Spiess, H. W., & Sillescu, H. (1981) *J. Magn. Reson. 42*, 381.
Stamatoff, J. B., Fever, B., Guggenheim, H., Tellez, G., & Yamane, T. (1982) *Biophys. J.* (in press).
Sunder, S., Cameron, D. G., Casal, H. L., Boulanger, Y., & Mantsch, H. H. (1981) *Chem. Phys. Lipids 28*, 137.
Tardieu, A., Luzzati, V., & Reman, F. C. (1973) *J Mol. Biol. 75*, 711.
Verkleij, A. D., Ververgaert, P. H. J., van Deenen, L. L. M., & Elbers, P. F. (1972) *Biochim. Biophys. Acta 288*, 326.
Vogel, H., & Jahnig, F. (1981) *Chem. Phys. Lipids 29*, 83.
Wittebort, R. J., Schmidt, C. F., & Griffin, R. G. (1981) *Biochemistry 20*, 4223.

Purification of a Skeletal Growth Factor from Human Bone†

John R. Farley and David J. Baylink*

ABSTRACT: A skeletal growth factor was isolated and purified from demineralized human bone matrix. A dose of 6 μg/mL of the purified factor significantly increased the proliferation rate of embryonic chick bone cells in serum-free culture (292% of controls, $p < 0.0001$) but had no effect on embryonic chick skin cells plated at the same initial density. The factor is sensitive to inactivation by trypsin and urea, but not by collagenase, 20% butanol, or 1% mercaptoethanol. It is also resistant to inactivation by heat (stable for 15 min at 75 °C) and extremes of pH (stable for 30 min at 4 °C from pH 2.5 to 10.0). Purification of the active factor by selective heat and acid precipitations, molecular sieve column chromatography, and preparative polyacrylamide gel electrophoresis provided a material that was homogeneous by the criteria of high-pressure liquid chromatography, polyacrylamide gel electrophoresis, and isoelectric focusing. The apparent molecular weight is 83 000. The purified factor increases bone cell proliferation at doses comparable to other mitogens: 0.3 μg/mL (3.6 nM) significantly increases DNA synthesis to 231% of controls ($p < 0.001$). The purified factor was also active on cultured embryonic chick bones, enhancing the growth rate of tibiae and femurs, as measured by increased dry weight (185% of controls, $p < 0.025$) and [^{3}H]proline incorporation (164% of control, $p < 0.001$), respectively.

The coupling of bone formation to resorption was first suggested by clinical observations (Harris & Heaney, 1969) and subsequently verified by in vivo studies with rats (Thompson et al., 1975; Baylink & Liu, 1979). It is our concept that this skeletal coupling provides a counterregulatory mechanism for the local control of bone volume and that faulty coupling may lead to osteoporosis (Ivey & Baylink, 1981). Previous studies from this laboratory established that (a) coupling depends on a local mechanism—it occurs in vitro (Howard et al., 1980), (b) coupling is mediated by an increase in osteoblast number (Baylink et al., 1980), (c) a factor in culture medium that has been conditioned by resorbing bone in vitro will increase both the rate of bone cell proliferation and the growth of embryonic bone (Drivdahl et al., 1980a), and (d) a similarly active factor can be extracted from embryonic chick bone (Drivdahl et al., 1980b). The factor from these latter two sources has been interpreted to be a putative coupling factor that is present in bone matrix and released by bone resorption to increase osteoblast number and thereby increase the bone formation rate. In the present studies we sought to determine whether a similar putative coupling factor was present in adult human bone matrix.

Experimental Procedures

Chemicals and Supplies. Type II collagenase was purchased from Worthington Biochemicals. BGJ_b culture medium, FCS,[1] 16-mm multiwell tissue culture plates, and all other tissue culture supplies were obtained from Gibco. Sephadex G-200 and Sephadex G-75 were purchased from Pharmacia. Acrylamide and other electrophoresis supplies, as well as ampholytes, were purchased from Bio-Rad Laboratories. [^{3}H]Proline and [^{3}H]thymidine were obtained from New England Nuclear. EGF and insulin were purchased from Collaborative Research, Inc.

Isolation and Culture of Embryonic Chick Calvarial Cells. This procedure was adapted from a method developed for the isolation of fetal rat calvarial cells (Dziak & Brand, 1974), and has been described in detail (Drivdahl et al., 1980a). Briefly, a population of cells is prepared from the frontal and parietal bones of 16-day-old embryonic chicks by sequential collagenase digestion. The cells are washed, suspended in serum-free BGJ_b medium, and plated into multiwell dishes (16-mm diameter wells) at a density of 250–350 cells/mm^2 in a total volume of 1.0 mL for incubation at 37 °C in 5% CO_2 and 95% air. For several of the experiments described below cells were plated in microwell dishes (6-mm diameter wells) at a density of 500 cells/mm^2 in a total volume of 0.25 mL. Histological examination of the calvaria before collagenase digestion showed that virtually all of the cells surrounding the embryonic bone were alkaline phosphatase positive. Since this enzyme is regarded as a specific marker for osteoblasts and their progenitors (and for chondrocytes, although they are not found in this site) (Puzas et al., 1981), we believe that the cells in these cultures are predominantly members of the osteoblast cell line. (Marrow cells and osteoclasts, which are relatively few in number, are unlikely contaminants since they are confined to the developing endosteal space which does not

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[1] Abbreviations: ^{3}H-TdR, tritiated thymidine; hSGF, human skeletal growth factor; cSCF, putative chick skeletal coupling factor; CM, conditioned medium; EGF, epidermal growth factor; FGF, fibroblast growth factor; FCS, fetal calf serum; BME, mercaptoethanol; IEF, isoelectric focusing; PTH, parathyroid hormone; BSA, bovine serum albumin; HSA, human serum albumin; EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography.

