Stimulation of DNA Synthesis in Rat A10 Vascular Smooth Muscle Cells by Threonine-59 Insulin-like Growth Factor I

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The clonal smooth muscle cell line A10, derived from fetal rat aorta, binds ^{125}I-insulin-like growth factor I at a Type 1 insulin-like growth factor receptor. Threonine-59 insulin-like growth factor I, multiplication stimulating activity, and insulin inhibit the binding with IC_{50} = 10 nM, 84 nM, and 500 nM, respectively. Insulin in high concentrations (>5 μM) completely inhibits ^{125}I-insulin-like growth factor I binding to A10 cells. Threonine-59 insulin-like growth factor I and insulin stimulate [^{3}H]thymidine incorporation into DNA in A10 cells that had been growth arrested by incubation in serum-free media (DMEM/0.1% BSA) for 24–36 hours. The stimulation produced by the peptides is 50–60% of the stimulation produced by 10% fetal calf serum. Low levels of serum (0.1 and 0.5%) also stimulate DNA synthesis, and the effects of Threonine-59 insulin-like growth factor I and low serum are additive. The ED_{50} for the effects of Threonine-59 insulin-like growth factor I, multiplication stimulating activity, and insulin are 6.8 ± 0.3 nM, 36 ± 2.5 nM, and 360 ± 242 nM, respectively. Incubation of A10 cells for 24 hours with Threonine-59 insulin-like growth factor I or serum increases the protein content per culture dish by 85 ± 21 and 183 ± 26%, respectively (mean ± SEM). Thus, both protein levels and DNA synthesis are increased by incubation with peptides. However, Threonine-59 insulin-like growth factor I does not increase the number of cells in serum starved cultures, although 10% fetal calf serum does. Platelet-derived growth factor also stimulates DNA synthesis in A10 cells, but epidermal growth factor and acidic fibroblast growth factor do not. The effects of platelet-derived growth factor and Threonine-59 insulin-like growth factor I are additive. DNA synthesis begins 12 hours after the addition of Threonine-59 insulin-like growth factor I or serum to growth-arrested A10 cells. Threonine-59 insulin-like growth factor I can be removed from the cells after 8 hours, and maximal stimulation of DNA synthesis still occurs. Thus, a minimum 8-hour exposure of A10 cells to Threonine-59 insulin-like growth factor I is necessary to initiate the events required for the cells to progress from G1 to S phase. These data suggest that insulin-like growth factor I stimulates DNA synthesis in the A10 rat vascular smooth muscle cell line in the absence of serum and other exogenously added growth factors. However, insulin-like growth factor I alone apparently is not sufficient for cells to progress from S phase to cell division. (*Circulation Research* 1986;59:171–177)

INSULIN-LIKE growth factors I and II (IGF I and II) are polypeptides, purified from human serum, which share remarkable sequence homology with insulin.[1,2] IGF I, which is identical to human somatomedin C,[3] IGF II, and the rat IGF II analog, multiplication stimulating activity (MSA), promote growth in hypophysectomized animals (for review, see Froesch et al[4]). Since the plasma concentration of IGF I is influenced by growth hormone levels,[4] the potential role of IGF I in the pathogenesis of growth disorders is of considerable interest.

The concept that atherosclerotic lesions develop after abnormal proliferation of smooth muscle cells stimulated by increased release of platelet products, including platelet-derived growth factor (PDGF) and arachidonic acid metabolites, was first proposed by Ross and Glomset.[5] This hypothesis stimulated many studies of abnormal platelet and endothelial cell function in diabetic patients (for review, see Colwell et al[6]). Studies by Merimee and coworkers documenting the absence of microvascular and atherogenic complications in diabetic growth hormone deficient dwarfs[7,8] indicate that growth hormone or IGF I may also play a role in the progression of these diseases. While IGF I levels in diabetic patients with or without retinopathy are identical to the levels in nondiabetic subjects, serum levels of IGF I were doubled in a small subgroup of patients with accelerated progression of their retinopathy.[9]

IGF I and its analogs weakly stimulate DNA synthesis in many *in vitro* cell systems. However, the observation by Stiles and coworkers that quiescent BALB/c 3T3 cells require transient exposure to PDGF and continuous exposure to IGF I to progress to DNA synthesis showed that the combined action of platelet products and IGF I could be required for the proliferation of some cells.[10] Thus, in view of the potential role of IGF I in diabetic vascular disease, it is important to examine the effects of IGF I in vascular cells. Recent studies have shown that IGF I stimulates the uptake of ^{3}H-thymidine into DNA in primary cultures of aortic smooth muscle cells[11–13] and retinal capillary endothelial cells[13] when cells are grown in suboptimal amounts of serum or are preincubated with PDGF.

