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Induction of the neural cell adhesion molecule and neuronal aggregation by osteogenic protein 1

(neural development/transforming growth factor β/neuroblastoma)

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ABSTRACT **The neural cell adhesion molecule (N-CAM) plays a fundamental role in nervous system development and regeneration, yet the regulation of the expression of N-CAM in different brain regions has remained poorly understood. Osteogenic protein 1 (OP-1) is a member of the transforming growth factor β superfamily that is expressed in the nervous system. Treatment of the neuroblastoma–glioma hybrid cell line NG108-15 for 1–4 days with recombinant human OP-1 (hOP-1) induced alterations in cell shape, formation of epithelioid sheets, and aggregation of cells into multilayered clusters. Immunofluorescence studies and Western blots demonstrated a striking differential induction of the three N-CAM isoforms in hOP-1-treated cells. hOP-1 caused a 6-fold up-regulation of the 140-kDa N-CAM, the isoform showing the highest constitutive expression, and a 29-fold up-regulation of the 180-kDa isoform. The 120-kDa isoform was not detected in control NG108-15 cells but was readily identified in hOP-1-treated cells. Incubation of NG108-15 cells with an antisense N-CAM oligonucleotide reduced the induction of N-CAM by hOP-1 and decreased the formation of multilayered cell aggregates. Anti-N-CAM monoclonal antibodies also diminished the formation of multilayered cell aggregates by hOP-1 and decreased cell–cell adhesion when hOP-1-treated NG108-15 cells were dispersed and replated. Thus, hOP-1 produces morphologic changes in NG108-15 cells, at least in part, by inducing N-CAM. These observations suggest that OP-1 or a homologue may participate in the regulation of N-CAM during nervous system development and regeneration.**

The neural cell adhesion molecule (N-CAM) belongs to the immunoglobulin superfamily and mediates cell–cell interactions in the developing and adult nervous system through homophilic binding (1–3). Three isoforms of N-CAM (180, 140, and 120 kDa) are derived from a single gene by alternative splicing and are differentially expressed during nervous system development (4–6). The pattern of the expression of N-CAM is important in neural tissue organization (2), neuronal migration (7), nerve–muscle adhesion (8, 9), retinal formation (10, 11), synaptogenesis (12), and neural regeneration (13, 14), but little is known about how N-CAM expression is regulated.

Osteogenic protein 1 (OP-1) is a bone morphogen, is a member of the transforming growth factor β (TGF-β) superfamily, is identical to bone morphogenetic protein 7, and is also referred to as decapentaplegic-Vg-related family 7 (15–17). OP-1 was identified in a human hippocampus cDNA library and is expressed in a small number of tissues including brain (15, 18). The actions of OP-1 in the nervous system are unknown, but several members of the TGF-β superfamily are believed to play an important role in the early stages of neural development (17, 19). Here, we show that OP-1 is a powerful morphogen and inducer of N-CAM in the neuroblastoma–glioma hybrid cell line NG108-15.

MATERIALS AND METHODS

Cell Culture. NG108-15 cells of passage numbers 21–30 were subcultured in poly(D-lysine)-coated (1 mg/ml) 9.6-cm² 6-well trays (Corning) at a density of 50,000 cells per well in chemically defined medium, as described (20), except that 25 nM Na_3VO_4 was included with other trace elements. After 2 days, the medium was replaced and supplemented with recombinant human OP-1 (hOP-1; 0–300 ng/ml) for 4 successive days. hOP-1 was isolated from conditioned medium from transfected Chinese hamster ovary cells by anion-exchange chromatography followed by reverse-phase HPLC to >98% purity, as determined by SDS/PAGE (21). In some experiments 327 nM N-CAM antisense or sense phosphorothioate oligonucleotides (22) (Oligos Etc., Ridgefield, CT) corresponding to positions −12 to +11 of rat N-CAM cDNA (23) were added simultaneously with hOP-1 (40 ng/ml). Cell viability was assessed by the exclusion of trypan blue.

