Factors Controlling the Proliferative Rate, Final Cell Density, and Life Span of Bovine Vascular Smooth Muscle Cells in Culture

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ABSTRACT   Low density vascular smooth muscle (VSM) cell cultures maintained on extracellular-matrix(ECM)-coated dishes and plated in the presence of either plasma or serum will proliferate actively when serum-containing medium is replaced by a synthetic medium supplemented with three factors: high density lipoprotein (HDL, 250 µg protein/ml); insulin (2.5 µg/ml) or somatomedin C (10 ng/ml); and fibroblast growth factor (FGF, 100 ng/ml) or epidermal growth factor (EGF, 50 ng/ml). The omission of any of these three factors from the synthetic medium results in a lower growth rate of the cultures, as well as in a lower final cell density once cultures reach confluence. When cells are plated in the total absence of serum, transferrin (10 µg/ml) is also required to induce optimal cell growth.

The effects of the substrate and medium supplements on the life span of VSM cultures have also been analyzed. Cultures maintained on plastic and exposed to medium supplemented with 5% bovine serum underwent 15 generations. However, when maintained on ECM-coated dishes the serum-fed cultures had a life span of at least 88 generations. Likewise, when cultures were maintained in a synthetic medium supplemented with HDL and either FGF or EGF, an effect on the tissue culture life span by the substrate was observed. Cultures maintained on plastic underwent 24 generations, whereas those maintained on ECM-coated dishes could be passaged repeatedly for 58 generations. These experiments demonstrate the influence of the ECM-substrate not only in promoting cell growth but also in increasing the longevity of the cultures.

Previous studies have shown that bovine vascular smooth muscle (VSM) cells maintained on dishes coated with extracellular matrix (ECM) proliferate equally well when exposed to either plasma or serum (13). Among the plasma components that could affect the proliferation of VSM cells are the high (HDL) and low density lipoproteins (LDL), both of which have been reported to be mitogenic for that cell type when cultures are exposed to lipoprotein-deficient serum (LPDS; 3, 5, 28) or to serum from abetalipoproteinemic subjects (24). Insulin, epidermal growth factor (EGF), and fibroblast growth factor (FGF) might also affect VSM cell proliferation, inasmuch as these agents have been shown to be mitogenic for a wide variety of cell types maintained under serum-free conditions (1, 2, 31) as well as for VSM cell cultures exposed to plasma (16, 18). Transferrin could also be required (1, 2, 31) to make iron available to the cells.

To define which of these factors are important for proliferation of VSM cells, we have studied the proliferative response of low density VSM cell cultures that were plated in the presence of serum or plasma, and then exposed to a synthetic medium supplemented with LDL, HDL, insulin, and FGF, and have compared the effects of the substrate (either ECM or plastic) on the proliferative response to these agents. Our results indicate that VSM cells maintained on ECM-coated dishes proliferate actively when exposed to a serum- or plasma-free synthetic medium supplemented with HDL, FGF, and pharmacological concentrations of insulin. FGF could be replaced by EGF, and insulin could be replaced by very low concentrations of somatomedin C. A requirement for transferrin became apparent when cells were plated in the total absence of serum. The omission of any of these components from the synthetic medium resulted in a lower growth rate as well as in a shorter

life span of the cells. Because HDL, transferrin, somatomedin C, and EGF are normal constituents of plasma (2, 4, 20, 31, 37), it is likely that they represent the plasma factors to which VSM cells respond when maintained on ECM-coated dishes.

MATERIALS AND METHODS

Materials

FGF was purified from bovine brains as described (7). Brain FGF yielded a single band on polyacrylamide gel electrophoresis at pH 4.5 and on an isoelectric focusing column (total volume 110 ml, pH 3.4–11.0). All the activity focused within a single peak with an isoelectric point of 9.2 to 9.6.

EGF was purified as described by Savage and Cohen (32). Somatomedin C was a generous gift from Dr. Van Wyk (University of North Carolina, Chapel Hill, N. C.). Crystalline bovine serum albumin was obtained from Schwarz/ Mann Div., Becton, Dickinson & Co. (Orangeburg, N. Y.). Carrier-free ^{125}I-Na was obtained from Amersham Corp. (Arlington Heights, Ill.). Lactoperoxidase, glucose oxydase type 5, chloramine-T, insulin, transferrin, and soybean trypsin inhibitor were obtained from Sigma Chemical Co. (St. Louis, Mo.). Dulbecco's modified Eagle's medium (DMEM, H-16) was obtained from Grand Island Biological Co. (Grand Island, N. Y.). Calf serum and fetal calf serum were obtained from Irvine Serum Co. (Irvine, Calif.). Tissue culture dishes were from Falcon Labware, Div. of Falcon Plastics (Oxford, Calif.), Gentamicin from Schering Corporation (Kenilworth, N. J.), and Fungizone from E. R. Squibb & Sons, Inc. (Princeton, N. J.).

Preparation of Plasma and Serum

Bovine plasma and serum were prepared as described previously (14, 29, 30, 34). Protein concentration was determined by the method of Lowry et al. (25) as modified by Maxwell et al. (26). Final protein concentrations were adjusted to 60 mg/ml by diluting plasma or serum with Ringer solution.

Preparation of LDL, HDL, and Lipoprotein-deficient Serum

Human and bovine LDL ($1.019 < d < 1.063$ g/cm^3), HDL ($1.07 < d < 1.21$ g/cm^3), and lipoprotein-deficient serum (LPDS, ($d < 1.25$ g/cm^3) were obtained from human and bovine plasma be differential ultracentrifugal flotation (20, 23). To remove contaminating plasma proteins, the LDL and HDL preparations were washed by recentrifugation in solutions of densities 1.063 and 1.210, respectively. Protein concentrations were determined as described above. LPDS protein concentration was adjusted to 60 mg/ml by dilution with Ringer solution.

The purified HDL and LDL preparations were analyzed by double immunodiffusion to determine the degree of cross-contamination of the HDL preparation by LDL and vice-versa (34, 35). When HDL or LDL preparations were analyzed by double immunodiffusion, 0.2 μg of HDL protein gave a single precipitin line against rabbit anti-human α_1-apoprotein (anti-HDL; Behring Diagnostics, American Hoechst Co., Somerville, N. J.). With this antiserum, no precipitin line could be observed with LDL, even at a 500-fold higher protein concentration (100 μg protein). Likewise, LDL (0.2 μg protein) gave a single precipitin line when tested against rabbit anti-human β-apoprotein (anti-LDL; N. L. Cappel Laboratories Inc., Cochranville, Pa.), whereas HDL at 500-fold higher protein concentration did not give a precipitin line. These results demonstrate that HDL preparations contained <0.2% LDL, if any at all, and vice versa (34, 35). To eliminate the possibility of a contamination by plasma proteins, the purity of the LDL and HDL preparations was analyzed by slab gel electrophoresis (10–18% and 5–18%, respectively; exponential polyacrylamide gel gradient containing 0.1% sodium dodecyl sulfate) with or without prior delipidation with tetramethyl urea (35). When the electrophoretic patterns of HDL and LDL preparations were compared with that of plasma or LPDS, no contamination by plasma proteins was observed.

Cell Culture Conditions

Cultures of bovine corneal endothelial cells were established from steer eyes as described (9, 15). Stock cultures were maintained on tissue culture dishes in DMEM supplemented with 10% fetal calf serum, 5% calf serum, 50 μg/ml Gentamicin, and 2.5 μg/ml Fungizone. FGF (100 ng/ml) was added every other day until the cells were nearly confluent. Plastic dishes coated with an ECM produced by corneal endothelial cells were prepared with either detergent treatment (0.5% Triton in phosphate-buffered solution [PBS]), as already described (8, 14, 39), or base treatment (NH$_4$OH, 0.02 M in distilled water). When base treatment was used, confluent corneal endothelial cell cultures were first washed

with distilled water and then exposed to 0.02 M NH$_4$OH in distilled water for 5 min, followed by washing with PBS. ECMs treated both ways were equally capable of supporting the growth of various cell types seeded on them (8, 14, 39). The presence of nuclei or cytoskeletal elements (actin and vimentin) could not be detected on the denuded ECMs when plates were examined by phase-contrast microscopy, scanning electron microscopy, or indirect immunofluorescence, using either specific antibodies directed against actin or vimentin or the benzimidole derivative Hoechst 33258 for nuclear staining.

