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Insulin-like growth factor I/somatomedin C: A potent inducer of oligodendrocyte development

(2',3'-cyclic nucleotide 3'-phosphodiesterase/insulin/myelination/nutrition)

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ABSTRACT **Cell cultures established from cerebrum of 1-day-old rats were used to investigate hormonal regulation of the development of oligodendrocytes, which synthesize myelin in the central nervous system. The number of oligodendrocytes that developed was preferentially increased by insulin, or by insulin-like growth factor I (IGF-I), also known as somatomedin C. High concentrations (5 μg/ml) of insulin were required for substantial induction of oligodendrocyte development, whereas only 3.3 ng of IGF-I per ml was needed for a 2-fold increase in oligodendrocyte numbers. At an IGF-I concentration of 100 ng/ml, oligodendrocyte numbers were increased 6-fold in cultures grown in the presence of 10% fetal bovine serum, or up to 60-fold in cultures maintained in serum-free medium. IGF-I produced less than a 2-fold increase in the number of nonoligodendroglial cells in the same cultures. Type I IGF receptors were identified on oligodendrocytes and on a putative oligodendrocyte precursor cell population identified by using mouse monoclonal antibody A2B5. These results indicate that IGF-I is a potent inducer of oligodendrocyte development and suggest a possible mechanism based on IGF deficiency for the hypomyelination that results from early postnatal malnutrition.**

Oligodendrocytes play a critical role in nervous system function by synthesizing and maintaining myelin in the central nervous system (CNS) (1). Very little is known about the stimuli or regulatory signals that promote the development of oligodendrocytes, but such information might provide the basis for approaches to promote repair in demyelinating disorders.

We studied tissue cultures established from cerebrum of 1-day-old rats, in which oligodendrocytes follow a developmental time course similar to that observed *in vivo* (2–4). We previously obtained evidence suggesting that insulin supports oligodendrocyte development (4), and we show here that insulin increases the number of oligodendrocytes that develop in the cultures. Because supraphysiological concentrations of insulin were required for the promotion of oligodendrocyte development (4), we suspected that insulin might act by cross-reacting with receptors for other insulin-like peptides, such as insulin-like growth factors (IGFs), also known as somatomedins (5–8), rather than by binding to insulin receptors. Our results show that IGF-I (somatomedin C) is a potent inducer of oligodendrocyte development *in vitro* and provides a mechanism for the reported effects of growth hormone on myelination in rats and mice. Our results also suggest a possible mechanism based on decreased levels of IGF-I for the hypomyelination observed in undernutrition.

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MATERIALS AND METHODS

Cell Explantation and Culture. Cells were explanted by mechanical dissociation of cerebrum of 1-day-old strain LEC rats as described (3, 4) and were inoculated into polystyrene tissue culture dishes, glass microscope slide chambers, or dishes containing glass coverslips. Glass surfaces were previously treated with D-polylysine (10–50 μg/ml) or polylysine followed by fibronectin (1 μg/cm^2; purified by method B of ref. 9). Cells were maintained as mixed cultures without separation of individual cell types. Unless otherwise noted, culture medium was Eagle's minimum essential medium supplemented with glucose to a total of 6 g/liter, 0.1 mM of each nonessential amino acid, antibiotics, and 10% fetal bovine serum. For serum-free culture, cells were maintained in the presence of 10% fetal bovine serum for the first 16–24 hr of culture to allow the cells to attach (10, 11), and the cells were then rinsed and refed with serum-free culture medium. Serum-free medium (modified from ref. 10) consisted of equal parts of Dulbecco's modified Eagle's medium (high-glucose formula) and Ham's F-12 medium, supplemented with glucose to a total of 6 g/liter/15 nM triiodothyronine/30 nM sodium selenite/transferrin (50 μg/ml). Insulin was absent except where noted. In all cases, medium was changed on the fourth day after explantation and every second day thereafter. Insulin or IGF-I was replenished with each medium change after the start of treatment.

