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Original Article

Analysis of a unique molecule responsible for regeneration and stabilization of differentiated state of tissue cells

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ABSTRACT In Wolffian regeneration in the newt, a functional lens can be regenerated through cellular transdifferentiation of the pigmented epithelium of the mid-dorsal marginal iris. A novel monoclonal antibody, 2NI-36 mAb, generated in our laboratory has been utilized as a highly useful probe to study newt lens regeneration. The antigen molecule against this 2NI-36 mAb (2NI-36) became temporarily undetectable only at the site of lens regeneration. Moreover, the ventral iris pieces expressed the ability to differentiate a lens when pretreated with this monoclonal antibody and implanted in lentectomized eyes (Eguchi, *Cell Differ. Dev. 25, Suppl.*, 1988). We have investigated the distribution of 2NI-36 in newt tissues. 2NI-36 was not specific to iris pigmented epithelium and distributed in many different kinds of mesodermal tissues, including dermis, blood vessel, mesonephros and so forth. 2NI-36 was also detected in either cell surface or intercellular spaces of cultured pigmented epithelial cells when they organized an epithelial cell sheet. Western blot analysis showed that 2NI-36 had the molecular weight of 50-200kD and was completely digested by trypsin, suggesting that 2NI-36 was a glycoprotein with many carbohydrate chains. It was also revealed by Western blot analysis that all the tissues in which 2NI-36 could be detected expressed this molecule similar to that in the iris epithelium. We expect that 2NI-36 is a glycoprotein expressed by various newt tissues and is functional to stabilize the differentiated state of each tissue cell in the same way as observed in the iris pigmented epithelial cells.

KEY WORDS: *regeneration, regeneration-responsive molecule, pigmented epithelial cells, mesodermal tissues, stabilization in differentiation*

Introduction

Lens regeneration in the newt is well known as Wolffian regeneration, in which a functional lens can be regenerated from the mid-dorsal margin of the iris through the transdifferentiation of pigmented epithelial cells (PECs) (Reyer, 1954, 1977; Scheib, 1965). In the eye *in situ*, the site of lens regeneration is strictly limited to the mid-dorsal margin of the iris and the ventral iris cannot regenerate any lens except in the case of administration of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) into the eye (Eguchi and Watanabe, 1973). Even when isolated iris pieces are implanted in lentectomized host eyes, the pieces derived from the ventral half of the iris are incapable of lens formation, showing clear contrast to the dorsal half (Sato, 1930; Mikami, 1941). However, both dorsal and ventral iris PECs can invariably transdifferentiate into lens cells to form lentoids when dissociated and cultured *in vitro*, strongly suggesting that PECs of both dorsal and ventral iris invariably retain the potential to transdifferentiate into lens cells, and that the dormant potential of PECs to transdifferentiate into lens cells must be repressed in the ventral iris (Eguchi *et al.*, 1974; Abe and Eguchi, 1977). Moreover, it was also demonstrated that the dorsal half of retinal pigmented epithelium was capable of lens formation (Sato, 1951). Eguchi and Okada first demonstrated that the progeny of retinal PECs isolated from 8- to 9-day-old chick embryos could transdifferentiate into lens cells (Eguchi and Okada, 1973). It was also shown by further cell culture studies that the ability of PECs to transdifferentiate into the lens is not limited to the newt, but is common in many kinds of vertebrates including human (Eguchi, 1979, 1986, 1988).

Abbreviations used in this paper: 2NI-36 mAb, 2NI-36 monoclonal antibody; BSA, bovine serum albumin; CBB-R250, Coomassie brilliant blue R-250; ECM, extracellular matrix; FCS, fetal calf serum; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; PBS, Ca^{2+}- and Mg^{2+}-free phosphate buffered saline; PECs, pigmented epithelial cells; SDS, sodium dodecyl sulfate; WE3 mAb, WE3 monoclonal antibody.

