

## Evidence for endogenous proteases, mRNA level and insulin as multiple mechanisms of N-cadherin down-regulation during retinal development

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### Summary

Our previous studies of the role of cell adhesion in retinal development have focused on the expression and function of N-cadherin, the predominant calcium-dependent intercellular adhesion protein of neural tissues. During the course of retinal development, N-cadherin expression undergoes significant qualitative and quantitative changes in its pattern of expression, most prominently a sharp down-regulation of expression throughout most of the retina. The present studies were directed at investigating the epigenetic mechanisms that could mediate this loss of N-cadherin from the retina. Using an *in vitro* intact retinal organ culture system, results were obtained which suggest that insulin enhances the down-regulation of N-cadherin expression in a protein-synthesis-dependent fashion. Furthermore, the metalloprotease inhibitor 1,10-phenanthroline inhibits the loss of N-cadherin from the retina. While N-cadherin is down-regulated in organ culture, other cell adhesion molecules, which are not down-regulated *in vivo*, are also not down-regulated in organ culture. The

defined organ culture medium conditioned by the retina accumulates both a soluble  $90 \times 10^3 M_r$  N-terminal fragment of N-cadherin as well as a number of secreted proteases. Both of these components are also shown to be present *in vivo* in the vitreous humor. Northern blot analysis indicates a single mRNA encoding N-cadherin in the retina and no evidence for a second message that could encode the  $90 \times 10^3 M_r$  fragment. However, the amount of N-cadherin mRNA detectable on northern blots decreases during development. The results reported here suggest that the down-regulation of N-cadherin that occurs during retinal development is possibly mediated by multiple mechanisms, which include turnover at the cell surface mediated by endogenous proteolysis, reduced levels of N-cadherin mRNA and modulation by growth factors.

Key words: N-cadherin, retina, insulin, protease, mRNA, cell adhesion, development.

### Introduction

Cadherins are a family of related proteins, which function as calcium-dependent cell adhesion molecules in a wide variety of tissues and species (Takeichi, 1988). Immunohistochemical mapping studies have shown that, during embryonic development, cadherins undergo complex spatiotemporal changes in their patterns of expression which are suggestive of a role in tissue modelling during morphogenesis (Thiery et al., 1984; Damjanov et al., 1986; Hatta et al., 1987; Duband et al., 1988). While several members of the cadherin family have been well characterized both functionally and structurally, little is known regarding the genetic and epigenetic mechanisms by which expression of these developmentally important molecules is regulated during embryogenesis and histogenesis.

N-cadherin, the predominant cadherin type in ner-

vous tissues, has been independently identified and studied in several laboratories and has been called by several names, including gp130/4.8 (Grunwald et al., 1982), N-cadherin (Hatta and Takeichi, 1986), A-CAM (Volk and Geiger, 1986) and N-calcam (Crittenden et al., 1987). Our studies have focused on the calcium-dependent adhesiveness mediated by N-cadherin and its role in ocular development. In our earlier studies, the formation of calcium-dependent adhesions among embryonic chick neural retina cells was shown to be involved in the formation of histotypic aggregates, to be under developmental regulation and to be associated with the protein now generally referred to as N-cadherin (Grunwald et al., 1980, 1981, 1982). More recent studies of the role of N-cadherin in retinal development have shown that the calcium-dependent adhesive system mediated by N-cadherin is most active at early phases of retinal histogenesis, and that as

assayed functionally, immunohistochemically and biochemically, N-cadherin is sharply down-regulated during later stages of retinal histogenesis (Lagunowich et al., 1988a; Matsunaga et al., 1988; Lagunowich and Grunwald, 1989a). This quantitative down-regulation is accompanied by a qualitative shift in expression such that by the time of hatching N-cadherin is only found in association with the zonula adherens junctions at the outer limiting membrane region of the neural retina (Lagunowich et al., 1988a; Matsunaga et al., 1988; Lagunowich and Grunwald, 1989a). Other ocular tissues such as the pigment epithelium, cornea, lens and sclera each show independent regulation of N-cadherin expression such that, at any one time during ocular development, expression may be up-regulated, down-regulated or held at steady-state levels depending on the stages in the development of these particular components of the eye (Lagunowich and Grunwald, 1989a).

These complex patterns of expression, which are presumably required for the normal development of these ocular tissues, have prompted us to explore mechanisms by which the expression of N-cadherin is regulated during ocular development. One of the most striking changes that occurs is the rapid loss of the majority of N-cadherin from the developing retina during later stages of histogenesis. The present studies were specifically directed at investigating this period of retinal development, when N-cadherin expression is sharply down-regulated, and elucidating the regulatory mechanisms that may be responsible. The results of these studies indicate that down-regulation of N-cadherin expression may be mediated in part by growth factors such as insulin, degradation via endogenous proteases and decreased levels of mRNA. Preliminary accounts of portions of the present results have been published in abstract form (Lagunowich et al., 1988b, 1990; Grunwald et al., 1990).

## Materials and methods

### Reagents

Bovine pancreatic insulin, sodium selenite and human transferrin were obtained from Sigma. Protease inhibitors were obtained from the following sources:  $\Sigma$ -amino-caproic acid, antipain, aprotinin, benzamidin and leupeptin, Sigma; 1,10-phenanthroline, Aldrich; phosphoramidon, Boehringer Mannheim; phenylmethylsulfonyl fluoride (PMSF), Bethesda Research Labs. Cycloheximide was from Calbiochem.

### Retina and vitreous body dissection and organ culture

Eyes from day-9 White Leghorn chick embryos (Shaw Hatcheries, Oxford, PA) were dissected into HBSG (Hepes buffered saline with glucose containing 10 mM Hepes [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] pH 7.4, 0.15 M NaCl, 3 mM KCl, 1 mg ml<sup>-1</sup> glucose and 10  $\mu$ g ml<sup>-1</sup> phenol red) with 2 mM CaCl<sub>2</sub> (HBSG/CaCl<sub>2</sub>). Eyes were bisected along the choroid fissure, the vitreous body was removed and the neural retina (NR) was removed from the underlying pigmented epithelium. Vitreous bodies were freed of any contaminating tissue, washed thoroughly in cold HBSG, and subjected to centrifugation at 10,000 revs

minute<sup>-1</sup> for 30 minutes to separate the gelatinous vitreous body matrix from the vitreous fluid. The resulting supernatant, referred to as vitreous humor, was used for further analysis. For organ culture, the resulting neural retina halves from each eye were transferred to one well of a sterile 24-well culture dish. After excess HBSG/CaCl<sub>2</sub> was removed, the well was flooded with 1 ml per retina of Eagle's minimal essential medium MEM (Sigma) which was the basal medium used in all experiments. The culture plate was placed on a rotating platform (72 revs minute<sup>-1</sup>) in a humidified incubator maintained at 37°C in an atmosphere containing 5% CO<sub>2</sub>, for the specified culture time. ITS (premixed insulin, transferrin and selenium; Collaborative Research) was used at a dilution of 1:100 which resulted in a final concentration of 5  $\mu$ g ml<sup>-1</sup> insulin, 5  $\mu$ g ml<sup>-1</sup> transferrin, and 5 ng ml<sup>-1</sup> selenium. Where indicated, the individual components insulin, transferrin and selenium were used at concentrations of 6  $\mu$ g ml<sup>-1</sup>, 6  $\mu$ g ml<sup>-1</sup>, and 6 ng ml<sup>-1</sup>, respectively. The protein synthesis inhibitor, cycloheximide, was used at a concentration of 10  $\mu$ g ml<sup>-1</sup>. At the conclusion of the culture period, the contents of each well were transferred to a 1.5-ml microcentrifuge tube and centrifuged for 5 minutes at 11,000 g. The supernatant containing conditioned culture medium was removed from the tissue in the pellet and both fractions were stored at -20°C until use.