Immunocytochemistry and Cell Identification. Immunochemical staining was carried out on cells that had been fixed for 20 min in 4% paraformaldehyde/0.1 M Na phosphate, pH 7.5, and then equilibrated with 96% (vol/vol) glycerol in the same buffer and permeabilized by freezing and thawing to allow antibody staining of intracellular antigens (modified from ref. 12; H. deF. Webster, personal communication). Cells were then stained by routine procedures of indirect immunofluorescence or avidin–biotin complex (13) immunoperoxidase staining. The following antibodies were used: mouse monoclonal antibody A2B5 (anti-tetrasialoganglioside; ref. 14), mouse monoclonal antibody against galactocerebroside (GC) (15), and rabbit antisera against GC (16), 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase; nucleoside-2',3'-cyclic-phosphate 2'-nucleotidohydrolase, EC 3.1.4.37) (17), or myelin basic protein (MBP) (18). For immunofluorescence staining, monoclonal antibodies were undiluted or 1:2 diluted culture supernatant or 1:30 diluted ascites fluid from hybridoma tumor-bearing BALB/c mice; rabbit antisera were diluted 1:50. Ten-fold higher dilutions were used for immunoperoxidase staining. Culture supernatant or ascites fluid from P3X63Ag8 mouse myeloma cells or nonimmune rabbit sera was used as control.

Abbreviations: CNS, central nervous system; GC, galactocerebroside; IGF, insulin-like growth factor; MBP, myelin basic protein; CNPase, 2',3'-cyclic nucleotide 3'-phosphodiesterase.

Flow Cytometry. Cells in suspension were fixed and stained by the indirect immunofluorescence method and analyzed on an Ortho Cytofluorograf cell sorter connected to a Data General MP200 computer. Cells were suspended with 0.25% trypsin/0.09% disodium EDTA, triturated gently, and diluted in culture medium containing $CaCl_2$ (0.87 mol of $CaCl_2$ per mol of EDTA, plus an additional 0.9 mM $CaCl_2$) and 10% normal goat serum. An aliquot was retained for cell counting, and the remainder of the cells were collected by centrifugation, fixed, permeabilized, and immunostained as described above. For analysis, the cell sorter was gated to single cells, and fluorescence intensity data were collected for at least 7000 control (nonimmune serum) and 7000 antibody-stained cells from each culture. A fluorescence intensity threshold was set at which 99.7–99.9% of the control cells were negative, and the percentage of the cells in the population that were positively stained by the diagnostic antiserum was determined. Comparison with cell numbers from hemocytometer counts revealed the total number of antigen-positive cells in each culture. In each experiment, the flow cytometry results were confirmed by (*i*) fluorescence microscopic examination of aliquots of the same cell suspension analyzed by flow cytometry; and (*ii*) microscopic examination of untrypsinized microscope slide or coverslip cultures maintained under identical culture conditions and immunostained *in situ* with the same antisera.

Microscopic Scoring of Antigen-Positive Cells. In some experiments, numbers of antigen-positive cells were determined from untrypsinized cultures grown on microscope slides and stained *in situ* by the immunoperoxidase method. Cells were scored at ×400 magnification. At least 400 cells were scored in each sample.

Cell Sorting. Cells for receptor identification experiments were isolated in 95–99% purity by fluorescence-activated cell sorting of live unfixed immunofluorescence-stained cells after culture without insulin or IGF-I. Antibody against GC, a myelin lipid that is diagnostic for oligodendrocytes in the CNS and present on the cell surface (19), and A2B5 mouse monoclonal antibody (14) were used for staining. Cells were sorted with a Becton-Dickinson FACS-IV cell sorter and cultured an additional 2 days to allow recovery from the trypsinization and cell sorting procedures.

Receptor Assays. Radioligand binding assays were performed on monolayers of A2B5-positive or GC-positive cells at 4°C exactly as described (20, 21), using 0.6–3.5 ng per ml (0.1 to 0.5 nM) of ^{125}I-labeled IGF-I in the absence or presence of graded concentrations of unlabeled IGF-I or insulin. Nonspecific binding was defined as radioactivity that remained bound in the presence of 750 ng of unlabeled IGF-I per ml. Affinity cross-linking of ^{125}I-labeled IGF-I to receptors was carried out as described (20) using 0.1 M disuccinimidyl suberate as the cross-linking reagent. After cross-linking, the cells were solubilized, electrophoresed in a 7.5% polyacrylamide gel (22) containing dithiothreitol and 0.1% $NaDodSO_4$, and the gels were dried and fluorographed.

