4. The interdisciplinary use of language by mathematicians and metallurgists can be a problem. Two-dimensional mathematical surfaces are used to represent surfaces, interfaces, and grain boundaries of crystals. We reserve the word "boundary" to mean the one-dimensional curves (straight or otherwise) that are spanned by portions of surfaces. In this report, therefore, a grain boundary is a surface (and not a "boundary").
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11. The prescribed boundary problem allows one to use the technique of barriers to see directly that surface free energy cannot be decreased by having the surface slope or zigzag toward the middle. Any surface T composed of a horizontal half-plane and a vertical half-plane sharing their bounding line is uniquely minimizing since it is (a translation of) a tangent cone to the cylinder W or its central inversion (*9*). Therefore no minimizing surface S' can cross such a two–half-plane surface T on the interior of S'. A family of such T's brought up against the prescribed boundary from in front and above shows that any minimizing surface S' having the prescribed boundary must lie on or behind the top part of S and on or below the horizontal part of S. Similarly, a family of T's brought up against the boundary from in back and below the horizontal part of S shows that any such minimizing surface S' must lie on or in front of the lower part of S and on or above the horizontal part of S. Therefore, the horizontal part of S is pinned by T's from above and below and must be in each and every minimizing surface S' having the same boundary as S. The remainder of S' is thus split effectively into two pieces, a top one and a bottom one. It only remains to show that these pieces are the cylindrical parts of S. But this in turn follows from the assumption on the radii of curvature of C_1 and C_2 and the arguments made previously, since the surface free energy for each piece of S' is at least $2\gamma_v$ times the projected area of that piece plus the z-integral of the lengths of its intersection with horizontal planes. (The factor of 2 arises from the fact that, if there are noncylindrical parts in these remaining pieces, they will cover their projection on the horizontal plane at least twice.)
12. If W is a right cylinder, the above proof goes over fairly directly, though the integrals are more complicated; if W is not a right cylinder, one can do a linear transformation of space to convert W to a right cylinder, solve the problem, and transform back again.
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The Generation of Insulin-Like Growth Factor-1–Sensitive Cells by Growth Hormone Action

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Insulin-like growth factor-1 (IGF-1), a mitogenic polypeptide, is usually considered the sole effector by means of which growth hormone increases tissue mass. However, growth hormone, but not IGF-1, directly promotes the differentiation of cultured preadipocytes to adipocytes. Adipocytes newly differentiated from precursor cells in response to growth hormone were shown to be much more sensitive to the mitogenic effect of IGF-1 than the precursor cells. The result of IGF-1 action is therefore a selective multiplication of young differentiated cells (clonal expansion). This supports the concept of a dual effector system in which the preferred target cells of IGF-1 action are created by the direct action of growth hormone.

GROWTH HORMONE SPECIFICALLY promotes the differentiation of cloned lines of preadipose 3T3 cells into adipose cells (*1*). This is the result of a direct action of the hormone on the cells; IGF-1 (insulin-like growth factor-1 or somatomedin C), which has been regarded as an obligatory intermediate effector of the hormone in the promotion of growth (*2–4*), does not promote this differentiation (*5, 6*). To account for the direct and the IGF-mediated effects of growth hormone in animals, we proposed a dual effector theory (*7*) based on the concept that growth of tissues commonly occurs in two stages: (i) differentiated cells are formed from their precursors and (ii) the number of young differentiated cells is increased through limited multiplication (clonal expansion). The dual effector theory states that both stages are promoted by growth hormone: the first, directly by the hormone, and the second, indirectly, through its intermediate effector IGF-1. Although these two effects of the hormone cannot be easily distinguished in animal tissues, they can be distinguished in cell cultures. We show that cells with marked sensitivity to IGF-1 are produced by the prior action of growth hormone.

Preadipose 3T3-F442A cells were grown in 35-mm dishes containing the Dulbecco-Vogt modification of Eagle's medium supplemented with 5% cat serum and 0.5% calf serum. For experiments, cells grown to confluence in this medium were fed with modified conversion medium (*6*) containing 1.5% cat serum and 1.0% calf serum but lacking insulin to enable the cells to respond to IGF-1. The concentration of serum was the lowest compatible with good multiplication and adipose differentiation; the effects produced by added growth hormone and IGF-1 are increments over a relatively low background, some of which may be due to the presence of both proteins in the serum supplement.

The effect on adipose conversion produced by the addition of the two proteins is shown in Fig. 1. As the measure of differentiation, we used the activity of glycerophosphate dehydrogenase, a sensitive marker of the adipose phenotype (*8–10*). In the absence of added growth hormone, the differentiation of preadipose 3T3 cells was completely unresponsive to IGF-1, up to a concentration of 300 ng/ml. The cells did not develop glycerophosphate dehydrogenase, and although IGF-1 exerted a mitogenic action detectable by [^{14}C]thymidine incorporation (see below), it had no detectable effect on the total cell protein content per dish. No adipose cells were formed (Fig. 1D).