Treatment of ECM with dithiothreitol (0.1 M) or 10% β-mercaptoethanol did not affect its ability to support cell growth. Because both of these treatments are known to destroy the activity of platelet-derived growth factor completely (33), it is unlikely that the growth-promoting effect of the ECM is due to traces of this factor which could be adsorbed onto it.

Primary cultures of bovine VSM cells were prepared from the vascular media of a bovine aortic arch (18, 19). Stock cultures were maintained on tissue culture dishes in DMEM supplemented with 10% bovine serum and 50 μg of Gentamicin and 2.5 μg of Fungizone/ml. FGF (100 ng/ml) was added every other day until the cells were nearly confluent.

Cell Seeding in the Presence of Plasma or Serum or in Their Absence

Cell monolayers from stock plates were dissociated by exposure (2–3 min, 24°C) to a solution containing 0.9% NaCl, 0.01 M sodium phosphates (pH 7.4), 0.05% trypsin, and 0.02% EDTA (STV solution, Difco Laboratories, Detroit, Mich.). When cells rounded up, they were resuspended in DMEM supplemented with 5% bovine plasma or serum to inhibit further proteolysis. When cells were seeded in the absence of plasma or serum, they were resuspended in DMEM supplemented with either soybean trypsin inhibitor (1 mg/ml) or HDL (500 μg protein/ml). Because the plating efficiency of cells resuspended in DMEM supplemented with HDL was higher than that achieved when soybean trypsin inhibitor was used (90 vs. 40% plating efficiency), neutralization of residual traces of trypsin was routinely performed using HDL rather than soybean trypsin inhibitor. The cell suspension was then spun down, and the cell pellet resuspended in DMEM supplemented with 5% bovine plasma, serum, and 5% LPDS, or in DMEM containing 500 μg protein/ml HDL. An aliquot of the cell suspension was then counted in a Coulter counter (Coulter Electronics, Hialeah, Fla.) and cells were distributed at an initial cell density of 1 × 10^4 cells/35-mm dish, as described below.

Cell Growth Measurement and Determination

For cell growth measurements, cells were seeded, as described above, at an initial density of 1 × 10^4 cells/35-mm dish on either plastic dishes or dishes coated with an ECM. In the case of cultures exposed to various concentrations of bovine serum and plasma, seeding was done in the presence of DMEM supplemented with 5% bovine serum. 6 h later, a set of triplicate plates was trypsinized and cells were counted to determine the plating efficiency. Plates were then washed twice with DMEM, and after this 2 ml of DMEM containing the appropriate concentration of serum or plasma was added to the plates. In the case of cultures exposed to different concentrations of LPDS, a similar procedure was used, except that cells were plated initially in 5% LPDS. 6 h later, plates were washed as described above, and medium containing the various concentrations of LPDS was then added to the plates. When cultures were to be maintained in serum-free medium, seeding of the cultures was done either in the presence of DMEM supplemented with 5% calf serum or plasma or in DMEM supplemented with transferrin alone. When seeding was done in the presence of serum or plasma, the plates were washed twice 6 h after seeding. 2 ml of DMEM supplemented with various concentrations of HDL, LDL, FGF, EGF, or insulin, present alone or in various combinations, was then added to the plates. Cells maintained in DMEM supplemented with combinations of various factors responded equally well when preplated in the presence of either plasma or serum. Addition of FGF, insulin, or EGF took place every other day at the concentrations indicated in the figure legends. Lipoprotein (either HDL or LDL) and transferrin was added only once (day 0) at the indicated concentrations. Triplicate plates were trypsinized and counted with a Coulter counter every other day. Morphological appearance of the cultures was analyzed by phase-contrast microscopy, and pictures were taken once the cultures maintained on ECM or plastic became confluent.

Culture Life Span Determination

The proliferative life span of VSM cells was determined by subcultivating the cultures at weekly intervals. Cells were seeded at an initial density of 10^4 cells/ 35-mm dish. When cells were seeded in the presence of serum (Figs. 7–9), the plates were washed 6 h later and DMEM supplemented with various factors was

then added to them. When cells were seeded in the total absence of serum (Fig. 11 B), they were exposed to DMEM supplemented with transferrin (10 µg/ml), HDL (250 µg protein/ml), insulin (2.5 µg/ml), and FGF (100 ng/ml) or EGF (50 ng/ml). In both cases, triplicate plates were trypsinized 8 h after seeding and counted to determine the plating efficiency. After 7 d, triplicate cultures were trypsinized to determine the final cell density. Cultures were counted and transferred weekly at a cell density of 10^4 cells/35-mm dish. The number of generations was determined from the initial cell density 8 h after seeding and the number of cells harvested at each transfer.

RESULTS

Effect of Substrate on the Ability of Low Density VSM Cell Cultures to Respond to Increasing Concentrations of Serum, Plasma, or LPDS

Survival and proliferation of low density VSM cell cultures (10 cells/mm^2) maintained on plastic were a function of the serum concentration to which cells were exposed. Little or no proliferation was observed when cultures were exposed to low serum concentration (0.25-1%). Above 1%, the average doubling time of the cultures decreased as a function of serum concentration to which cells were exposed (Fig. 1 A). Cultures exposed to low plasma concentration (0.25 and 0.5%) did not survive. Plasma concentrations higher than 1% allowed cells to survive and to proliferate slowly. At plasma concentrations as high as 10% (Fig. 1 A), a threefold increase in final cell density was observed. When cultures were exposed to low concentrations of LPDS (0.25-5%), cells did not proliferate. At higher concentrations of LPDS (5-10%), cells died (Fig. 1 A).

When cells were maintained on ECM-coated dishes and exposed to increasing concentrations of plasma or serum (0.25-

10%), they proliferated vigorously and at a similarly fast rate (Fig. 1 B) which became optimal when cultures were exposed to 5% serum or plasma. In contrast, cultures exposed to increasing concentrations of LPDS proliferated slowly. The average population doubling time was similar, regardless of whether cultures were exposed to low (0.25%) or high (5%) LPDS concentrations (Fig. 1 B). Toxic effect was observed at LPDS concentrations >5%. The difference in growth-rate between cultures exposed to plasma or serum vs. LPDS indicates that lipoproteins could be involved in the proliferation of VSM cells.

Effects of Increasing Concentrations of HDL and LDL on the Proliferation of VSM Cells Maintained on ECM-coated Dishes and Exposed to Plasma or Serum-free Medium

To test the hypothesis that lipoproteins promote the proliferation of VSM cells, low density cultures (10 cells/mm^2) maintained on ECM-coated dishes were exposed to medium supplemented with increasing concentrations of LDL or HDL. As shown in Fig. 2, human HDL at concentrations ranging from 10 to 1,000 µg protein/ml stimulated cell proliferation. Saturation was observed at concentrations of 250 µg protein/ml. Bovine HDL was more potent than human HDL at concentrations below saturation, but in both cases saturation was observed at 250 µg protein/ml. In contrast to HDL, high concentrations (above 100 µg protein/ml) of human LDL were toxic for the cells. Similar results were observed with bovine LDL (unpublished results). At low concentrations of LDL (5-
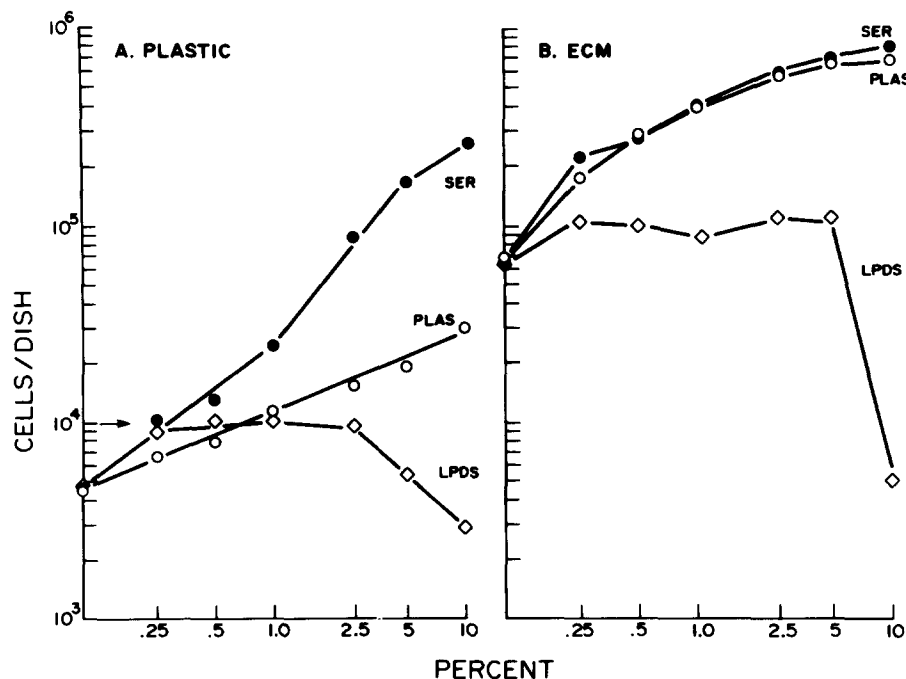