Materials. For some experiments, IGF-I was partially purified from Cohn Fraction IV-1 of human plasma as described (23). Preparations used for cell culture were ≈0.1% pure, and preparations for radioiodination had a specific activity of 7500 units/mg and were judged to be >80% pure. IGF-I was iodinated to an activity of 150–250 mCi/mg (1 Ci = 37 GBq) as described (23). For most experiments, synthetic IGF-I, purchased from Amgen Biologicals (Thousand Oaks, CA), was used. Synthetic IGF-I is identical to natural human IGF-I except for the substitution of threonine for methionine at position 59. We have found the synthetic IGF-I to be similar to natural IGF-I in radioreceptor and radioimmunoassays, mitogenic activity on fibroblasts, and with respect to biological activity and receptor-binding characteristics in our experimental systems (unpublished observations). Synthetic IGF-I was used in experiments unless noted otherwise.

Second antibodies for immunofluorescence and immunoperoxidase staining were purchased from Cappel Laboratories (West Chester, PA) and Vector Laboratories (Burlingame, CA), respectively, and Lab-Tek microscope slide culture chambers were obtained from Miles. Bovine insulin and other biochemicals were purchased from Sigma. Other supplies and reagents were of the best grade available.

RESULTS

Experiments were carried out in cell cultures established from cerebrum of 1-day-old rats, before significant numbers of oligodendrocytes are present in cerebrum. Oligodendrocytes, identified by the presence of the myelin components GC, CNPase, or MBP, appear during the first week in culture and increase rapidly in number, following a developmental time course similar to that observed *in vivo* (2, 4). We previously observed that addition of insulin (5 μg/ml) to the culture medium increases the CNPase activity of the cultures, suggesting that insulin promotes oligodendrocyte development (4).

Cell number was then examined by immunofluorescence staining and flow cytometry after growth in the presence of insulin or IGF-I for 10–18 days. As shown in Fig. 1, insulin (5000 ng/ml) increased the number of CNPase-positive cells (oligodendrocytes) in the cultures by 3- to 6-fold. A lower concentration (by a factor of 250) of IGF-I (20 ng/ml) also increased the number of oligodendrocytes in the cultures at all time points studied, although the increase was not as great as that seen with insulin. Sister cultures assayed at the same time points showed insulin- or IGF-I-dependent increases in CNPase activity that were similar to the increases in oligodendrocyte number (data not shown). Results of the microscopic analysis of immunofluorescence-stained untrypsinized coverslip or microscope slide chamber cultures grown under the same conditions were consistent with the flow cytometry results (not shown).

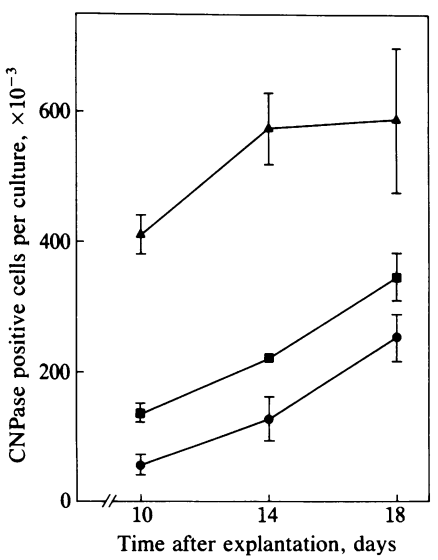

FIG. 1. Number of oligodendrocytes in rat cerebral cell cultures exposed to insulin at 5000 ng/ml (▲), IGF-I at 20 ng/ml (■), or neither (●). Partially purified IGF-I was used. Treatments were started at the time the cells were explanted. At the times shown, the cells were harvested, counted, immunostained, and analyzed by flow cytometry to determine the number of CNPase-positive cells (oligodendrocytes) per 10-cm² culture dish. Data are the mean ± SEM determined from analysis of at least 7000 antiserum-treated cells and 7000 control cells from each of three cultures per condition.