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communicate with the external bone surface.) We should note, however, that less than half of the cells in culture are alkaline phosphatase positive, and we do not know if this activity is lost during the collagenase digestion. Although this question may be moot, because alkaline phosphatase deficient cells from bone have been shown to have osteogenic potential (Ashton et al., 1980), we cannot dismiss the possibility of heterogeneity in our cultures.

Isolation of Other Cell Types. Embryonic chick skin, kidney, muscle, cartilage, bone, and liver cells were obtained by digesting rinsed tissues from 17-day-old embryonic chicks with collagenase (2 mg/mL). The cells obtained between 20 and 120 min at 37 °C were collected by centrifugation, rinsed, suspended in BGJ_b medium, and plated with 1% added FCS at an initial density of 25 cells/mm^2 in 16-mm diameter multiwell dishes. After 2 days in culture, they were changed to serum-free BGJ_b medium, rinsed, and incubated for an additional 24 h before the factor was added.

Assay for Cell Proliferation. The proliferation rate of cells in monolayer culture was assayed by the incorporation of ^{3}H-TdR into Cl_3CCOOH-precipitable material during a 2-h incubation. Our assay was adapted from the method of Gospodarowicz (Gospodarowicz et al., 1978) and has been described in detail (Puzas et al., 1981). Briefly, the chick calvarial cells were plated and cultured overnight, and then effectors were added for an 18–66-h incubation (the standard incubation was 18 h). ^{3}H-TdR (1 μCi/mL) was added to the cells 2 h before the end of this culture period (i.e., in the standard incubation, cells are labeled between 16 and 18 h). The radioactive medium was then removed, and the cells were rinsed in cold phosphate-buffered saline. A Q-tip, moistened in 12.5% Cl_3CCOOH, was used to swab each culture well, trapping the cellular material in the cotton fibers. The cotton tips were then washed twice in 12.5% Cl_3CCOOH and once in 95% ethanol (10 min for each wash) and counted in scintillation vials. A minimum of six wells were assayed for each sample (i.e., six separate data points for each determined mean). The standard deviation was usually 10% of the value determined (examples of variation within control groups are included under Results). This assay does assume that de novo synthesis of DNA in our culture system reflects cell proliferation; but, as shown below (Results), changes in the rate of ^{3}H-TdR incorporation do reflect changes in cell number.

In order to monitor purification (see below), one unit of skeletal growth factor activity is defined as the amount of material required to obtain a 100% increase in the cell proliferation rate, above control values, when ^{3}H-TdR incorporation is assayed between 16 and 18 h after addition of the factor, with chick calvarial cells plated in multiwells at an initial density of 350/mm^2. As a positive control for cell responsiveness, we expect to see 280–350% of control ^{3}H-TdR incorporation with 1% added FCS. Because intraassay variation (usually ±10%) was less than interassay variation (typically ±25%), all data in this paper are based on intraassay comparisons between controls and test agents.

Isolation and Culture of Embryonic Chick Tibiae and Femurs. Tibiae and femurs were dissected from 9-day-old embryonic chicks and cultured in serum-fee BGJ_b medium, with or without added factor, as previously described (Howard et al., 1980). Bone formation was determined by comparing increased dry weights or [^{3}H]proline incorporation in the treated and control bones, after 7 days in culture.

Purification of hSGF. Human femoral heads (about 80 g each) were obtained at hip replacement surgery and stored at −20 °C. The bones were frozen in liquid nitrogen, crushed, and repeatedly rinsed in 25 mM P_i buffer, pH 7.2, to remove all contaminating serum and marrow. The washed bone fragments were then suspended in a small volume of 10% EDTA at pH 7.2 (about 10 mL/100 g original weight of bone) and dialyzed against the same solution at 4 °C. After 7–14 days, the demineralized bone matrix and surrounding protein solution were homogenized in a Waring blender, and the soluble extract was retained for further purification. (Throughout the purification, activity was assayed by bone cell proliferation in vitro, as described above, and aliquots from the various stages of purification were analyzed in a single assay to assess the extent of purification for each step.) This crude extract of human bone matrix was then heated for 15 min at 75 °C, cooled on ice, and centrifuged at 18000*g* for 20 min. The precipitate was discarded, and the supernatant was acidified (at 4 °C with constant stirring) with HCl to a final pH of 3.0. The acid-insoluble material was removed by centrifugation as above. The acid-soluble protein solution was neutralized with NaOH, dialyzed against 2.5 mM P_i, pH 7.2, and concentrated to a small volume (e.g., 3–10 mL) with Aquacide (Calbiochem) for molecular sieve column chromatography on Sephadex G-200 (total column volume 240 mL). Fractions were monitored for protein by absorbance at 280 nm and assayed for bioactivity (^{3}H-TdR assay). The active fractions were pooled and concentrated with Aquacide to a final concentration of 1.0–2.5 mg/mL. This material, which was used for most of the studies detailed below, showed one major band of protein on polyacrylamide gel electrophoresis, representing 85–90% of the stainable protein (Coomassie Blue R-250), and three minor bands of larger molecular weight. Activity could be recovered from the position of the major band on unstained gels. This material was homogeneous by the criteria of HPLC, polyacrylamide gel electrophoresis (using a range of gel concentrations), and IEF.