The continuously cultured, clonal smooth muscle cell line, A10, which was derived from fetal rat aorta,

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has proved to be a valuable tool in studying the electrophysiological properties of vascular smooth muscle.[14] We have characterized the stimulation of A10 cell DNA synthesis by IGF I in order to see if this stable cell line could be used as a model system for the investigation of the effects of IGF I on vascular smooth muscle. We now show that IGF I stimulates DNA synthesis in A10 cells in the absence of serum or exogenously added PDGF. Insulin and MSA are also active but less potent than IGF I. PDGF also stimulates A10 DNA synthesis, and the effects of PDGF and IGF I are additive. Prolonged exposure of A10 cells to IGF I is required for stimulation of DNA synthesis to occur. These cells appear to be a good model system for studying the effects of IGF I in vascular smooth muscle.

Materials and Methods

Dulbecco's Modified Eagle Medium (DMEM) containing 1000 mg D-glucose/L and all other tissue culture reagents were obtained from Grand Island Biological Co. (GIBCO). ^{125}I-insulin (receptor grade) and ^{3}H-thymidine (20 Ci/mmol) were from New England Nuclear Corp. ^{125}I-iodine (carrier free) was from Amersham Corp. [Thr 59] IGF I was from Amgen, partially purified PDGF (50,000 U/mg) and EGF from Biomedical Technologies, Inc. Coomassie Brilliant Blue protein dye reagent was from BioRad. Purified human IGF I was from Dr. R.E. Humbel, and purified acidic fibroblast growth factor was from Dr. K. Thomas. Insulin (porcine) was from Elanco. Multiplication stimulating activity (MSA) was the kind gift of Dr. Michael Czech. All other biochemicals were from Sigma.

The smooth muscle cell line, A10, derived from the thoracic aorta of embryonic rats was kindly provided by Dr. Richard Vandlen. Cells were grown in DMEM containing 10% fetal calf serum (FCS), 100 units of penicillin G and 100 μg of streptomycin sulfate/ml at 37° C in a humidified, 8.5%-CO_2-in-air atmosphere. To subculture the cells, confluent monolayers were washed with phosphate-buffered saline (PBS), treated with 0.1% trypsin–0.04% EDTA, placed in an equal volume of medium, and centrifuged at 600g for 5 minutes. Cells were seeded into T-75 flasks at an initial density of 2×10^4 cells/cm^2. For experimental purposes, cells were grown to confluence in 24 well tissue culture plates. The IGF receptor binding characteristics and the efficacy and potency of IGF in stimulating DNA synthesis in A10 cells remained stable from passage 16 through at least passage 150. The cells are hypertetraploid and the modal chromosome numbers of the A10 cultures at passage 56 were 98 and 100, which is similar to the 96 reported originally by Kimes and Brandt.[14]

^{125}I-IGF I Receptor Binding. ^{125}I-IGF I (specific activity, 85 Ci/g) was prepared by the method of Zapf et al.[15] ^{125}I-insulin or ^{125}I-IGF I (30 nCi) were incubated with cells in 16-mm wells in a final volume of 0.2 ml 0.1 M Hepes, pH 8, containing 0.12 M NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 8 mM glucose, and 0.1% bovine serum albumin (BSA) at 4° C for 17–20 hours. Cells were washed with three 1-ml aliquots of cold incubation buffer, then lysed with 1% sodium dodecyl sulfate (SDS) and counted. Specific binding of ^{125}I-IGF I, defined as that which is inhibited by 0.3 μM [Thr 59] IGF I, is 50% of the total binding.

^{3}N-Thymidine Incorporation into DNA. Cells were grown to confluence in 16-mm wells, washed two times with 1 ml DMEM/0.1% BSA, then incubated in 1 ml DMEM/0.1% BSA (unless otherwise indicated) for 24–36 hours at 37° C. Cells remained viable even after 72 hours of incubation in DMEM/0.1% BSA as determined by trypan blue exclusion. Cells were incubated with peptides or 10% FCS in 0.5 ml DMEM/0.1% BSA containing 1 Ci/ml ^{3}H-thymidine (20 Ci/mmol). After 24 hr (unless described otherwise) at 37° C, the cells were washed 4 times with 1 ml phosphate-buffered saline, then lysed by the addition of 0.3 ml of 0.4 N NaOH. The lysates were supplemented with calf thymus DNA (0.2 mg), then neutralized with 0.3 ml of 0.4 N HCl. DNA was precipitated by the addition of 1.2 ml absolute ethanol (−20° C). After 30 minutes at −20° C, the precipitates were collected on Whatman GF/F filters, and wells and filters were washed 5 times with 2 ml ice cold 70% ethanol. Filters were cut and shaken with 10 ml Aquasol II (New England Nuclear Corp.) for 2 hours before counting.