Fig. 2 shows that IGF-I significantly increased the number of oligodendrocytes per culture at all concentrations from 3.3 to 100 ng/ml. Oligodendrocyte numbers were increased >2-fold over control in the presence of IGF-I at 3.3 ng/ml, and increased 6-fold in the presence of IGF-I at 100 ng/ml. In the same cultures, the total number of nonoligodendroglial cells (CNPase-negative cells) was increased only 1.4-fold. Insulin at 33–100 ng/ml produced less than a 2-fold increase in the number of oligodendrocytes (Fig. 2), in contrast to the much greater increase seen with insulin at 5000 ng/ml (Fig. 1).

The experiments described above were carried out in culture medium containing 10% fetal bovine serum. Since serum contains significant amounts of endogenous IGF-I and factors that can affect IGF-I action, we also tested the effects of IGF-I and insulin on oligodendrocyte development in serum-free medium. Representative immunoperoxidase-stained cultures are shown in Fig. 3. Cells survived and grew in the absence of IGF-I or insulin, and oligodendrocytes could be detected by immunoperoxidase staining with antibodies against GC, CNPase and MBP, but oligodendrocyte numbers were small, accounting for <0.5% of the total cell number (Figs. 3 and 4). Addition of IGF-I at 5–100 ng/ml greatly increased the number of oligodendrocytes in the cultures (Figs. 3 and 4). IGF-I (5 ng/ml) increased oligodendrocytes numbers ≈1.4-fold, whereas IGF-I at 100 ng/ml produced up to a 60-fold increase in the number of cells that stained with antisera against MBP, CNPase, or GC (Fig. 4). In the same cultures, the number of nonoligodendroglial cells was increased <1.8-fold (data not shown). These data demonstrate that no additional serum components are required for IGF-I promotion of oligodendrocyte development. Insulin also increased oligodendrocyte numbers, but even at 5000 ng/ml, it was less effective than IGF-I at 100 ng/ml (Fig. 4).

In the experiment of Fig. 4, we found significantly different numbers of cells positive for GC, CNPase, and MBP in the cultures treated with IGF-I at 100 ng/ml ($P < 0.05$, Student's t test), but no significant differences in GC, CNPase, and MBP staining were found within the other treatment groups. In other experiments performed under similar conditions, IGF-I at 100 ng/ml or insulin at 5000 ng/ml dramatically increased oligodendrocyte numbers with no significant difference in the numbers of GC-, CNPase, and MBP-positive cells (not shown). Thus, neither IGF-I nor insulin substantially affects the expression of these three antigens relative to each other in oligodendrocytes.

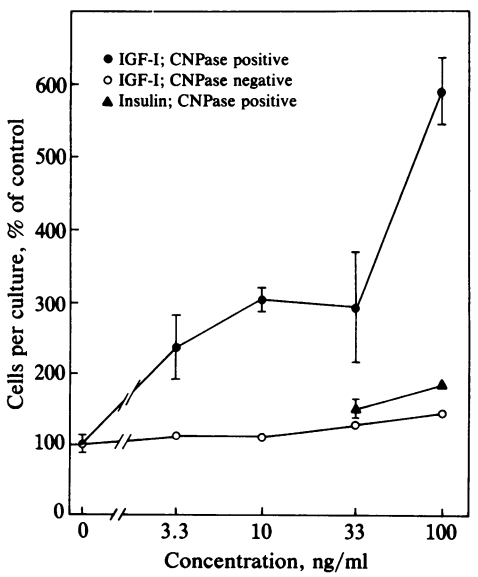

FIG. 2. Dependence of oligodendrocyte development on concentration of insulin or IGF-I. Cells were cultured for 14 days in the presence of the indicated concentrations of insulin or IGF-I, starting on the day of explantation, and then harvested, counted, stained with antiserum against CNPase, and analyzed by flow cytometry. Data are the mean ± SEM from triplicate cultures.