### Protein gel electrophoresis and immunoblotting

One-dimensional SDS-PAGE was carried out according to Laemmli (1970) on gradients of 5-10% acrylamide. Tissue samples were used directly, while the conditioned medium samples corresponding to a given experimental condition, owing to their dilute state, were concentrated prior to use. Retina organ culture conditioned medium was precipitated by addition of five volumes of ethanol and incubation at -20°C overnight. The precipitate was recovered by centrifugation at 3,000 revs minute<sup>-1</sup> for 15 minutes at 4°C and resuspended in SDS-PAGE sample buffer. Following electrophoresis, proteins were transferred to nitrocellulose according to Towbin et al. (1979). Following transfer, the nitrocellulose sheet was cut in half, the upper half was processed for immunodetection while the lower half was stained with amido black for total protein detection in order to assure equal sample loading. For immunostaining the blot was blocked with 5% non-fat dry milk in TBS/CaCl<sub>2</sub> (0.01 M Tris-HCl, pH 7.5, 0.15 M NaCl, 2 mM CaCl<sub>2</sub>). For detection of N-cadherin, this was followed by incubation for 16 hours with the NCD-2 monoclonal antibody in blocking solution (1:500 dilution of conditioned medium). The NCD-2 hybridoma line, which was the generous gift of M. Takeichi, Kyoto, Japan, secretes a monoclonal rat IgG which specifically recognizes chick N-cadherin (Hatta and Takeichi, 1986). For detection of other cell adhesion molecules, primary antibodies used were 2A10, which is specific for the  $\beta_1$ -subunit of avian integrin (Chu and Grunwald, 1991a,b); monoclonal antibody G4, which was the generous gift of S. Chang, Philadelphia; and anti-N-CAM monoclonal antibodies H28 and P61, which were the generous gifts of G. Rougon, Marseille. Following incubation with primary antibody, the blots were washed three times with TBS/CaCl<sub>2</sub> and then were incubated for 8 hours with blocking solution containing alkaline phosphatase-conjugated goat anti-rat IgG (1:5000, Fisher Biotec) for NCD-2, H28 and P61, or anti-mouse conjugate for 2A10 and G4. The blots were then washed three times in TBS/CaCl<sub>2</sub> and developed in 0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, and 5 mM MgCl<sub>2</sub> with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium to visualize immunoreactive bands. The specificity of the NCD-2 monoclonal antibody for N-cadherin, revealing a

single  $130 \times 10^3 M_r$  band on immunoblots of fresh retinal tissues, has been previously described (Hatta and Takeichi, 1986; Lagunowich and Grunwald, 1989a). Thus to conserve space, only the relevant regions of immunoblots are illustrated.

#### Substrate gels for determination of proteolytic activity

Retina organ culture conditioned medium was prepared as described above. Samples in SDS-PAGE buffer without reducing agents were resolved on 8% polyacrylamide gels copolymerized with  $0.9 \text{ mg ml}^{-1}$  gelatin (Heussen and Dowdle, 1980). Following electrophoresis, gels were washed twice in 2.5% Triton-X-100 for 30 minutes to remove SDS. The gels were then rinsed twice with distilled water and once with TBS/CaCl<sub>2</sub>. The gels were then incubated in TBS/CaCl<sub>2</sub> at 36°C for 24 hours with gentle shaking. After incubation, the gels were stained with Coomassie blue dye overnight and then destained to visualize bands of proteolysis.

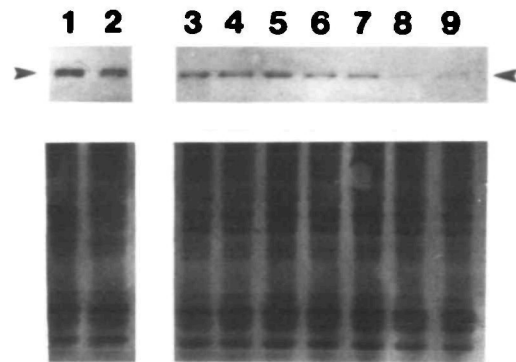
#### Northern blot analysis

Total cellular RNA was isolated by the guanidinium thiocyanate method according to Chomczynski and Sacchi (1987). Briefly, neural retinas or livers were homogenized in 4 M guanidinium thiocyanate buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarkosyl, 0.1 M 2-mercaptoethanol) and total cellular RNA was isolated by phenol fractionation and centrifugation. RNA was precipitated by freeze-thawing in isopropanol and quantitated on an LKB spectrophotometer. Total RNA ( $1.5 \mu\text{g}$  per lane as indicated) was separated by electrophoresis on a 2% agarose-3% formaldehyde gel and blotted directly onto nitrocellulose overnight in  $10 \times \text{SSC}$  buffer (Maniatis et al., 1982). Northern blot analysis was done using a cDNA probe corresponding to the N-terminal region of N-cadherin. The probe was produced by *Eco*R1 digestion of the lambda-N2 full-length cDNA coding for chicken N-cadherin, which was the generous gift of M. Takeichi, Kyoto, Japan. The probe was purified by agarose gel electrophoresis and was labelled with [ $\alpha$ -<sup>32</sup>P]dCTP by random priming and treatment with the Klenow fragment of *E. coli* DNA polymerase I according to the manufacturer's protocol (Pharmacia, Cat. no. 27-9250-01). Prehybridization was done for 6 hours at 42°C in a solution consisting of 0.05 M NaH<sub>2</sub>PO<sub>4</sub>, 10% Denhardt's, 1% SDS, 50% formamide and  $10 \mu\text{g ml}^{-1}$  herring sperm DNA in  $6 \times \text{SSC}$ . For hybridization the labelled probe was added directly to the prehybridization solution and incubated at 42°C for 18 hours. Following hybridization the blots were washed for 1 hour at 65°C in 0.1% SDS,  $2 \times \text{SSC}$ . Blots were then exposed to Kodak X-OMAT film for 72 hours at  $-70^\circ\text{C}$ .