Autoradiography of Labelled Nuclei. Cells were incubated in serum-free media for 36 hours, then incubated with peptide or FCS for 12 hours. ^{3}H-Thymidine was added to pulse label cells for 4 hours, and then cells were washed 4 times with phosphate-buffered saline. Cells were washed with 1 ml ethanol:glacial acetic acid (3:1), with 3 × 1 ml 5% trichloracetic acid, and with 1 ml methanol at 0° C. Dried cells were lightly coated with Ilford Nuclear Research K5 Photographic Emulsion: 1% glycerol (1:1), and exposed for 6 days in a light-tight N_2 evacuated box with dessicant. Plates were submerged in Microdex developer for 3 minutes, rinsed in 1% acetic acid, fixed in 24% sodium thiosulfate for 5 minutes, rinsed excessively with H_2O, and dried. Cells were counterstained with a modified Giemsa's solution. The percent of labelled cells was determined by counting 350–400 cells from 4 different fields of each well. Under these conditions, the localization of the label was exclusively nuclear.

Protein Assay. Serum-starved cells were incubated with peptides or FCS for 24 hours, then washed once with phosphate-buffered saline. Cells were trypsinized (0.1%, 5 minutes), washed with phosphate-buffered saline, then suspended in 0.3 ml phosphate-buffered saline. Cells were disrupted (Branson Sonifier) and assayed for protein by the method of Bradford,[16] using bovine serum albumin as standard.

Determination of Cell Number. Confluent monolayers of cells in 6-well tissue culture plates (Nunclon) were serum starved as described above for 40 hours. The medium was then replaced with 2 ml DMEM/0.1% BSA containing 10% fetal calf serum or 50 nM [Thr 59] IGF I (Day 0). After 24 hr (Day 1) cells being treated with [Thr 59] IGF I were supplemented with an

additional 50 nM peptide. On subsequent days, total and viable cell counts were performed. Cells were washed once with 3 ml PBS, treated with 0.5 ml of 0.1% trypsin–0.04% EDTA for 2 minutes at 20° C and rinsed from the well with an equal volume of PBS/0.1% fetal calf serum. Cells were collected by a 30-second centrifugation in a microfuge and resuspended in 0.25–0.6 ml PBS/1% fetal calf serum, depending on the cell density. Fifty microliters of 0.4% trypan blue was added to 0.25 ml cell suspension for 5 minutes. The total and nonviable cells were counted with a hemacytometer.

Degradation of ^{125}I-IGF I. [Thr 59] IGF I (50 nM) and ^{125}I-IGF I (0.5 μCi/ml) were incubated with serum-starved A10 cells as described above for the thymidine incorporation assay. The media was removed and stored at −20° C until chromatography. An aliquot (50 μl) was chromatographed on a Supelcosil C18 column (Supelco) in 0.05 M phosphoric acid buffered to pH 2.5 with triethylamine. The peptide was eluted with a linear acetonitrile gradient (0 to 100%, 40 minutes). Intact ^{125}I-IGF I elutes at 25 minutes, while the largest peak of degraded material eluted at 4 minutes.

The radioimmunoassay of IGF I was performed as previously described,[17,18] except that [Thr 59] IGF I was used as a standard.

Results

The recombinant-DNA-derived peptide, [Thr 59] IGF I, is biologically active in many cell systems[19] and is a valuable tool in studying the activity of IGF I. We previously showed that [Thr 59] IGF I is equipotent to human IGF I in stimulating glucose transport in the skeletal muscle cell line BC3H1.[20] We now report use of this peptide to study the activity of IGF I in A10 cells.

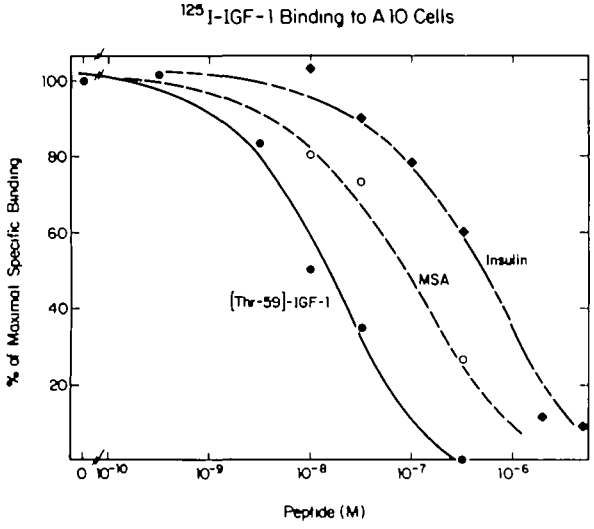

FIGURE 1. *Inhibition of ^{125}I-IGF I binding to A10 cells by [Thr 59] IGF I, MSA, and insulin. Confluent cultures (8 × 10^4 cells) were incubated with ^{125}I-IGF I (0.2 nM) and competing peptides at 4° C for 17–20 hours. Each point represents the average of duplicate determinations for 3 experiments or 1 experiment (MSA).*