Human growth hormone promoted substantial differentiation even in the absence of added IGF-1, but when IGF-1 was added, the specific activity of cellular glycerophosphate dehydrogenase increased up to 4.5-fold (Fig. 1A). IGF-1 also substantially increased the protein content of the cultures in which the differentiation had been promoted by growth hormone (Fig. 1C). As a result, the total enzyme activity per culture undergoing adipose conversion increased up to tenfold after IGF-1 was added (Fig. 1B).

The combined effect of the hormone and IGF-1 could be the result of either more advanced differentiation within each adipose cell or an increase in the number of adipose cells. The proportions of adipose and nonadipose cells were therefore scored by counting cells containing or lacking fat droplets. This measurement is complicated by the fact that the addition of IGF-1 to growth hormone–treated cells increases the amount of lipid per fat cell. Using the dye Nile red, which is very specific and sensitive for lipid (*11*), to stain the living cells, we could easily identify fat cells containing small amounts of lipid. Cells exposed to IGF-1 alone did not acquire lipid droplets, but in growth hormone–treated cultures the addition of IGF-1 increased the proportion of adipose cells about 3.5-fold (Fig. 1D).

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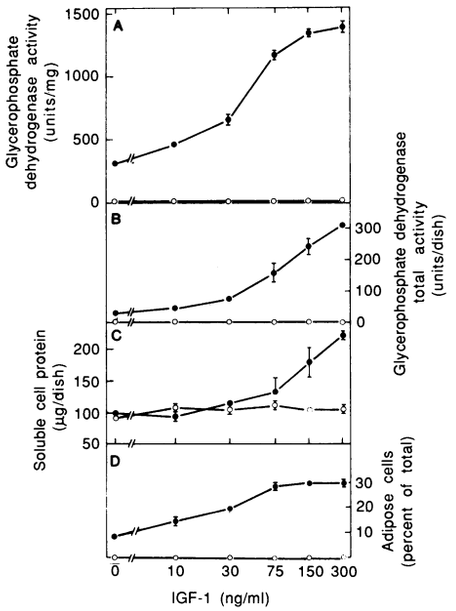

Fig. 1. The effect of IGF-1 on 3T3-F442A cells stimulated by growth hormone to undergo adipose differentiation (●), in the presence of human growth hormone (25 ng/ml) and (○), in the absence of growth hormone. (A) Specific activity of glycerophosphate dehydrogenase. (B) Total activity of glycerophosphate dehydrogenase. (C) Total cellular protein. (D) Number of cells becoming adipose. Cells (2×10^4) were inoculated into 35-mm dishes in the Dulbecco-Vogt modification of Eagle's medium supplemented with 5% cat serum and 0.5% calf serum. At confluence (5 to 6 days) the cells were fed with 3 ml of medium supplemented with 1.5% cat serum, 1% calf serum, $10^{-6}M$ biotin, transferrin (5 μg/ml), triiodothyronine ($2 \times 10^{-9}M$), mouse epidermal growth factor (30 ng/ml), partially purified with Bio-Gel P-10 (*23*) and 40 μ*M* β-mercaptoethanol. Additions of human growth hormone (to 25 ng/ml) were made at confluence, and IGF-1 was added, at varying concentrations, 1 day later. Eight days after the addition of growth hormone, cells were harvested. Cell monolayers were washed with phosphate-buffered saline (*p*H 7.4) and harvested in 0.3 ml of buffer containing 25 m*M* tris-HCl and 1 m*M* EDTA (*p*H 7.5). Any detached cells in the culture medium were recovered by centrifugation and added to the cell harvest. Cells were sonicated for 10 seconds at 30W with the microtip of a sonifer (Branson model 185). The extract was then centrifuged for 30 minutes at 78,000*g* at 4°C, and the supernatants were stored at −70°C. Assay of glycerophosphate dehydrogenase activity was as previously described (*9*) by measurement of oxidation of dihydronicotinamide adenine dinucleotide (NADH) at 340 nm. One unit of enzyme activity corresponds to the oxidation of 1 nmol of NADH per minute. The protein concentration of the supernatants was determined with Coomassie brilliant blue G-250 (*24*). All points on the graph represent averages of duplicate dishes. The bars indicate the range of the duplicates; where bars are absent, duplicate values fell within the symbol. The IGF-1 was the cloned product of Ciba-Geigy; the cloned products of Amgen and KabiVitrum were used in other experiments and gave results similar to those shown here. The human growth hormone used was the cloned product of Genentech.