HPLC was performed on a Waters Associates apparatus equipped with a Model 440 absorbance detector (measuring at 278 nm). Molecular sieve columns I-125 (exclusion limit 80 000) and I-250 (exclusion limit 230 000) were used both individually and in series.

Polyacrylamide gel electrophoresis was performed on a Pharmacia GE-4 electrophoresis apparatus using 5–15% gels and (Hedrick & Smith, 1968) stained with Coomassie Blue R-250.

IEF was performed on Pharmacia FBE-3000 flat bed apparatus in a granular Sepharose medium (Sephadex G-75) with Biolyte 3/10 ampholytes (2% ampholyte concentration). By means of a grid, we obtained fractions from the focused gel, and these were eluted with 2.5 mM P_i buffer, pH 7.2, and dialyzed for 72 h against the same buffer for determination of protein concentration and bioactivity (^{3}H-TdR incorporation assay).

Chemical Methods. Protein concentrations were determined by the dye-binding method of Bradford (1976). Activities of bone extracts and effector solutions (i.e., ^{3}H-TdR assay) were determined by the reciprocal analyses of cell proliferation data; 1/percent stimulation was plotted vs. reciprocal factor concentration, and activity was calculated from the apparent K_m (e.g., the concentration required to obtain half the maximum effect). The kinetic nomenclature of Segel (1975) is used throughout.

Statistical Analysis of Data. All data are given as mean ± standard deviation, except where otherwise noted, and comparisons used Student's two-tailed *t* test for significance.

Results

Purification of hSGF. The hSGF activity in extracts of

Table I: Purification of Human Skeletal Growth Factor

fraction	volume (mL)	total protein[a]	total act.[b] (units)	sp act. (units/mg)	purification
crude extract[c]	360	2586	4.0×10^5	154	
heated extract	355	471	3.6×10^5	763	5.0-fold
acid-soluble extract	380	97.9	3.3×10^5	3343	21.7-fold
G-200 peak[d]	104	22.7	7.9×10^4	3498	22.7-fold
polyacrylamide gel electrophoresis gel slices[e]	3.7	0.51	2.1×10^3	4116	27.1-fold

[a] Protein measured by method of Bradford (1976). [b] Activity determined from reciprocal plots of percent stimulation (^{3}H-TdR incorporation) vs. concentration of hSCF. One unit of activity increases ^{3}H-TdR incorporation 100% above control levels. [c] Four femoral heads (322 g wet weight) were used in this preparation. [d] Only 70% of the acid-soluble extract was applied to the G-200 column. [e] Only 2.5 mg of the G-200 peak was applied to the gels.

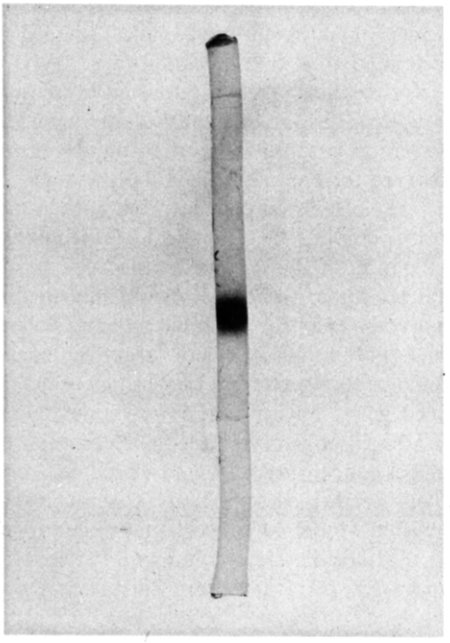

FIGURE 1: Photograph of 7.5% polyacrylamide gel containing 150 μg of purified hSGF. The same single band was detectable with concentrations as low as 10 μg. Single bands were also seen on 5%, 10%, 12.5%, and 15% gels.

demineralized human bone matrix was purified more than 27-fold to apparent homogeneity as shown in Table I. The estimate of purification (e.g., 27-fold) is undoubtedly too low, since the calculations assume that all of the activity in crude extracts is attributable to hSGF. Because we cannot account for the extent of nonspecific effects on cell proliferation from adding protein to a protein-free media, we can only be certain that hSGF accounts for *less* than 3.7% of the extractable protein in our preparation of bone matrix. In this regard, the stimulation of ^{3}H-TdR incorporation seen with HSA at 100 μg/mL (Table II) could represent such a nonspecific effect. Separate experiments using chick calvarial cells in microwell culture dishes demonstrated no effect of added casein at 10 μg/mL, but a significant stimulation of ^{3}H-TdR incorporation (140 ± 19% of control, $p < 0.005$) at 200 μg/mL. In the same experiment, purified hSGF stimulated ^{3}H-TdR incorporation to 208 ± 17% ($p < 0.001$) at 2.5 μg/mL. With regard to Table I, the material recovered from polyacrylamide gel electrophoresis was homogeneous by the following criteria: (a) Reelectrophoresis on polyacrylamide gels containing 5–15% acrylamide (Hedrick & Smith, 1969) revealed only one band of protein, even when 10 times the minimum detectable

