Entactin/Nidogen: Synthesis by Bovine Corneal Endothelial Cells and Distribution in the Human Cornea

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Purpose. The purposes of this study were to determine whether entactin/nidogen (E/N) is synthesized and secreted by corneal endothelial cells and to characterize the distribution of E/N in the human cornea.

Methods. Cultured bovine corneal endothelial cells were metabolically labeled with [35S]methionine. Newly synthesized E/N was detected in cell lysates and culture medium by immunoprecipitation, using monoclonal anti-E/N antibodies, polyacrylamide gel electrophoresis, and autoradiography. The presence of E/N in the subendothelial extracellular matrix was demonstrated by Western blot analysis of solubilized extracellular matrix proteins. The distribution of E/N in normal human corneas was studied by indirect immunofluorescent staining of frozen sections, using monospecific anti-E/N antibodies.

Results. E/N was detected in the basement membrane (BM)-like extracellular matrix deposited by corneal endothelial cells, as well as in cell lysates and culture medium. Immunofluorescence studies revealed the presence of E/N in both the epithelial and endothelial BM and to a much lower extent in the stroma. E/N was detected throughout the thickness of the epithelial BM, but its staining decreased in intensity toward the central part of the cornea. In the endothelial BM (Descemet’s membrane), E/N fluorescence was limited to its most posterior portion, produced postnatally.

Conclusions. Corneal endothelial cells synthesize and secrete E/N. E/N was found in both the epithelial and endothelial basement membranes but was primarily localized to the posterior portion of Descemet’s membrane and the periphery of the epithelial BM. The authors suggest that E/N may be important in healing processes of corneal injuries and in the pathogenesis of diseases involving the postnatal region of Descemet’s membrane. Invest Ophthalmol Vis Sci. 1994;35:495-502.

Basement membranes (BM) are thin layers of specialized extracellular matrix (ECM) composed of collagen and noncollagenous proteins. They form the natural supporting structure upon which cells migrate, proliferate and differentiate. Their functions include filtration, cell adhesion, polarity induction, and tissue scaffolding. The major molecular constituents of BMs are collagen type IV, laminin, heparan sulfate proteoglycans, and entactin/nidogen (E/N).1,2 E/N is a 150-kDa glycoprotein first isolated from a mouse endodermal cell line M1536-B3 and named entactin and later from Engelbreth Holm Swarm tumor where it was designated as nidogen.3,4 The identity of these two preparations was confirmed after the discovery of their complete sequences.5,6 Recent studies revealed that E/N is composed of three globular domain structure interconnected either by a rod or a thin segment. E/N forms a tight complex with laminin, interacts with itself, as well as with type IV collagen and fibronectin.7,8 E/N contains an Arg-Gly-Asp sequence and supports cell adhesion, influencing their polarity, migration and persistence in culture. It was proposed to play a role in hemostasis and possibly in wound healing through specific binding to fibrinogen and was also shown to stimulate chemotaxis and phagocytosis.9-11
The cornea is composed of three main layers separated by two basement membranes. On the outside, the corneal epithelium is supported by the epithelial basement membrane, which separates it from the central zone (the Bowman's layer and the corneal stroma). The inner layer, the corneal endothelium, is separated from the stroma by the endothelial basement membrane (Descemet's membrane). The anterior region of Descemet's membrane is thought to be formed prenatally and the posterior part found in close contact with the endothelial cells is formed postnatally. Immuno-histochemical studies revealed the presence of E/N in the epithelial BM, Descemet's membrane and stromal plaques of the mouse cornea. In another study, E/N mRNA was identified in the stromal region of the mouse embryo cornea. In the current study we investigated the distribution of E/N in human corneas by immunofluorescence and demonstrated its biosynthesis by corneal endothelial cells maintained in culture. E/N synthesized by these cells was deposited into the subendothelial BM and secreted to the culture medium.

