Growth Hormone Antiserum Suppresses the Growth Effect of Diabetic Serum

Studies on Rabbit Aortic Medial Cell Cultures

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SUMMARY

Growth medium containing serum from young diabetic subjects caused a significant stimulation both of cell proliferation and of the outgrowth in cultures of rabbit aortic medial cells above that noted with normal human sera. The addition to the sera of guinea-pig human growth hormone antibody caused a marked inhibition of these stimulatory effects. The growth effect of rabbit serum was not affected by the human growth hormone antiserum.

Reinvestigation of the effect of human growth hormone disclosed that the same increase as observed in growth with the diabetic sera could be obtained with a growth hormone concentration of 0.2 ng. per milliliter medium.

The present results strongly suggest that the increased stimulatory effect of normolipemic human diabetic serum on growth and cell proliferation of aortic medial cell cultures is due to increased serum growth hormone concentration. DIABETES *26*:798-803, August, 1977.

The high frequency of coronary artery disease among diabetics has been considered as an expression of a particularly severe degree of atherosclerosis. However, data from epidemiologic, clinical, and autopsy studies suggest that the changes occurring in coronary arteries and other large arteries of the body are caused by a combination of diabetic macroangiopathy and atherosclerosis.[1-7]

Animal models, which facilitate measurement of the growth of arterial medial cells in cultures, have been widely used in atherosclerosis research,[8-10] and one of these models has proved useful in the study of diabetic large-vessel disease.[11] It was shown in previous studies that nonlipemic serum from diabetic rabbits or diabetic patients accelerates the growth of medial cells from rabbit aorta.[11,12] It has also been proposed that the increased secretion of growth hormone obtaining in diabetic patients may be a causal factor in the development of diabetic angiopathy.[13,14]

Recently in-vitro studies have demonstrated that addition of small amounts of human growth hormone to serum from normal subjects enhances the growth of arterial medial cell cultures.[15] In the present study the effect of growth hormone antibody added to diabetic serum has been studied on growth and cell proliferation of rabbit aortic medial cell cultures. The results indicated that growth hormone may be the factor responsible for the excessive growth effect of diabetic serum.

MATERIAL AND METHODS

The methods employed have been described in detail elsewhere.[11,12] Thoracic aortas were collected from rabbits weighing 2-3 kg. The tunica media of each aorta was coined into primary explants with a diameter of 2 mm. and then incubated in Basal Medium of Eagle (BME) with 10 per cent rabbit serum.

Sera for the Growth Media

Human diabetic serum was collected from 10 young subjects with classic juvenile diabetes mellitus (table 1). The average age was about 27 years and the duration of diabetes varied from one-half to 15 years. The diabetics were male, nonobese, insulin-treated patients. Retinopathy was present in one of the diabetic subjects.

Sera from eight nondiabetic males of comparable age and weight were used as a control group (table 2).

Venous blood (50 ml.) was withdrawn from the diabetic and control subjects after overnight fasting. The usual insulin doses (NPH 50) for the diabetic patients were given 12 hours (eight patients) and 24 hours (two patients) before blood sampling. The serum was treated and stored at −25° C. as earlier

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TABLE 1

Diabetic subjects

No.	Age (yr.)	Duration (yr.)	Retinopathy	Percentage of ideal body weight (%)	Glucose (mg./100 ml.)	Cholesterol (mg./100 ml.)	Triglycerides (mg./100 ml.)	Growth hormone (ng./ml.)
1.	21	10	—	90	271	186	58	1.6
2.	22	½	—	92	198	144	50	1.1
3.	23	4	−	103	372	148	42	2.0
4.	27	4	−	106	145	203	58	0.8
5	27	15	+	97	120	198	67	1.1
6.	28	13	−	90	152	227	103	2.5
7.	29	½	−	88	136	169	64	1.0
8.	29	1	−	95	100	214	64	0.6
9.	31	6	−	98	271	200	60	1.9
10.	31	6	−	108	182	186	58	1.0
Mean	26.8	6.0		96.7	194.7	187.5	64.2	1.4
S.E.M.	1.14	1.63		2.22	26.99	8.54	5.64	0.19

described.[12] Normal rabbit serum was obtained commercially (Grand Island Biological Co., Scotland).

