4×10^{12} *hv* cm^{-2} sec^{-1} for 10 seconds. This means that each chloroplast absorbed about 6×10^5 quanta. The number of grains associated with one chloroplast varied from 28 to 200. So we have one visible grain for something like 3×10^3 quanta absorbed.

WILLIAM ARNOLD, STELLA PERDUE
JIM AZZI

Biology Division,
Oak Ridge National Laboratory,
Oak Ridge, Tennessee 37830

References and Notes

1. R. Hill, *Proc. R. Soc. Lond.* **127**, 192 (1939).
2. C. E. K. Mees, *The Theory of the Photographic Process* (Macmillan, New York, 1946).
3. H. Molisch, *Sitzungsber. Akad. Wiss. Wien Math.-Naturwiss. Kl.* **127**, 449 (1918).
4. D. A. Walker, *Plant Physiol.* **40**, 1157 (1965).
5. G. A. Boyd, *Autoradiography* (Academic Press, New York, 1955); W. D. Gude, *Autoradiographic Techniques* (Prentice-Hall, Englewood Cliffs, N.J., 1968).
6. It is a pleasure to thank R. Irwin, D. E. Foard, W. D. Gude, and R. F. Kimball for help and advice. Research sponsored by the U.S. Atomic Energy Commission under contract with the Union Carbide Corporation.

11 January 1974; revised 4 March 1974

Expression of the Familial Hypercholesterolemia Gene in Heterozygotes: Mechanism for a Dominant Disorder in Man

Abstract. *Studies in cultured fibroblasts indicate that the primary genetic abnormality in familial hypercholesterolemia involves a deficiency in a cell surface receptor for low density lipoproteins (LDL). In normal cells, binding of LDL to this receptor regulates cholesterol metabolism by suppressing cholesterol synthesis and increasing LDL degradation. In cells from heterozygotes, a 60 percent reduction in LDL receptors leads to a concentration-dependent defect in regulation, so that attainment of equal rates of cholesterol synthesis and LDL degradation in normal and heterozygous cells requires a two- to threefold higher concentration of LDL in the heterozygote. The identification of this genetic regulatory defect in fibroblasts of heterozygotes makes available an in vitro system for studying the effects of a dominant mutation on gene expression in mammalian cells.*

Although nearly 500 different dominantly inherited mutations are known to cause disease in man, very little information exists regarding the biochemical mechanisms by which these mutant genes act (*1*). Unlike recessive disorders for which many of the basic enzyme defects are known, dominant disorders must involve a type of gene product which in a 50 percent deficiency is capable of producing clinical symptoms in heterozygotes. For this reason, it has been suggested that in many dominant disorders the mutations are likely to involve abnormalities not in enzymes, but in key nonenzymic proteins such as those that have been postulated to regulate complex metabolic pathways (*1*).

Evidence for the role of such regulatory proteins in eukaryotes is lacking since in vitro systems for demonstrating their presence and for studying the functional effect of their alteration by mutation are not generally available. Moreover, biochemical genetic studies of the possible involvement of regulatory proteins in dominant disorders have been hampered by the rarity of individuals homozygous for dominant genes, thus forcing investigators to study heterozygotes in whom biochemical expression of the regulatory role of the mutant protein is complicated by the presence of the normal gene product.

We have developed a cell culture system for the study of familial hypercholesterolemia, a dominantly inherited disorder that appears to involve a genetic abnormality in a regulatory protein and for which homozygotes are available (*2–4*). This culture system makes use of the observation that in normal human fibroblasts the rate of cholesterol synthesis is regulated by the presence of low density lipoproteins (LDL) in the culture medium (*3*). In order to suppress cholesterol synthesis, LDL must first bind to a specific high affinity receptor on the cell surface (*4*), and this binding results in inhibition of the synthesis of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase), the rate-controlling enzyme in the cholesterol biosynthetic pathway (*4*).