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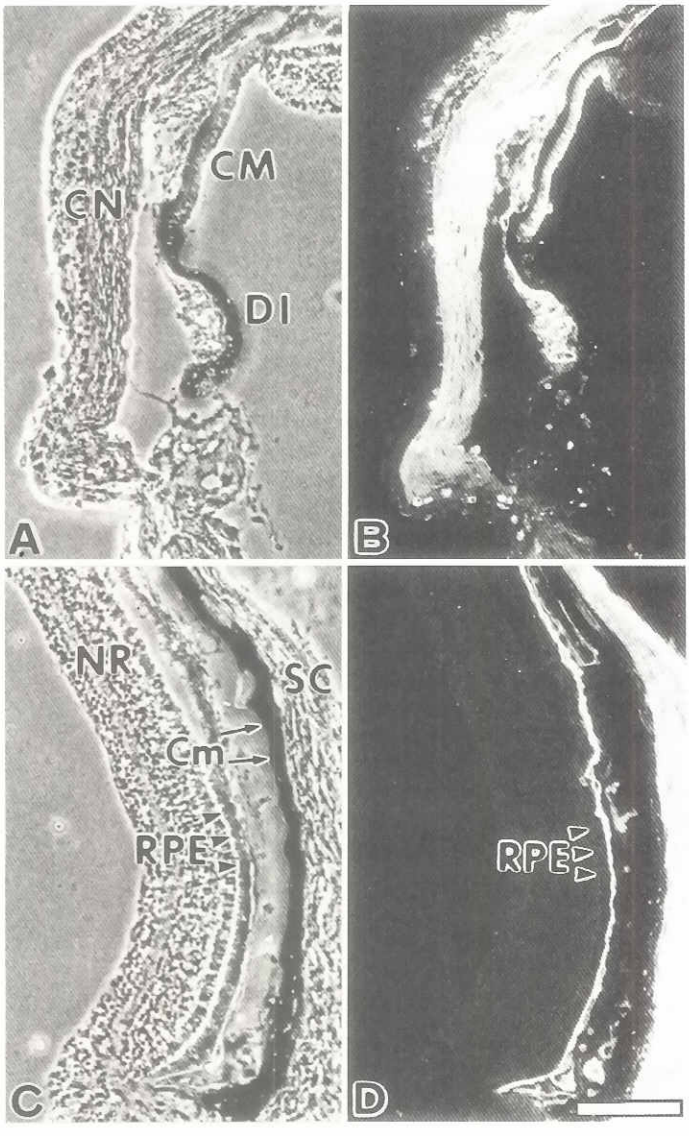


Fig. 1. Immunohistochemical staining of the lentectomized eye (2 days) with 2NI-36 mAb. (A and C) *Phase-contrast micrograph.* **(B and D)** *Fluorescence micrograph. 2NI-36 mAb reacted with several tissues in eye in situ (B and D). The choroid membrane is indicated by arrows and the retinal pigmented epithelium is indicated by arrowheads. CM, Ciliary muscle; Cm, Choroid membrane; CN, Cornea; DI, Dorsal iris; NR, Neural retina; RPE, Retinal pigmented epithelium; SC, Screa. Bar: 250 μm.*

According to the results obtained from these cell culture studies, we have presumed the presence of molecules responsible for stabilization of the differentiated state of each PEC in the pigmented epithelium. This presumption has been supported by the existence of such a molecule, which was discovered using a monoclonal antibody against the newt iris tissue (Eguchi, 1988). Soon after lentectomy, the antigen molecule against this monoclonal antibody (2NI-36 mAb), which was designated as 2NI-36, began to decay only in the dorsal margin of the iris pigmented epithelium, the site of lens regeneration. 2NI-36 temporarily became undetectable in the dorsal iris at around day 5 after lentectomy, corresponding to the stage at which PECs of the dorsal marginal iris begin to dedifferentiate and to synthesize DNA (Eguchi and Shingai, 1971; Yamada, 1977). It was particularly important that the pieces dissected from the ventral iris, from which lens regeneration never occurred in the eye *in situ*, became capable of differentiating lenses when pretreated with 2NI-36 mAb for 5 days and then implanted into the lentectomized eyes (Eguchi, 1988).