Assays for Cell Adhesion. *Aggregation during long-term cell culture.* Cells were cultured in the absence or presence of hOP-1 for 2–4 days and examined by phase-contrast microscopy at ×100 magnification. Multilayered aggregates, defined as clumps of 10 or more tightly adherent cells growing above the cell monolayer, were counted in at least 20 randomly selected fields from each well of subconfluent cells by two observers. Counts were averaged for each well and differed by 5–15%.

Short-term replating assay. NG108-15 cells were cultured for 5 days in the absence or presence of hOP-1 (40 ng/ml), mechanically dissociated, and resuspended in calcium-free Krebs–Ringer buffer containing 10 mM Hepes (pH 7.4). The cell suspensions were added to 4.5-cm² 12-well trays and incubated for 1 h at 37°C on a rotary shaker set at 60 rpm. The cells were then allowed to adhere without stirring for an additional hour at 37°C. Two observers determined the number of single cells and groups of two or more cells from two fields viewed at a magnification of ×100. In some experiments, the replating assay was carried out in the absence or presence of Fab fragments (7 μg/ml) of anti-N-CAM monoclonal antibody (mAb) H28.123 (24) (AMAC, Westbrook, ME).

Immunofluorescence of N-CAM. NG108-15 cells were cultured for 3 days on poly(D-lysine)-coated glass coverslips with defined medium containing hOP-1 (0–40 ng/ml). Cells were fixed with acetone at −20°C for 20 min, incubated for

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Abbreviations: N-CAM, neural cell adhesion molecule; OP-1, osteogenic protein 1; hOP-1, recombinant human OP-1; TGF-β, transforming growth factor β; mAb, monoclonal antibody.

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1 h with mAb H28.123, washed for three 5-min periods with Tris-buffered saline, and incubated for 1 h with a goat anti-rat fluorescein-conjugated antibody. Control and OP-1-treated cells were photographed at a magnification of ×100 by using identical exposure times.

Western Blot Analysis of N-CAM Isoforms. NG108-15 cells were cultured for 4 days with medium containing hOP-1 (0–300 ng/ml). SDS sample buffer was added to solubilize the cells, and 120 μg of protein from each well was separated by SDS/PAGE (25) on a 5–15% gel and transferred electrophoretically to nitrocellulose (Schleicher & Shuell) (26). The nitrocellulose membranes were blocked with 10 mM Tris acetate, pH 7.5/0.2% bovine serum albumin/0.1% Tween 20 (blocking buffer) for 30 min at room temperature, incubated for 2 h with mAb H28.123 (0.1–0.5 μg/ml), washed for three 5-min periods with blocking buffer, and then incubated for 1 h at room temperature with ^{125}I-labeled goat anti-rat IgG (ICN; 0.05 μCi/ml; 1 Ci = 37 GBq) in blocking buffer. The membranes were then washed for three 5-min periods with blocking buffer and subjected to autoradiography. Autoradiographs of gels loaded with a range of protein dilutions for each sample were analyzed by quantitative densitometry.

RESULTS

Morphologic Effects of OP-1. Subconfluent control NG108-15 cells had polygonal cell bodies, extended short spike-like processes, and made few contacts with neighboring cells (Fig. 1*a* and *b*). Treatment of NG108-15 cells with hOP-1 (0.1–300 ng/ml) induced an orderly dose-dependent change in cell morphology. After 1 day, hOP-1-treated cells underwent a rounding of the soma, an increase in phase brightness, and an extension of short neurites. After 2 days, cells treated with hOP-1 began to form epithelioid sheets, which served as a nidus for the growth of multilayered aggregates after the third day of treatment. By 4 days, the great majority of hOP-1-treated cells were associated in tightly packed multilayered aggregates (Fig. 1 *c–e*). This sequence of events occurred without any associated changes in DNA synthesis, cell division, or cell viability (unpublished data), making it unlikely that the morphologic changes were secondary to cell differentiation or a toxic effect of hOP-1. No morphologic changes were observed when cells were incubated with the diluent of hOP-1 (2×10^{-7}% trifluoroacetic acid/0.2–2 mM ethanol).