FIGURE 1   Proliferation of bovine VSM cells maintained on plastic or on ECM and exposed to different concentrations of serum, plasma, or LPDS. VSM cells were seeded, as described in Materials and Methods, at 1 × 10^4 cells/35-mm dish (arrow) on either plastic (A) or ECM-coated dishes (B) and maintained in the presence of DMEM supplemented with 5% bovine serum, 5% bovine plasma, or 5% LPDS. After 6 h, the medium was removed and the cultures were washed twice with DMEM. Cells previously incubated in DMEM supplemented with 5% bovine serum or plasma were then exposed to DMEM supplemented with increasing concentrations, ranging from 0.5 to 10% of either serum (SER, ●) or plasma (PLAS, ○). Cells previously exposed to DMEM supplemented with 5% LPDS were then exposed to increasing concentrations of LPDS ranging from 0.5 to 10% (◇). After 6 d, the cultures were trypsinized and counted. All determinations were done in triplicate. The standard deviation in the different determinations did not exceed 10% of the mean.

25 µg protein/ml), a small mitogenic effect was observed (Fig. 1). However, this effect was much less than that observed with similar concentrations of human or bovine HDL. Because lipoproteins from human plasma are better defined than lipoproteins of bovine origin, we decided to continue this study using human rather than bovine HDL.
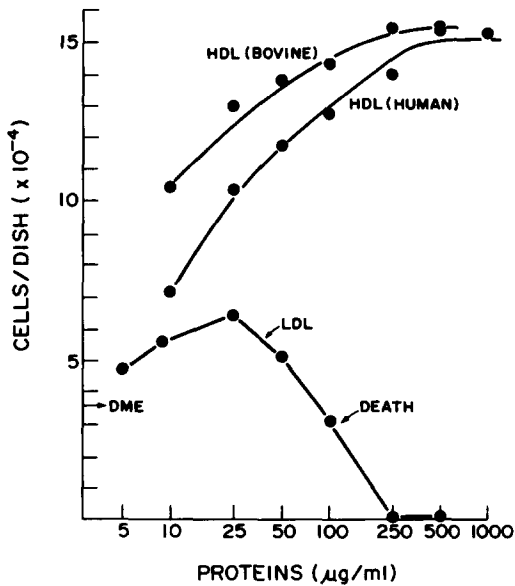

FIGURE 2   Effect of increasing concentrations of HDL or LDL on the proliferation of bovine VSM cells maintained on ECM-coated dishes and exposed to serum- or plasma-free medium. VSM cells were seeded as described in Materials and Methods at 1 × 10⁴ cells/35-mm dish on ECM-coated dishes. Cultures were exposed to DMEM (*DME* on fig.) supplemented with increasing concentrations (ranging from 10 to 1,000 µg protein/ml) of human or bovine HDL or increasing concentrations (ranging from 5 to 500 µg protein/ml) of human LDL. On d 6, cell cultures were trypsinized and counted. The final cell density of cultures exposed to DMEM alone was 3.5 × 10⁴ cells/35-mm dish (arrow). All determinations were done in triplicate. The standard deviation in the different determinations did not exceed 10% of the mean.

Effect of Increasing Concentrations of EGF, Insulin, or FGF on the Proliferation of VSM Cells Maintained on ECM-coated Dishes and Exposed to Plasma or Serum-free Medium and Supplemented with Optimal Concentrations of HDL

The final cell density of the cultures, even when exposed to optimal HDL concentrations, was always much less than that of cultures exposed to optimal plasma or serum concentration (1.5 × 10⁵ cells vs. 5–7 × 10⁵ cells/35-mm dish). This indicates that factors other than HDL are required to obtain optimal cell proliferation.

Among the factors other than HDL that could be involved in the proliferation of VSM cells are EGF, insulin, and FGF. To determine whether any of these agents could have an additive effect in conjunction with HDL, cultures exposed to medium supplemented with optimal concentration of HDL (250 µg protein/ml) were exposed to increasing concentrations of either of these agents (Fig. 3). In all three cases, increased cell proliferation, as reflected by the final cell density of the cultures after 6 d, was observed. EGF was active at concentrations ranging from 1 to 500 ng/ml, with a maximal effect at 50 ng/ml; insulin was active from 10 ng/ml to 10 µg/ml, with a maximal effect at 2.5 µg/ml; FGF was active from 1 to 200 ng/ml, with a plateau observed at 100 ng/ml (Fig. 3). However, the final cell density of the cultures was in all cases still two- to threefold lower (2.2–2.4 × 10⁵ cells/35-mm dish) than that of cultures exposed to optimal concentrations of plasma or serum (5–7 × 10⁵ cells/35-mm dish).

Growth Rate of Low Density VSM Cell Cultures Maintained on ECM-coated Dishes and Exposed to Serum-free Medium Supplemented with Optimal Concentrations of Either HDL, FGF, and Insulin or HDL, EGF, and Insulin

In an effort to maximize the growth rate of the cultures, the effect of various combinations of HDL, FGF, EGF, and insulin
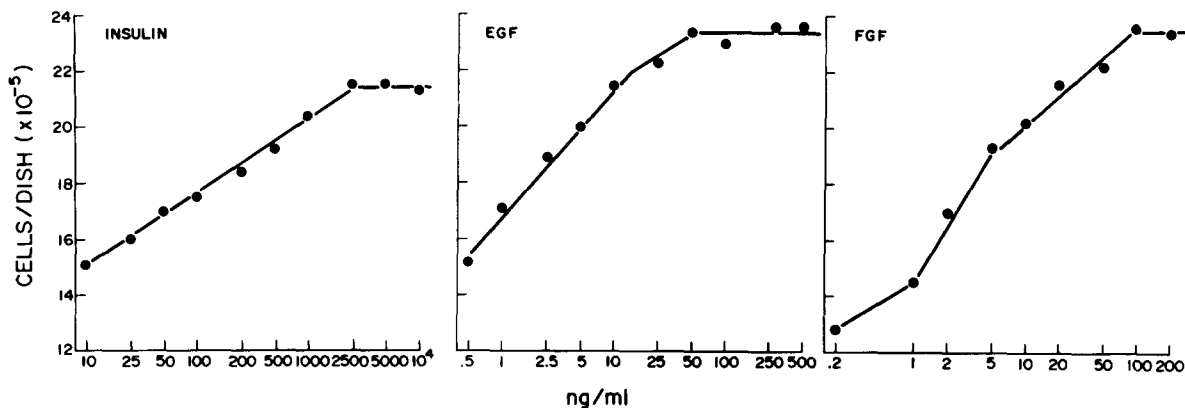

FIGURE 3   Effect of increasing concentrations of insulin, FGF, and EGF on the rate of proliferation of VSM cells exposed to a plasma- or serum-free medium supplemented with human HDL. VSM cells were seeded at 1 × 10⁴ cells/35-mm dish on ECM-coated dishes in the presence of DMEM supplemented with 5% bovine serum. 6 h later, the dishes were washed twice with DMEM, and fresh DMEM supplemented with HDL (250 µg protein/ml) and with insulin at concentrations ranging from 10 ng to 10 µg/ml, FGF at concentrations ranging from 0.5 to 500 ng/ml, or EGF at concentrations ranging from 0.2 to 200 ng/ml was added to the dishes. Insulin, EGF, or FGF was added every other day. After 6 d, the cultures were trypsinized and counted. All determinations were done in triplicate. The standard deviation in the different determinations did not exceed 10% of the mean.