METHODS

Cells

Institutional review board approval was obtained, and the ARVO Statement for the Use of Animals in Ophthalmic Research was followed. Cultures of bovine corneal endothelial cells were established from steer eyes as previously described. Cells were cultured in Dulbecco's modified Eagle's medium (glucose 1 g/l) supplemented with 10% bovine calf serum, penicillin (50 U/ml), and streptomycin (50 µg/ml) at 37°C in 10% CO2 humidified incubators. Partially purified brain derived basic fibroblast growth factor (100 ng/ml) was added every other day during the phase of active cell growth. Early-passaged cells (second through fifth passages) were used in the experiments and both subconfluent, actively growing cells and confluent, resting cultures were examined. Cultures of bovine aortic endothelial cells were obtained from steer aorta, as previously described. Cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% bovine plasma, 5% fetal calf serum. Bovine eyes were obtained from calves sacrificed in a government-approved slaughter house. The bovine ocular parts were otherwise routinely discarded.

Preparation of ECM

Bovine corneal endothelial cells were maintained as described above, except that 5% dextran T-40 was included in the growth medium. Six to eight days after the cells reached confluence, the subendothelial ECM was exposed by dissolving (3 minutes at 22°C) the cell layer with a solution containing 0.5% Triton X-100 and 20 mM NH4OH in phosphate-buffered saline (PBS), followed by four washes in PBS. The ECM remained intact, free of cellular debris, and firmly attached to the entire area of the tissue culture dish. The presence of nuclei, cytoskeletal elements and serum proteins could not be detected in the denuded ECM.

Antibodies

Anti-E/N polyclonal antibodies were prepared by immunizing rabbits with purified bovine kidney E/N. Affinity-purified antibodies were prepared using E/N coupled to affinity matrix discs (Nalge Company, Rochester, NY) and bound antibodies were eluted with 0.1 M glycine, pH 2.5. To ensure antibody specificity further, E/N was transferred from sodium dodecyl sulfate (SDS)-polyacrylamide gels to polyvinylidene difluoride membranes (Millipore, Bedford, MA) by semidry electrophoresis transfer. A strip containing E/N was cut from the transfer membrane and reacted with affinity-purified antibodies prepared as described above. Antibodies were then eluted from the strip using 0.1 M glycine pH 2.5 to a final concentration of 425 µg/ml, and the pH was neutralized. The monoclonal antibody, Mab A9 (5.7 mg/ml), was developed by immunizing mice with the collagenase resistant residue of human glomerular basement membrane, using standard procedures. Reactivity and specificity of both the monoclonal and polyclonal anti-E/N antibodies was demonstrated by Western blotting and immunofluorescence as previously described. Rabbit anti-bovine plasma fibronectin antiserum was generously provided by Dr. Denis Gospodarowicz (University of California, San Francisco). This antibody gave by immunoelectrophoresis one precipitin line against whole bovine plasma or serum, and cross-reacted with fibronectin from several species.

Immunoblot Detection of E/N

ECM was partially solubilized in hot SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer or extracted by incubation (2 hours at 37°C) with collagenase (50 µg/ml, CLS II; Cooper Biomedical, Malvern, PA), trypsin (50 µg/ml, Sigma Chemical Co., St Louis, MO), 6 M urea, or 6 M guanidine HCl. Insoluble material was removed by centrifugation, and the supernatant was diluted 1:1 with sample buffer, boiled, and subjected to SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane and reacted with polyclonal or monoclonal anti-E/N antibodies (1:250) followed by staining with alkaline phosphatase-conjugated goat antirabbit immunoglobulin G (IgG) or goat antimouse IgG, respectively, NBT, and BCIP, as described.

Immunoprecipitation of Corneal E/N

The immunoprecipitation procedure described herein is a modification of a previously described methodol-
ogy.23 Cells cultured in 10-cm dishes were radiolabeled for 16 hours in 3 ml methionine-free Dulbecco's modified Eagle's medium supplemented with 0.5% calf serum and 50 μCi/ml [35S]methionine (> 1000 mCi/mmol, Amersham, UK).24 The cells were washed twice with PBS, pH 7.2, scraped into 1 ml cell lysis buffer (0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 0.01 M Tris HCl, pH 7.4, 1 mM ethylenediamine tetraacetate, and 0.25 mM PMSF), and homogenized by repeated aspiration through a 1-ml syringe. Cell nuclei and debris were pelleted by a 2-minute centrifugation in an Eppendorf centrifuge. The supernatant was incubated (2 hours, 24°C) with 10 μl of the anti-E/N antibodies (monoclonal or polyclonal) or with 10 μl of either normal rabbit serum or antifibronectin antibodies. Protein A Sepharose (100 μl) was added for overnight incubation at 4°C. The pellet was washed three times with cell lysis buffer and boiled for 3 minutes with 30 μl SDS-PAGE sample buffer.23 The supernatant was diluted with SDS-free cell lysis buffer and subjected again to the immunoprecipitation protocol described above. The final pellet was washed with protein A wash buffer solution (100 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1 mM ethylenediamine tetraacetate) and boiled in Laemmli SDS-PAGE sample buffer and the supernatant applied to 10% SDS-PAGE. This technique facilitates the identification of the appropriate protein by eliminating most of the background. Gels were dried and exposed to Kodak (Rochester, NY) X-Omat film at −70°C for autoradiography.25 Molecular weight determination was made using the following globular protein standards (Sigma Chemical Co.) for calibration: carbonic anhydrase (24,000 Da), egg albumin (45,000 Da), bovine albumin (54,000 Da), phosphorylase B (88,000 Da), beta galactosidase (115,000 Da), and myosin (180,000 Da).