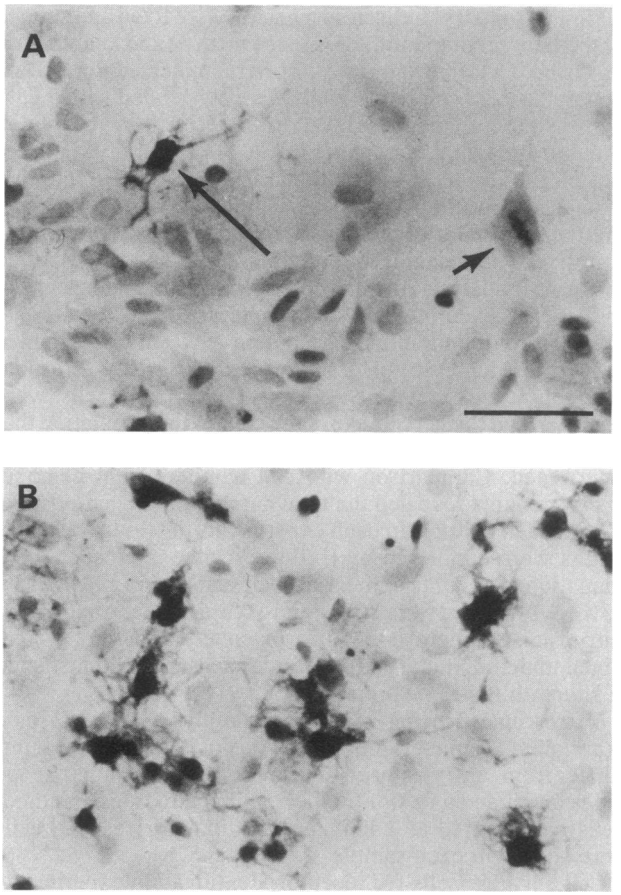

FIG. 3. IGF-I induction of oligodendrocyte development in serum-free culture medium. Cells were cultured without (*A*) or with (*B*) IGF-I at 100 ng/ml, beginning 18 hr after explantation. Fifteen days after explantation, cells were fixed, stained by the avidin–biotin complex immunoperoxidase method with antiserum against MBP, and counterstained with hematoxylin. In *A*, a single immunoperoxidase-stained oligodendrocyte (long arrow) is visible against a background of hematoxylin-stained nuclei of MBP-negative cells. Most microscope fields from these control cultures contained no MBP-positive cells. One MBP-negative cell is in mitosis (short arrow). MBP-positive oligodendrocytes are abundant in cultures supplemented with IGF-I at 100 ng/ml (*B*). (Bar = 50 μm.)

Oligodendrocytes were assayed for the presence of IGF receptors by measuring specific radioligand binding and by affinity cross-linking studies. Two physiochemically distinct IGF receptors are known: the type I IGF receptor is a heteromer in excess of 330 kDa with an IGF-binding subunit of ≈130 kDa, whereas the type II receptor is a single polypeptide of 220 kDa (21, 24–26).

Oligodendrocytes were collected in 95–99% purity by fluorescence-activated cell sorting after immunofluorescence staining of live unfixed cells with mouse monoclonal antibody against GC. Assays were also carried out on 95–99% pure A2B5-positive putative oligodendrocyte precursors (11, 14). Fig. 5 shows that both GC-positive cells (oligodendrocytes) and A2B5-positive cells exhibit specific IGF-I binding. Binding of ^{125}I-labeled IGF-I to both cell types was reduced 24–26% by 10 ng of unlabeled IGF-I per ml (1.3 nM), demonstrating that binding is to a high-affinity IGF receptor and not to insulin receptors. Radioligand binding to A2B5-positive cells was also displaced 40% by insulin at 10,000 ng/ml, suggesting that the receptors are type I IGF receptors,

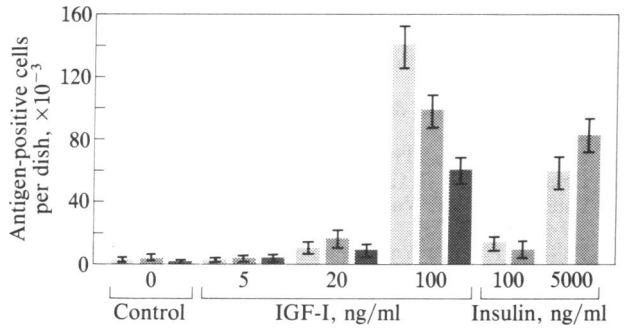

FIG. 4. Stimulation of oligodendrocyte numbers in serum-free culture medium. Insulin or IGF-I was added 18 hr after explantation. Fifteen days after explantation, triplicate cultures in 10-cm^2 dishes for each condition were suspended with trypsin/EDTA for cell counting in a hemocytometer, and additional cultures in microscope slide chambers were stained by the avidin–biotin complex immunoperoxidase method with antibodies against GC, CNPase, or MBP and scored under the microscope to determine the percentage of cells positive for each of the three oligodendroglial antigens. The figure shows the total number of cells per 10-cm^2 dish that were positive for GC (light stippling), CNPase (medium stippling), or MBP (heavy stippling). (Cultures treated with insulin were not examined for MBP expression.) Data are from the same experiment shown in Fig. 3.