on VSM cell proliferation has been studied. As shown in Fig. 4A, VSM cell cultures maintained on ECM-coated dishes plated in the presence of serum and then exposed to serum-free medium supplemented with 250 μg protein/ml HDL, 2.5 μg/ml insulin, and 100 ng/ml FGF had an average doubling time during their logarithmic growth phase of 15 h. This doubling time was similar to that of cultures exposed to optimal concentrations of serum (10%). The main difference between both types of culture was in their final cell density, which was lower (5.4 × 10⁵ cells/35-mm dish) in the case of cultures exposed to HDL, insulin, and FGF than in the case of cultures exposed to 10% serum (7.2 × 10⁵ cells/35-mm dish). This difference is the result of a decreased growth rate after 5 d in culture, when VSM cells are exposed to the medium supplemented with insulin, HDL, and FGF. The omission of any one of these factors (HDL, insulin, or FGF) resulted in a slower growth rate, as well as in a lower final cell density. This indicates that all three factors have an additive effect upon one another and must be present simultaneously to induce optimal cell growth. Neither FGF nor insulin, either singly or in combination, had a significant effect on cell growth (Fig. 4A). When the ability of EGF (50 ng/ml) (Fig. 4C) to substitute for FGF (Fig. 4B) was tested, it was found to be as potent as FGF. Likewise, we have observed that somatomedin C at low concentration (10 ng/ml) can fully substitute for insulin (Fig. 5). The morphological appearance of VSM cell cultures maintained in serum-free medium and exposed to the various factors, either singly or in combination, is shown in Fig. 6. Low density cell cultures maintained on ECM-coated dishes and exposed to DMEM alone (Fig. 6A) or to DMEM supplemented with EGF (Fig. 6B) or FGF (Fig. 6D) were composed to

sparse, spindly cells. When exposed to insulin alone, VSM cells flattened out (Fig. 6C). Only with HDL did cells proliferate actively, in some places forming a monolayer composed of enlarged cells (Fig. 6E). But the cells never covered whole dishes, and denuded areas were conspicuous. Addition of either FGF or EGF (Fig. 6F and G) to cultures exposed to HDL markedly increased the cell density of the cultures, as reflected by the increased packing of the cells. The cell density increased even more when insulin was present (Fig. 6H and I) and cultures became confluent. Under these conditions (HDL, insulin, and FGF or EGF), the morphological appearance of confluent cultures was not markedly different from that of cultures exposed to optimal serum concentration (Fig. 6J).

Effect of the Substrate and Medium Supplements on the Life Span of VSM Cell Cultures Passaged Repeatedly at Low Cell Density

EFFECT OF THE SUBSTRATE ON SERUM-FED CULTURES: To investigate the effect of the substrate on the life span of VSM cell cultures, we seeded cultures at low cell density (10 cells/mm²) on plastic vs. ECM-coated dishes, exposed them to DMEM supplemented with 5% calf serum, and passaged them repeatedly every 6 d. As shown in Fig. 7A, cells maintained on plastic underwent 15 generations before senescence. Addition of FGF (100 ng/ml) increased the longevity of the cultures to a total of 25 generations (Fig. 7B). In contrast, when similar serum-fed cultures were maintained on ECM-coated dishes, cells divided actively and cultures could be passaged for 80-88
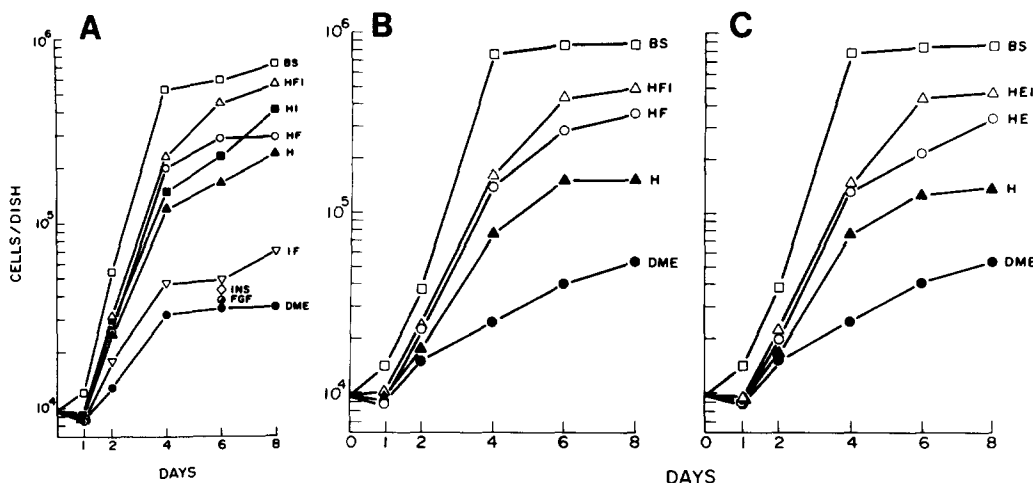