Cultured cells from homozygotes with familial hypercholesterolemia are almost completely deficient in this cell surface receptor for LDL and therefore are unable to bind the lipoprotein with high affinity (*4*). Consequently, LDL fails to suppress HMG CoA reductase activity in these cells, and cholesterol is overproduced (*2, 3*). In addition to regulating cholesterol synthesis, the LDL receptor also plays a major role in regulating LDL degradation as shown by the observation that LDL bound to the high affinity receptor in normal cells is degraded by proteolysis to a material that is soluble in trichloroacetic acid (*4*). Since the cells from the homozygotes are deficient in high affinity LDL binding, they are also unable to degrade LDL when it is present at low concentrations in the culture medium (*4*).

As a result of our studies in homozygotes, three biochemical tests are now

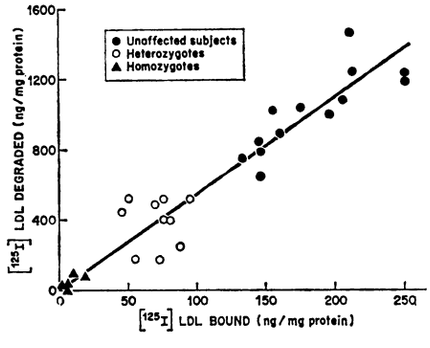

Fig. 1 (left). Relation between LDL binding and LDL degradation in cells from unaffected subjects, heterozygotes, and homozygotes with familial hypercholesterolemia. Each point is derived from the values for LDL binding and degradation given in Table 1.

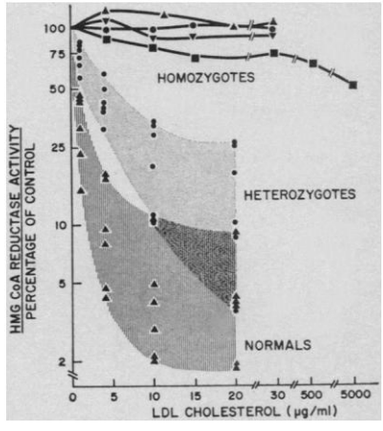

Fig. 2 (right). Effect of varying concentrations of LDL on HMG CoA reductase activity in cells from unaffected subjects, heterozygotes, and homozygotes with familial hypercholesterolemia. Cells were prepared in dishes, varying amounts of LDL were added, and after 24 hours, extracts for measurement of HMG CoA reductase activity were made as described in the legend to Table 1. Results are expressed as activity (amount of mevalonate formed per milligram of cell protein per minute) of cells grown in the presence of LDL divided by activity of cells grown in the absence of LDL; the ratio is multiplied by 100 to give the percentage of the control. The concentration of LDL is given in terms of its cholesterol content, which is twice the protein content.

available to assay the functional product of the familial hypercholesterolemia gene in fibroblasts: (i) measurement of the extent of [^{125}I]LDL binding, (ii) measurement of the rate of degradation of [^{125}I]LDL to ^{125}I-labeled acid-soluble material, and (iii) measurement of LDL-mediated suppression of HMG CoA reductase activity (*2–4*).

To elucidate a mechanism by which a dominant gene in single dose is able to produce derangement in cellular metabolism in diploid cells, we now report studies of the three functions of the LDL receptor in cultured cells from heterozygotes with familial hypercholesterolemia. Table 1 shows the results of assays for LDL binding, LDL degradation, and LDL-mediated suppression of HMG CoA reductase activity in fibroblasts derived from 13 unaffected subjects, 11 heterozygotes, and 5 homozygotes with familial hypercholesterolemia. Of the 11 heterozygotes, 6 were obligate gene carriers by virtue of their being parents of homozygotes and manifesting the typical clinical features of the phenotype (*5*); the remaining 5 were classified as heterozygotes on the basis of a typical clinical and genetic picture (that is, a serum cholesterol of 300 to 500 mg per 100 ml, tendinous xanthomas, and a family pedigree showing three-generation transmission of the trait). Clinical features of four of the homozygotes have been previously reported (*2*).