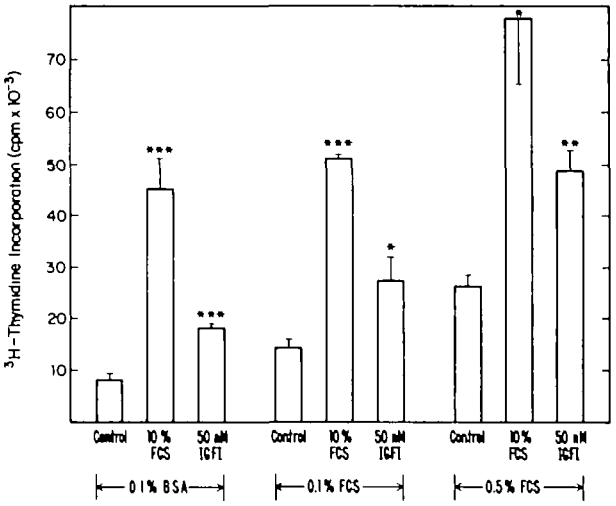

FIGURE 2. *Stimulation of DNA synthesis in A10 cells by [Thr 59] IGF I. Confluent monolayers were incubated for 40 hours with DMEM/0.1% BSA, DMEM/0.1% fetal calf serum, or DMEM/0.5% fetal calf serum. The medium was changed to the appropriate control containing either 10% fetal calf serum or 50 nM [Thr 59] IGF I. After 8 hours, ^{3}H-thymidine was added and the incubation continued for an additional 16 hours. Cells were digested and DNA precipitated as described in "Materials and Methods." Data are expressed as the mean ± SEM for triplicate samples. *p < 0.05; **p < 0.01; ***p < 0.005, different from control.*

^{125}I-IGF I binds to confluent A10 cells with high affinity (Figure 1). [Thr 59] IGF I, MSA, and insulin inhibit the binding with IC_{50} equal to 10 nM, 84 nM, and 500 nM, respectively. Purified human IGF I is equipotent to [Thr 59] IGF I as an inhibitor of ^{125}I-IGF I binding (data not shown). High concentrations of insulin (>5 μM) inhibit 100% of the binding of ^{125}I-IGF I to A10 cells. Specific binding of ^{125}I-insulin is 20% that observed with ^{125}I-IGF I in confluent cells. Specific binding of ^{125}I-MSA to A10 cells is not observed under these conditions (data not shown).

[Thr 59] IGF I and insulin stimulate the incorporation of ^{3}H-thymidine into DNA in confluent A10 cells that had been incubated in serum-free media for 24 hours prior to the addition of growth factor (Figure 2). In several experiments, the stimulation produced by the peptides is 50–60% that produced by 10% fetal calf serum. Autoradiography shows that incubation of cells with [Thr 59] IGF I or serum increases the percentage of cells with labelled nuclei by 2.2- and 3.7-fold, respectively. The percent of nuclei labelled during a 4-hour pulse with ^{3}H-thymidine after 12 hours in the absence or presence of growth factor or serum is 16.0 ± 2.7, 36.0 ± 2.5, and 59.5 ± 4.1 for DMEM/0.1% BSA, 50 nM [Thr 59] IGF I, and 10% fetal calf serum, respectively (mean ± SD, n = 6).

When A10 cells are growth inhibited by preincubation in the presence of low levels of serum, the basal incorporation of ^{3}H-thymidine into DNA increases with increasing amounts of serum, while the amount of incorporation in the presence of 10% fetal calf serum

