating the growth of the cells, rather than the origin of the explants. Under these experimental conditions the effects of diabetic serum and hyperlipemic serum on culture size were of the same order of magnitude. It was not possible to detect an additive growth effect when diabetic serum was added to hyperlipemic serum, although the autographic results seem to indicate some additive effect on the proliferation. It is important to remember that culture size may be the result of other factors besides cell proliferation, such as cell hypertrophy, cell migration, and changes in the amount of intercellular substances. In these studies, therefore, proliferation was only one factor contributing to the enlargement of the cultures.

The increase in cell size after growth in diabetic serum may be due to the increased glucose concentration. Morrison et al.[31] reported an increase in cell size after incubation for a few hours in a high-glucose concentration, indicated by the increase in the total water content and decrease in the inulin space in intima-media preparations from rabbit aortas. In our rapid-growth-phase experiments the final glucose concentrations of the media containing diabetic serum and the media containing normal serum with added glucose were only slightly higher than those of the control media—14 mg./100 ml. and 26 mg./100 ml. higher, respectively—but the period of observation

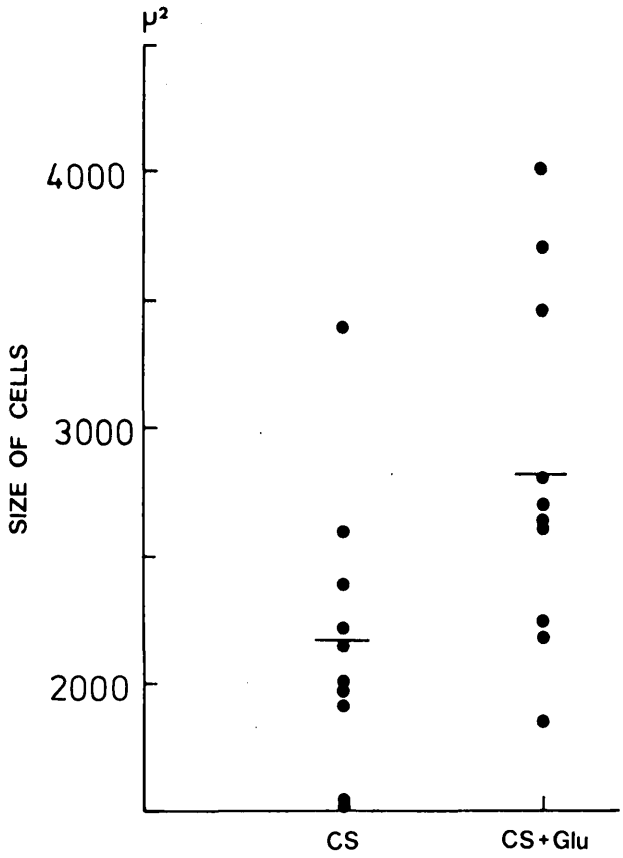

FIG. 5. The size of normal arterial medial cells in the stationary-growth phase. Experiment 1. CS=control serum, CS + GLU = control serum + glucose.

was several weeks rather than a few hours. If the effect of glucose is a consequence of time and concentration, our results are reconcilable with those of Morrison et al.[31] and Arnquist.[32] In the stationary-growth-phase studies, in which the incubation time was only 12 days, we observed an increase in cell size only in cultures grown in normal serum with added glucose. The short incubation period may account for the lack of effect on cell size of the diabetic serum.

The media supplemented with hyperlipemic serum of the same type as that used in previous studies of the effect of blood lipids on aortic smooth-muscle cells[16,17,19,21] resulted in smaller cells than did the control media. This finding has not been reported before. The fact that Bouin's solution causes more shrinkage of the tissue than formalin accounts for the clear-cut difference between the cell size in experiments 1 and 2 of the rapid-growth-phase studies.

The present study indicates that cells propagated in diabetic serum are clearer, without vacuoles, than those grown in normal serum and especially those grown in hyperlipemic serum. This suggests that diabetic serum and hyperlipemic serum affect the structures in the cells in different ways. A study of the ultrastructure of aortic smooth-muscle cells grown in various media is in progress in order to quantitate precisely these observations.

Of course, at the moment it would be hazardous to transfer these in-vitro results directly to the clinical and pathologic macroangiopathy in diabetes mellitus. Many factors have been cited as responsible for the development of the diabetic micro- and macroangiopathy. Recently Lundbaek et al.[33] have put forward the hypothesis that growth hormone may be a causal factor in the development of diabetic angiopathy.

At present, additional tissue-culture experiments using media containing various concentrations of glucose, insulin, and growth hormone, and including an analysis of the apoprotein pattern, are in progress.

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