Immunofluorescence Studies of N-CAM. The striking induction of cell aggregation by hOP-1 suggested that this morphogen might regulate the expression of cell adhesion molecules. Since N-CAM plays a major role in neuronal cell adhesion, we conducted immunofluorescence studies with mAb H28.123, which recognizes all three isoforms of N-CAM (24). This antibody decorated the periphery and processes of hOP-1-treated cells but did not react with untreated cells (Fig. 2 *a* and *b*). Regions of cell–cell contact showed dense granular areas of immunoreactivity, consistent with the presence of N-CAM clusters (27).

Differential Expression of N-CAM Isoforms. To determine whether hOP-1 differentially increases the expression of the three N-CAM isoforms, we cultured NG108-15 cells for 4 days in the presence of increasing concentrations of hOP-1 and performed Western blots on whole-cell extracts (Fig. 2*c*). Control NG108-15 cells expressed the 140-kDa isoform to a greater extent than the 180-kDa isoform, whereas the 120-kDa isoform could not be detected, even in lanes containing up to 100 μg of protein. Treatment of NG108-15 cells with hOP-1 resulted in a dose-dependent increase in the expression of the 180-kDa and 140-kDa isoforms and the induction of the 120-kDa isoform. The increase in the expression of the 180-kDa isoform was significantly greater than that of the 140-kDa isoform (28.6 ± 7.5 times vs. 6.3 ± 1.8 times; mean ± SEM; $P < 0.02$), perhaps because constitutive expression of the 140-kDa isoform was closer to its maximal attainable level.

hOP-1 produced half-maximal induction of N-CAM at a concentration of ≈10 ng/ml, and maximal induction at ≈100 ng/ml (Fig. 2*d*); the dose–response curve for the induction of N-CAM by hOP-1 was similar to that for its induction of multicellular aggregates (Fig. 1*e*). The actions of hOP-1 appeared to be specific; hOP-1 did not change the expression of several other cytoskeletal and membrane proteins, as determined by Western blot analysis using antibodies against neurofilament proteins, microtubule-associated protein 2, tau, glial fibrillary acidic protein, and N-cadherin (gift from J.