Table II: Comparison of hSGF Activity with Other Mitogens

mitogen	mitogen concn	effect on ^{3}H-TdR incorporation[a]	significance
hSGF at 0.3 μg/mL	3.6 nM	231 ± 35%	$p < 0.001$
hSGF at 1.0 μg/mL	12.0 nM	268 ± 42%	$p < 0.001$
hSGF at 10 μg/mL	120.0 nM	525 ± 59%	$p < 0.001$
insulin at 1 μg/mL	166 nM	229 ± 30%	$p < 0.001$
insulin at 10 μg/mL	1667 nM	256 ± 29%	$p < 0.001$
EGF at 0.1 μg/mL	16.5 nM	153 ± 18%	$p < 0.001$
EGF at 1.0 μg/mL	165.0 nM	207 ± 17%	$p < 0.001$
BSA at 10 μg/mL	151.0 nM	95 ± 14%	none
HSA at 10 μg/mL	150.0 nM	123 ± 19%	none
HSA at 100 μg/mL	1500 nM	258 ± 56%	$p < 0.001$
FCS at 66 μg/mL	0.1%	336 ± 57%	$p < 0.001$

[a] Standard cell proliferation assay (see Experimental Procedures); all values given as mean ± SD in percent of control, where control ($n = 12$) values are 100 ± 11%.

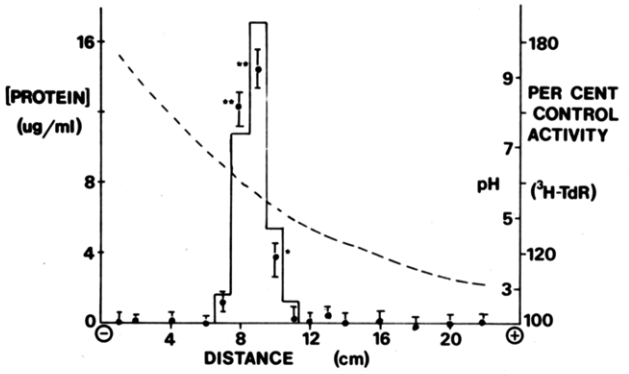

FIGURE 2: Profiles of protein concentration, pH, and bioactivity (^{3}H-TdR incorporation) from isoelectric focusing in a granular bed of Sephadex G-75 (procedural details under Experimental Procedures). Dotted line shows pH gradient, solid line shows protein concentration, and solid circles indicate bioactivity expressed as percent of control activity ± SEM. Asterisk indicates significant difference from controls, $p < 0.01$; two asterisks indicate $p < 0.001$.

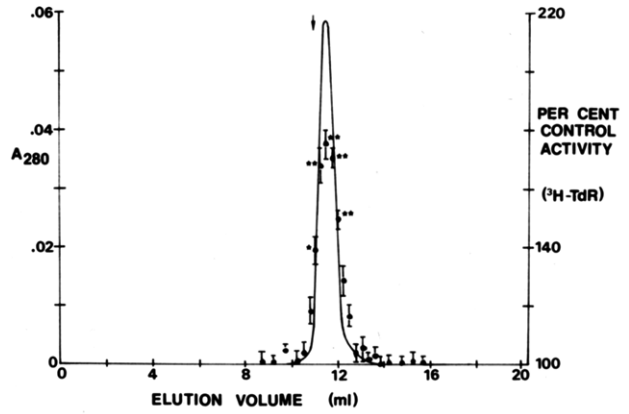

FIGURE 3: Profiles of protein concentration (measured by absorbance at 280 mM) and bioactivity (^{3}H-TdR incorporation) from HPLC on molecular seive columns I-125 and I-250 used in series. Solid line indicates absorbance at 280 nM, and solid circles indicate bioactivity ± SEM. Asterisk indicates significant difference from control activity, $p < 0.01$; two asterisks indicate $p < 0.001$. Arrow indicates void volume. To obtain this profile 25 μL of purified hSGF was injected at a solvent (2.5 mM P_i, pH 7.2) flow rate of 1 mL/min, and fractions were collected at 12-s intervals. Aliquots were added to the chick calvarial cells at a final dilution of 1/20 for determining bioactivity.

amount of protein was applied (Figure 1). The data obtained from these gels indicate that hSGF has a molecular weight of 83 000. (b) IEF, using ampholytes with an effective range of pH 3–10, also revealed only one protein band (Figure 2),