All the experiments were performed after the cultures had developed to the "stationary growth phase," in which the growth rate was generally much lower than in the "initial growth phase." The stationary phase of growth was reached after four weeks of incubation in an ordinary growth medium, BME with 10 per cent normal rabbit serum. At this point of time the cultures were divided into experimental and control groups, care being taken to match each experimental culture with a control culture of the same area from the same aorta. For this matching, 216 cultures were selected from about 300 specimens obtained from four rabbit aortas.

In the experimental period the cultures were grown in media containing serum from normal subjects or diabetic patients with the addition of serum from either normal or growth-hormone-immunized guinea pigs. The guinea-pig serum (100 μl.) was added in a dilution of 1:60 to the growth medium 60 minutes before the start of the experiment, i.e., at the point of time when the ordinary medium was replaced by one of the experimental media. On competitive binding analysis with ^{125}I-hGH, the binding capacity of the antiserum was found to be 600 μg. per milliliter undiluted serum. Consequently the growth hormone antiserum was added in a concentration of about 10,000 times more than necessary for binding the amount of growth hormone present in the diabetic sera with the highest levels.

The composition of the growth media in the experimental period was as follows:

BME with 5 per cent rabbit serum including:
nondiabetic human serum (5 per cent)
and normal guinea-pig serum dilution
or
nondiabetic human serum (5 per cent)
and guinea-pig antiserum against human growth hormone
or

TABLE 2

Nondiabetic subjects

No.	Age (yr.)	Percentage of ideal body weight (%)	Glucose (mg./100 ml.)	Cholesterol (mg./100 ml.)	Triglycerides (mg./100 ml.)	Growth hormone (ng./ml.)
1.	24	110	101	256	138	0.1
2.	24	98	105	149	90	0.0
3.	25	101	80	185	80	0.2
4.	26	102	88	180	108	0.6
5.	26	103	111	183	90	1.3
6.	27	88	95	225	88	0.2
7.	31	97	87	219	112	0.7
8.	35	107	102	171	120	0.4
Mean	27.3	100.8	96.1	196.0	103.3	0.4
S.E.M.	1.36	2.37	3.71	12.21	6.96	0.15

diabetic human serum (5 per cent)
and normal guinea-pig serum dilution
or
diabetic human serum (5 per cent)
and guinea-pig antiserum against human growth hormone.

The influence of human growth hormone antibody on the growth effect of incubation medium containing 10 per cent rabbit serum was also analyzed on 20 pairs of aortic medial cell cultures in the stationary phase of growth.

In another set of experiments the effect of human growth hormone (Nanormon,* Nordisk Insulinlaboratorium, Copenhagen) on the arterial medial cell cultures described before[5] was reinvestigated. In comparison with a Wilhelmi preparation (no. HS 1216c) the two components were found to be immunologically identical over a large range of concentrations. In a series of experiments the hormone was added to BME with 10 per cent rabbit serum in a final concentration of 0, 25, 75, 200, 500, and 1,000 pg./ml. (BME plus 10 per cent rabbit serum was used for dissolving the growth hormone). Cultures (20) from two aortas were incubated with each of the various growth hormone concentrations.

Chemical Methods

Glucose, cholesterol, and triglycerides were estimated with an AutoAnalyzer. Growth hormone was measured by a single-antibody technique using wick-chromatography.[16]

Measurements

The growth rate of the cultures was followed in the experimental period by determination of their area at days 0, 2, and 4. The area of the cultures was measured by a point-counting technique as described previously[12] without knowledge of the type of incubation medium, and the increase in area was expressed as a percentage of the area at day 0. The mean per cent increase in the area of cultures grown in each of the sera was then calculated.