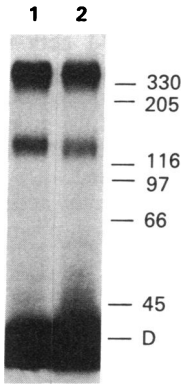

FIG. 6. Affinity cross-linking of ^{125}I-labeled IGF-I to A2B5-positive cells or GC-positive oligodendrocytes. Cells were isolated in >95% purity by fluorescence-activated cell sorting on day 13 after explantation, exposed to ^{125}I-labeled IGF-I at 2 ng/ml, and treated with the cross-linking agent disuccinimidyl suberate. Cells were then homogenized, electrophoresed on an $NaDodSO_4$/polyacrylamide gel under reducing conditions, and fluorographed to detect ^{125}I. Lane 1, A2B5-positive cells; lane 2, GC-positive cells. Molecular size markers are given in kDa. D, dye front.

which can bind insulin at high concentrations, and not type II IGF receptors, which do not bind insulin (24–26).

The identity of the IGF receptors of A2B5-positive cells and GC-positive cells was confirmed by affinity cross-linking experiments. As shown in Fig. 6, gel electrophoresis revealed the >330-kDa and 130-kDa bands labeled with ^{125}I-labeled IGF-I expected for type I receptors (24–26), as well as free ^{125}I-labeled IGF-I (7.6 kDa) migrating with the dye front.

DISCUSSION

The data presented here show that IGF-I is a potent inducer of oligodendrocyte development in tissue cultures of rat cerebral cells. In culture medium containing 10% fetal bovine serum, IGF-I at 100 ng/ml induced up to a 6-fold increase in oligodendrocyte numbers, whereas in serum-free medium, IGF-I induced up to a 60-fold increase. Oligodendrocyte development was not a simple linear function of IGF-I concentration. There was a sharp increase in oligodendrocyte numbers with IGF-I at 100 ng/ml (Figs. 2 and 4), and this was a consistent finding, both in serum-containing and in serum-free medium. IGF-I preferentially affects oligodendrocytes, having little effect on nonoligodendroglial cell numbers.

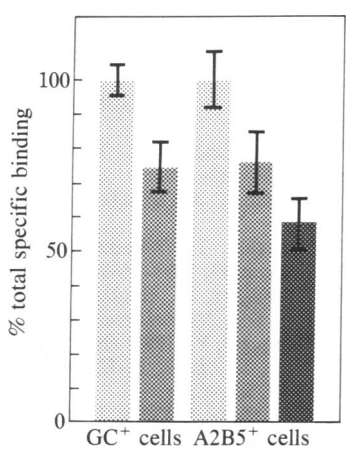

FIG. 5. Presence of specific high-affinity IGF receptors on GC-positive oligodendrocytes or A2B5-positive cells purified by fluorescence-activated cell sorting. Cells were isolated in 95–99% purity 23 days after explantation (GC-positive cells) or 10 days after explantation (A2B5-positive cells) and assayed at 4°C for binding of ^{125}I-labeled IGF-I. The figure shows specific binding (total binding minus nonspecific binding) of ^{125}I-labeled IGF-I in the absence (light stippling) or in the presence (medium stippling) of unlabeled IGF-I at 10 ng/ml, or in the presence of unlabeled insulin at 10,000 ng/ml (heavy stippling). Data are shown as percentage of total specific binding ± SEM for triplicate determinations.