For immunoprecipitation of E/N from culture medium, antibodies were first incubated with protein A Sepharose (100 μl). The immobilized antibodies were then added to the culture medium followed by immunoprecipitation, SDS-PAGE, and autoradiography, as described above.

Immunofluorescence

Tissue sections of two to three mm, dissected from the periphery of six donor's cornea (human donors aged 15 to 40 years) left after using the cornea center for transplantation—or corneas considered unsuitable for transplantation—or corneas not used for transplantation—and corneas considered unsuitable for transplantation were used in this study. Sections were incubated with anti-E/N monoclonal antibodies diluted 1:20 in 2% BSA in PBS for 1 hour at 24°C. After three washes with PBS, sections were stained with fluorescein-conjugated rabbit antimouse IgG (1:60, Dako Inc., Glostrup, Denmark) for 30 minutes, washed as described above and counterstained with 0.1% Evans blue in saline. To determine the level of nonspecific staining, tissue sections were treated as described above, omitting application of the primary antibodies.21

RESULTS

Production of E/N by Corneal Endothelial Cells and Its Deposition into the Subendothelial ECM

We investigated whether E/N is produced by corneal endothelial cells maintained in vitro and secreted into the culture medium and subendothelial ECM. For this purpose, ECM was solubilized in boiled SDS-PAGE sample buffer containing 0.1 M dithiothereol and subjected to SDS-PAGE followed by Western blot analysis using monoclonal anti-E/N antibodies (MAb A9). As demonstrated in Figure 1, ECM derived E/N (lane 3) appeared primarily as a 150-kDa protein. In some experiments lower molecular weight proteins, representing degradation fragments of E/N, were detected apart from the intact 150-kDa protein, as also seen in the E/N preparation purified from bovine kidney (Fig. 1, lane 2). A similar pattern of proteins was previously seen in E/N preparations from Engelbreth Holm Swarm tumor, bovine kidney and cultured cell lines.3,21,25 Similar results were obtained when ECM was extracted with 6 M guanidine or 6 M urea and then applied for SDS-PAGE.

In subsequent experiments corneal endothelial cells were metabolically labeled with [35S]methionine and both the cell lysate and conditioned medium were subjected to immunoprecipitation with anti-E/N antibodies followed by SDS-PAGE. Cultured vascular endothelial cells were similarly treated, as the presence of E/N in the vascular subendothelial BM has been clearly demonstrated in various tissues.21 Newly synthesized [35S]methionine labeled E/N was immuno-precipitated from cell lysates (Fig. 2) and conditioned medium (Fig. 3), but there was no precipitation by nonimmune rabbit IgG (Fig. 3, lane 1). Although the ECM is, to a large extent, not solubilized by the cell lysis solution applied in this study, the possibility that some ECM components were extracted into the cell lysate can not be excluded. There was little or no precipitation of labeled E/N by the polyclonal anti-E/N antibodies. This may be due to the small amount of...
FIGURE 1. Presence of E/N in the extracellular matrix produced by corneal endothelial cells. ECM produced by confluent corneal endothelial cells maintained in 10-cm culture dishes was solubilized in 0.5 ml hot SDS-PAGE sample buffer. Fifty microliters of the solubilized material were applied onto a 10% polyacrylamide gel followed by Western blot analysis. Proteins were detected by successive incubations with monoclonal anti-E/N antibodies and alkaline phosphatase-conjugated goat antimouse IgG, as described in Methods. Lane 1, molecular weight standards; lane 2, E/N purified from bovine kidney; lane 3, solubilized corneal ECM. The position of E/N is marked by an arrow.