## Results

#### Down-regulation of retinal N-cadherin occurs in vitro as well as in vivo

In order to facilitate analysis of intrinsic and extrinsic mechanisms regulating expression of retinal N-cadherin, an in vitro organ culture system was developed to maintain intact retinal tissues under defined serum-free conditions. In preliminary studies, several basal media were tested for their ability to maintain viable, well-differentiated trypsin-dissociated retinal neurons in monolayer cultures. Using criteria of glial and neuronal cell survival and neurite growth, Eagle's Minimal



**Fig. 1.** Comparison of N-cadherin expression in vivo and in vitro. Neural retina tissue samples, either freshly dissected or harvested from organ culture, were electrophoresed and transferred to nitrocellulose as described in Materials and Methods. Blots were cut in half and the upper portion was processed for immunodetection of N-cadherin while the lower portion was stained for total protein with amido black. Lanes 1 and 2 represent retinas from day-9 and -10 chick embryos, respectively (in vivo controls) while lanes 3-9 represent retinas from day-9 chick embryos which were maintained in organ culture for 0.25, 4, 8, 12, 16, 20 and 24 hours, respectively. The arrowheads indicate the position of N-cadherin.

Essential Medium (MEM) was found to provide the minimal nutritional requirements for the maintenance of viable cultures (Roark and Grunwald, unpublished results). Further studies were conducted using organ cultures of intact retinas, since analysis of N-cadherin expression in monolayer culture would be complicated by the need to use proteolytic enzymes to dissociate the cells, a process which removes surface N-cadherin (Grunwald et al., 1982; Cook and Lilien, 1982; Hatta and Takeichi, 1986). The level of N-cadherin expression, either in vivo or in vitro, was determined by gel electrophoresis and immunoblotting followed by detection with monoclonal antibody NCD-2. Since we had previously determined that expression of N-cadherin in vivo decreases significantly after embryonic days 9-10 as retinal cells become postmitotic and the histological layers of the retina become established (Lagunowich and Grunwald, 1989a), neural retinas from 9-day chick embryos were cultured in vitro and assayed for N-cadherin. The level of N-cadherin expressed by retinal tissues decreased during in vitro organ culture more rapidly than it does in vivo (Fig. 1). While the amount of N-cadherin changes very little from day 9 to 10 of embryonic development in vivo (Fig. 1, lanes 1 and 2), the decrease of N-cadherin expression is accelerated in vitro over the same time course (Fig. 1, lanes 3-9). While the level of N-cadherin changes little over the first 8 hours of culture, by 12 hours a decrease is apparent and by 24 hours little N-cadherin remains. The decrease in the expression of N-cadherin is not due to a general decrease in protein content as determined by total protein staining patterns, which remain constant during this time period (Fig. 1, lower panels).



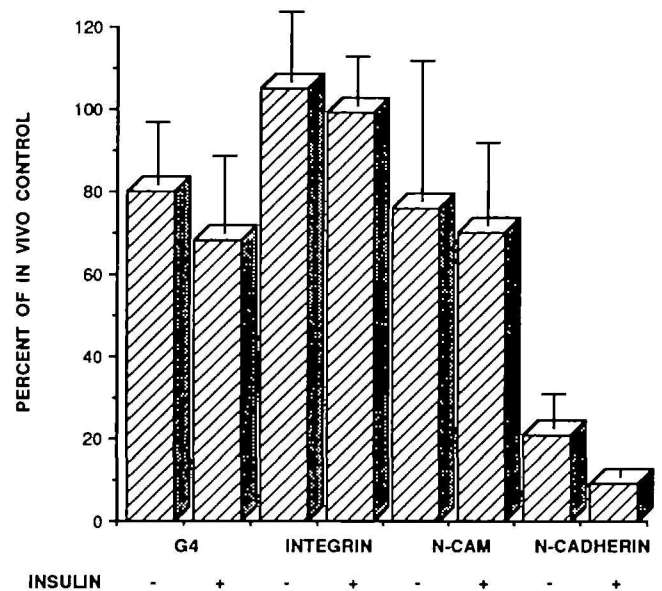
**Fig. 2.** Insulin potentiates the decrease in expression of N-cadherin *in vitro*. Retinal tissues, either freshly dissected or harvested from organ culture, were processed for electrophoresis, blotting and immunostaining to detect N-cadherin as described in Materials and Methods. Lanes 1 and 2 represent retinas from day-9 and -10 chick embryos, respectively. Lanes 3-7 represent neural retinas from day-9 chick embryos maintained in organ culture for 16 hours in MEM alone (lane 3) or in MEM with the addition of the following: lane 4, ITS (1:100); lane 5,  $6 \mu\text{g ml}^{-1}$  insulin; lane 6,  $6 \mu\text{g ml}^{-1}$  transferrin; lane 7,  $6 \text{ ng ml}^{-1}$  selenium. The arrowheads indicate the position of N-cadherin.

*The in vitro decrease of N-cadherin expression is enhanced by insulin*

We hypothesized that this accelerated decrease of N-cadherin during *in vitro* organ culture might be due to the absence of some essential component from the minimal basal medium which could be necessary for maintenance of high N-cadherin levels. During the preliminary studies using dissociated cell cultures, it was found that addition of insulin, transferrin and selenium together (ITS) to the basal MEM medium enhanced the survival, growth and differentiation of retinal cells *in vitro* (Roark and Grunwald, unpublished data). These culture additives were then tested for their effects on expression of N-cadherin during organ culture with intact retinas, to determine if the level of N-cadherin expression would remain high under these conditions. Contrary to expectations, it was found that rather than help maintain high N-cadherin levels the effect of including ITS in the culture medium was an even greater decrease in retinal N-cadherin expression (Fig. 2, lane 4) compared to cultures containing MEM alone (Fig. 2, lane 3). Similar results to those with ITS were obtained with ITS+, which is similar to ITS with the addition of linoleic acid and bovine serum albumen carrier (data not shown). No proteolytic activity could be detected in the ITS supplement using the azocoll colorimetric assay (data not shown) and we therefore hypothesized that the accelerated decrease of N-cadherin expression was perhaps mediated by one or more of the specific ITS components. We thus tested the individual components of ITS for their effect on N-cadherin expression. Of all the components tested, only insulin was found to be necessary and sufficient for an enhanced rate of N-cadherin disappearance (Fig. 2, lane 5) while transferrin and selenium had no such effect (Fig. 2, lanes 6 and 7).

*Other adhesion proteins are stably expressed in retinal organ culture*

As no general decrease in protein level was observed during retinal organ culture, but the level of N-cadherin detected decreased sharply, it became of interest to determine whether other cell surface adhesion proteins known to be expressed in the retina, but not down-

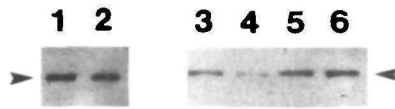


**Fig. 3.** Down-regulation of cell surface adhesion proteins is specific to N-cadherin. Retinal tissues from 9-day chick embryos were maintained in organ culture in the absence (-) or presence (+) of insulin, and the tissues were recovered and processed for SDS-PAGE and immunoblotting as described in Materials and Methods. The tissue extracts on the blots were probed with monoclonal antibodies recognizing cell surface adhesion proteins G4, integrin, N-CAM and N-cadherin, as indicated. The developed blots were quantitated by laser densitometry. The results are the means of at least three independent experiments,  $\pm$  the standard error.

regulated *in vivo* as is N-cadherin, were stably expressed under the culture conditions employed. Thus retinal organ cultures similar to those described above were maintained in the presence and absence of insulin, the tissues were collected, and cell adhesion protein expression was assayed by electrophoresis and immunoblotting for expression of N-CAM, G-4 and  $\beta_1$ -integrin in addition to N-cadherin (Fig. 3). Compared to the level of each protein expressed by the retina *in vivo* at the time of tissue removal and establishment of the organ cultures, the level of N-cadherin remaining at the end of the organ culture period was again found to be much reduced while the level of the other proteins was much less affected, either in the presence or absence of insulin. Thus the down-regulation of N-cadherin observed in retinal organ culture, as well as the insulin-dependent enhancement of this down-regulation, are specific effects on N-cadherin and are not artifacts of the culture conditions.