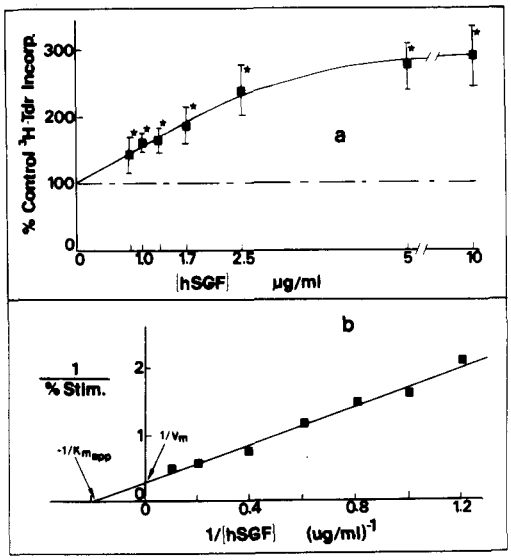

FIGURE 4: Dose/response curve for hSGF activity on bone cell proliferation in vitro. (a) Percent of control ^{3}H-TdR incorporation vs. hSGF concentration. Data points are mean ± SD for 6 determinations (12 determinations for controls). (☆) Difference from controls, $p < 0.01$; (★) difference from controls, $p < 0.001$, where controls were 100 ± 8%. (b) Replot of data from (a) in reciprocal form, where 1/percent stimulation indicates 1/foldness of stimulation. $K_{m_{app}}$ = 6.7 µg/mL and V_m = 430% of control.

as did (c) HPLC, using molecular sieve filtration columns (Figure 3). hSGF recovered from these analyses (HPLC, polyacrylamide gel electrophoresis, and IEF) was fully active on the cells (Figures 1–3). For most of the studies described below hSGF from the G-200 column peak was used. Reciprocal analyses of cell proliferation data gave the same value for $K_{m_{app}}$ with this fraction as with purified hSGF. We, therefore, believe that the minor contaminants present in the G-200 peak were neutral, with respect to cell proliferation. A dose/response curve with reciprocal analysis for the effect of the purified factor on cell proliferation is shown in Figure 4. $K_{m_{app}}$ is about 6.7 µg/mL, and $V_{m_{app}}$ is about 430% of control. This value of $K_{m_{app}}$ was much less variable than the value observed for $V_{m_{app}}$ with different preparations of chick calvarial cells. This suggests that different cell preparations differed more in their rates of basal serum-free proliferation (as observed) than in their responsiveness to the factor.

Stability of hSGF. hSGF is resistant to inactivations by heat (Figure 5) and extremes of pH (data not shown; no effect of 30-min incubation in P_i buffer at pH 2.5–10, with subsequent dialysis). Since the aliquots of hSGF used in these studies were treated (e.g., heated or acidified) and then reequilibrated to normal conditions (2.5 mM P_i buffer, pH 7.2) before addition to the cells, we can only conclude that hSGF activity was not irreversibly inactivated during these procedures. The hSGF activity was destroyed by trypsin. As shown in Figure 6, 60 min at 37 °C with 5 units of trypsin resulted in a complete loss of activity. Also shown in Figure 6 are the effects of 6 M urea, 4-h exposure to collagenase (5 units at 37 °C), 20% butanol, and 1% mercaptoethanol on hSGF activity. Of these, only 6 M urea resulted in a detectable inactivation.

Comparison with Other Mitogens. As shown in Table II, we saw a highly significant effect of hSGF on calvarial cell proliferation at doses as low as 0.3 µg/mL, corresponding to a concentration of 3.6 nM. A significant increase in ^{3}H-TdR incorporation with this dose of hSGF was confirmed in two additional experiments. Table II also contains data on the effects of insulin, EGF, BSA, HSA, and FCS on calvarial cell

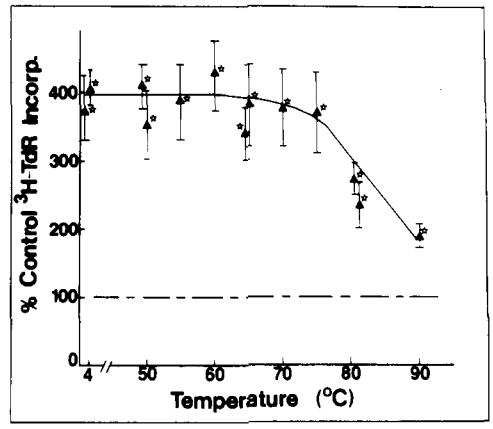

FIGURE 5: Effect of heat on hSGF activity. Purified hSGF heated at indicated temperatures (15 min in 2.5 mM P_i, pH 7.2), cooled, and assayed for effect on bone cell proliferation at 10 µg/mL. Data points are mean ± SD of 6 determinations (12 determinations for controls). (☆) Values different from controls, $p < 0.001$, where controls were 100 ± 13%.