The use of four different rabbit aortas resulted in four different growth rates in the cultures (see also ref. 12). In order to compare the results it was therefore necessary to normalize the data in the following manner: the mean growth rates of the control cultures from four aortas were termed A_1, A_2, A_3, and A_4, respectively. The growth rates obtained from aortas 2, 3, and 4 were referred to that of aorta 1 by multiplying their growth rates in diabetic as well as control sera by factors A_1/A_2, A_1/A_3, and A_1/A_4, respectively. The same procedure was used on data obtained from the mitosis counts and in the supplementary experiments with growth hormone.

Autoradiography

Cell proliferation in the experiments with anti-growth-hormone serum was assessed from the percentage of cells incorporating ^{3}H-methyl-thymidine as identified by autoradiography. The analysis was performed during the first two days of growth in the experimental period. The various growth media were supplemented with 0.2 μCi. ^{3}H-methyl-thymidine per milliliter (Radiochemical Centre, Amersham, London) and the analyses carried out as previously.[12] Cell counts were made on the peripheral monolayer of the cultures at a total magnification of 200 without knowledge of the experimental group. Countings were performed in each case on four cultures, two incubated with and two without added growth hormone antiserum. The autoradiographic analysis was carried out with sera from six diabetics and three controls.

Mitosis Studies

In other cultures the percentage of cells in mitosis after completion of the experimental phase with anti-growth-hormone serum was determined histologically as described previously.[12] The number of mitotic and nonmitotic bodies was obtained from counts on the peripheral parts of cultures grown for four days in the experimental media. The number of figures was blindly determined on four cultures incubated in the individual sera, two with and two without addition of growth hormone antiserum and for a total of 150-200 cells in each culture. Sera from 10 diabetics and eight controls were included in the study.

Statistical Methods

Statistical comparison between paired and non-paired cultures was performed with the Student's *t*-test. The ratio of labeled and nonlabeled cells was determined by the X^2-test.[17] A 2p-value less than 0.05 was considered as significant.

RESULTS

The concentrations of cholesterol, triglycerides, glucose, and growth hormone in the diabetic and control serum are given in tables 1 and 2. The glucose concentration was significantly higher in the diabetic than in the control serum ($2p < 0.01$). The concentration of serum growth hormone was approximately three times as high in the diabetics as in the controls (1.4 ng./ml. against 0.4 ng./ml., $2p < 0.01$). The

*Nanormon contains 40 mg. glycine, 5 mg. sodium bicarbonate, and 4 mg. of mannitol per 2 mg. growth hormone.

level of serum cholesterol was the same but triglycerides were higher in serum from the controls than from the diabetics (tables 1 and 2, 2p <0.01).

The rate of growth of the arterial medial cell cultures was significantly greater in the diabetic sera than in the control sera (figure 1, table 3, 2p <0.01).

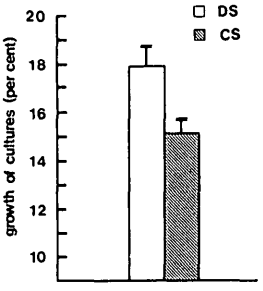

FIGURE 1

Growth effect of human diabetic and control sera on rabbit aortic medial cell cultures. One bar = 1 S.E.M. DS = diabetic serum. CS = control serum.

TABLE 3

Growth of cultures

	Number of individual sera (N)	Increase in culture-area (%) (mean ± S.E.M.)	Statistical comparison (*t*-test) 2p-values
Diabetic	10	17.96 ± 0.82	DS/CS 2p < 0.01
Diabetic sera + anti-hGH	10	14.71 ± 0.70	DS/DS + anti-hGH* 2p < 0.01
Control sera	8	15.11 ± 0.61	CS/CS + anti-hGH* 2p > 0.05
Control sera + anti-hGH	8	14.24 ± 0.77	DS + anti-hGH/CS + anti-hGH 2p > 0.05

*Paired comparison: DS = diabetic serum, CS = control serum, anti-hGH = human growth hormone antiserum.

This rate of growth of the arterial myomedial cells decreased when the diabetic serum was supplemented with growth hormone antiserum (figure 2, left part, table 3, 2p <0.01, paired comparison), and the growth rate was now comparable to that found in serum from nondiabetics (figure 3, table 3).