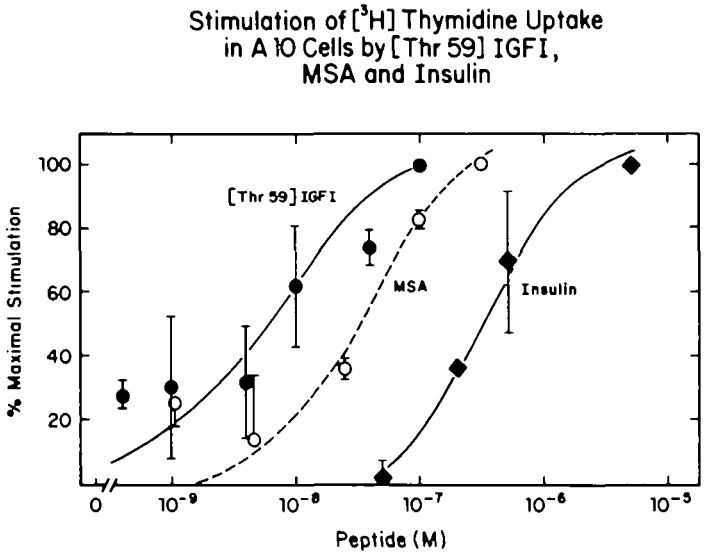

FIGURE 3. *Stimulation of ^{3}H-thymidine incorporation into DNA by [Thr 59] IGF I, MSA, and insulin. Confluent cells were serum starved for 24 hours, then incubated with peptides and ^{3}H-thymidine for 24 hours. Cells were digested and DNA precipitated as described in "Materials and Methods." Assays were done in triplicate. Data are expressed as the mean ± SD for 3 experiments or 2 experiments (MSA).*

remains relatively constant (Figure 2). [Thr 59] IGF I stimulates ^{3}H-thymidine incorporation into DNA to the same relative extent in the presence of 0.1% BSA, 0.1% fetal calf serum, and 0.5% fetal calf serum (2.2-fold, 1.9-fold and 1.9-fold, respectively [Figure 2]). Thus, [Thr 59] IGF I and low levels of serum have an additive effect on DNA synthesis in A10 cells.

Maximal amounts of [Thr 59] IGF I, MSA, and insulin stimulate ^{3}H-thymidine incorporation into DNA to the same extent. The ED_{50} for the effects of [Thr 59] IGF I, MSA, and insulin on ^{3}H-thymidine incorporation into DNA are 6.8 ± 0.3 nM, 36 ± 5 nM, and 360 ± 242 nM, respectively (Figure 3). The ED_{50} for [Thr 59] IGF I in 0.1% BSA and 0.1% fetal calf serum is identical (data not shown).

Incubation of A10 cells with [Thr 59] IGF I, insulin or serum for 24 hours significantly increases the protein content per culture dish (Figure 4). In three such experiments, [Thr 59] IGF I or serum increased the amount of protein by 85 ± 21 and 183 ± 26%, respectively (mean ± SEM). Thus, both protein levels and

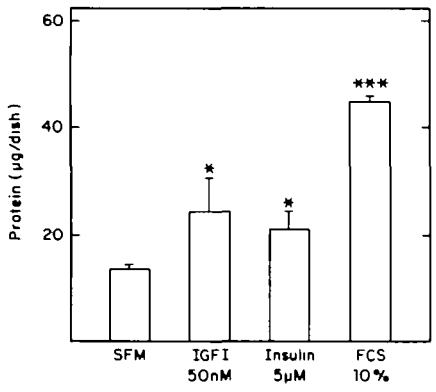

FIGURE 4. *Stimulation of total protein per culture by [Thr 59] IGF I, insulin, and FCS. Cells were serum starved for 24 hours, incubated with peptide or FCS for 24 hours, then assayed for protein as described in "Materials and Methods." Data are expressed as the mean ± SD for 3 determinations.* *$p < 0.05$; ***$p < 0.001$, *different from control (SFM).*

DNA synthesis are increased after incubation with peptide.

A10 cells made quiescent by a 40-hour incubation in DMEM/0.1% BSA were incubated for 2 days in fresh media in the presence or absence of [Thr 59] IGF I or 10% fetal calf serum. The number of viable A10 cells remains constant during this period in the absence of added serum or growth factor (Figure 5). The number of viable A10 cells increases 2.4-fold after a 2-day reexposure of quiescent, serum-starved cells to 10% fetal calf serum (Figure 5). Thus, even after extended serum starvation, A10 cells will divide when full growth media is reintroduced. In contrast, a 2-day exposure of these cells to [Thr 59] IGF I does not increase cell number (Figure 5). Thus, while [Thr 59] IGF I stimulates DNA synthesis in these cells, it is not sufficient to carry cells from S phase to cell division.

A partially purified preparation of PDGF stimulates ^{3}H-thymidine incorporation into DNA in A10 cells in the absence of serum (Table 1). The effects of PDGF and [Thr 59] IGF I are additive, and together they stimulate incorporation to an extent that approaches that of 10% fetal calf serum (Table 1). The ED_{50} for [Thr 59] IGF I stimulation is not altered in the presence of 1 unit/ml PDGF (data not shown). Purified acidic fibroblast growth factor (0.5 pM to 5 nM) and epidermal growth factor (2 nM) do not stimulate ^{3}H-thymidine incorporation.