These findings strongly suggested that 2NI-36 cross-reacted with 2NI-36 mAb must be required for stabilization of the differentiated state of the newt iris PECs, and that the loss of 2NI-36 may be closely related to the initiation of dedifferentiation of dorsal iris PECs prior to transdifferentiation. In this study, we have examined the distribution and biochemical character of 2NI-36 as the first step to understanding the developmental function of this molecule.

Results

Distribution of 2NI-36

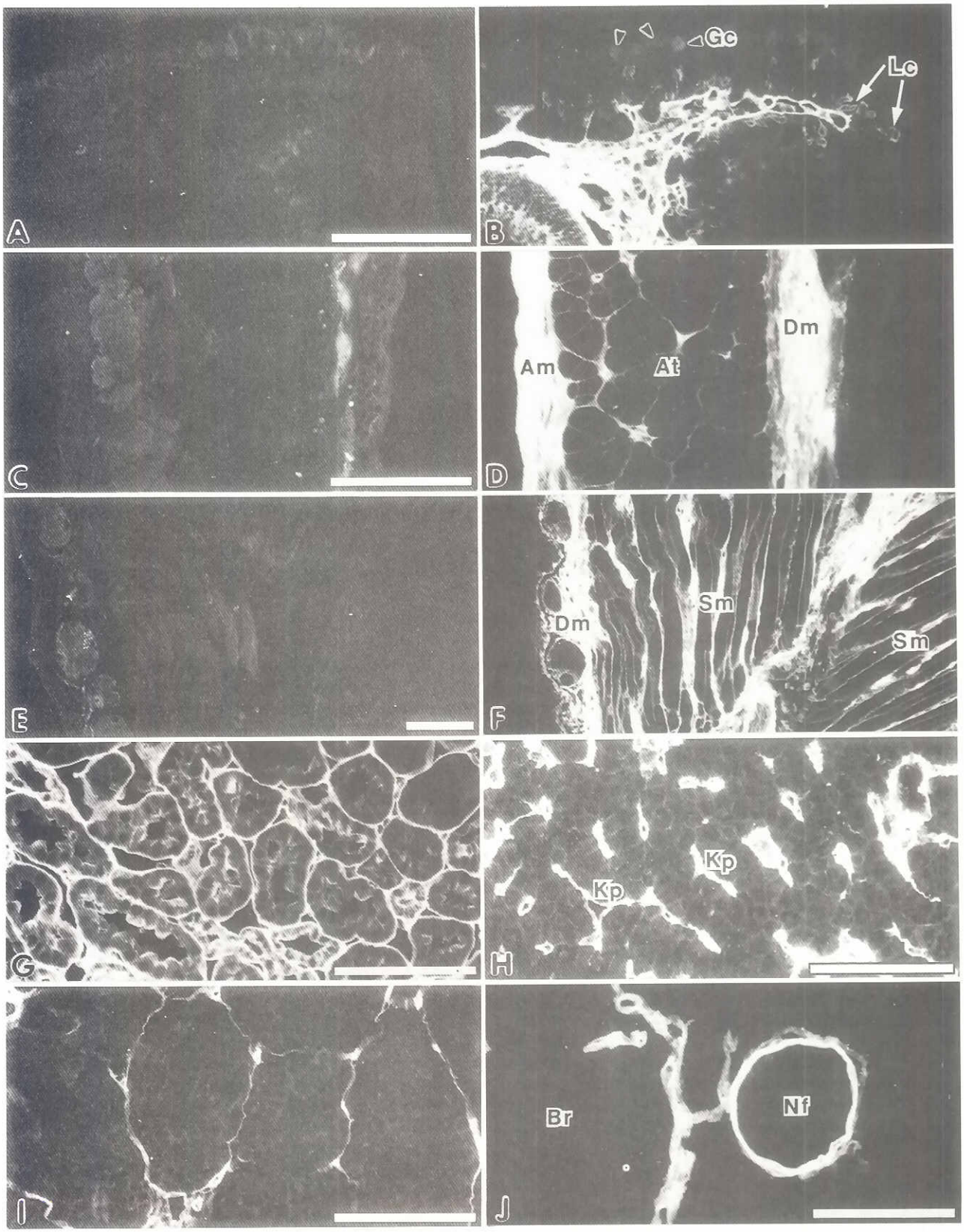
Cryosections of lentectomized eyes were stained with 2NI-36 mAb. 2NI-36 mAb was not specific to iris pigmented epithelium, but also cross-reacted with iris stroma including blood vessels (Figs. 1A and B). 2NI-36 mAb also cross-reacted with cornea, ciliary muscle, screa and the choriocapillary layer which adjoined Bruch's membrane (Fig. 1). However, 2NI-36 mAb did not cross-react with the neural retina at all (Fig. 1C and D).