*The in vitro decrease in N-cadherin expression is dependent on protein synthesis*

The assay for N-cadherin utilized in these studies detects the steady-state amount of the protein present at a given time and does not reflect the relative contributions of synthesis and degradation to the steady-state level. While insulin caused an enhanced rate of N-cadherin disappearance, it had no inhibitory

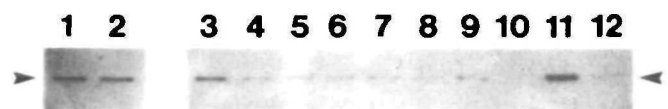


**Fig. 4.** Cycloheximide inhibits both the basal and insulin-potentiated decrease of N-cadherin expression *in vitro*. Retinal tissues, either freshly dissected or harvested from organ culture, were processed for electrophoresis, blotting and immunostaining to detect N-cadherin as described in Materials and Methods. Lanes 1 and 2 represent retinas from day-9 and -10 chick embryos, respectively. Lanes 3-6 represent retinas from day-9 chick embryos which were maintained as organ cultures for 16 hours in the presence (lanes 4 and 6) or absence (lanes 3 and 5) of  $6 \mu\text{g ml}^{-1}$  insulin and in the presence (lanes 5 and 6) and absence (lanes 3 and 4) of  $10 \mu\text{g ml}^{-1}$  cycloheximide. The arrowheads indicate the position of N-cadherin.

effect on general protein synthesis as determined by metabolite incorporation studies with intact retinas in organ culture (data not shown). In order to distinguish the relative contribution of anabolic and catabolic kinetic components to the steady-state level of N-cadherin, organ culture experiments were repeated in the presence of the protein synthesis inhibitor, cycloheximide, which effectively blocks new synthesis of retinal proteins (Grunwald et al., 1980). With cycloheximide included in the culture medium, the amount of N-cadherin detected after the period of organ culture would reflect the fate of N-cadherin already synthesized at the beginning of the experiment. The effect of cycloheximide was tested on both the basal rate of N-cadherin decrease which occurs in MEM alone as well as on the enhanced rate of decrease that occurs in the presence of insulin. The expectation was that in the absence of new N-cadherin synthesis, the disappearance of pre-existing N-cadherin would be more evident. Contrary to this expectation, cycloheximide had the clear and surprising effect of not only inhibiting the basal rate of N-cadherin disappearance in MEM alone (Fig. 4, compare lanes 3 and 5) but also negated the enhancing effect of insulin (Fig. 4, compare lanes 4 and 6). In fact, the amount of N-cadherin remaining at the end of organ culture was comparable to that found *in vivo* (Fig. 4, compare lanes 1 and 2 with 5 and 6). Since little if any new N-cadherin could be added to existing pools in the presence of cycloheximide, these results indicate that both the enhanced decrease of N-cadherin *in vitro* as well as the further enhancement of the decrease in N-cadherin in retinas treated with insulin are due to an active process which requires the synthesis of one or more proteins critical to this process.

*The in vitro decrease of N-cadherin expression is inhibited by 1,10-phenanthroline, a metalloprotease inhibitor*

Two non-mutually exclusive possibilities for protein synthesis-dependent processes that could affect the stability of N-cadherin and result in decreased expression are (1) post-translational modifications of N-cadherin which could affect its stability, and (2) the role of endogenous proteases in its degradation. Regarding

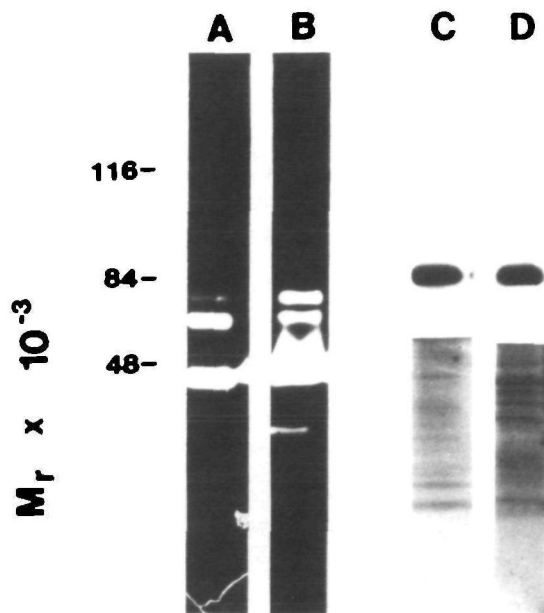


**Fig. 5.** The effect of protease inhibitors on expression of N-cadherin. Retinal tissues, either freshly dissected or harvested from organ culture, were processed for electrophoresis, blotting and immunostaining to detect N-cadherin as described in Materials and Methods. Lanes 1 and 2 represent retinas from day-9 and -10 chick embryos, respectively. Lanes 3-12 represent retinas from day-9 chick embryos which were maintained as organ cultures for 16 hours in the absence (lane 3) or presence (lanes 4-12) of  $6 \mu\text{g ml}^{-1}$  insulin. The cultures included either no further additions (lanes 3 and 4) or the following protease inhibitors: lane 5, 0.5 mM antipain; lane 6, 100 i.u.  $\text{ml}^{-1}$  trasyolol (aprotinin); lane 7, 0.5 mM leupeptin; lane 8, 10 mM 6-amino caproic acid; lane 9, 2 mM benzamide; lane 10, 0.4 mM PMSF; lane 11, 0.3 mM 1,10-phenanthroline; lane 12, 0.3 mM phosphoramidon. The arrowheads indicate the position of N-cadherin.

the former possibility, in results reported elsewhere, we have recently determined that N-cadherin is a sulfoprotein and a phosphoprotein, that these post-translational modifications occur in a tissue- and age-specific fashion, and that the state of phosphorylation of N-cadherin correlates with its interaction with the cytoskeleton as determined by non-ionic detergent extractability (Lagunowich and Grunwald, 1990, 1991). To examine the latter possibility, i.e. that endogenous proteases could play a role in the metabolism of N-cadherin, retinal organ cultures were maintained in the presence of a number of inhibitors of various classes of proteases (Fig. 5). Of the inhibitors examined, which were each tested individually, only 1,10-phenanthroline, an inhibitor of metalloproteases, was found to have a protective effect, equal to that of cycloheximide, with respect to the level of N-cadherin expression during organ culture (Fig. 5, lane 11). Retinas maintained in organ culture in the presence of 1,10-phenanthroline, even when insulin was also included in the medium, retained a level of N-cadherin comparable to that found *in vivo* (Fig. 5, lanes 1 and 2). As 1,10-phenanthroline, a zinc ion chelator, inhibits metalloproteases by virtue of depleting the enzyme of its zinc cofactor, experiments were conducted in which excess zinc was added back to 1,10-phenanthroline-containing retina organ cultures. While zinc by itself had no effect on expression of N-cadherin in control cultures (without 1,10-phenanthroline), zinc was found to reverse the protective effect of 1,10-phenanthroline on N-cadherin expression such that the amount of N-cadherin detected resembled that found in retinas cultured without 1,10-phenanthroline (data not shown).