Table 1. LDL binding, LDL degradation, and LDL-mediated suppression of HMG CoA reductase activity in cultured fibroblasts derived from unaffected subjects, heterozygotes, and homozygotes with familial hypercholesterolemia. Cell lines were derived from explants of skin, maintained in logarithmic growth (*3, 4*), and studied between passage 5 and 20. Each confluent cell monolayer in a 60-mm petri dish was washed with phosphate-buffered saline 24 hours before the experiment, and then 2 ml of fresh Eagle's minimum essential medium containing 5 mg of lipoprotein-deficient human serum (*3*) was added. Binding and degradation were determined by incubating each monolayer with 5 μg of protein per milliliter of [^{125}I]LDL (specific activity, 150 to 220 count/min per nanogram of protein in different experiments) in the absence and presence of 500 μg of protein per milliliter of native LDL. After 6 hours at 37°C, the [^{125}I]radioactivity that remained bound to the cells after extensive washing was measured, and the content in the medium of ^{125}I-labeled material soluble in trichloroacetic acid was determined (*4*). The values, which represent high affinity [^{125}I]LDL binding and degradation, were calculated by subtracting the number of nanograms of ^{125}I bound or degraded in the presence of native LDL from that obtained in its absence (*4*). Each value represents the mean of triplicate determinations. The LDL-mediated suppression of HMG CoA reductase activity was determined as follows. After 24 hours growth in lipoprotein-deficient serum, the cells were treated with identical volumes of buffer containing 2.5 μg of protein per milliliter of native LDL or of buffer alone. After an additional 24 hours, cell extracts were prepared and HMG CoA reductase activity was determined as described (*2, 3*). Results are expressed as the ratio of the activity (picomoles of mevalonate formed per milligram of cell protein per minute) of cells grown in the presence of LDL to those grown in the absence of LDL; this ratio was multiplied by 100 to give the percentage of control. Each value is the mean of duplicate determinations; S.E.M., standard error of the mean.

Age (years)	[^{125}I]LDL distribution (ng/mg per 6 hours)			HMG CoA reductase (% of control)
	Sex	Bound	Degraded	
		Unaffected subjects		
Newborn	M	212	1240	5
Newborn	M	254	1190	4
6	M	143	639	
10	M	152	929	
11	M	204	1029	
25	F	142	816	
28	F	142	759	
30*	F	208	1470	18
31	M	188	996	8
31†	F	161	857	18
31	F	176	934	
34	M	253	1232	
40	M	131	762	9
Mean ± S.E.M.		182 ± 12	898 ± 66	10 ± 2.5
		Heterozygotes		
19	M	49	521	
29‡	F	74	513	
31	F	79	396	48
36‡	F	71	472	41
37‡	M	74	395	
38‡	F	71	179	52
38	M	96	517	
40‡	M	54	182	43
44‡	F	87	245	40
48	M			30
63	F	44	452	
Mean ± S.E.M.		70 ± 5.2	387 ± 43	42 ± 3.0
		Homozygotes		
6	F	0	0	95
6	F	19	95	84
10	M	9.4	86	91
12	F	2.1	20	100
23	F	2.4	1.0	99
Mean ± S.E.M.		6.6 ± 3.5	40 ± 21	94 ± 2.9

* Hypercholesterolemia secondary to hypothyroidism; serum cholesterol = 353 mg/100 ml. † Hypercholesterolemia secondary to nephrotic syndrome; serum cholesterol = 594 mg/100 ml. ‡ Obligate heterozygote (that is, a parent of one of the homozygotes).

High affinity [^{125}I]LDL binding, measured at a nonsaturating level of LDL (5 μg of LDL protein per milliliter), was reduced in the cells of all homozygotes tested, averaging 3.6 percent of the normal values (Table 1). Since our previous data have demonstrated that the homozygotes show no specific binding at concentrations of LDL as high as 500 μg/ml, this binding defect appears to be due to a deficiency in the number of functional receptor molecules rather than to the production of a receptor with decreased affinity (*4*). Thus, any binding observed in the heterozygote cells can be attributed to the product of the normal allele. In the heterozygotes, binding was reduced to approximately 40 percent of the normal values, suggesting that in these cells the single normal allele produces nearly one-half the normal number of LDL receptor molecules (Table 1). The functional significance of this diminished binding in heterozygotes was reflected in a mean decrease of 40 percent in the rate of LDL degradation and in an approximately half normal suppression of HMG CoA reductase activity (Table 1).