As to the control sera the growth of the aortic medial cells was about the same with or without growth hormone antiserum (figure 2, right part, table 3).

Control experiments comprising incubation medium with 10 per cent rabbit serum with and without human growth hormone antiserum were also performed. It was not possible to detect an influence of human growth hormone antibody on the growth effect of the rabbit serum (13.5 per cent ± 0.94 S.E.M. against 13.4 ± 1.07 S.E.M.).

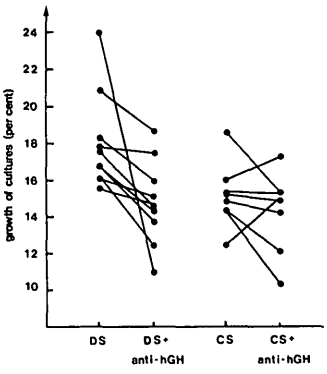

FIGURE 2

Growth of rabbit aortic medial cell cultures propagated in human diabetic and control sera with and without added guinea-pig human growth hormone antibody. Paired comparison. DS = diabetic serum. CS = control serum. Anti-hGH = guinea-pig human growth hormone antibody.

Cell Proliferation

In accordance with the observation on growth rates the number of mitotic figures was increased significantly in the presence of diabetic sera as compared with control sera (table 4, 2p <0.01). Correspondingly, for diabetic and control sera supplemented with growth hormone antiserum the number of mitotic cells observed was significantly less than with the diabetic and control sera without antiserum (table 4, 2p <0.001, paired comparison).

However, the proportion of mitotic figures was the same in cultures propagated in diabetic and control sera that contained growth hormone antiserum (table 4 [12.62 per cent against 12.65 per cent]).

The incorporation of ^{3}H-methyl-thymidine was increased appreciably after incubation in diabetic serum (table 5, p <0.005). The presence of growth hormone antiserum resulted in a significantly lower number of labeled cells in cultures grown in diabetic as well as in control sera (table 5, p <0.005 and p <0.025).

Cultures propagated in either diabetic or control sera with human growth hormone antibody exhibited the same degree of incorporation of ^{3}H-methyl-thymidine (32.5 per cent against 28.8 per cent).

Supplementary Growth Hormone Experiments

The effect on growth of adding human growth hormone in various amounts to a medium consisting

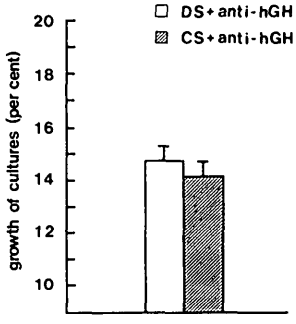

FIGURE 3

Growth of rabbit aortic medial cell cultures propagated in human diabetic and control sera with added guinea-pig human growth hormone antibody. one bar = 1 S.E.M. DS = diabetic serum. CS = control serum. Anti-hGH = guinea-pig human growth hormone antibody.

TABLE 4

Mitosis studies

	Number of sera N	Total number of cells counted N	Percentage of mitotic bodies mean (S.E.M.) %	Statistical comparison (*t*-test)	2p-values
DS	10	3,346	19.21 (1.08)	DS / CS	< 0.01
DS + anti-hGH	10	3,651	12.62 (1.03)	DS / DS + anti-hGH	< 0.001*
CS	8	2,903	15.33 (0.99)	CS / CS + anti-hGH	< 0.001*
CS + anti-hGH	8	2,459	12.65 (0.46)	CS + anti-hGH / DS + anti-hGH	> 0.1

*Paired comparison: CS = control serum, DS = diabetic serum, anti-hGH = human growth hormone antiserum.

of BME with 10 per cent rabbit serum is shown on figure 4. The growth response was significantly enhanced at a growth hormone level of 200 pg. or more per milliliter incubation medium (2p <0.05, paired comparison).

DISCUSSION

In earlier studies an accentuation of the growth of aortic medial cells was shown to occur in serum from alloxan-diabetic rabbits and young diabetic patients[11,12] but the growth factor(s) in the diabetic serum was not identified.