*Similar extracellular proteases and soluble  $90 \times 10^3 \text{M}$ , N-terminal fragments of N-cadherin are found both in vitro and in vivo*

Since the organ cultures were established with intact retinal explants that had no exposure to exogenous proteases, and the cultures were maintained under



**Fig. 6.** In vitro and in vivo detection of proteases and N-cadherin fragments. Samples of retinal organ culture conditioned medium and of vitreous humor were prepared and analyzed by electrophoresis followed by immunoblotting or substrate gel analysis as described in Materials and Methods. Substrate gel electrophoresis of samples from embryonic day 13 neural retina conditioned medium (lane A) and embryonic day 13 vitreous humor (lane B) show similar comigrating bands of proteolytic activity. Immunoblots of embryonic day 13 conditioned medium (lane C) and vitreous humor (lane D) using the monoclonal antibody NCD-2 both demonstrate the presence of the  $90 \times 10^3 M_r$  fragment of N-cadherin. The lower portions of the immunoblots were stained with amido black to illustrate relative amounts of total protein.

defined serum-free conditions, the possibility that the retina tissue itself might be the source of endogenous proteases was examined. Conditioned medium from retinal organ cultures was harvested and assayed for proteolytic activity on copolymerized acrylamide-gelatin gels. Several distinct bands of proteolytic activity were detected (Fig. 6, lane A). That the presence of proteolytic activity is not simply due to lysis of retinal cells with concomitant release of intracellular proteases into the medium is indicated by the observation that lysates of retinal tissue, when run on similar substrate gels, contain so many proteases that the result is an uninterpretable smear (data not shown). The presence of discrete bands in the conditioned medium sample indicates that the retina produces and secretes a small number of specific proteases into the medium.

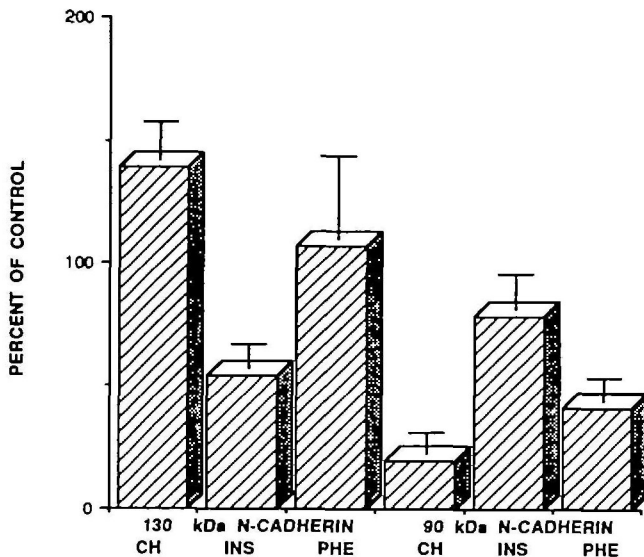
If the decrease in expression of N-cadherin is indeed due at least in part to proteolysis, such degradation of N-cadherin should result in the appearance of fragments in the conditioned medium. Indeed, the secretion of a  $90 \times 10^3 M_r$  fragment of N-cadherin into retina conditioned medium has been observed previously and the isolation of such fragments and their application in antibody neutralization assays was instru-

mental in the original identification of the parental  $130 \times 10^3 M_r$  N-cadherin protein (Grunwald et al., 1982). The presence of such a fragment of N-cadherin was confirmed under the present culture conditions, where it is found as a  $90 \times 10^3 M_r$  soluble fragment which is immunoreactive with the NCD-2 antibody (Fig. 6 lane C). Recognition by this antibody indicates that it is an N-terminal fragment corresponding to a major portion of the extracellular domain.

To investigate the relationship of release of the N-terminal fragment of N-cadherin or secretion of proteases by retinal tissue in in vitro organ culture to events in vivo, we sought to determine whether these components could be directly isolated from in vivo sources. We reasoned that as the retina appeared to secrete these products into the surrounding medium in culture, a likely place to look for such products in vivo would be the vitreous body of the eye. As the vitreous body is immediately surrounded by the neural retina in situ, it may serve as a sink for neural retina metabolites. The presence of proteases in the day 13 vitreous body, and their comparison with those found in day 13 retina organ culture conditioned medium, was examined by substrate gel electrophoresis (Fig. 6). Four bands of proteolytic activity were observed in extracts of vitreous humor, three of which comigrated with the conditioned medium bands (Fig. 6, lane B). In order to test for the presence of N-cadherin fragments in vitreous humor, and to compare any such fragments with those found in vitro, samples were resolved by gel electrophoresis, immunoblotting and detection with the NCD-2 monoclonal antibody (Fig. 6). The NCD-2 antibody detected a similar band on immunoblots of vitreous samples, which comigrated with the  $90 \times 10^3 M_r$  band from conditioned medium (Fig. 6, lane D). While comparable amounts of both the  $90 \times 10^3 M_r$  N-cadherin fragment and the proteases were detected on the immunoblots and substrate gels respectively, it should be recalled that the vitreous samples were used straight while the conditioned medium samples were concentrated 50-fold prior to analysis. Thus the concentrations of these proteins in vivo is quite high relative to the levels accumulated during the brief period of in vitro organ culture. No intact  $130 \times 10^3 M_r$  N-cadherin nor any N-cadherin fragments other than the  $90 \times 10^3 M_r$  one were detected on the immunoblots of either conditioned medium or vitreous humor.