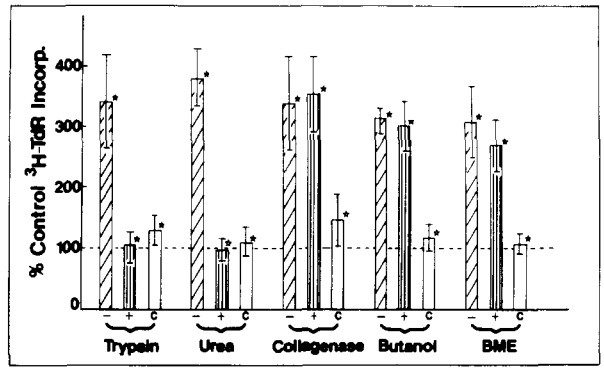

FIGURE 6: Potential inhibitors of hSGF activity. The hSGF was incubated with the following: 5 units of trypsin, 60 min at 37 °C; with 6 M urea, 30 min at 37 °C; with 5 units of collagenase, 4 h at 37 °C; with 20% butanol, 24 h at 4 °C; with 1% mercaptoethanol, 4 h at 4 °C. Residual activity (^{3}H-TdR incorporation assay) was determined by using treated hSGF (+) (vertical striped bars) and untreated hSGF (−) (diagonally striped bars) at 10 µg/mL. Effector controls, without added hSGF, were also tested (c) (open bars). Samples containing trypsin and collagenase were heated (75 °C) 15 min to inactive the enzymes after treatment, and these samples, as well as those containing mercaptoethanol, urea, and butanol, were dialyzed before application to the cells. Data are shown as mean ± SD of six determinations. (★) Values different from control (no additions), $p < 0.001$, where controls were 100 ± 13%; (☆) values different from untreated (−) hSGF, $p < 0.001$.

proliferation in vitro. It should be noted that (a) insulin and EGF, while also active at nanomolar concentrations, have a smaller maximum effect than hSGF and (b) BSA and HSA are only active at concentrations in excess of 10 µg/mL.

Cell Specificity of hSGF. As shown in Table III hSGF is effective at increasing the proliferation rate of low density cultures of bone and cartilage cells under serum-free conditions. It had no effect on identically treated skin, muscle, kidney, or liver cells. The data comparing calvarial and skin cells in response to hSGF are also shown in Figure 7a in terms of counts per minute of ^{3}H-TdR incorporated into Cl_3CCOOH-insoluble material. When skin cells were plated at a much higher initial density (as shown in Figuer 7b), a significant effect of hSGF was observed.

Effect of hSGF on Embryonic Bone. When purified hSGF was added to calvarial cells at 5 µg/mL, it increased the ^{3}H-TdR incorporation rate to 360% of controls (at 18 h) and increased cell number to 161% of controls (at 24 h). The larger increase in ^{3}H-TdR incorporation than in cell number

Table III: Comparison of hSGF Activity on Skeletal Cells and Bone

chick assay system	parameter	effect[a]	significance
calvarial cells[b]	^{3}H-TdR	292 ± 27%	$p < 0.001$
femoral (bone) cells[b]	^{3}H-TdR	218 ± 16%	$p < 0.001$
cartilage (sternum) cells[b]	^{3}H-TdR	248 ± 29%	$p < 0.001$
calvarial cells[c]	cell number	161 ± 7%	$p < 0.001$
calvarial cells[c]	^{3}H-TdR	369 ± 33%	$p < 0.001$
tibiae[d]	dry weight	185 ± 28%	$p < 0.001$
femurs[d]	[^{3}H]proline	164 ± 7%	$p < 0.001$

[a] Percent of control activity, mean ± SEM (n = 6 for cells, 10 for bones). [b] Cells from 17-day-old embryonic chicks (Experimental Procedures) plated at 25 cells/mm^2 and grown 48 h in 1% FCS and then 24 h serum free before addition of hSGF (6 μg/mL). All cells (including skin, kidney, liver, and muscle cells; see text) reached comparable densities. [c] Cells plated at 300/mm^2 (Experimental Procedures). Cell number was determined by releasing cells with trypsin/EDTA and counting in a hemocytometer. hSGF was added at 5 μg/mL. [d] Nine-day-old embryonic chick tibiae and femurs cultured as described under Experimental Procedures; treated with hSGF at 5 μg/mL. BSA, added at the same dose, had no effect on either parameter. [^{3}H]Proline incorporation in this system is predominantly into collagen [see Howard et al. (1980)].