A10 cells made quiescent by serum starvation begin DNA synthesis 12 hours after addition of [Thr 59] IGF I or serum (Figure 6). The maximal rate of DNA synthesis is increased 5-fold and 8.8-fold in cells incubated with [Thr 59] IGF I and serum, respectively. In order to determine the portion of this 12-hour period during which the cells require constant exposure to growth factor or serum for DNA replication to occur, [Thr 59] IGF I or serum was added to quiescent A10 cells (Time 0), and at various times, the cells were washed and incubated in fresh serum-free media. Twelve hours after Time 0, ^{3}H-thymidine was added for 2 hours and the incorporation into DNA was measured. Maximal DNA synthesis occurs in cells incu-

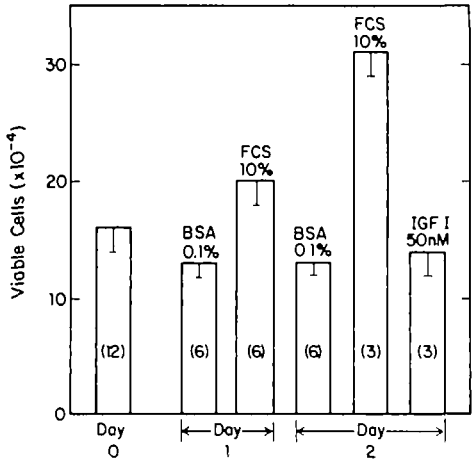

FIGURE 5. *Stimulation of cell division by reexposure to serum after serum starvation. Cells were serum-starved for 40 hours, then the media was changed to DMEM/0.1% BSA containing 10% fetal calf serum or 50 nM [Thr 59] IGF I (Day 0). After 24 hours (Day 1), cells treated with [Thr 59] IGF I were supplemented with an additional 50 nM peptide. On subsequent days, cells were washed and counted as described in "Materials and Methods." Data are expressed as the mean ± SD for the number of determinations given in parenthesis. The percentage of viable cells (as determined by trypan blue exclusion) is 85–90% or 90–95% for cells incubated with or without fetal calf serum, respectively.*

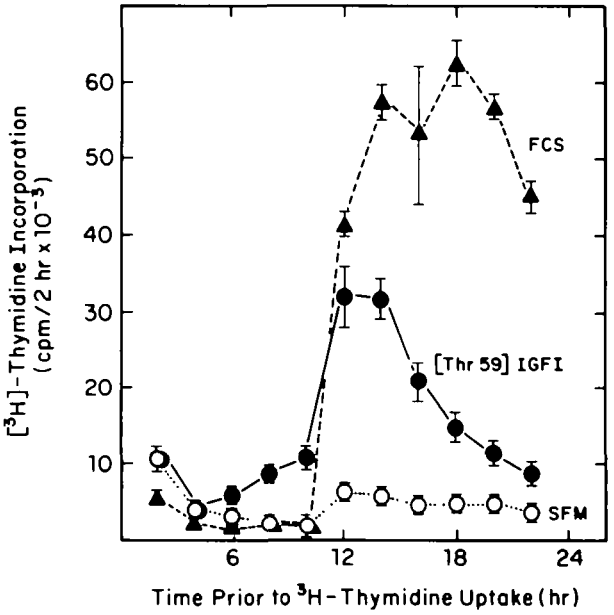

FIGURE 6. *³H-Thymidine incorporation into DNA at various times after addition of growth factor. Cells were serum-starved for 36 hours, then incubated with [Thr 59] IGF I (50 nM) or 10% FCS. At the times shown, ³H-thymidine was added and after 2 hours the cells were digested and the DNA precipitated. Data are the mean ± SD for 3 determinations. Similar results were obtained in one other experiment.*

bated with [Thr 59] IGF I for 8 hours and in serum-free media for 4 hours (Figure 7). However, some stimulation of DNA synthesis is seen even after a 4-hour transient exposure to peptide and 8 hours in serum-free media. The rate of DNA synthesis after 8 hours of exposure to [Thr 59] IGF I or serum followed by 4 hours in serum-free media is identical. However, DNA synthesis in cells exposed to serum for 10 hours and serum-free media for 2 hours is higher than in cells exposed to [Thr 59] IGF I for 10 hours (Figure 7, $p < 0.01$).