The promotion of oligodendrocyte development in our system is mediated by type I IGF receptors. Insulin had a significant effect in promoting oligodendrocyte development only at high concentrations, at which it can bind to type I IGF receptors, and not at lower concentrations, which would be sufficient for binding to insulin receptors. Type II IGF receptors cannot be involved in the insulin response because they do not bind insulin (24–26). Furthermore, binding of IGF-I to type I IGF receptors was detected in both oligodendrocytes and A2B5-positive cells, whereas binding to type II receptors could not be detected. We cannot exclude the possibility, however, that type II receptors are also present, since their lower binding affinity for IGF-I may prevent their detection in our assays. Whether or not type II receptors are present, oligodendrocyte development might be subject to regulation by IGF-II as well as by IGF-I, because IGF-II can bind to type I receptors, although not as effectively as IGF-I (24–26).

Our data are consistent with IGF-I action by an increase in cell proliferation, a promotion of cell differentiation, or both. We detected type I IGF receptors on A2B5-positive cells. In postnatal rat optic nerve cultures, A2B5 antibody stains an immature cell type that is capable of differentiating into either an astrocyte or an oligodendrocyte, depending on culture conditions (11, 27). A2B5 antibody also stains neurons (14), which were absent in these cultures as determined by absence of immunostaining with mouse monoclonal antibody against neurofilament protein (data not shown). It is not known whether the A2B5-positive cells in our cerebral cell cultures are identical with the A2B5-positive bipotential glial progenitor cells in optic nerve cultures, but if they are, IGF-I might promote the development of these cells into oligodendrocytes. This might occur by an increase in the proliferation rate of the cells, in the probability of their developing into oligodendrocytes rather than astrocytes, or in the rate at which they progress through their developmental program. The absence of detectable oligodendrocyte death or disinte-

gration in the cultures in the presence or absence of IGF-I makes it unlikely that IGF-I acts simply by supporting oligodendrocyte survival. We have also identified type I IGF receptors on GC-positive oligodendrocytes. Thus, the effects of IGF-I may be due, in part, to promoting proliferation or maturation of oligodendrocytes that have already reached the stage at which GC is expressed.

IGFs act as intermediates in the action of growth hormone, which does not exert its growth-promoting effects by direct interaction with target tissues (5–8). Previous studies have shown that growth hormone affects myelination in rats and mice. When newborn rats are made growth hormone-deficient by injection of antiserum against growth hormone, CNS myelination is reduced by 70–90%, possibly because of a deficit in the number of oligodendrocytes (28). Secondly, Snell dwarf mice, which are genetically deficient in growth hormone and several other pituitary hormones, myelinate poorly, and the defect is reversible by growth hormone (29, 30). Recently, Almazan *et al.* (31) have reported that growth hormone increases the MBP content of brain cell aggregates *in vitro*. Because the effects of growth hormone are mediated by IGFs, studies demonstrating a growth hormone requirement for myelination suggest a requirement for IGF. Consistent with this prediction and with the results reported here, Lenoir and Honegger (32) have shown that IGF-I promotes DNA synthesis and the development of myelin-associated enzyme activity in fetal rat brain cell cultures. Together, these findings suggest that IGFs promote myelination.

IGF-I regulation of oligodendrocyte development may bring myelination under the control of other factors, unrelated to growth hormone, that influence IGF concentrations. IGF levels are not under the direct control of growth hormone at all ages or under all physiological conditions. Control of IGF levels by growth hormone does not begin until shortly after birth in rats (33), begins at some undetermined time perinatally in humans (5), and brain IGF-I levels apparently become uncoupled from growth hormone in adult rats (34). The late onset of growth hormone control of IGF levels in humans may explain the apparent absence of a myelin deficiency in human pituitary dwarfs: myelination may be complete before IGF levels fall. In contrast, IGF levels are regulated by nutritional status at all ages, both pre- and postnatally, and IGF levels are depressed in malnutrition in spite of normal or even increased growth hormone levels (5, 7, 35, 36). Early postnatal undernutrition in rats and humans profoundly and preferentially depresses CNS myelination, and the deficit can be irreversible (reviewed in ref. 37). The critical period for an irreversible myelin deficit corresponds to the period of oligodendrocyte formation, not to the period of actual myelin synthesis (37). The total number of oligodendroglial cells (including immature as well as mature cells) is apparently normal in undernourished rats (38), but myelination is delayed and reduced in amount (37, 38). Thus, the primary effect of undernutrition might be to interfere with oligodendrocyte development and maturation. Our results suggest that the myelin deficiency in malnutrition is the direct result of a deficiency of IGF.

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