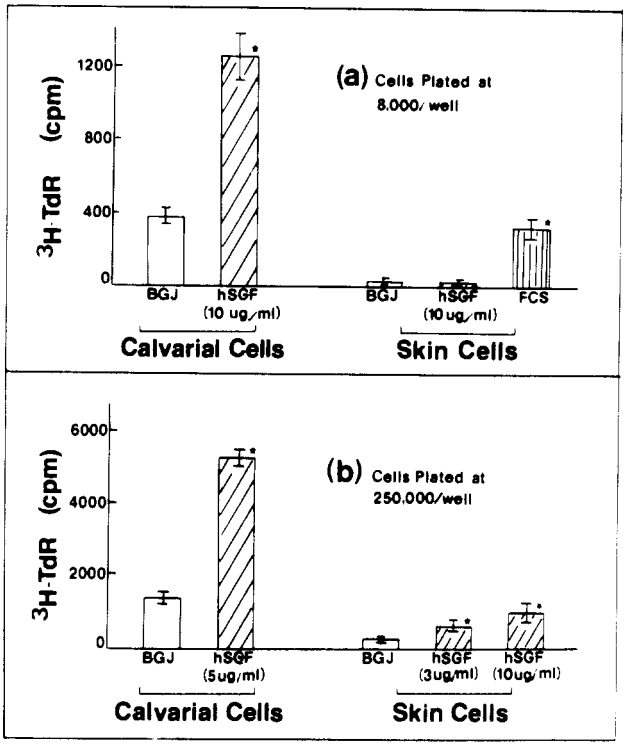

FIGURE 7: hSCF activity on cell proliferation in vitro; calvarial vs. skin cells. Values are shown as the mean ± SD of 12 determinations. (★) Values different from untreated controls, $p < 0.001$. (a) ^{3}H-TdR incorporation (cpm) in 16-day-old embryonic chick calvarial and skin cells with no additions (open bars), 10 μg/mL added hSGF (diagonally striped bars), and 1% added FCS (vertically striped bar). These cells were plated at a density of 8000/16-mm diameter well and cultured 48 h in 1% FCS and then 24 h serum free, before application of effectors. (b) ^{3}H-TdR incorporation in chick cells as in (a), with no additions (open bars) and added hSGF (diagonally striped bars). The cells were plated at 250 000/16-mm well and cultured 24 h serum free before the addition of the factor.

could mean (a) that hSGF has its effect on a subpopulation of cells that account for most of the basal ^{3}H-TdR incorporation, (b) that ^{3}H-TdR incorporation, measured at its peak, is not a valid index of cell number, which reflects the rate of cell proliferation integrated over time, (c) that ^{3}H-TdR incorporation is only influenced by cell proliferation but cell number is also influenced by cell death, or (d) that hSGF induces cells to enter S phase but prolongs the cell cycle. We need additional information to evaluate these possibilities. These data are shown in Table III. Table III also demonstrates that embryonic chick tibiae and femurs cultured for 7 days in the presence of 5 μg/mL purified hSCF grew faster than untreated controls.

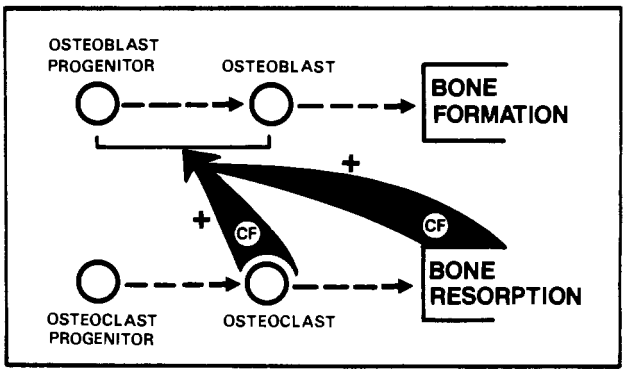

FIGURE 8: Theoretical model for coupling in bone. Details and interpretation are provided under Discussion.

Discussion

It is now generally accepted that bone volume is at least partially controlled by the coupling of bone formation to resorption (Baylink & Liu, 1979; Ivey & Baylink, 1981). Previous studies from this laboratory have established that this coupled increase in bone formation is partially mediated by an increase in osteoblast number (Baylink et al., 1980) and that coupling can occur in vitro (Howard et al., 1980). This latter observation is important because it implies that the mechanism of coupling is local and intrinsic to bone. Further investigations revealed a putative coupling factor in culture medium conditioned by coupling in vitro (Drivdahl et al., 1980a) and in extracts of chick bone (Drivdahl et al., 1980b). These observations from in vivo and in vitro studies have been incorporated into the model shown in Figure 8.

It is our concept that a bone resorption stimulus, such as PTH, increases osteoclast activity, increasing the bone resorption rate and releasing coupling factor. Our experimental observations require that the coupled increase in bone formation be proportionate to the increase in bone resorption, so the coupling factor could be released from bone matrix (assuming a uniform distribution in the matrix). Alternatively, the coupling factor could be released from osteoclasts in proportion to their activity; however, we have not been able to identify coupling activity in extracts of osteoclasts, so we favor the former possibility. Once it is released, coupling factor acts to increase the proliferation rate of osteoblast progenitors, resulting in increased numbers of osteoblasts and in increased bone formation rate. This model predicts that coupling factor should increase both the rate of osteoblast progenitor cell proliferation and the bone formation rate, as the activities in conditioned medium and the chick bone extract do. It also predicts that the release of coupling factor should be concomitant with resorption; the appearance of the factor in conditioned medium seems to be. Finally, this model predicts that coupling factor should interact locally and selectively with osteoblast progenitor cells, but this has not been tested. We should note that this model describes coupling in embryonic chick bone. Before we can apply it to coupling in general, we need to identify a similar coupling activity in adult bone.