*The retention of intact N-cadherin and the accumulation of its soluble N-terminal fragment in organ culture are inversely related*

Since insulin, cycloheximide and 1,10-phenanthroline were found to affect the disappearance of intact  $130 \times 10^3 M_r$  N-cadherin, the effect of these factors on the appearance of the  $90 \times 10^3 M_r$  N-cadherin fragment was examined. If the  $90 \times 10^3 M_r$  fragment is indeed derived from the intact parental molecule by proteolysis, then this precursor-product relationship should result in opposite effects of the three independent treatments on the levels of the  $130 \times 10^3 M_r$  and  $90 \times 10^3 M_r$  N-cadherin forms. Thus retinal organ cultures were



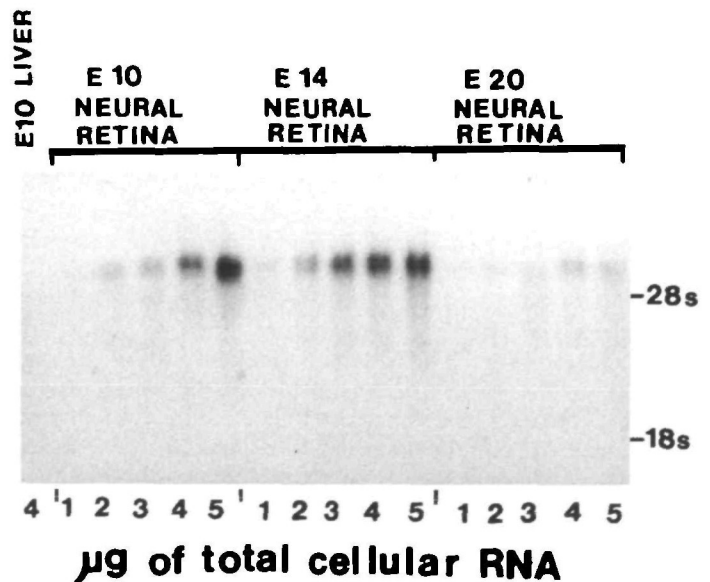
**Fig. 7.** The disappearance of N-cadherin and the appearance of its soluble fragment are inversely affected by organ culture treatments. Retinas from 9-day embryos were maintained in organ culture in control medium or medium containing cycloheximide (CH), insulin (INS) or 1,10-phenanthroline (PHE) and the tissue and conditioned medium were recovered and separately assayed for N-cadherin and its soluble fragment by SDS-PAGE and immunoblotting as described in Materials and Methods. The developed blots were quantitated by laser densitometry. The results are the means of at least five independent experiments, expressed as a percentage of the untreated control cultures,  $\pm$  the standard error.

established in control medium or in the presence of cycloheximide, insulin or 1,10-phenanthroline, and both the retinal tissue as well as the conditioned medium was recovered and analyzed by SDS-PAGE and immunoblotting for the presence of N-cadherin and its fragment. The results of these organ culture studies are shown in Fig. 7. As seen in previous experiments, treatment with either cycloheximide or 1,10-phenanthroline resulted in a greater retention of  $130 \times 10^3 M_r$  N-cadherin relative to the untreated control cultures, while treatment with insulin resulted in a lower retention of the intact protein. With respect to the  $90 \times 10^3 M_r$  N-cadherin fragment, treatment with either cycloheximide or 1,10-phenanthroline resulted in a decreased accumulation of the fragment. While cultures treated with insulin accumulated greater levels of the  $90 \times 10^3 M_r$  N-cadherin fragment than the cycloheximide or 1,10-phenanthroline treated cultures, the level was slightly less than that accumulated in untreated cultures. The latter could result if insulin is having effects in addition to those directly affecting the breakdown of N-cadherin, such as depressing the overall synthesis of N-cadherin, thus reducing the total amount available for breakdown (see discussion). Taken as a whole, however, these results demonstrating opposite relative effects on N-cadherin and its fragment of three independent treatments of the organ cultures are consistent with the accumulation of the  $90 \times 10^3 M_r$

N-cadherin fragment at the expense of intact  $130 \times 10^3 M_r$  N-cadherin.

*A single mRNA corresponding to  $130 \times 10^3 M_r$  N-cadherin is detected in neural retina and decreases during development*

Even if the hypothesis of a proteolytic origin for the  $90 \times 10^3 M_r$  N-cadherin fragment were correct, the possibility remains that some fraction of the  $90 \times 10^3 M_r$  form of N-cadherin could be synthesized *de novo* and secreted directly as a soluble protein. If this were the case, an mRNA corresponding to such a protein should be demonstrable among the total mRNA population of the retina, in addition to the one corresponding to the  $130 \times 10^3 M_r$  transmembrane form. To investigate the possible existence of such a hypothetical mRNA, northern blot analyses were conducted using appropriate cDNA probes corresponding to the N-terminal portion of N-cadherin (Hatta et al., 1988; Nose et al., 1990). This is the region of N-cadherin which is recognized by the NCD-2 antibody, and which is present in both the  $90 \times 10^3 M_r$  fragment and intact  $130 \times 10^3 M_r$  N-cadherin. The results of northern blot analyses utilizing total RNA from 10, 14 and 20 day embryonic chick retinas demonstrate that a single mRNA is detected at all three stages of development over a range of total RNA concentrations (Fig. 8). The electrophoretic mobility of the mRNA detected corresponds to the single 4.3 kb mRNA previously reported



**Fig. 8.** Northern blot analysis of N-cadherin mRNA levels in chicken neural retina. Total cellular RNA from embryonic day 10, 14 and 20 chick neural retinas were resolved on agarose gels and analyzed by northern blotting with the N-terminal *EcoR1* fragment of the lambda-N2 cDNA probe as described in Materials and Methods. A range of equivalent total RNA levels from 1-5  $\mu$ g was used for each developmental age of retina. Total cellular RNA from liver was used as a negative control. The 28S and 18S ribosomal RNA positions are noted on the gel.

as that corresponding to  $130 \times 10^3 M_r$  N-cadherin (Hatta et al., 1988). No hybridizing bands were detected using samples of total RNA from chick embryo liver, a tissue which does not express N-cadherin. Thus no evidence exists for a second mRNA which could possibly encode the  $90 \times 10^3 M_r$  N-cadherin fragment. Interestingly, the amount of N-cadherin message detected was much less in total RNA extracts of 20 day chick embryo retinas than at the earlier ages. This result suggests that, in addition to proteolytically mediated turnover of N-cadherin which we postulate here, the overall down-regulation of N-cadherin during retinal development may also be mediated in part by decreased transcription and/or message stability.

## Discussion

The formation of histotypic aggregates *in vitro* from dissociated retinal cells involves calcium-dependent adhesions of the type mediated by N-cadherin (Grunwald et al., 1980). The ability of dissociated embryonic chick neural retina cells to form histotypic aggregates *in vitro* decreases over the course of retinal development (Moscona, 1962; Grunwald et al., 1981). Subsequent immunohistochemical and biochemical analyses of N-cadherin expression during retinal development demonstrated that this loss of histotypic aggregation competence is paralleled by down-regulation and redistribution of N-cadherin in the developing retina (Matsunaga et al., 1988; Lagunowich and Grunwald, 1988a; Lagunowich and Grunwald, 1989a). Thus modulation of N-cadherin expression *in vivo* appears to be programmed as part of the normal differentiation process of the retina. The organ culture system used for the present studies allows for the maintenance of the retina under defined conditions without the need for cell dissociation and the concomitant introduction of exogenous proteases or the requirement for N-cadherin to be resynthesized.