FIGURE 4   Comparison of the growth rate of low-density VSM cell cultures maintained on ECM-coated dishes and exposed to either medium supplemented with bovine serum or medium supplemented with HDL, insulin, and FGF or EGF, added either singly or in combination. (A) VSM cells were seeded at 1 × 10⁴ cells/35-mm dish on ECM-coated dishes in the presence of DMEM supplemented with 5% bovine serum. 6 h later, dishes were washed twice with DMEM and fresh DMEM supplemented with 5% bovine serum (BS, □); HDL, FGF, and insulin (HFI, △); HDL and insulin (HI, ■); HDL and FGF (HF, O); insulin and FGF (IF, ▽); or HDL (H, ▲), insulin (INS, ◇), and FGF (◐) alone. The growth rate of cultures exposed to various concentrations of HDL, FGF, and insulin, either alone or in combination, was compared with that of cultures maintained in either serum-free medium (DMEM, ●; DME on fig.) or medium supplemented with 5% bovine serum (BS, □). The concentration of HDL added was 250 μg protein/ml, whereas the concentrations of insulin and FGF were 2.5 μg/ml and 100 ng/ml, respectively. HDL was added only once at day 0, whereas FGF was added every other day and insulin every 4 d. Every other day, triplicate plates representing each condition were trypsinized and counted. The standard deviation in the different determinations did not exceed 10% of the mean. (B and C) VSM cells were seeded as described above. 6 h later, dishes were washed twice with DMEM. Fresh DMEM supplemented with 5% bovine serum (BS, □), HDL, FGF, and insulin (HFI, △), HDL and FGF (HF, O), HDL alone (H, ▲), or DMEM alone (●) was then added to the dishes. The growth rate of the cultures was then compared with that of cultures in which EGF (E) was added instead of FGF. The concentrations of HDL, insulin, and FGF were as in A. The concentration of EGF was 50 ng/ml. Schedule of addition of the various factors was as in A. EGF was added every other day (C).
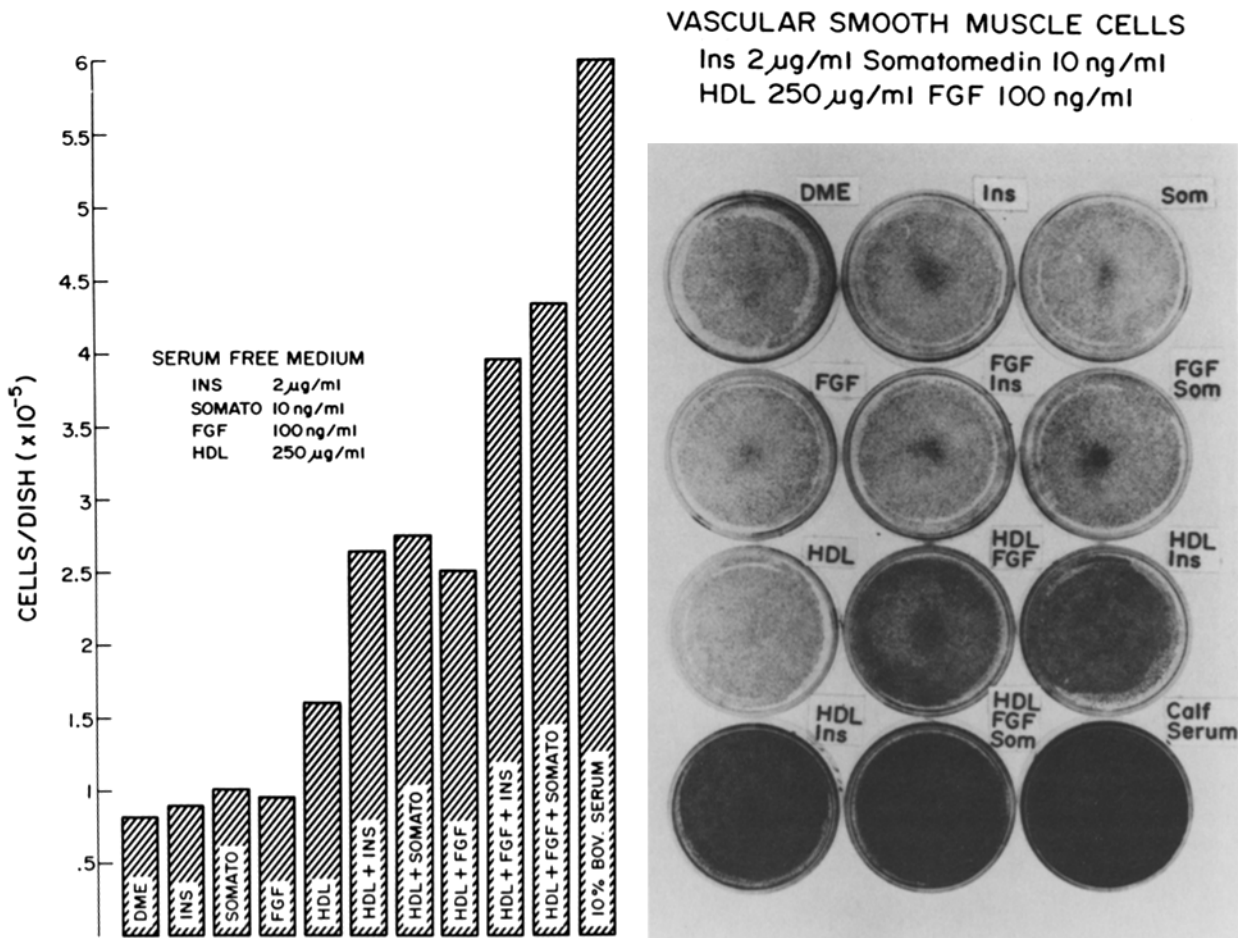
FIGURE 5 Comparison of the effect of somatomedin C and insulin on the proliferation of bovine VSM cells maintained in the presence of medium that either is or is not supplemented with HDL, FGF, and insulin. VSM cells were seeded at 1 × 10⁴ cells/35-mm dish on ECM-coated dishes in the presence of DMEM supplemented with 5% bovine serum. 6 h later, dishes were washed twice with DMEM, and fresh DMEM (DME on fig.) was or was not supplemented with HDL (250 μg protein/ml), somatomedin C (Somato, 10 ng/ml), insulin (Ins, 2.5 μg/ml), FGF (100 ng/ml), or 10% bovine serum (Bov. Serum, 10%) was added to the dishes. FGF and somatomedin C were added every other day. Insulin was added every 4 d. Serum or HDL was added only once at day 0. After 6 d, triplicate plates were trypsinized and counted. (A) One set of plates was fixed with 10% formalin and stained with 0.1% crystal violet (B).
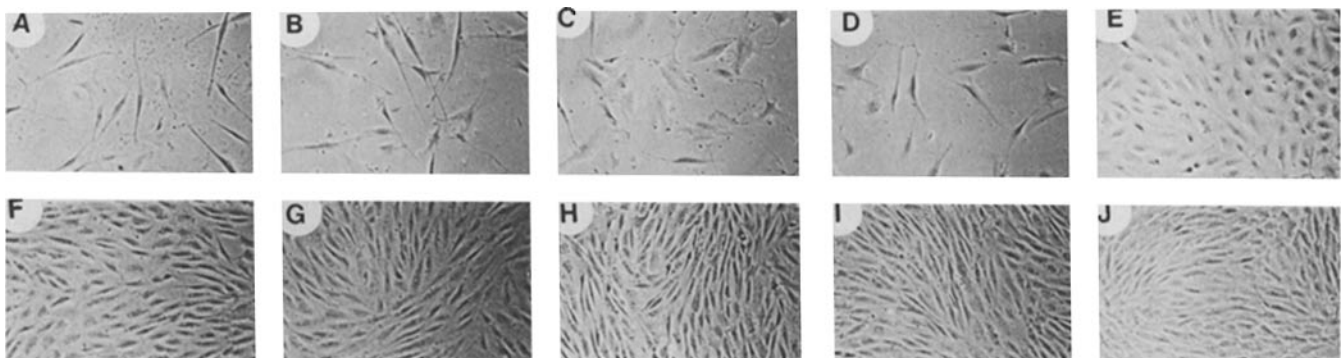

FIGURE 6 Morphological appearance of bovine VSM cells maintained on ECM-coated dishes and exposed to DMEM alone (A), EGF (B), insulin (C), FGF (D), HDL (E), HDL and FGF (F), HDL and EGF (G), HDL, insulin, and FGF (H), HDL, insulin, and EGF (I), or 5% bovine serum (J). Cultures were seeded and maintained as described in Figs. 4 and 5. Concentrations and the schedule of addition of the various factors to the culture media were the same as those described in Figs. 4 and 5. Pictures were taken on day 6. Pictures were taken with a Nipon phase-contrast photomicroscope. × 100.

generations before becoming senescent, regardless of whether FGF was present (Fig. 7 B) or not (Fig. 7 A).

EFFECTS OF HDL, LDL, INSULIN, AND FGF OR EGF ON CELLS CULTURED ON SYNTHETIC MEDIUM: The effects

of HDL, LDL, insulin, and FGF or EGF on the longevity of VSM cell cultures passaged at low cell density and seeded in the presence of serum on either plastic or ECM-coated dishes are shown in Figs. 8 and 9. Cells maintained on plastic and
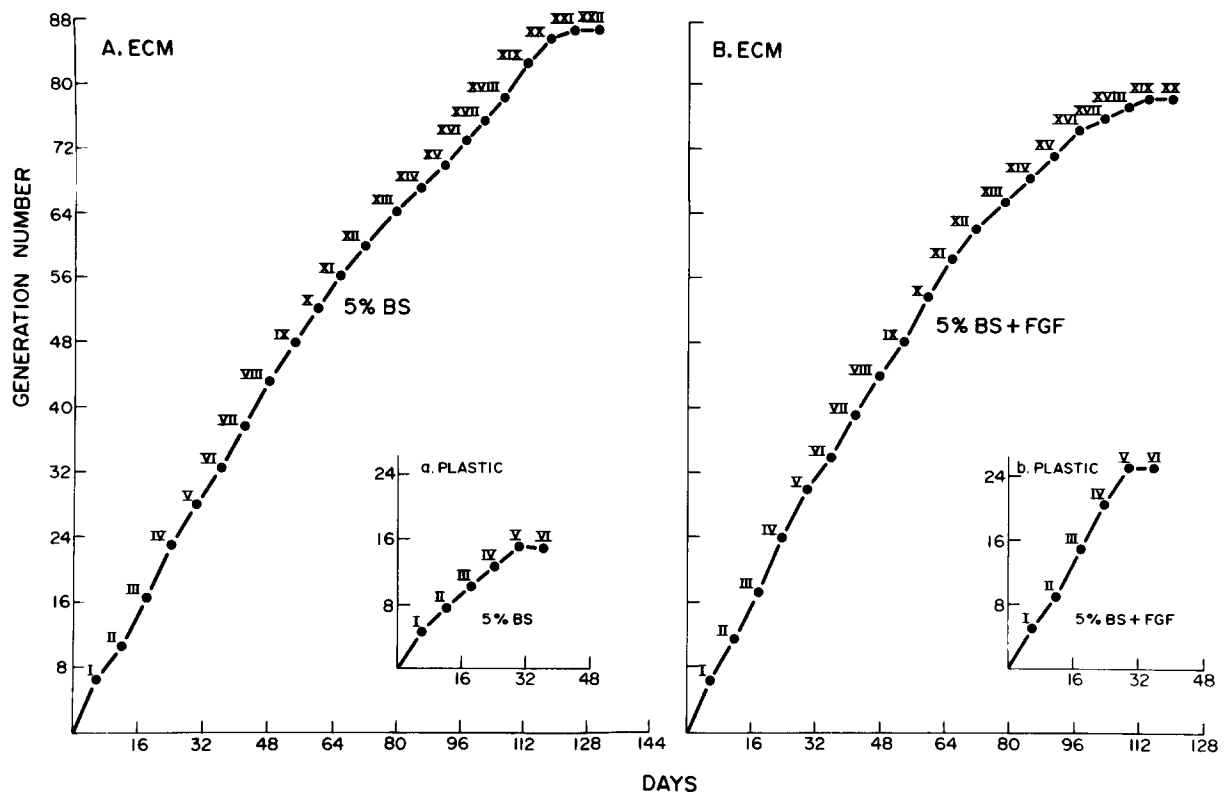
FIGURE 7   Comparison of the effect of the substrate (ECM vs. plastic) upon which VSM cells are maintained on the life span of the culture. VSM cells were maintained and passaged on plastic dishes (a and b) or on dishes coated with an ECM (A and B). Cultures were exposed to DMEM supplemented with 5% bovine serum (BS) and in the presence (B and b) or absence (A and a) of FGF (100 ng/ml) added every other day. The cultures were seeded at 1 × 10⁴ cells/35-mm dish and passaged every 6-7 d. The number of generations was determined from the initial cell density 8 h after seeding, and the number of cells harvested at each transfer. Each point represents a single transfer. Roman numerals indicate the passage number.
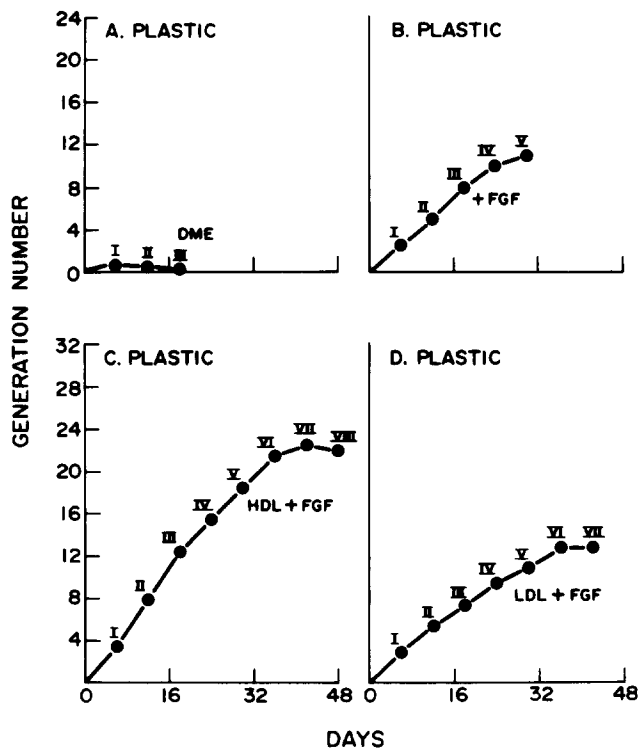