[Thr 59] IGF I (50 nM) and ^{125}I-IGF I were incubated with serum-starved A10 cells for 24 hours, then the media was removed and analyzed by reverse-phase HPLC. Less than 40% of the radioactivity coelutes with intact ^{125}I-IGF I, suggesting that there is extensive degradation of the peptide during the incubation (data not shown). In some experiments, 50% of the [Thr 59] IGF I is degraded even after 8 hours of incubation. However, an additional dose of [Thr 59] IGF I added 8

TABLE 1. *Stimulation of ³H-Thymidine Incorporation into DNA in A10 Cells by PDGF and [Thr 59] IGF I*

	³H-Thymidine incorporation (cpm × 10⁻³)*
DMEM/0.1% BSA	5.4 ± 1.4 (6)
[Thr 59] IGF I (50 nM)	16.0 ± 1.2 (3)†
PDGF (1 U/ml)	12.3 ± 0.6 (3)†
PDGF + [Thr 59] IGF I (1 U/ml + 50 nM)	26.4 ± 2.1 (3)†
Fetal calf serum (10%)	37.1 ± 0.5 (3)†

Confluent cultures of A10 cells were incubated in DMEM/0.1% BSA for 40 hours. Cells were incubated with or without peptides or serum for 8 hours before addition of ³H-thymidine, then for an additional 16 hours before ethanol precipitation.
*Mean ± SD (n), *n* = number of determinations.
†$p < 0.001$, different from DMEM/0.1% BSA.

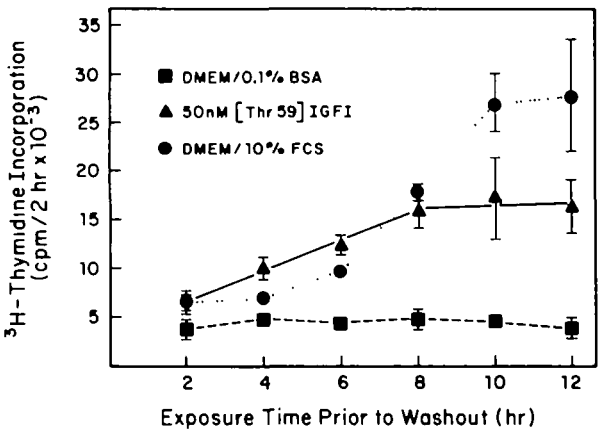

FIGURE 7. *³H-Thymidine incorporation into DNA after transient exposure to growth factor. Cells were serum-starved for 36 hours, then [Thr 59] IGF I (50 nM) or FCS (10%) was added. At the times indicated, cells were washed 2 times with DMEM/ 0.1% BSA and fresh DMEM/0.1% BSA was added. Twelve hours after the addition of peptide or FCS, ³H-thymidine was added for 2 hours before digestion and DNA precipitation. Data are expressed as the mean ± SD for 3 determinations. Similar results were obtained in one other experiment.*

hours after the initial dose does not increase the response of A10 cells to the peptide (data not shown).

Serum-free media that had been incubated with A10 cells contains material which binds to an antibody which recognizes human IGF I (antibody kindly provided by Drs. L. Underwood and J.J. Van Wyk and the National Hormone and Pituitary Program). Confluent A10 cells were incubated with DMEM/0.1% BSA, the media was removed and analyzed by radioimmunoassay. Little or no IGF I-like material is observed during the first 24 to 48 hours. However, between 48 and 67 hours, 9.1 ng-equivalents/ml IGF I-like material is secreted by the cells.

Discussion

The A10 cell line, isolated and characterized by Kimes and Brandt[14] from fetal rat aorta, has many of the properties of smooth muscle cells in culture. The specific activities of myokinase and creatine phosphokinase increase as the cells reach confluence. Confluent cultures of A10 cells contain the MM creatine phosphokinase isoenzyme that is characteristic of muscle tissue. Confluent cells produce synchronous, spontaneous, overshooting action potentials, and ultrastructural analysis reveals surface vesicles, thin filaments parallel to the long axis of the cells, and well developed endoplasmic reticulum and basement membranes that are characteristic of muscle cells. In addition, these cells contain multiple types of voltage-regulated calcium channels[21] and receptors for the vasoactive peptide, atrial nutriuretic factor.[22] Thus, these cells may represent a good model system for vascular smooth muscle. These cells have some advantages over primary cultured cells, including ease in handling and morphological and biochemical stability after multiple passages. In our studies, the characteristics of the stimulation of DNA synthesis by [Thr 59] IGF I and the properties of the IGF receptors have remained stable from Passage 16 through Passage 150. However, it must be carefully established that such clonal cell lines mimic the biological properties of interest of primary cultured lines.

High concentrations of insulin completely inhibit the binding of ^{125}I-IGF I to A10 cells. This is characteristic of the Type 1 IGF receptor as described by Massague and Czech,[23] for which MSA and insulin are 5–10 and 50–100 fold less potent than IGF I, respectively, as inhibitors of ligand binding. In contrast, the Type 2 IGF receptor has a higher affinity for IGF II and MSA than for IGF I, and insulin does not inhibit ligand binding to the Type 2 receptor even at micromolar concentrations.[23] Since ^{125}I-MSA does not bind to A10 cells under these conditions, these cells apparently have little or no Type 2 IGF receptor.