We then investigated the distribution of 2NI-36 in various newt tissues. In brain, pancreas, stomach and intestine, 2NI-36 mAb did not react with any functional parenchyma, but reacted with blood vessels which traversed these tissues (Fig. 2A and B). Fig. 2B shows that strong immunohistochemical staining occurred only in the blood vessels and the lymphatic vessels in the intestinal villus. Lymphocytes and the goblet cells which secreted mucus were slightly stained (Fig. 2A and B). Moreover, 2NI-36 was strongly detected in mesodermal tissues; for example, dermis, skeletal muscle, heart (data not shown), mesonephros and Kupffer cells, all of which originate from the mesoderm (Fig. 2C-H). As for muscles, the strong expression of 2NI-36 was observed in abdominal muscles underneath adipose tissue (Fig. 2C and D) and also in both epimysium and perimysium (Fig. 2E and F). As shown in Fig. 2D, I and J, 2NI-36 mAb cross-reacted with various connective tissues; for example, the external regions of adipose tissue, the basement membrane of the testis and the perineurium of nerve fibers.

Expression of 2NI-36 by PECs cultured in vitro

Eguchi had already confirmed *in situ* localization of 2NI-36 on PECs (Eguchi, 1988). Hence, we examined the expression and localization of 2NI-36 in cultured PECs in the early phase of cell