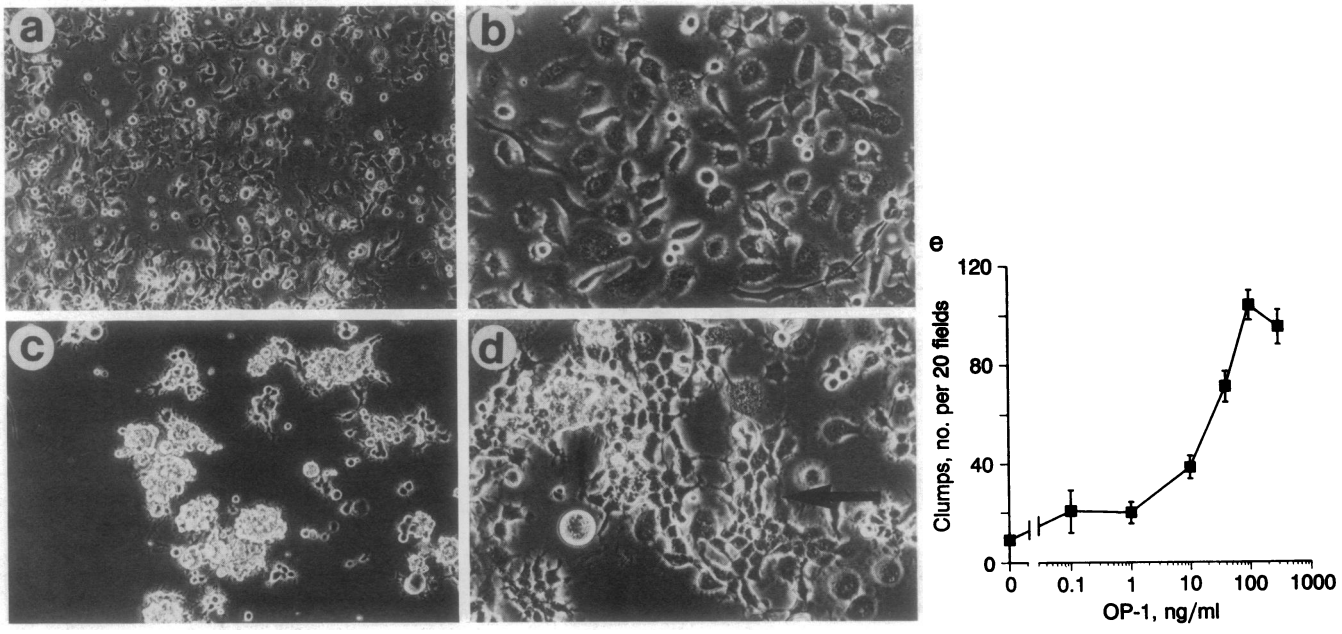

FIG. 1. Effect of hOP-1 on NG108-15 cell morphology. (*a* and *b*) Control cells. (*c* and *d*) Cells treated for 4 days with hOP-1 (40 ng/ml) showing an epithelioid sheet (large arrow) and a multilayered aggregate (small arrow). (*e*) Number (mean ± SEM) of multilayered aggregates (clumps) from six experiments. Cells were photographed under phase-contrast microscopy. (*a* and *c*, ×75; *b* and *d*, ×150.)