While N-cadherin is down-regulated *in vitro* as well as *in vivo*, loss of N-cadherin *in vitro* occurs at an accelerated rate. The observed enhancement in the rate of N-cadherin disappearance from the retina upon removal from its normal environment may be due to changes in retinal physiology following altered interactions with its normal neighbors such as the vitreous humor and the pigment epithelium. These tissues have been shown to affect the development of their neighbors via hormonal, inductive and nutritive factors that alter cell-cell interactions and cell differentiation (Newsome, 1972; Beebe et al., 1980).

Our observation of a possible role for insulin in the down-regulation of N-cadherin is consistent with other observed effects of insulin in the differentiation of embryonic rat and chick retina cells. Insulin receptors are present in the retina (Saviolakis et al., 1985; Peterson et al., 1986) and insulin has been shown to induce precocious development of both cholinergic synaptic transmission and choline acetyltransferase activity in retinal cells (Puro and Agardh, 1984;

Peterson et al., 1986; Kyriakis et al., 1987). Insulin-specific mRNA has been localized in the Müller glial cells of the rat retina (Das et al., 1987). Thus, insulin may provide a signal that enhances the rate at which the retina proceeds along its normal developmental program, one consequence of this being a decrease in N-cadherin expression. Compared to the overall changes which occur in N-cadherin mRNA and protein levels, the effect of insulin may make a minor contribution to the expression of this adhesion protein. Nevertheless, even the two-fold difference observed in some experiments may be significant in terms of cellular behavior, since such behavior need not necessarily change as a linear function of cell adhesion molecule concentration.

N-cadherin has been shown to require calcium for function. The calcium-dependence of N-cadherin function is most likely due to a conformational change that occurs upon removal of calcium. This results not only in the inactivation of the molecule as a cell adhesive ligand but also results in an altered susceptibility to degradation by exogenously added proteases, such as trypsin, chymotrypsin and pronase, and to chemical modifications such as iodination (Grunwald et al., 1982; Cook and Lilien, 1982). The endogenous metalloprotease that appears to be responsible for degradation of N-cadherin does so in the presence of calcium, and thus may have a different substrate specificity than these other exogenous pancreatic and bacterial enzymes. The identification of the protease will thus be of interest and is a point that we are presently pursuing. This endogenous proteolytic activity may explain previous observations by us and others that retinal tissue in organ culture sheds a  $90 \times 10^3 M_r$  fragment of N-cadherin into the medium (Grunwald et al., 1982; Cook et al., 1984). Indeed, Cook et al. demonstrated that following radioiodination of intact cell surface N-cadherin, a soluble  $90 \times 10^3 M_r$  radiolabelled fragment of N-cadherin could be recovered from conditioned culture medium. Their observation is consistent with our hypothesis regarding a role for proteolysis in N-cadherin turnover. A similar fragment of Cell Cam 120/80, a human epithelial cell analogue of E-cadherin, is capable of competitively inhibiting the adhesive function of intact cell cam 120/80 (Wheelock et al., 1987). This suggests that N-cadherin fragments may be of functional significance perhaps in the modulation of N-cadherin-mediated adhesions.

All of the above cited work demonstrating the existence of soluble N-terminal fragments of cadherins, and much of the work described in the present report, was done *in vitro*. The question may thus be raised as to the relevance of these observations to regulation *in vivo*. Several of the results of the present study, however, mitigate against these phenomena being merely *in vitro* artifacts. First, the accelerated down-regulation of N-cadherin that we observe in organ culture appears to be specific to N-cadherin, at least when compared to the three other cell surface adhesion molecules examined, G4, N-CAM and integrin. Thus we conclude that the sharp down-regulation of N-cadherin that we observe *in vitro* may not be general-



ized to all surface or adhesion proteins even under our culture conditions, and is thus likely to be brought about by similar mechanisms to those that cause the down-regulation of N-cadherin observed *in vivo*. Second, our demonstration of the presence of the  $90 \times 10^3 M_r$  N-terminal fragment of N-cadherin *in vivo* in the vitreous humor is also strong evidence that such phenomena are not merely cell culture artifacts. While serum proteins are known to accumulate in the vitreous (Beebe et al., 1986), N-cadherin and its fragment are undetectable in chick embryo serum (Paradies and Grunwald, unpublished observations). Thus the most likely source of the  $90 \times 10^3 M_r$  fragment of N-cadherin is the retina itself, as is the case in organ culture. The experiments described here, to the best of our knowledge, provide the first evidence that such N-terminal fragments of cadherins can be found *in vivo*.

It has been shown that several neuronal cell types secrete proteases (Krystosek and Seeds, 1981a,b, 1984; Moonen et al., 1982; Soreq and Miskin, 1983; Alvarez-Buylla and Valinsky, 1985; Pittman, 1985; Pittman and Williams, 1988). This protease activity is greatest in the vicinity of the growth cones and during the time of active cell migration. The secretion of plasminogen activators (Alvarez-Buylla, 1985; Krystosek and Seeds, 1984) as well as metalloproteases (Pittman, 1985; Pittman and Williams, 1988) has been described. Pittman and Williams (1988) have demonstrated that metalloprotease secretion by neurites of peripheral neurons may be involved in neurite growth through collagen-rich matrices. Hawkins and Seeds (1986) have proposed that proteases are involved in detachment of growth cones from the substratum as it moves along the surface. Proteases have been demonstrated to be bound at the cell surface and localized to regions of specialized cell contacts (Reich, 1977; Saksela and Rifkin, 1988). While these studies have concentrated on cell-substrate adhesion, the present results indicate that similar mechanisms may be operative for the regulation of cell-cell adhesion as well.

In the present study, three independent factors were shown to affect the expression of intact  $130 \times 10^3 M_r$  N-cadherin: insulin, cycloheximide and 1,10-phenanthroline. These same three treatments were used to correlate the disappearance of intact  $130 \times 10^3 M_r$  N-cadherin with the appearance of the soluble  $90 \times 10^3 M_r$  fragment with the expectation that the effects on the  $130 \times 10^3 M_r$  and  $90 \times 10^3 M_r$  components would be complementary. This expectation was supported by the results with cycloheximide and 1,10-phenanthroline, where a clear inverse relationship exists between the retention of intact N-cadherin and the accumulation of its N-terminal fragment, whereas the relationship following insulin treatment, although also complementary, was less dramatic. The results indicate that while 1,10-phenanthroline and cycloheximide may be directly affecting the breakdown of N-cadherin by inhibiting the synthesis and/or function of the responsible protease, the effects of insulin may be less direct or more complex. For example, as discussed above, insulin has been demonstrated to affect several parameters of

retinal development which indicate that normal developmental profiles are accelerated by insulin. The studies cited above as well as the present studies have indicated that down-regulation of N-cadherin as well as its mRNA are part of the normal program of retinal development. Thus the effects of insulin may be complex and may cause a decrease in the level of N-cadherin through mechanisms other than or in addition to proteolysis. This possibility is consistent with all the results of the present study and could explain why there is not an overaccumulation of the N-cadherin fragment in insulin-treated cultures as compared to controls.