Fig. 2. Immunohistochemical staining of each tissue with 2NI-36 mAb. *Figs. A, C and E show control experiments with unused culture medium for hybridoma instead of 2NI-36 mAb.* **(A and B)** *Small intestinal villus.* **(C and D)** *Abdominal skin.* **(E and F)** *Limb.* **(G)** *Mesonephros.* **(H)** *Liver.* **(I)** *Testis.* **(J)** *Brain and perineurium. Lymphocytes are indicated by arrows and goblet cells are indicated by arrowheads. Am, abdominal muscle; At, Adipose tissue; Br, Brain; Dm, Dermis; Gc, Goblet cell; Kp, Kupffer cell; Lc, Lymphocyte; Nf, Nerve fiber; Sm, Skeletal muscle. Bar: 200 μm.*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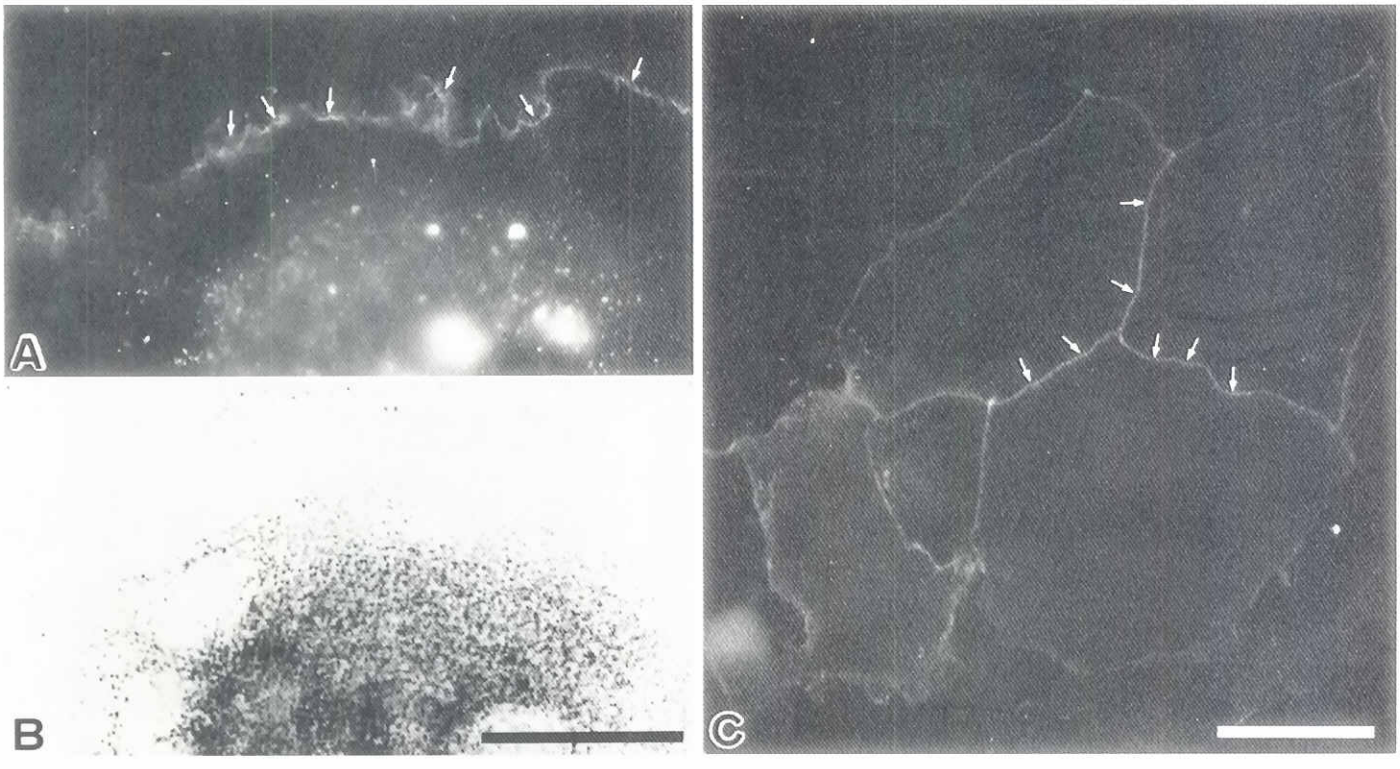

Fig. 3. Immunofluorescent localization of 2NI-36 in cultured PECs. (A and C) *Fluorescence micrograph.* **(B)** *Transmission micrograph. Cell surface and cell-cell adhesive sites of cultured PECs are indicated by arrows. Fluorescence at the bottom right of figure A is autofluorescence of newt pigment granules. Bar: 100 μm.*

culture. PECs from newt eyes were cultured in modified Leibovitz medium L-15 containing 10% fetal calf serum (FCS) at 25°C for 20 days. Shortly after plating cells, 2NI-36 could not be detected in individual iris PECs. However, when PECs well attached to the substrate and stabilized, 2NI-36 became detectable on the surface of each cell (Fig. 3A), and when PECs grew into organized epithelial cell sheets, it was detected at boundary lines of adherent cells (Fig. 3C). We also confirmed that well-differentiated PECs expressing 2NI-36 had numerous pigment granules exhibiting autofluorescence (Figs. 3A and B). These results indicated that 2NI-36 was synthesized by the PECs themselves and was localized at their cell surface.

Western blot analysis of tissue extracts

We characterized 2NI-36 by Western blot analysis. 2NI-36 in the iris had the broad molecular weight range of 50-200kD (Fig. 4A). In addition, we examined whether the molecule expressed by various tissues cross-reacting with 2NI-36 mAb were identical to 2NI-36 of the iris or not. Regardless of the difference of tissues, the molecule from various tissues had the same molecular weight as 2NI-36 of the iris (Fig. 4B), suggesting that the same 2NI-36 exists in various newt tissues. 2NI-36 was not detected in extracts of tissues which were immunohistologically not cross-reacted with 2NI-36 mAb (Fig. 4B).