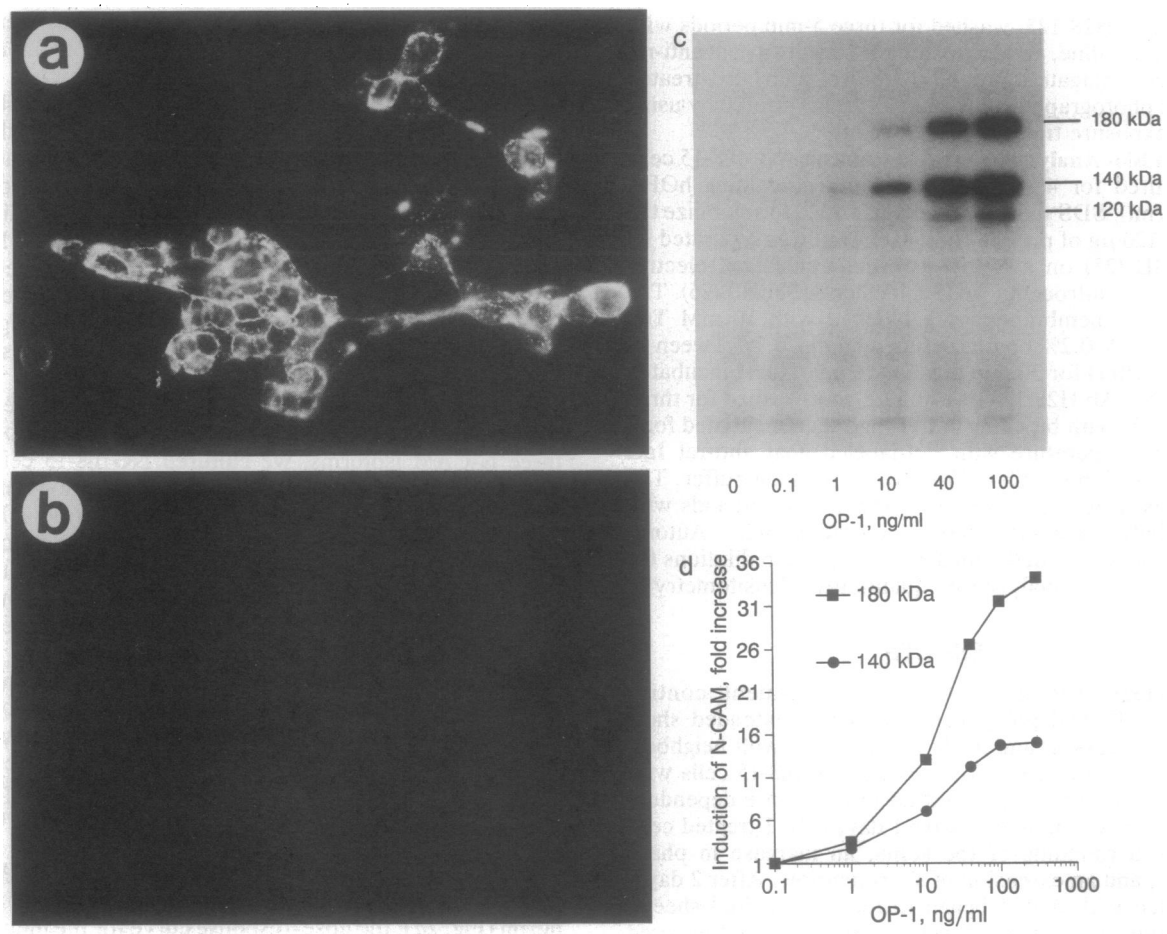

FIG. 2. Induction of N-CAM by hOP-1 in NG108-15 cells. (*a*) Anti-N-CAM immunofluorescence of hOP-1-treated cells. (*b*) Control cells. (*c*) Western blot of whole-cell extracts from cells treated with the indicated concentrations of hOP-1. (*d*) Dose–response curve for the induction of the 180-kDa and 140-kDa isoforms in a representative experiment that was repeated five times with similar results.

Lilien, Clemson University, Clemson, SC) (unpublished data).

Inhibition of OP-1 Effects by Antisense N-CAM and Anti-N-CAM Antibodies. To determine whether the induction of N-CAM is responsible for hOP-1-induced neural cellular aggregation, we cultured NG108-15 cells with hOP-1 in the presence of N-CAM antisense phosphorothioate oligonucleotides (22). Concentrations of antisense oligonucleotides that inhibited the induction of N-CAM by hOP-1 (Fig. 3*a*) also diminished the formation of multilayered cell aggregates (Fig. 3*b*). By contrast, similar concentrations of the corresponding sense oligonucleotide were without effect.

It was not possible to block completely the effects of hOP-1 using the N-CAM antisense oligonucleotide. At concentra-