FIGURE 8   Culture lifetime of bovine VSM cell cultures maintained on plastic and exposed to DMEM (DME) alone (A) or to DMEM supplemented with FGF (B) HDL and FGF (C), or LDL and FGF (D). Cultures were seeded (1 × 10⁴ cells/35-mm dish) in the presence of

exposed to DMEM alone had a short life span in culture and did not markedly replicate (Fig. 8A). Addition of FGF markedly increased the longevity of the cultures, which underwent 12 generations (Fig. 8B). In the presence of HDL and FGF, the life span of the culture was extended to 24 generations (Fig. 8C). Low concentrations (30 µg protein/ml) of LDL together with FGF (Fig. 8D) did not markedly increase the longevity of the cultures beyond that observed when cultures were exposed to FGF alone (Fig. 8B).

When cells were maintained on ECM-coated dishes instead of plastic (Fig. 9) and exposed to DMEM alone, the longevity of the cultures was greatly increased because they could now be passaged during 14 generations (Fig. 9A). Addition of FGF did not significantly increase their longevity (Fig. 9B). This contrasts with its effect on the longevity of cultures maintained on plastic (Fig. 8A and B). When cultures were exposed to DMEM supplemented with optimal concentrations of HDL and FGF, the cultures underwent 43 generations (Fig. 9C), whereas when exposed to HDL, FGF, and insulin the total number of generations undergone by the cells was 51 (Fig.

serum (5%). 6 h later, the plates were washed twice and the medium was replaced with DMEM alone or DMEM supplemented with FGF, HDL and FGF, or LDL and FGF. FGF was added at a concentration of 100 ng/ml every other day. HDL (250 µg protein/ml) and LDL (50 ng protein/ml) were added only once at each transfer and on day 0. The number of generations was determined from the initial cell density 8 h after seeding and the number of cells harvested at each transfer. Each point represents a single transfer. Roman numerals indicate the passage number.

FIGURE 9 Culture lifetime of bovine VSM cell cultures maintained on ECM-coated dishes and exposed to DMEM (*DME*) alone (*A*) or to DMEM supplemented with FGF alone (*B*), HDL and FGF (*C*), HDL, FGF, and insulin (*D*), HDL and EGF (*E*), or HDL, EGF, and insulin (*F*). Cultures were seeded (1 × 10⁴ cells/35-mm dish) in the presence of serum (5%). 6 h later, the plates were washed twice and the medium was replaced with DMEM alone or DMEM supplemented with the various factors shown in panels *A–F*. HDL (250 µg protein/ml) was added only once at each transfer, whereas FGF (100 ng/ml) or EGF (50 ng/ml) was added every other day. Insulin (2.5 µg/ml) was added every 4 d. The number of generations was determined from the initial cell density 8 h after seeding, and the number of cells harvested at each transfer. Each point represents a single transfer. Roman numerals indicate the passage number.
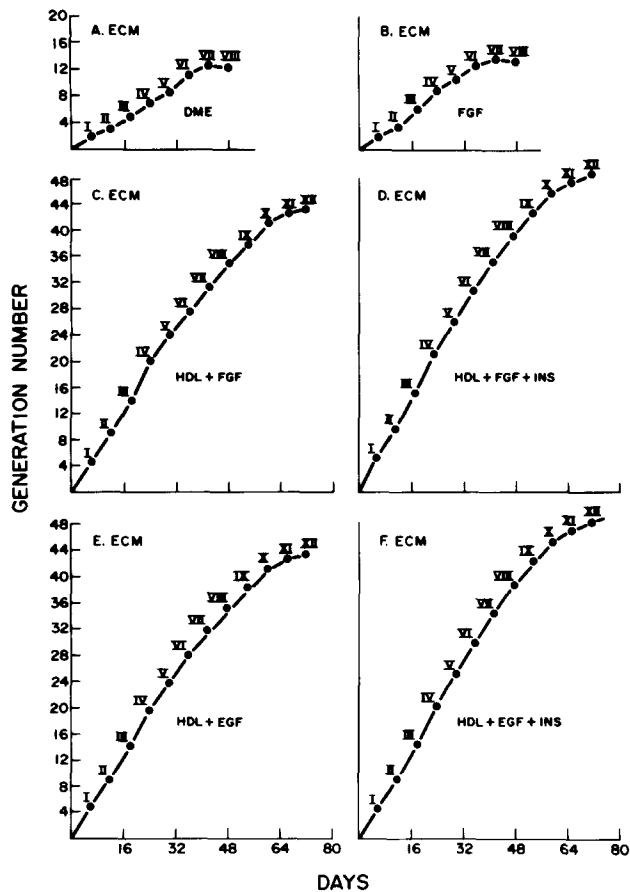
9 *D*). Cultures exposed to HDL and EGF or HDL, insulin, and EGF (Fig. 9 *E* and *F*) had a life span in culture similar to their counterparts exposed either to HDL and FGF or to HDL, insulin, and FGF. This is further demonstration that EGF can substitute for FGF.

Growth Rate and Life Span of Low-density VSM Cell Cultures Passaged in the Total Absence of Serum

The results described above demonstrate that VSM cells can proliferate actively when exposed to a synthetic medium supplemented with HDL, insulin or somatomedin C, and FGF or EGF. However, proliferation in response to these various factors required that seeding take place in the presence of either serum or plasma. This requirement may reflect the neutralizing effect of serum or plasma on the residual traces of trypsin bound to the cell surface or the need for transferrin, which is the main iron-carrying protein present in plasma. This

protein, known to adsorb strongly to various substrates (27), has been shown by others (1, 2, 31) to be required by various cell types grown in serum-free medium.