Trypsin treatment

After 2NI-36 was fixed on nitrocellulose membranes by usual Western blot methods, blotted protein was treated by trypsin. 2NI-36 was completely digested by trypsin, but was not digested by bovine serum albumin (BSA) at all (Fig. 5), suggesting that 2NI-36 might be a glycoprotein with many carbohydrate chains. We also confirmed that 2NI-36 was not digested by collagenase (data not shown).

Discussion

It is highly reasonable to assume that stability of the differentiated state of PECs in the newt iris must be different between the dorsal and ventral regions due to different situations of cell-cell adhesion and communication, cell-substrate interactions and so on. The dormant potential of transdifferentiation of the ventral PECs into lens cells might be completely repressed by those *in vivo* conditions and regulatory mechanisms which are in effect when PECs construct an intact iris tissue. 2NI-36 temporarily decays at the site of lens regeneration, and the ventral iris pieces treated with 2NI-36 mAb can transdifferentiate lenses in lentectomized eyes (Eguchi, 1988). Judging from these studies, we can assume that 2NI-36 must participate in stabilization of the differentiated state of PECs in the eye *in situ* and the temporary loss of this molecule may be an essential process of dedifferentiation of the iris PECs.

As for the changes in the cell surface of the newt iris after lentectomy, it has been observed that glucosyltransferase and neuroaminidase disappear from iris PECs (Yamada, 1977). In the process of dedifferentiation of mid-dorsal iris PECs, PECs release N-acetylglucosamine due to the activity of N-acetylglucosaminidase

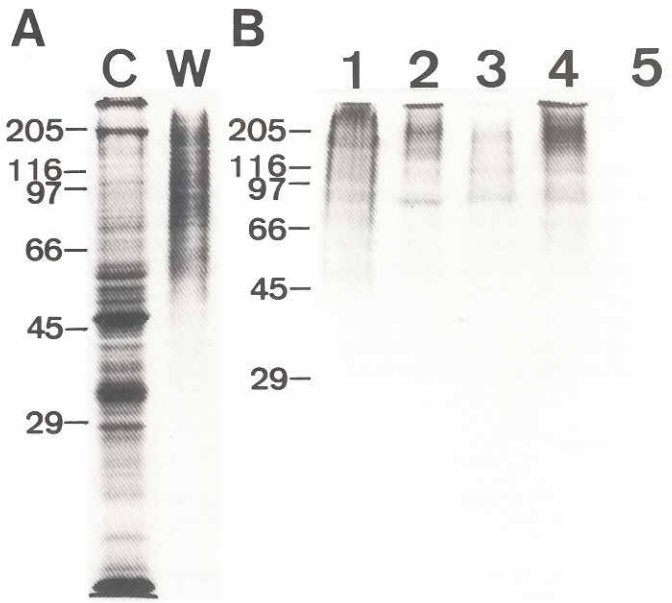

Fig. 4. Western blot analysis of 2NI-36. *Samples were electrophoresed on 12% SDS-polyacrylamide gel, blotted to a nitrocellulose membrane and probed with 2NI-36 mAb. Molecular weight markers are shown at the left of each figure. A: Western blot analysis of iris. Lane C, Coomassie brilliant blue staining; Lane W, Western blot staining. B: Western blot analysis of several tissues. Lane 1, Iris; Lane 2, Eyeball; Lane 3, Heart; Lane 4, Mesonephros; Lane 5, Neural retina.*

(Idoyaga-Vargas and Yamada, 1974) and lose acidic mucopolysaccharides and glycoproteins (Zalik and Scott, 1972, 1973; Zalik *et al.*, 1976). While at the present stage of our study it is rather difficult to relate these findings to our result, the temporary decay of 2NI-36 in the dorsal iris PECs after lentectomy, our present observation has clearly suggested that 2NI-36 might be synthesized by the PECs themselves and transported to their own cell-cell adhesive site to stabilize the differentiated state of PECs (Fig. 3). Therefore, the disappearance of 2NI-36 must be one of the essential processes for the initiation of dedifferentiation of iris PECs.