The results of the northern blotting studies indicate the existence of a single mRNA encoding N-cadherin in the retina. These results are in agreement with previously reported studies using brain and heart tissue (Hatta et al., 1988). While in theory it is possible that premature termination during translation of a single mRNA may lead to the production of a truncated form of N-cadherin, there is no evidence to support this. Our data represent the first report of comparative northern blot studies of N-cadherin mRNA between different developmental stages within the same tissue. Interestingly, the results indicate that a decreased level of N-cadherin mRNA is in part responsible for the lower levels of N-cadherin expressed at later developmental stages. The rapid loss of N-cadherin from the retina late in development may thus be a combined result of a rapid rate of N-cadherin turnover, mediated by proteolysis, coupled with down-regulation of transcription.

The results of the present study derive from experiments done utilizing both freshly obtained tissues as well as tissues maintained *in vitro*, and as discussed above, the results under both circumstances are in general agreement where a comparison has been possible. The main difference found between the two sets of conditions, however, is with respect to the rate of down-regulation of N-cadherin *in vitro*, which occurs at an accelerated pace when compared to the *in vivo* time course. Recently, we have initiated experiments to determine whether changes in mRNA levels occur during organ culture which could explain this discrepancy. Indeed, in experiments comparing *in vitro* and *in vivo* levels of both total recoverable RNA as well as recoverable specific mRNA, preliminary results indicate that both these parameters are significantly reduced following the period of organ culture. The results suggest that the accelerated decrease in N-cadherin which occurs *in vitro* results from a combination of reduced N-cadherin mRNA levels coupled with continuing protein turnover. Thus the *in vitro* situation again appears similar to the case *in vivo*, although as demonstrated by the present study the down-regulation of N-cadherin mRNA *in vivo* does not occur until later in development. Further work will be required to determine if the observed decreases of N-cadherin mRNA observed *in vivo* and *in vitro* are due to decreased transcription, increased mRNA turnover, or both, and why the process is accelerated *in vitro*.

Just prior to the submission of this manuscript, a

paper was published by Volk et al. (1990) which describes the presence of a number of different fragments of A-CAM, which is presumed to be identical to N-cadherin, in cultures of chick embryo lens cells. The authors suggest that the generation of these fragments could occur via endogenous proteolytic cleavage although no direct evidence was provided for the existence of such proteases. The conclusions reached both by Volk et al. and by the present study regarding the possible role of endogenous proteases are in agreement. However, there are several significant differences in the approaches taken and the results obtained which bear comment: (1) Volk et al. used trypsin to generate single cells for their culture studies, and included serum in the medium, while in the present study we used organ cultures of intact retinas which were never exposed to exogenous proteases and which were maintained under defined conditions. We have previously shown that despite extensive washing, enzyme dissociated cells can retain bound and active residual trypsin which is capable of degrading cell surface N-cadherin (Grunwald et al., 1980). In addition, serum is believed to contain both proteases and protease inhibitors. It is for this reason that our present studies were carried out under conditions that avoid any ambiguity as to the source of the proteolytic activity. (2) Furthermore, the use of intact tissues as opposed to dissociated cells allows for the analysis of phenomena more closely approximating the *in vivo* situation. (3) Volk et al. concluded that no proteases were secreted by the lens cells into the medium, whereas we have demonstrated that the retina indeed secretes proteases that accumulate in the organ culture conditioned medium. (4) Finally, Volk et al. studied the lens, whereas we have studied the retina. We have previously shown that differences exist between lens and retinal N-cadherin, including relative molecular mass, phosphorylation and detergent solubility (Lagunowich and Grunwald, 1990, 1991). It will thus be of interest to determine to what extent the regulation of N-cadherin by endogenous proteases may differ between different tissues.

## Conclusion

The expression pattern of N-cadherin described here and previously (Matsunaga et al., 1988; Lagunowich and Grunwald, 1989a,b) suggests a changing role for N-cadherin during the three major phases of retinal histogenesis. (1) During early chick development, N-cadherin is expressed in the neural plate and remains in the neural tube, from which the retina develops, but is not found in the overlying ectoderm. This tissue specificity suggests that N-cadherin may be important for the separation of early neural tissue from the ectoderm during neurulation, which also is suggested by recent experiments where ectopic N-cadherin expression has led to altered neurulation patterns (Detrick et al., 1990; Fujimori et al., 1990). (2) Later in development, N-cadherin continues to be expressed at

a high level during early retinal histogenesis. Its pericellular expression pattern among all retinal cells indicates that it serves to hold cells together as the retina is transformed from a homogenous neuroepithelium into a multilaminar array of alternating neuronal cell body and synaptic layers. N-cadherin may thus contribute during this period to adhesive interactions not only between cell bodies but also during neurite elongation, both processes which N-cadherin has been shown to mediate *in vitro* (Grunwald et al., 1982; Hatta and Takeichi, 1986; Bixby et al., 1987; Matsunaga et al., 1988; Drazba and Lemmon, 1990; Bixby and Zhang, 1990). This active phase of retinal histogenesis, which is characterized by extensive migration of cells and elaboration of neurites, involves a continuous making and breaking of contacts as the mature retinal cytoarchitecture unfolds. A high rate of synthesis of N-cadherin, coupled with its rapid proteolytic turnover at the cell surface, may provide a mechanism for maintaining overall tissue integrity while permitting a considerable degree of plasticity. (3) Finally, later in development, retinal cells cease division, the characteristic histological layers have been generated, and synaptogenesis ensues. At this stage N-cadherin is only detected in the adherens junctions of the retinal outer limiting membrane, a condition that persists at least through hatching. Thus a third specific function of N-cadherin may be to maintain the integrity of these specialized junctions. It is not yet clear if the continued expression of N-cadherin in this region of the retina is due to continued high levels of localized synthesis or is due to the differential stability of a specific subpopulation of N-cadherin. We are pursuing both of these possibilities, the former by *in situ* hybridization, and the latter through analysis of cytoskeletal association and post-translational modifications such as phosphorylation and sulfation of N-cadherin, which we have recently shown to occur in an age- and tissue-specific fashion during chick embryo development (Lagunowich and Grunwald, 1990, 1991).

The results of the present study suggest that N-cadherin may be a substrate for a specific endogenous metalloprotease that may be involved in the epigenetic regulation of N-cadherin at the cell surface. Our demonstration of the modulation of N-cadherin expression by insulin, endogenous proteases and decreased mRNA levels suggests three possible mechanisms whereby the dynamic expression patterns of cell adhesion proteins in the embryo may be regulated. Further studies are required to confirm a role for these processes in the regulation of N-cadherin expression and to determine how these and other factors contribute to the distinct patterns of regulation observed during the development of different tissues.

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#### Note added in proof

Recently we have carried out pulse-chase metabolic turnover studies which provide direct biochemical evidence for the existence of a precursor-product relationship between intact cell surface membrane-associated N-cadherin and its soluble N-terminal fragment. Furthermore, we have recently purified the soluble N-terminal fragment, and we have carried out in vitro cell culture experiments demonstrating that it retains functional binding activity and serves as a substrate for both cell adhesion and neurite growth. The detailed results will be published elsewhere (Paradies and Grunwald, in preparation).