To distinguish between these possibilities, cells after being trypsinized were resuspended in media containing plasma (5%) or HDL (500 µg/ml) and seeded on ECM-coated dishes (Fig. 10 *A* and *B*). When cells were seeded in the presence of serum and exposed 6 h later to serum-free medium, transferrin was not required for proliferation (Fig. 10 *A*). In contrast, when cells were seeded in the absence of serum, addition of transferrin (10 µg/ml) was required for cells to respond to HDL. A comparison of Fig. 10 *A* and *B* demonstrates that the final cell density of the cultures was similar when cells were seeded on ECM-coated dishes in the presence of serum and exposed 6 h later to a synthetic medium supplemented with HDL, insulin, and FGF or when cells were seeded in total absence of serum and exposed directly to a synthetic medium supplemented with transferrin, HDL, insulin, and FGF. The concentration of transferrin required to obtain an optimal mitogenic effect of HDL was 2.5 µg/ml. Minimal effect was observed at concentrations <0.1 µg/ml (Fig. 10 *C*). The growth rate of low density VSM cell cultures maintained on ECM-coated dishes and seeded in total absence of serum is shown in Fig. 11 *A*. Maximal growth-rate was observed when cells were exposed to transferrin (10 µg/ml), insulin (2.5 µg/ml), and FGF (100 ng/ml) or EGF (50 ng/ml). The deletion from the medium of any of these factors resulted in a decreased growth rate (Fig. 11 *A*). Cultures maintained on ECM-coated dishes and exposed to serum-free medium supplemented with a combination of transferrin, HDL, insulin, and FGF or EGF could be passaged repeatedly at low cell density and in the total absence of serum (Fig. 11 *B*). The life span of such cultures was 58 generations.

DISCUSSION

Most normal cells in culture require either serum or plasma to attach, survive, and proliferate when maintained under tissue culture conditions. Previous studies have shown that, in the case of VSM cells maintained on ECM-coated dishes, the cells proliferate equally well in the presence of either plasma or serum (13). This ECM, as characterized in previous studies, is composed of collagen types I, III, and IV and V, the ratio of types I:III:IV and V being 3:16:1 (36). Associated with the collagens are proteoglycans and glycosaminoglycans that consist mostly of heparan sulfates (90%) together with small amounts of chondroitin sulfates (10%). Fibronectin and laminin are also present (11, 12). The corneal ECM, which is composed of laminin, glycosaminoglycans, as well as of collagen types III, IV and V, and fibronectin, therefore, has the biochemical composition of a basal lamina composed of a lamina lucida and a lamina densa (10, 11).

To investigate the factors present in plasma that could affect the proliferation of VSM cells, we have followed the innovative approach of Gordon Sato and his colleagues (1, 2, 31), who have shown that the serum or plasma requirement for growth of a number of cell lines can be satisfied by the addition of specific hormones and growth factors to synthetic media. We were further encouraged to take this approach by previous observations that indicated that VSM cells maintained on ECM-coated dishes needed much less serum or plasma to proliferate actively than when they were maintained on plastic (13). It is therefore possible that VSM cells, when maintained on ECM-coated dishes, could survive and still be responsive to plasma factors even when maintained in a well-defined syn-

SERUM FREE MEDIUM
INS 2μg/ml
FGF 100ng/ml
HDL 250μg/ml
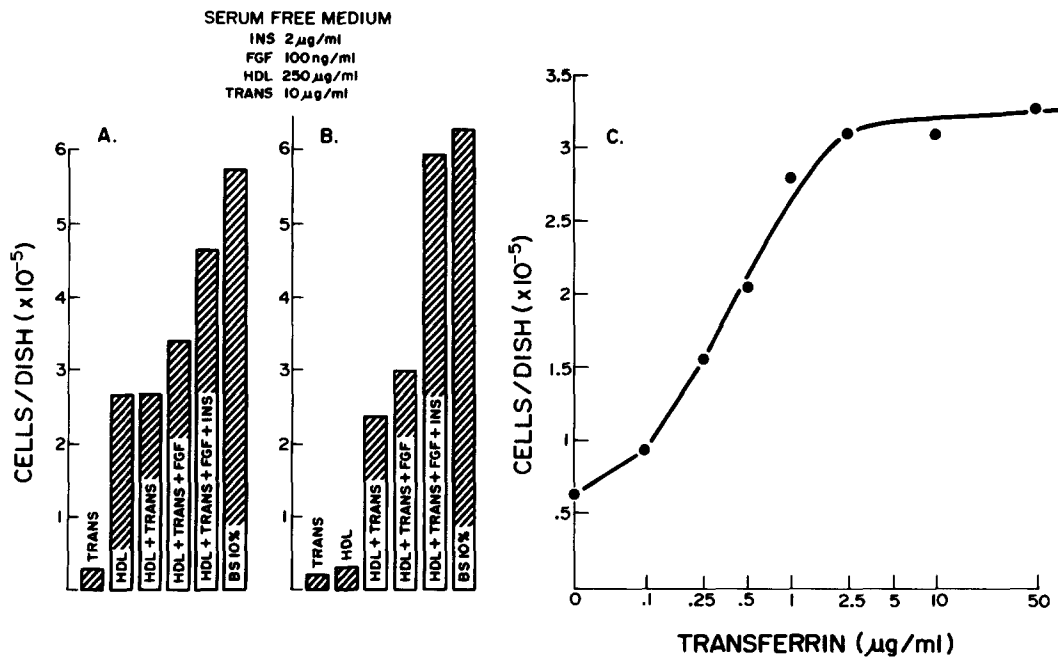TRANS 10μg/ml

TRANSFERRIN (μg/ml)

FIGURE 10   Effect of transferrin on the proliferation of bovine VSM cells seeded on ECM-coated dishes in the presence or absence of serum for 6 h and then exposed to serum-free medium. (A) VSM cells were seeded at 1 × 10⁴ cells/35-mm dish on ECM-coated dishes in the presence of DMEM supplemented with 5% bovine serum. 6 h later, dishes were washed twice with DMEM, and fresh DMEM that either is or is not supplemented with transferrin (TRANS, 10 μg/ml), HDL (250 μg/ml), FGF (100 ng/ml), insulin (Ins, 2.5 μg/ml), or 10% bovine serum (BS, 10%) was added to the dishes. HDL and transferrin were added once on day 0; insulin was added every 4 d; FGF was added every other day. After 6 d, triplicate plates were trypsinized and counted. (B) Same as A, except that after trypsinization cells were resuspended in medium containing 500 μg/ml HDL. Aliquots containing 1 × 10⁴ cells/35-mm dishes were then seeded in the presence of DMEM and in the total absence of serum. Transferrin, HDL, FGF, or calf serum was then added as described in A. After 6 d, triplicate plates were trypsinized and counted. (C) Effect of increasing concentrations of transferrin on the proliferation of VSM cells seeded in the absence of serum, as described in B, and exposed to medium supplemented with 250 μg/ml HDL. HDL and transferrin were added at the indicated concentrations only once (day 0). 6 d later, triplicate plates were trypsinized and counted. The standard deviation in the different determinations did not exceed 10% of the mean.

thetic medium unsupplemented with either plasma or serum.

Our results demonstrate that VSM cells seeded on ECM-coated dishes in the presence of plasma or serum can actively proliferate when exposed 6 h later to a synthetic medium supplemented with HDL, insulin, and FGF or EGF. An absolute requirement for transferrin becomes obvious only when cells are seeded and passaged in total absence of serum. Because transferrin, HDL, insulin, and EGF are present in plasma (FGF alone being absent), they may represent the plasma constituents involved in the control of the proliferation of VSM cells when such cells are maintained on ECM-coated dishes. The concentrations at which insulin was mitogenic are clearly pharmacological. However, because insulin can be replaced by somatomedin C, and because insulin is known to have a low affinity for somatomedin binding sites (37, 38), it may be that the high insulin concentrations required are due to its weak interaction with the somatomedin binding sites. This possibility is further supported by the failure of insulin and somatomedin C to have an additive effect on cell proliferation when they are added together at optimal concentrations (unpublished observation). This suggests that they interact through a common binding site. It is therefore likely that the mitogenic activity of insulin on VSM cells is not directly mediated through its interaction with its own receptor sites but through its interaction with somatomedin C binding sites.