Growth hormone has been suggested to be a causal factor in the development of diabetic vascular disease,[13,14] and we have previously reported that the addition of growth hormone accelerated the growth of cultures of aortic myomedial cells.[15]

The sensitivity of the myomedial cells to growth hormone was found in the present study to be considerably higher than observed previously.[15] However, in the present study better care was taken to diminish absorption of growth hormone to surfaces by keeping adequate protein concentration in the dilution medium. Augmentation of growth could already be shown at a hormone level of 0.2 ng./ml. medium. It is noteworthy, however, that a growth hormone value

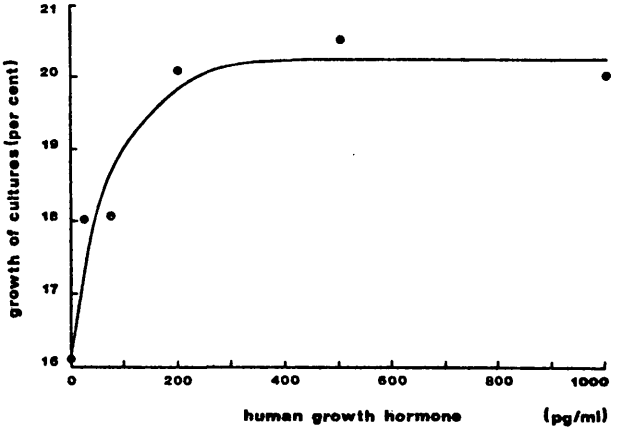

FIG. 4. Stimulation of growth of rabbit aortic medial cell cultures with various doses of human growth hormone in the incubation medium. The cultures were exposed for four days.

TABLE 5

Autoradiography

	CS	CS + anti-hGH	DS	DS + anti-hGH
Total number of cells counted	951	794	1,863	1,582
Percentage of labeled cells	34.5%	28.8%	43.0%	32.5%
Statistical comparison	CS / DS	CS / CS + anti-hGH	DS / DS + anti-hGH	CS + anti-hGH / DS + anti-hGH
p-values (χ^2-test)	< 0.005	< 0.025	< 0.005	> 0.1

CS = control serum, DS = diabetic serum, anti-hGH = human growth hormone antiserum.

of 0.2 ng./ml. medium is about the same as that obtained when diabetic serum containing 4 ng. growth hormone per milliliter has been diluted into the growth medium, and increased growth rates are attained.

The present studies of the effect of diabetic and nondiabetic sera were performed to test the possibility that serum growth hormone could be the factor responsible for accelerated growth. This was accomplished by the use of growth hormone antibody. A stimulatory effect of diabetic serum on aortic myomedial cell growth was found, as in earlier studies,[11,12] and this effect was abolished by growth hormone antibodies. Moreover, the growth effect of diabetic and control sera was the same after the addition of growth hormone antiserum.

The percentage of mitotic figures and ^{3}H-methylthymidine-labeled cells was also increased in cultures grown in diabetic serum, and these results are in accordance with earlier observations.[11,12] Growth hormone antibody supplementation significantly inhibited these effects. Cell proliferation was reduced to the same low level in cultures grown in diabetic and normal serum.

The present results, obtained with a specific growth hormone antibody, suggest that it is the growth hormone in the diabetic serum that is the factor responsible for the growth effect.

The effect of guinea-pig growth hormone antiserum cannot be due to an unspecific inhibiting influence of guinea-pig serum proteins, since normal guinea-pig serum was added to all control media.

The currently held view is that growth hormone does not stimulate essential growth of tissues directly but leads to the generation of secondary hormonal agent(s), somatomedin(s), acting at the cellular level.[18] Presumably, the growth of arterial medial cells in the present study was stimulated directly by the growth hormone present in diabetic serum. However, the possibility that the effect is due to somatomedin produced locally in the cultures cannot be excluded. This possibility is currently under investigation. Considering the limitations associated with working with in-vitro systems, the present results are compatible with the concept of growth hormone as a causal factor in the development of diabetic angiopathy.

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