A plot of the relation of LDL binding to LDL degradation in the cells of all three genotypes demonstrated a trimodal distribution for both parameters with no overlap among the three groups (Fig. 1). A single regression line fits the data, supporting the conclusion that the decreased degradation of LDL in familial hypercholesterolemia is a secondary consequence of decreased binding to the LDL receptor.

The manner in which the defect in LDL binding affects the regulation of HMG CoA reductase activity in the cells of the three genotypes is shown in more detail in Fig. 2. Homozygotes showed no significant suppression of enzyme activity at LDL levels up to 50- to 100-fold higher than those producing 90 percent suppression in normal cells. In the heterozygotes, the 60 percent reduction in the number of LDL receptors was functionally translated

into a relative, but not absolute resistance to the feedback suppression effect of LDL; that is, to achieve any given level of enzyme suppression, about two to three times the concentration of LDL was required in heterozygotes' cells as compared with normal cells. This result can be explained by the observation that suppression of HMG CoA reductase activity is directly proportional to the absolute number of receptor sites that contain bound LDL (*4*). Since in the heterozygote the number of functional receptor sites is reduced to about 40 percent of normal, the law of mass action would require that at levels of LDL below saturation approximately a 2.5-fold higher concentration of LDL be present in the heterozygote to produce the same absolute number of occupied receptor sites and hence the same suppressive effect as in the normal cells.

If, in the body, the LDL receptor plays a physiologically important role in regulating cholesterol synthesis and LDL degradation and if these processes are controlled at a level of LDL that occupies only a fraction of the total number of receptor sites, then one would predict that heterozygotes would manifest an absolute rate of cholesterol synthesis and LDL degradation similar to that in unaffected subjects, but at the expense of a two- to threefold elevation in serum LDL. This prediction is in precise agreement with a large body of clinical and metabolic data derived from study of such patients (*5*, *6*). On the other hand, if suppression of cholesterol synthesis to normal levels and the attainment of a normal rate of LDL degradation required complete saturation of all LDL receptors present in the normal cell, then heterozygotes would be unable to generate this number of occupied receptors at any LDL level and hence would be phenotypically indistinguishable from homozygotes, a situation at variance with clinical observations (*5*).

Thus, these cell culture data offer an explanation of the biochemical mechanism by which a gene that is clinically dominant can produce a much more severe syndrome in homozygotes than in heterozygotes. The data also provide new insight into the mechanism by which a genetic abnormality in a regulatory protein may produce a metabolic defect in human cells.

MICHAEL S. BROWN
JOSEPH L. GOLDSTEIN
Department of Internal Medicine, University of Texas Southwestern Medical School, Dallas 75235