If, as suggested by this experiment, the effect of IGF-1 is not on the process of differentiation itself, but on clonal expansion, it should be possible to demonstrate an effect of IGF-1 on multiplication of young adipose cells. Confluent cultures of preadipose cells received growth hormone; after 24 hours, IGF-1 (150 ng/ml) was added. Three days after the addition of IGF-1, 1 ml of medium was replaced with fresh medium containing the same concentrations of growth hormone and IGF-1. Twenty-six hours later, [^{14}C]thymidine was added. After 3 days, the cells were trypsinized and counted, and aliquots were precipitated with trichloroacetic acid and counted by liquid scintillation. In the absence of growth hormone, the addition of IGF-1 induced a 50% increase in the amount of ^{14}C incorporated; this is consistent with the known ability of IGF-1 to induce multiplication in different cell types. However, in the presence of growth hormone, the effect was much greater, amounting to about eightfold (Table 1). Clearly the action of growth hormone put the cells into a state much more susceptible to mitogenesis by IGF-1. Even in the absence of added IGF-1, the cultures that underwent adipose conversion incorporated somewhat more labeled thymidine than those that did not; it is not certain whether this could have occurred in the total absence of IGF-1, since some must have been present in the serum supplement.

From this experiment, the possibility could not be excluded that cells exposed to growth hormone became more sensitive to IGF-1, whether or not they had undergone differentiation. To examine this point, we repeated the experiment using autoradiography to reveal the location of the multiplying cells. In cultures inoculated with 10^4 preadipose cells, colony size reached about 100 cells at confluence. Susceptibility of the colonies to conversion is not uniform, and fat cell clusters formed by differentiation correspond to individual susceptible colonies. When cultures were grown in the presence of [^{14}C]thymidine and the incorporated ^{14}C was located by autoradiography, the clusters of labeled nuclei corresponded to clusters of adipose cells. Dense clusters of labeled nuclei were produced only when both growth hormone and IGF-1 were added (Fig. 2). In the absence of adipose conversion, the addition of IGF-1 increased the number of labeled nuclei only slightly and randomly.

These experiments did not determine whether IGF-1 acted on cells before, during, or after the action of growth hormone. This question was studied in two ways. First, cells were treated with IGF-1 for 4 days while they grew to confluence. The IGF-1 was then removed, the cells were washed

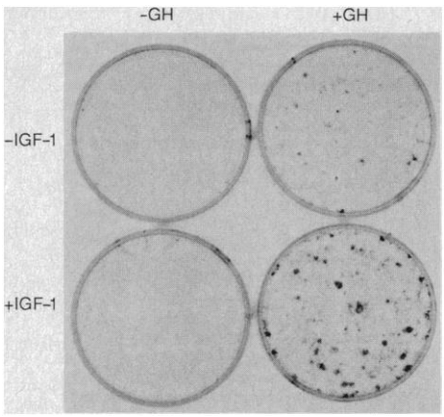

Fig. 2. Focal cell multiplication in differentiating cultures. Cultures were treated with growth hormone and IGF-1 and labeled as described in the legend to Table 1. After 2 days of labeling, the cultures were fixed and processed for autoradiography with NTB2 nuclear emulsion (*25*). Dishes were exposed to emulsion at 4°C and developed after 6 days.

extensively, growth hormone was added, and 4 days later [^{14}C]thymidine was added. Examination of such cultures by autoradiography after 3 days of labeling showed no more cell multiplication in the fat cell clusters than in clusters formed in the absence of preliminary IGF-1 treatment.

Second, we obtained from O. G. P. Isaksson an antiserum to human growth hormone which, when added together with growth hormone to preadipose cultures, was able to completely block hormonal action. For the experiment, this antiserum was added to cell cultures which had already been exposed to growth hormone for 5 days. If IGF-1 was now added together

Table 1. Effect of IGF-1 on the incorporation of [^{14}C]thymidine by preadipose and adipose cultures of 3T3-F442A cells. Cells were plated as described in Fig. 1. Additions of growth hormone (to 25 ng/ml) were made at confluence, and of IGF-1 (to 150 ng/ml) 1 day later. Three days after the addition of IGF-1, 1 ml of medium was replaced with fresh medium containing the same concentrations of hormone and IGF-1. Twenty-six hours later the cells were labeled by the addition of 1 μCi of [^{14}C]thymidine (53 mCi/mmol) to the 3 ml of medium. After 3 days of labeling, the cells were trypsinized and counted, and aliquots of cells were precipitated with trichloroacetic acid. The precipitates were deposited on glass fiber filters for counting by liquid scintillation. The values are the averages of duplicate samples together with the range of the duplicates.