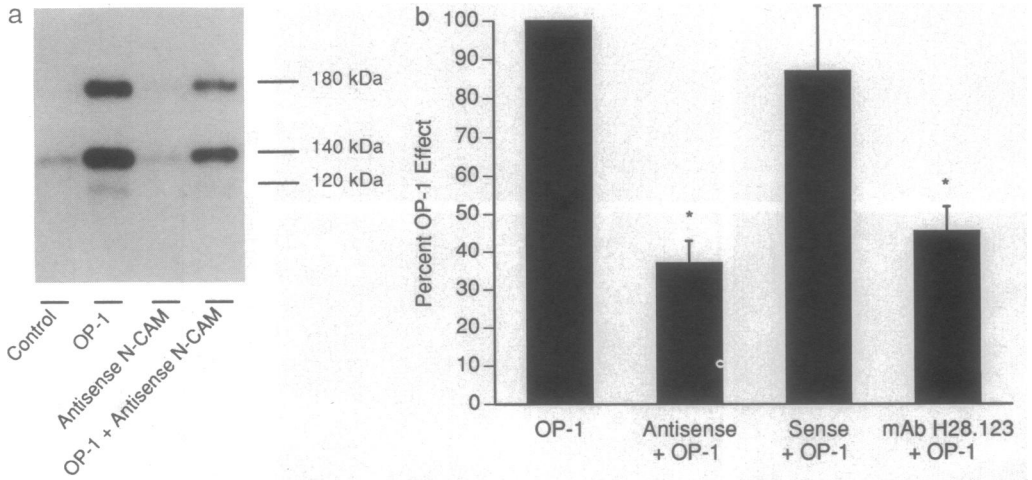

FIG. 3. Inhibition of hOP-1 effect by N-CAM antisense oligonucleotides and anti-N-CAM mAb. (*a*) Inhibition of hOP-1 induction of N-CAM by N-CAM antisense in a representative immunoblot of protein from cells treated as indicated. (*b*) Percent of hOP-1-induced aggregation in cells treated with 327 nM antisense oligonucleotide ($n = 6$), 327 nM sense oligonucleotide ($n = 4$), or anti-N-CAM mAb H28.123 (10 μg/ml; $n = 3$). Data are the mean ± SEM for the indicated number of paired experiments. Two days of treatment with hOP-1 (40 ng/ml) induced an average of 17.7 ± 5.6 clumps per 20 fields. *, $P < 0.001$ vs. OP-1.

tions >327 nM, both sense and antisense oligonucleotides induced cell clumping, even in the absence of hOP-1. However, at all oligonucleotide concentrations tested, the clumping induced by hOP-1 and antisense oligonucleotides was less than that induced by hOP-1 and the corresponding sense oligonucleotides. hOP-1-induced clumping was also inhibited significantly when NG108-15 cells were incubated with the anti-N-CAM mAb H28.123 (Fig. 3*b*). This effect was specific for the anti-N-CAM mAb, because hOP-1-induced cell clumping was not inhibited by mAb 12C5 raised against glial hyaluronate binding protein (unpublished data).

Adhesion of hOP-1-Treated Cells. The aggregation over several days of dividing cells could be governed by multiple factors under the regulatory control of OP-1. To better understand the relative contribution of increased levels of N-CAM to the morphologic changes produced by hOP-1, we performed short-term cell adhesion assays in the presence of specific anti-N-CAM antibodies (28). NG108-15 cells were cultured for 5 days with hOP-1 (40 ng/ml), incubated with anti-N-CAM mAbs, mechanically dissociated, and then replated in calcium-free buffer (Fig. 4). Only 11.0 ± 3.2% of control cells reestablished themselves in contact with neighboring cells, whereas 71.2 ± 2.8% of hOP-1-treated cells reformed in groups of two or more ($n = 5$, $P < 0.001$). The aggregation of dissociated hOP-1-treated cells was inhibited 50.9 ± 5.5% by Fab fragments of mAb H28.123 and 55.2 ± 2.8% by mAb 5A5, which reacts specifically with the polysialic acid moiety of N-CAM (29).

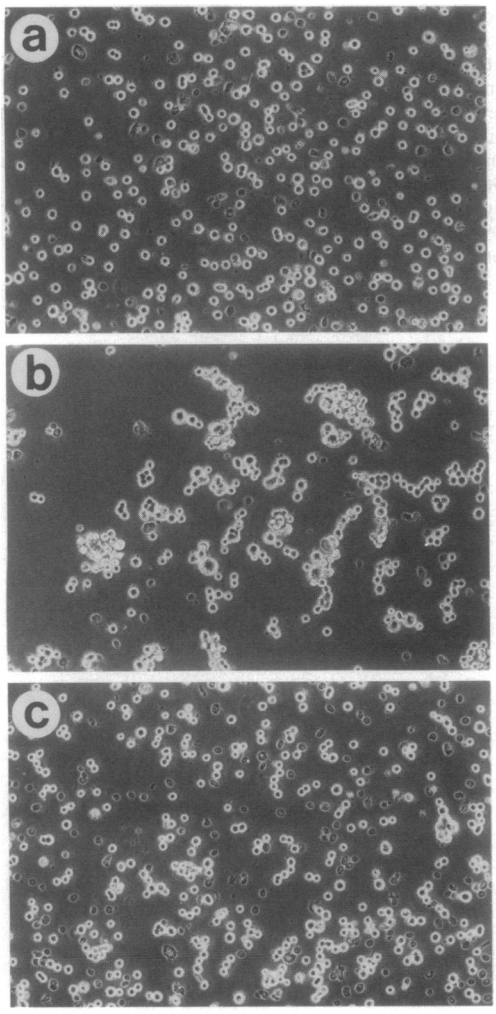

FIG. 4. Acute inhibition of cell adhesion by anti-N-CAM mAb. (*a*) Control cells. (*b*) hOP-1-treated cells. (*c*) hOP-1-treated cells incubated with mAb H28.123.