In the present studies we have described the purification of a potent growth factor from adult human bone that possesses the activities required of a coupling factor. The homogeneous

factor is a large molecular weight (83 000) protein and is capable of increasing both bone formation and bone cell proliferation in vitro. Because in adult humans the amount of bone resorbed per day is about 1.7 g (i.e., 500 mg of calcium) (Harris & Heaney, 1969; Wergedal & Baylink, 1974), the amount of hSGF released over the entire skeletal resorbing surface would be only 255 μg/day (calculated from the data in Table I, assuming that 50% of the crude activity is attributable to hSGF). This would represent a low local concentration at a given resorption site, relative to the lowest effective dose of about 0.3 μg/mL. It remains to be determined whether this factor is released during or in proportion to resorption or bears any relation to coupling in vivo. Indeed, we do not know that it acts as a growth factor in vivo. We do know that the effect on cell proliferation is relatively selective for embryonic bone and cartilage cells in vitro. The data in Figure 7 suggest that purified hSGF will enhance the proliferation rate of skin cells in culture, but only when they are plated at high density. This could mean that hSGF is not specific for bone and cartilage cells. Before such a conclusion can be made, we need to better characterize those cells that do respond. Still, we cannot exclude the possibility that the responsive cells in skin, as well as those in bone, have the potential to form bone under certain conditions, particularly since ectopic bone can form in soft tissue sites.

With regard to the effect of hSGF on bone cell proliferation in vitro, our data will support the following conclusions: (a) hSGF is large for a mitogen, but not uniquely so. Although insulin, somatomedin, EGF, and FGF are all less than 15 000, a colony stimulating factor has been described with M_r 73 000 (Gospodarowicz & Moran, 1976). More pertinent to these results is the fact that previous estimates of the molecular weight of cSCF were about 70 000 (Drivdahl et al., 1980b). It is possible that we are observing aggregates of hSGF, but we saw no change in the elution profile of hSGF on HPLC in 0.1 M NaCl, as opposed to 2.5 mM P_i, with concentrations as low as 5 μg/mL. (b) Purified hSGF is active as a mitogen at nanomolar concentrations, as are EGF, insulin, FGF, and somatomedin (Table II; Gospodarowicz & Moran, 1976).

We can also conclude from these studies that hSGF is different from any growth factor previously described in human, or, with the exception of cSCF, any other bone. The factors recently described (Canalis et al., 1980) as affecting bone cell proliferation and bone growth in rats are much smaller than hSGF. The bone morphogenetic protein described by Urist and others has been variously characterized as being 23 000 daltons (Urist et al., 1981) or 70 000 daltons (Nogami et al., 1977) but is reported to be insoluble in dilute acid and in aqueous solvents and therefore should not be identical with hSGF.

In summary, (a) we have identified a potent growth factor from human bone matrix, (b) at nanomolar concentrations in vitro this factor will increase bone cell proliferation and enhance the growth of embryonic bone, and (c) the effect on cell proliferation appears to be specific for bone and cartilage cells.

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References

Ashton, B. A., Allen, T. D., Howlett, C. R., Eagleson, C. C., Hattori, A., & Owen, M. (1980) *Clin. Orthop. Relat. Res. 151*, 294.

Baylink, D. J., & Liu, C. C. (1979) *J. Periodontol. (Special Issue) 50*, 43.

Baylink, D. J., Morey, E. R., Ivey, J. L., & Stauffer, M. (1980) in *Vitamin D: Molecular Biology & Clinical Nutrition* (Norman, A. W., Ed.) p 387, Marcel Dekker, New York.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248.

Canalis, E., Peck, W. A., & Raisz, L. G. (1980) *Science (Washington, D.C.) 210*, 1021.

Drivdahl, R. H., Howard, G. A., Puzas, J. E., & Baylink, D. J. (1980a) *Endocrinology (Baltimore) 106 (Suppl.)*, 866.

Drivdahl, R. H., Howard, G. A., & Baylink, D. J. (1980b) *Calcif. Tissue Int. 31*, 53.

Dziak, R., & Brand, J. S. (1974) *J. Cell. Physiol. 84*, 75.

Gospodarowicz, D., & Moran, J. S. (1976) *Annu. Rev. Biochem. 45*, 531.

Gospodarowicz, D., Bialecki, H., & Greenburg, G. (1978) *J. Biol. Chem. 253*, 3736.

Harris, W. H., & Heaney, R. P. (1969) *N. Engl. J. Med. 280*, 253.

Hedrick, J. L., & Smith, A. (1968) *Arch. Biochem. Biophys. 126*, 155.

Howard, G. A., Bottemiller, B. L., & Baylink, D. J. (1980) *Metab. Bone Dis. Relat. Res. 2*, 131.

Ivey, J. L., & Baylink, D. J. (1981) *Metab. Bone Dis. Relat. Res. 13*, 3.

Nogami, H., Oohira, A., Terashima, Y., & Urist, M. R. (1977) *Clin. Orthop. Relat. Res. 122*, 307.

Puzas, J. E., Drivdahl, R. H., Howard, G. A., & Baylink, D. J. (1981) *Proc. Soc. Exp. Biol. Med. 166*, 113.

Segel, I. H. (1975) *Enzyme Kinetics*, Wiley-Interscience, New York.

Thompson, E. R., Baylink, D. J., & Wergedal, J. E. (1975) *Endocrinology (Philadelphia) 97*, 283.

Urist, M. R., Conover, M. A., Triffitt, J. T., Lietze, A., Bauer, F. C. H., Finerman, G. A. M., Hanamura, H., & DeLange, R. (1981) *Trans. Annu. Meet.—Orthop. Res. Soc. 6*, 136.

Wergedal, J. E., & Baylink, D. J. (1974) *Am. J. Physiol. 226*, 345.