We have predicted that the molecule similar to 2NI-36 must be expressed by many other stable tissues besides the pigmented epithelium. Based on this prediction, our study has resulted in the following clear findings. 2NI-36 mAb is not specific to the iris pigmented epithelium, but cross-reacted with various tissues including cornea, iris stroma, ciliary muscle, choriocapillary layer and screa (Fig. 1). It is very interesting that 2NI-36 was strongly expressed between retinal pigmented epithelium and choriocapillary layer where extracellular matrix (ECM) of retinal pigmented epithelium exists, since ECM of PECs is essential to stabilization of the differentiated state of PECs. In addition to these ocular tissues, 2NI-36 was also expressed in dermis, skeletal muscle, heart (data not shown), mesonephros, blood vessels and Kupffer cells (Fig. 2). It is of interest that most of these tissues are mesodermal derivatives and stable tissues in which the growth potential of each cell organizing the tissue is repressed. It is possible for us to assume that the differentiated state of various cell types organizing tissues in general might be stabilized by 2NI-36.

Tassava *et al.* generated a monoclonal antibody, WE3 mAb, which reacted with the regenerate epithelium (Tassava *et al.*, 1986, 1987), and this group recently reported that this WE3 mAb reacted with many kinds of newt tissues (Goldhamer *et al.*, 1989). Interestingly, this distribution of antigen molecule against WE3 mAb is very similar to that of 2NI-36. But as regards distribution, the strict difference can be found. 2NI-36 mAb does not react with the wound epithelia and strongly reacts with cardiac muscle which does not express WE3 antigen molecule. Hence, 2NI-36 must be different from WE3 antigen molecule.

2NI-36 had the broad molecular weight of 50-200kD according to Western blot analysis and was digested by trypsin (Figs. 4 and 5), suggesting that 2NI-36 was a glycoprotein with many carbohydrate chains. Hence, we presumed that 2NI-36 might construct a similar structure as proteoglycan (PG), which has a single core protein and numerous carbohydrate chains. Recently, we found that 2NI-36 temporarily disappeared at the stump just before formation of the regeneration blastema during limb regeneration of the newt (Imokawa *et al.*, 1990). Around day 8 after amputation, at the stage just before degeneration of the muscle and bone of the stump, 2NI-36 was found to decay temporarily in the muscle, dermis and connective tissues. Judging from this result, in addition to the findings of lens formation from 2NI-36 mAb-treated ventral iris pieces and the wide distribution of 2NI-36 confirmed in the present study, we can assume that 2NI-36 might be responsible for stabilization of the differentiated state of each cell in various tissues, and its temporary decay might be closely related to degeneration of tissues in the process of regeneration. It can also be assumed that similar molecules must exist in other vertebrates and participate in the stabilization of the differentiated state of tissues, since 2NI-36 mAb was cross-reactive with *Xenopus laevis*, mouse and human tissues (Imokawa *et al.*, 1990). Some organs of these animals, in which 2NI-36-like molecules temporarily disappear after dissection, like newt eyes and limbs, must be capable of regeneration. We believe that it is very important to identify this 2NI-36 in order to understand the initial mechanism of regeneration.

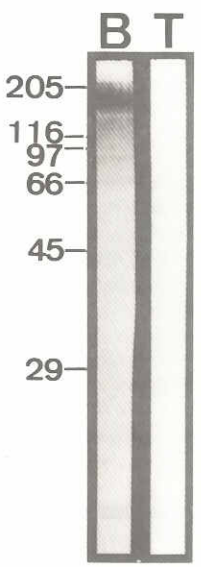

Fig. 5. Trypsin treatment of 2NI-36 on a nitrocellulose membrane. *Samples were electrophoresed on 12% SDS-polyacrylamide gel and blotted to a nitrocellulose membrane. Proteins on nitrocellulose membranes were treated by 1% BSA or 1% trypsin at 37°C for 2 h and probed with 2NI-36 mAb. Molecular weight markers are shown on the left. Lane B, No enzyme treatment (BSA treatment); Lane T, Trypsin treatment.*

Materials and Methods

Animals

Adult newts, *Cynops pyrrhogaster* were exclusively used in all experiments. They were collected from Tagarasu, Obama, Fukui prefecture, and maintained at 10°C.