E/N applied onto the gel and to the relatively low sensitivity of the polyclonal antiserum, as compared to the monoclonal anti-E/N antibodies. Under the same conditions we were not able to detect newly synthesized fibronectin in the corneal endothelial cell lysates (Fig. 2, lane 6).

Corneal endothelial cells were compared for their ability to synthesize E/N, 3 and 12 days after seeding. As demonstrated in Figure 4, E/N was produced and secreted into the culture medium by actively growing, subconfluent cultures (lane 1) and by resting cells, 5 to 6 days after reaching confluence (lane 3). E/N was not detected when nonimmune rabbit IgG was used instead of the anti-E/N antibodies (lanes 2 and 4). Low (~ 30- and 45-kDa) and high (> 180-kDa) molecular-weight proteins were nonspecifically precipitated under the experimental condition applied in the current study. Immunoprecipitation studies with metabolically labeled bovine aortic endothelial cells revealed the presence of E/N in cell lysates (Fig. 2, lane 2) and culture medium (not shown) in a manner similar to the corneal cells. These cell types were shown to exhibit similar structural and functional characteristics.

Immunolocalization of E/N in the Human Cornea

Monoclonal anti-E/N antibodies were used to localize E/N within frozen sections of normal human corneas. Positive E/N immunofluorescent staining was found in all nine corneas studied. E/N was observed throughout the entire thickness of the epithelial BM (Fig. 5). The staining exhibited a linear pattern with decreased intensity from the periphery (Fig. 5A, limbus) toward the center of the cornea (Fig. 5D). In two cases E/N staining was not detected in the central portion of the epithelial BM. Descemet's membrane E/N appeared to be concentrated in a fine line adjacent to the endothelial surface of the cornea. The rest of the Descemet's membrane was devoid of E/N staining (Fig. 6). No staining was observed when sections of human cornea were incubated with FITC-conjugated IgG alone. The stroma of the cornea showed diffuse faint streaks of E/N fluorescence (Fig. 6). No detectable difference in E/N fluorescence pattern were observed in corneas from donors of different ages.

FIGURE 2. Immunoprecipitation of E/N synthesized by cultured corneal and vascular endothelial cells. Confluent cultures of bovine vascular (lanes 1 to 3) and corneal (lanes 4 to 6) endothelial cells were subjected to metabolic labeling (16 hours) with [35S]methionine. The cell layer was solubilized with cell lysis buffer and subjected to immunoprecipitation with either affinity-purified polyclonal anti-E/N antibodies (lanes 1 and 4), monoclonal anti-E/N antibodies (lanes 2 and 5), or polyclonal antifibronectin antibodies (lanes 3 and 6), as described in Methods. Immunoprecipitable material was detected by 10% SDS-PAGE and autoradiography.
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that in the adult, E/N of Descemet's membrane is pro-
duced primarily by corneal endothelial cells. The meth-
odiology described in this study for the isolation and
identification of E/N may allow to investigate the ef-
effect of conditions simulating disease situations, as well
as that of various cytokines, inflammatory molecules
and therapeutic protocols on the rate of corneal E/N
synthesis.

This study is the first to report the distribution of
E/N in the human cornea. Immunofluorescence stud-
ies using monoclonal anti-E/N antibodies revealed the
presence of E/N both in the epithelial and endothelial
(Descemet's) basement membranes and of minute
amounts within the stroma. These findings are in ac-
cordance with E/N distribution as previously de-
scribed in the mouse cornea. In our studies, E/N
staining of the Descemet's membrane showed a single
linear fluorescence pattern restricted to its most poste-
rior portion. This layer of Descemet's membrane is the
latest to be produced by the corneal endothelium

FIGURE 3. Immunoprecipitation of E/N synthesized by cor-
neal endothelial cells and secreted into the culture medium.
Confluent cultures of bovine corneal endothelial cells were
metabolically labeled (16 hours) with [35S]methionine. The
culture medium was then subjected to immunoprecipitation
with either nonimmune rabbit IgG (lane 1), monoclonal
anti-E/N antibodies (lane 2), or polyclonal anti-E/N antibod-
ies (lane 3). Immunoprecipitable material was analyzed by
SDS-PAGE and autoradiography.