DISCUSSION

The major finding of this study is that hOP-1 causes striking formation of epithelioid sheets and multicellular aggregates in NG108-15 cells without arresting cell division. These morphologic changes were associated with a large induction of the three isoforms of N-CAM and could be diminished by expressing an anti-N-CAM antisense oligonucleotide or by incubating the cells with anti-N-CAM mAbs. These findings suggest that the induction of N-CAM by hOP-1 plays an important role in its morphologic actions in NG108-15 cells.

N-CAM mediates cell–cell adhesion through homophilic binding between adjacent cells (1–3); hence the formation of epithelioid sheets and cell aggregates in hOP-1-treated NG108-15 cells is not a surprising consequence of N-CAM induction. Indeed, overexpression of cell adhesion molecules by transfection of their corresponding cDNAs induces the formation of cell aggregates or epithelioid sheets in selected cell lines (30, 31). The failure of two anti-N-CAM mAbs to block completely the formation of NG108-15 cell aggregates raises the possibility that hOP-1 increases the expression of other calcium-independent cell adhesion molecules or modifies the sulfation, phosphorylation, or glycosylation of N-CAM (3, 32, 33).

Expression of the different isoforms of N-CAM is developmentally regulated. The 140-kDa isoform appears earliest in neural development followed by the 180-kDa and 120-kDa isoforms at later stages during cell differentiation and synaptogenesis (3). hOP-1 treatment of NG108-15 cells increased the constitutive expression of the 140-kDa isoform and produced a dramatic induction of the 180-kDa and 120-kDa isoforms. Hence, in NG108-15 cells, hOP-1 appears to shift the pattern of N-CAM expression from an early embryonic to an adult phenotype.

Differentiation of PC12 cells with nerve growth factor (34) and P19 embryonal carcinoma cells with retinoic acid (35) also increase N-CAM expression; however, both treatments arrest cell division. In contrast, hOP-1 induced N-CAM in NG108-15 without inhibiting cell division, and the differentiation of NG108-15 cells with dimethyl sulfoxide or forskolin did not induce N-CAM (unpublished data). Thus, hOP-1 appears to activate selectively a cellular program for the induction of cell adhesion molecules without triggering cell differentiation or growth arrest.

Although hOP-1 is a member of the TGF-β superfamily, its actions in NG108-15 cells appear to be specific. Treatment of NG108-15 cells with comparable concentrations of recombinant human TGF-β1 (Codon-Berlax-Bioscience, San Francisco) neither induced N-CAM nor caused cell aggregation (unpublished data). Moreover, the induction of N-CAM by hOP-1 in NG108-15 cells was considerably greater than that produced by TGF-β in 3T3 cells (36) or nerve growth factor in PC12 cells (34).

The NG108-15 cell line has been valuable in the study of neuronal signal transduction, synaptogenesis, and differentiation (37). Our findings suggest that this cell line may also prove useful in modeling some of the earliest events of neural tissue formation (2) and in studying the regulation and function of N-CAM. The ability of hOP-1 to induce N-CAM in NG108-15 cells suggests that this morphogen or a homologue may participate in the regulation of N-CAM expression during the development of brain and other tissues. Little is known about the expression of OP-1 during development, and it would be of great interest to determine whether the transient expression of N-CAM in a particular brain region coincides with that of OP-1. hOP-1 is effective *in vivo* in triggering the cellular cascade involved in endochondral bone

formation during development and regeneration (21); in view of the observation that N-CAM is induced after peripheral nerve injury (13, 14), it is conceivable that hOP-1 could also be effective in promoting neural regeneration.

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