Condition	[^{14}C]Thymidine incorporated per cell (cpm) $\times 10^3$	
	−GH	+GH
−IGF-1	6.7 (0.5)	15 (2)
+IGF-1	9.8 (1.1)	122 (5)

with [^{14}C]thymidine, an increased mitogenic effect was observed in the adipose cell clusters, demonstrating that the IGF-1 acted on cells that had already responded to growth hormone. As the effect was less marked than that obtained when the growth hormone and IGF-1 were present together for several days, these experiments do not rule out the possibility of an additional synergistic effect of IGF-1. However, because the cellular response to the hormone is not highly synchronized, the more pronounced effect observed when both agents are present is probably due to a greater likelihood that the IGF-1 will encounter the young adipose cells when they are most sensitive to it.

It is clear from these experiments that IGF-1 is a second effector in growth hormone action. Alone, it has a small mitogenic effect on preadipose cells, but it produces no differentiation. Together with growth hormone, it has a large mitogenic effect, but mainly on the differentiating cells. This results in clonal expansion of those cells, thereby selectively increasing their abundance. This process is completed within a few days, after which the adipose cells become mature enough so that they no longer multiply.

It has been demonstrated (*12, 13*) that insulin at supraphysiological concentrations has a mitogenic effect on cultures of Ob17 adipose cells. We found that all of the effects we describe for IGF-1 could also be produced by insulin at similar concentrations. Receptors for both IGF-1 and insulin increase with the differentiation of 3T3-L1 fibroblasts to young adipocytes (*14–16*). Although, in many cell types, mitogenic effects of both insulin and IGF are mediated by IGF receptors (*17*), in some cell types insulin receptors may also mediate mitogenic effects (*17–19*). However, since the serum concentration of IGF-1 is about 200 ng/ml, whereas that of insulin is usually about 1 ng/ml, it seems likely that clonal expansion in adipose tissue of the animal would be mainly the result of the action of IGF-1.

The dual effector theory of growth hormone action likely applies to other cell types. A direct effect of growth hormone on cartilage has been demonstrated in animals and in culture (*20–22*), although not yet clearly on prechondrocytes, as should be the case if the analogy to the adipose conversion is valid.

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Near-Total Glutathione Depletion and Age-Specific Cataracts Induced by Buthionine Sulfoximine in Mice

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The specific inhibitor of glutathione biosynthesis, L-buthionine sulfoximine (L-BSO), although relatively nontoxic in adult mice, induces severe glutathione depletion and age-specific pathological changes when repeatedly administered to male suckling mice. Dense cataracts developed when mice aged 9 to 12 days were given a series of injections of L-BSO, despite excellent survival and the absence of other significant long-term effects. By contrast, similar treatment of mice aged 14 to 17 days, although slightly less effective in reducing glutathione levels, resulted frequently in death, hind-leg paralysis, or impaired spermatogenesis, but did not produce cataracts. Administration of L-BSO to preweanling mice provides a novel model system for the induction of cataracts by depletion of lens glutathione and may enable the study of critical functions of glutathione in the lens and other growing tissues during early postnatal development.

GLUTATHIONE (GSH), γ-GLUTAMYLcysteinylglycine, the most abundant nonprotein thiol in living cells [0.5 to 10 m*M* (*1*)], is recognized to be a major intracellular antioxidant, a key component in the metabolism of cysteine and cysteine-containing proteins, and a broadly specific deactivator of potentially toxic electrophilic agents, via enzyme-catalyzed S-conjugation (*2*).

A number of compounds have been developed to alter the metabolism and intracellular concentration of GSH (*2*). One of the most widely used, in recent years, is buthionine sulfoximine (BSO) [(S-(*n*-butyl) homocysteine sulfoximine], a specific inhibitor of γ-glutamylcysteine synthetase (*3*), the enzyme that catalyzes the first step of GSH biosynthesis. Over 90 percent reduction of GSH levels has been achieved in cells cultured in the presence of D,L-BSO (*4*), a mixture of four stereoisomers, or the more specific preparation, L-BSO (L-buthionine-*S*,*R*-sulfoximine) (*5*). The consequences of such in vitro exposure include increased sensitivity to radiation, chemotherapeutic drugs, thermal stress, and oxidative agents (*4, 6*), inhibition of the biosynthesis of leukotriene C and prostaglandin E_2 in macrophages (*7*), and teratogenesis (*8*). On the other hand, treatment of adult mice with BSO has resulted in significant retention of GSH (at least 20 percent of control values) in most tissues and no grossly observable pathological effects (*9*). This and the large doses of BSO required for a significant effect on GSH levels in vivo have limited the usefulness of this highly specific drug for general physiological surveys of GSH function.

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