References and Notes

1. V. A. McKusick, in *Mendelian Inheritance in Man* (Johns Hopkins Press, Baltimore, Md., 1971), pp. vii–xiv; H. N. Kirkman, *Prog. Med. Genet.* **8**, 125 (1972); in *Congenital Malformations*, F. C. Fraser and V. A. McKusick, Eds. (Excerpta Medica, Amsterdam, 1969), pp. 209–214. As reviewed by Kirkman, basic defects have been identified at the molecular level in only a few dominant disorders, most notably in hereditary methemoglobinemia, several hemolytic anemias due to unstable forms of homoglobin, and possibly acute intermittent porphyria.
2. J. L. Goldstein and M. S. Brown, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2804 (1973); J. L. Goldstein, M. J. E. Harrod, M. S. Brown, *Am. J. Hum. Genet.* **26**, 199 (1974).
3. M. S. Brown, S. E. Dana, J. L. Goldstein, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2162 (1973); *J. Biol. Chem.* **249**, 789 (1974).
4. M. S. Brown and J. L. Goldstein, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 788 (1974); J. L. Goldstein and M. S. Brown, *J. Biol. Chem.*, in press. The following evidence indicates that the [^{125}I]radioactivity bound to the fibroblasts is initially in the form of intact LDL and that this bound material is released from the cell as it is degraded: (i) comparison of the time courses of binding and degradation indicates that binding precedes degradation; (ii) the ^{125}I-labeled material released from the cell is unable to bind when added to fresh cells; (iii) the bulk of the [^{125}I]radioactivity that remains bound to the cells is precipitable by trichloroacetic acid and by a monospecific antibody to LDL; and (iv) using isolated fibroblast membranes, we have been able to achieve LDL binding equal to that of intact cells under conditions in which LDL degradation does not occur.
5. A. K. Khachadurian, *Am. J. Med.* **37**, 402 (1964); M. S. Brown and J. L. Goldstein, *Adv. Intern. Med.*, in press.
6. B. Lewis and N. B. Myant, *Clin. Sci.* **32**, 201 (1967); S. M. Grundy and E. H. Ahrens, Jr., *J. Lipid Res.* **10**, 91 (19[illegible] T. Langer, W. Strober, R. I. Levy, *J. Clin. Invest.* **51**, 1528 (1972).
7. We thank S. E. Dana, G. Brunschede, J. Helgeson, and H. Bohmfalk for technical assistance. Supported by grants from the American Heart Association (72629), and the NIH (HL 16024 and GM 19258). J.L.G. is the recipient of a PHS research career development award (GM 70,277).

21 January 1974; revised 1 March 1974

Brain Cells in Culture: Morphological Transformation by a Protein

Abstract. *One type of embryonic rat brain cell having an epithelioid morphology in the monolayer culture can be transformed by brain extract into cells having extensive processes resembling mature astrocytes. The transforming factor is a protein with a molecular weight of 350,000. A partially purified sample showed that it is active at a concentration as low as* 1×10^{-8}M. *The transforming activity is high in adult brains but low in embryonic brains and tumors of the nervous system.*

Dissociated embryonic brains when grown in a monolayer contain two major types of immature cells: the bipolar neuroblasts and the flat epithelioid cells that support the growth of the neuroblasts. The latter cell type was suspected by some (*1*, *2*) to be glial precursors. Recently, several laboratories (*3*–*4*) reported that, under certain conditions, the flat brain cells observed in vitro can undergo morphological transformation into multipolar cells resembling mature astrocytes. We have obtained the flat cells in a relatively homogeneous population and inferred that, on the basis of the nondialyzability and heat lability of the activity (*3*), one of the factors responsible for the transformation is a macromolecule found in adult brain extracts. In this report we present evidence that the transforming factor is a protein.

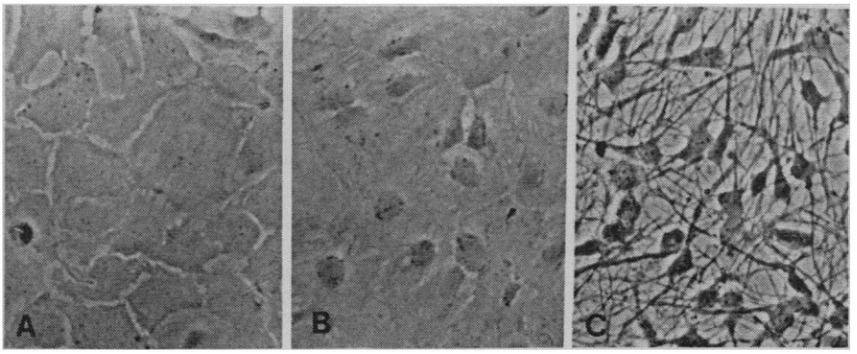

Fig. 1. Morphological transformation of embryonic brain cells in culture. (A) Cells in standard medium before testing, the morphology of which remained the same over the next few days if left undisturbed; (B) cells in the control medium for 20 hours; and (C) cells in the experimental medium for 20 hours containing brain extract. The poor contrast observed in (A) and (B) compared with (C) was due to the thinness of the cells and was not a result of poor photographic technique or interference by the media (phase contrast, × 200).