[Thr 59] IGF I is 60 times more potent than insulin in stimulating DNA synthesis in A10 cells. The concentrations of insulin and MSA required to stimulate DNA synthesis correlate well with the concentrations required to inhibit the binding of ^{125}I-IGF I to A10 cells. This suggests, but does not prove, that insulin and MSA stimulate DNA synthesis in A10 cells by interacting with the Type 1 IGF receptor. In work published while our studies were in progress, Clemmons[11] and King et al[13] showed that insulin was much less potent than IGF I in stimulating DNA synthesis in porcine and calf aortic smooth muscle cells, respectively.

[Thr 59] IGF I stimulates DNA synthesis in postconfluent A10 cells in the absence of other exogenously added growth factors, although this stimulation is only 50% that seen with 10% fetal calf serum. Our data indicate that A10 cells extensively degrade IGF I during a 24-hour incubation. However, this does not appear to account for the lower stimulation of thymidine incorporation into DNA observed with [Thr 59] IGF I than with serum since an additional dose of [Thr 59] IGF I added 8 hours after the initial dose does not increase the response. Alternatively, other growth factors contained in serum may be required to act in concert with IGF I to maintain maximal stimulation of growth. This latter hypothesis is supported by our finding that the effects of [Thr 59] IGF I and PDGF or low serum are additive and approach the stimulation seen with 10% fetal calf serum. EGF, which potentiates the effects of IGF in some cells,[10,11] does not greatly affect the activity of [Thr 59] IGF I in A10 cells. We have been unable to demonstrate that A10 cells bind ^{125}I-EGF (Cascieri, unpublished data), therefore, the poor response of A10 cells to EGF may be due to a deficiency in receptors.

Clemmons showed that DNA synthesis in porcine aortic smooth muscle cells is not stimulated by IGF I unless cells are pretreated with PDGF.[24,25] Similarly, IGF I stimulation of DNA synthesis in human arterial smooth muscle cells was only seen in the presence of 1% fetal calf serum.[12] Thus, in these two primary cell lines, PDGF or low serum potentiates the effects of IGF I, whereas in A10 cells the effects are additive. It is possible that some of the gene products normally increased in response to PDGF are produced constitutively in the clonal A10 cells such that the response to IGF I is not so clearly dependent on prior exposure to PDGF. Alternatively, A10 cells may produce low levels of growth factors, when incubated in serum-free medium, that are capable of stimulating DNA synthesis in an autocrine manner. Seifert et al[26] showed that aortic smooth muscle cells prepared from postnatal rats produce a PDGF-like molecule, while those prepared from adult rats do not. Subsequently, Nilsson et al[27] demonstrated that primary cultured adult arterial smooth muscle cells transiently produce a PDGF-like molecule when they are expressing a synthetic phenotype. Since A10 cells were cloned from fetal rats, it is possible that they produce such a PDGF type molecule. A10 cells do secrete an IGF I-like substance when incubated for prolonged periods with DMEM/0.1% BSA. Clemmons[25] recently reported that porcine aortic smooth muscle cells produce a similar substance and postulated that it may act to stimulate proliferation in an autocrine manner.

Confluent A10 cells rendered quiescent by serum deprivation require 12 hours to enter DNA synthesis after the addition of either IGF I or serum. However,

only an 8-hour period of exposure to IGF I is required to subsequently get maximum stimulation of DNA synthesis. Maximal stimulation by serum requires a 10-hour exposure. Density arrested BALB/c 3T3 fibroblasts require 12 hours after addition of PDGF and platelet-poor plasma to begin DNA synthesis.[28] These cells need only a transient exposure to PDGF, but, continuous exposure to serum during the 12-hour interval is required. IGF I and EGF can substitute for this serum requirement.[29] Maximal DNA synthesis is seen when IGF I is present in the media during the entire 12-hour period,[10,29] while EGF is required for only 6 hours out of the 12-hour interval.[10,30] It remains to be determined if the role of IGF I in stimulating proliferation of smooth muscle cells is the same or different from its role in fibroblasts.

Abnormal proliferation of smooth muscle cells is thought to be integral in the pathogenesis of diabetic atherogenesis.[6] The finding that IGF I and high doses of insulin stimulate DNA synthesis of vascular smooth muscle cells may indicate that the Type 1 IGF receptor plays a role in mediating these pathogenic processes. The fetal rat aorta smooth muscle cell line, A10, is extremely sensitive to IGF I and, therefore, may be a good model system to investigate the role of IGF I in these processes.

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