The effect of EGF, unlike that of insulin, is mediated by its interaction with high affinity cell surface binding sites (K_d 2.08 × 10⁻¹⁰ M, 6,000 sites/cell). These binding sites are specific for

EGF but not for FGF, because FGF neither competes with EGF for binding nor displaces bound EGF from the cell surface (unpublished observations). Although it may at first seem surprising that EGF can replace FGF, numerous cell types (16), particularly fibroblasts (17), have now been shown to respond to both mitogens. The possibility exists that both mitogens, although they interact with distinct binding sites, could activate similar pathways leading to cell proliferation.

The mitogenic effect of HDL over a wide range of concentrations and its lack of toxicity for VSM cells are in accord with previous observations with vascular endothelial cells (34). However, in the case of vascular endothelial cells exposed to a synthetic medium, the addition of HDL alone is capable of insuring an optimal growth rate similar to that observed with optimal concentration of serum or plasma (34). In the case of VSM cells, addition of HDL alone, even at high concentration, does not fully replace plasma or serum, as reflected by the growth rate of the cultures, which is suboptimal. The mitogenic effect of HDL on VSM cells vs. the cytotoxic effect of LDL agrees with earlier reports of Hessler et al. (22) who, using human aortic medial smooth muscle cells maintained in the presence of medium supplemented with LPDS, have reported a cytotoxic effect of LDL at concentrations as low as 80 μg cholesterol/ml of medium, a concentration to which cultured cells would be exposed if whole serum from a normalipemic human donor were added as 10% of the culture medium. A similar cytotoxic effect of LDL has also been reported in the case of vascular endothelial cell cultures derived from either
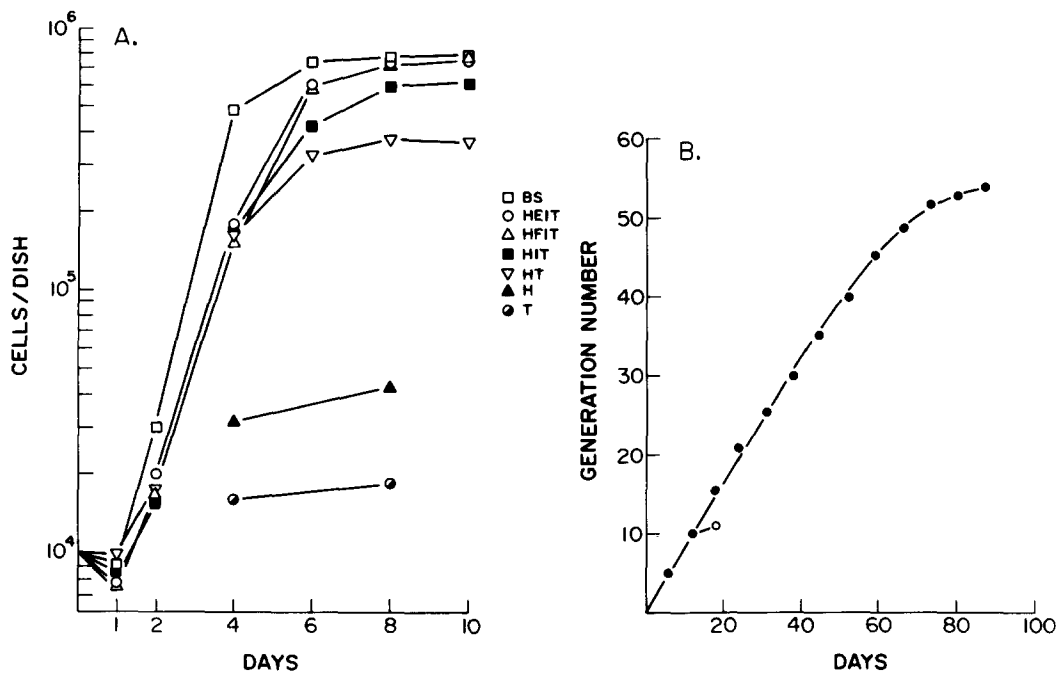
FIGURE 11 Growth rate and life span of low-density VSM cell cultures seeded on ECM-coated dishes and passaged in the total absence of serum. (*A*) VSM cells were seeded at 1 × 10⁴ cells/35-mm dish coated with ECM. Seeding was done as described in Fig. 10 *B*. Cells were exposed to 10% bovine serum (*BS*, □); transferrin (10 μg/ml, *T*, ◕); HDL (250 μg/ml, *H*, ▲); HDL and transferrin (*HT*, ▽); HDL, insulin (2.5 μg/ml), and transferrin (*HIT*, ■); HDL, FGF (100 ng/ml), insulin, and transferrin (*HFIT*, △); or HDL, EGF (50 ng/ml), insulin, and transferrin (*HEIT*, ○). The schedule of addition of the various factors was as described in Figs. 4 and 10. Triplicate plates representing each condition were trypsinized and counted every other day. Culture exposed to *HDL*, *FGF*, and transferrin had a final cell density of 4.86 × 10⁵ cells/dish, whereas cultures exposed to HDL, EGF, and transferrin had a final cell density of 5.28 × 10⁵ cells/dish. The standard deviation in the different determinations did not exceed 10% of the mean. (*B*) Life span of VSM cell culture seeded in the absence of serum and passaged repeatedly at low cell density. VSM cells were seeded initially at 1 × 10⁴ cells/35-mm dish coated with ECM. Seeding in the total absence of serum was done as described in Fig. 10 *B*. Every 6 d for the early passages and every 7 d for late passages, cultures were passaged at 1 × 10⁴ cells/35 mm dish (●). Cultures were exposed to DMEM supplemented with HDL (250 μg/ml), insulin (2.5 μg/ml), FGF (100 ng/ml), and transferrin (10 μg/ml). Similar life spans were observed when cultures were passaged in the presence of EGF (50 ng/ml) instead of FGF (unpublished data). Schedule of addition of the various factors was as described in Figs. 4 and 10 *B*. As soon as transferrin was omitted from the medium, cells no longer proliferated (○) and cultures therefore could no longer be passaged.

bovine aortic arch or human umbilical vein (21, 34).

The use of a synthetic medium supplemented with HDL, transferrin, insulin, and FGF or EGF allows VSM cells not only to proliferate actively but also to be passaged repeatedly. It therefore makes it possible to reach the goal of serial passage in a totally defined medium.

The substrate upon which VSM cells are maintained is an important factor in their response to the various factors considered in the present study. When cells are maintained on plastic and exposed to a synthetic medium, they will not proliferate. The addition of FGF, which in previous studies has been shown to replace the requirement for an ECM (13, 14), will allow the cells to proliferate and to respond to either HDL or LDL. Yet, the life spans of the cultures are far from impressive as, even when exposed to HDL and FGF, cells stopped proliferating after undergoing 23 generations. In contrast, when cells are maintained on ECM-coated dishes, even when exposed to a synthetic medium unsupplemented with any factor, cells can undergo 15 generations and, whereas the addition of FGF to the medium no longer has any effect on the life span of the cultures, the addition of HDL together with insulin and FGF or EGF will allow the cells to undergo 46 generations. The effect of the ECM in allowing cells to proliferate is even more impressive if one considers the case of cells exposed to synthetic medium supplemented with optimal concentrations of serum. Whereas cultures maintained on plastic can be maintained at

best for 16 generations, cultures maintained on ECM and exposed to serum can now be passaged for 88 generations. We therefore conclude that the ECM upon which VSM cells were maintained not only made cells sensitive to factors present in plasma but also increased considerably the life span of cultures exposed to a synthetic medium supplemented either with well-defined factors or with serum. This clearly demonstrates that environmental factors such as the substrate upon which cells rest affect the life span of cultured cells. Furthermore, because the life span of the cultures exposed to serum is longer than that of cultures exposed to a combination of HDL, insulin, and FGF or EGF, it is likely that there are other factor(s) present in serum which can increase it further.

Our observation that the close contact of the VSM cells with the ECM increases the longevity of the culture raises the possibility that it could also stabilize the phenotypic expression of VSM cell cultures with respect to their enzyme content and ultrastructural properties. Previous studies done by Fowler et al. (6) have shown that when calf aortic smooth muscle cells are grown in vitro under various conditions, striking alterations in enzyme contents, physical properties, and morphological appearances of lysosomes, endoplasmic reticulum, plasma membranes and, to a lesser extent, mitochondria were observed. Their results therefore demonstrate significant differences in specific cellular characteristics and functions of aortic smooth muscle cells grown in vitro compared with aortic cells *in situ.*

Whether such differences would be observed if cells were maintained on ECM-coated dishes rather than on plastic is an interesting question raised by these studies.

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