DISCUSSION

E/N is a major constituent of BM. Its ability to bind
different components of ECM and to modulate cell
adhesion and function suggest a role in stabilization of
BM. In the only study of E/N synthesis by corneal
cells, Dong and Chung examined the expression of the
E/N gene in the developing eye of the mouse embryo
using in situ hybridization. E/N messenger RNA was
present in stromal cells underlying the epithelium, but
was totally absent from corneal epithelial cells. No ref-
erence was made to corneal endothelial cells. No ref-
ence was made to corneal endothelium. Using an
in vitro cell system and monoclonal anti-E/N antibod-
ies we have demonstrated, for the first time, that E/N
is produced by corneal endothelial cells. Similar
amounts of E/N were synthesized by cells in sparse
cultures and by highly contact inhibited cells in con-
fluent cultures. The newly synthesized E/N was depos-
ited into the subendothelial BM-like ECM and se-
creted into the culture medium. It was similar in molec-
ular weight, antibody reactivity, and proteolytic
cleavage products to E/N isolated from other extra-
cellular matrices. These findings may indicate

FIGURE 4. Synthesis of E/N by corneal endothelial cells in
subconfluent and confluent cultures. Subconfluent (5 days
after seeding) (lanes 1 and 2), or confluent (11 days after
seeding) (lanes 3 and 4) corneal endothelial cells were meta-
bolically labeled (16 hours) with [35S]methionine. The cul-
ture medium was then subjected to immunoprecipitation
with monoclonal anti-E/N antibodies (lanes 1 and 3) or with
nonimmune rabbit IgG (lanes 2 and 4). Newly synthesized
E/N was visualized by autoradiography. Its position is
marked by an arrow.
FIGURE 5. Immunofluorescent localization of E/N in intact human corneal epithelial BM: successive portions of the same cornea from the periphery (A) toward the center portion of the cornea (D). E/N in the epithelial basement membrane showed a thin linear distribution. E/N staining in the intact cornea was of maximal intensity in the periphery (limbus, A) and gradually decreased (B, C) toward the center of the cornea (D). (Magnification X625.)

FIGURE 6. Immunofluorescent localization of E/N in Descemet's membrane of human cornea. In Descemet's membrane, E/N immunofluorescence was observed in its most posterior portion directly underlying the endothelial cell layer, whereas the region adjacent to the stroma was devoid of E/N. Corneal endothelial cells tended to peel off Descemet's membrane during tissue preparation and staining and hence were not detected in this section. Irregular foci of E/N fluorescence were seen in the stroma. (Magnification X625.)
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postnatally.12 The rest of the postnatal Descemet’s membrane, as well as the anterior embryonal part were negative for E/N staining. Such an uneven distribution of E/N between the anterior and posterior parts of the Descemet’s membrane was not observed by immunoelectron microscopy in the mouse cornea.13 This may reflect a preferential role of corneal endothelial cells in E/N synthesis in mammalians.

Unlike the asymmetric immunofluorescence pattern observed in Descemet’s membrane, we found E/N to be distributed throughout the thickness of the epithelial basement membrane. The intensity of staining for E/N was not uniform, however. It was intense in the periphery (limbus) and gradually decreased toward the center of the cornea. This regional difference may be due to a higher degree of differentiation of the peripheral epithelial cells. Alternatively, it may result from a better supply of substrates for E/N synthesis, or of inhibitors to E/N degradation in the vicinity of limbal vessels. A similar distribution pattern in the epithelial BM of the human cornea was previously described for type IV collagen and laminin with the former showing a decreased intensity toward the center of the cornea.27 The immunofluorescence staining pattern of E/N in the epithelial BM of the mouse cornea was similar to that observed in the human cornea.13 Immunelectron microscopy revealed, however, that E/N is located at the borderline between the lamina densa and the lamina lucida with a maximum slightly inside the lamina densa.13

This study confirms that E/N is a major constituent of corneal basement membranes. The function of E/N in the cornea is not clear. Its involvement in cell adherence, blood coagulation and chemotactic processes in various experimental systems9-11 may suggest a role in wound healing and epithelial cell migration. It may also be implicated in the pathogenesis of disease states affecting the postnatal portion of Descemet’s membrane.

Key Words
entactin, nidogen, human cornea, Descemet’s membrane, corneal endothelial cells

References