Cell culture

Whole eyes were isolated, immersed in 70% ethanol for 1 min and washed with modified Dulbecco's phosphate-buffered saline prepared by diluting the original solution with distilled water to 80% strength. Sterilization with 70% ethanol was repeated three times. The iris-rings (iris pars iridica) were then removed and treated with Dispase II solution consisting of the diluted Leibovitz L-15 medium (Leibovitz, 1963; Eguchi *et al.*, 1974) and Dispase II (Sanko Pure Chemical Co. Ltd.) for at least 3 h at 25°C. The adherent iris stroma was then completely removed from the iris epithelium. Iris epithelia cleanly isolated with Dispase II were dissociated with trypsin solution and cultured with diluted Leibovitz L-15 medium, essentially according to the method established by Eguchi and his colleagues (Eguchi *et al.*, 1974).

Antibodies and indirect immunofluorescence

The monoclonal antibody, 2NI-36 mAb, identifies 2NI-36 detected in the cell surface or intercellular spaces of the newt iris. It was previously described that 2NI-36 temporarily decayed at the site of lens regeneration at around day 5 after lentectomy (Eguchi, 1988). Unused culture medium for hybridoma was used on all control experiments of immunohistochemical staining.

For immunohistological observations, each newt tissue was fixed with 4% paraformaldehyde in 0.1M phosphate buffer (pH7.4) for 8 h at 4°C and washed 6 times with the same buffer. Fixed tissues were infiltrated with a graded series of sucrose solutions in 0.1M phosphate buffer (pH7.4) at 4°C and embedded in Tissue-Tek O.C.T. compound (Miles Scientific). They were then rapidly frozen in liquid nitrogen. Serial frozen sections of 10 μm in thickness were cut in a cryostat (Cambridge Instruments GmbH) at -20°C and collected on albumen-coated glass slides. Cryosections were treated with FCS diluted 1:10 with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) for 1 h and subsequently treated with 2NI-36 mAb for 1 h at room temperature. After washing in PBS for 30 min, the sections were treated with fluorescein isothiocyanate-conjugated anti-mouse IgG (Cappel Laboratories Inc.) diluted 1:50 with PBS for 1 h. Immunofluorescence-stained specimens thus prepared were observed under a fluorescence microscope.

Cultured iris epithelial cells on cover glasses were fixed with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) for 3 h at 4°C and washed 5 times with PBS. Fixed cells were treated with FCS diluted 1:10 with PBS for 1 h. The cells were subsequently treated with 2NI-36 mAb for 1 h at room temperature. The cells were then treated with a 1:200 dilution of biotinylated sheep anti-mouse IgG (Amersham) for 1 h, followed by a 1 h treatment with fluorescein-streptavidin (Amersham). The cells were mounted and observed under a fluorescence microscope.

Western blot analysis

Tissue samples were solubilized by sodium dodecyl sulfate (SDS) and electrophoresed on 12.0% SDS-polyacrylamide gels according to the method described by Laemmli (1970). The electrophoresed proteins were transferred onto nitrocellulose membranes (Schleicher & Schuell, BA85), which were processed according to Towbin *et al.* (1979) with horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad laboratory) to determine the samples. The binding of 2NI-36 mAb to the specific protein was visualized in 20 mM Tris-HCl buffer (pH7.4) containing 0.6% 4-chloro-1-naphthol (Bio-Rad laboratory), 20% methanol, and 0.01% H_2O_2. Proteins in the SDS-polyacrylamide gel were stained with Coomassie brilliant blue R-250 (CBB-R250, 0.1% in 40% methanol/10% acetic acid, Sigma Chemical Co.).

Trypsin treatment

After the samples were transferred onto nitrocellulose membranes by Western blot methods, proteins on nitrocellulose membranes were digested for 2 h at 37°C by 1% (w/v) trypsin (Sigma Chemical Co.) in PBS. In the control experiments, 1% (w/v) BSA (Fraction V, Sigma Chemical Co.) was used instead of 1% (w/v) trypsin. After the trypsin treatment, the membrane was rinsed 5 times with PBS and processed as mentioned above to determine the samples.

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