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Insulin-like growth factor-I stimulates neurogenesis in chick retina by regulating expression of the α6 integrin subunit

José M. Frade[1], Elisa Martí[1,‡], Paola Bovolenta[1], M. Ángeles Rodríguez-Peña[2], David Pérez-García[1], Hermann Rohrer[3], David Edgar[4] and Alfredo Rodríguez-Tébar[1,*]

[1]Cajal Institute for Neurobiology, CSIC, Avenue Doctor Arce 37, E-28002 Madrid, Spain
[2]Biomedical Research Institute, CSIC, Arturo Duperier 8, E-28033 Madrid, Spain
[3]Max-Planck-Institute for Brain Research, Deutschordenstrasse 46, D-60528 Frankfurt/M, Germany
[4]Department of Human Anatomy and Cell Biology, University of Liverpool, Liverpool L69 3BX, UK

‡Present address: Biological Laboratories, Harvard University,16 Divinity Avenue, Cambridge, Massachusetts 02138, USA
*Author for correspondence (e-mail: Rodriguez@cajal.csic.es)

SUMMARY

Insulin-like growth factor I (IGF-I) strongly stimulates the generation of differentiated neurons in cultures of neuroepithelial cells of the embryonic chick neural retina in the presence of a laminin-1 tissue culture substrate. Treatment of cultured neuroepithelial cells with IGF-I rapidly up-regulated the mRNA coding for the α6 integrin subunit whereas specific reduction of α6 subunit levels by treatment with an α6 integrin antisense oligonucleotide resulted in reduced neuronal differentiation in vitro. Although IGF-I immunoreactivity is seen throughout the neural retina, expression of IGF-I mRNA is confined to the pigment epithelium during the period of neurogenesis in vivo. Neutralization of the endogenous IGF-I with a blocking antibody down-regulated levels of α6 integrin mRNA and reduced the production of differentiated retinal neurons in vivo. These data indicate a role for IGF-I in the generation of retinal neurons mediated by the interaction of laminin with its α6 integrin subunit-containing receptor.

Key words: retina development, neuron differentiation, insulin-like growth factor I, α6 integrin, chick

INTRODUCTION

The development of neurons and other cell types largely relies on environmental cues. Both growth factors and extracellular matrix (ECM) components act synergistically on neurons to play a crucial role in many processes of their development. It is of particular interest to investigate the function of extracellular factors in the vertebrate retina, since lineage analysis studies have demonstrated that the differentiation of retinal cells is lineage-independent (Turner and Cepko, 1987).

Insulin-like growth factor I (IGF-I) is known to promote growth and differentiation of a large variety of cell types, although the molecular basis of its various actions remains largely unknown (Cohick and Clemmons, 1993). The role of IGF-I in the development of the nervous system is even less well understood, although a number of observations indicate that this factor is implicated in the control of neuron number and differentiation (Rohrer, 1990). The physiological importance of endogenous IGF-I for neuron development is shown by a reduction in neuron number upon elimination of IGF-I, either by knockout of the *igf-1* gene (Beck et al., 1995) or by neutralization of IGF-I by antibodies (Zackenfels et al., 1995). Expression of both IGF-I and the IGF-I receptor are developmentally regulated in the brain. Highest expression of IGF-I mRNA has been observed at later stages of neuronal development, coinciding with differentiation, maturation, dendritic outgrowth and synaptogenesis (Bondy, 1991; Lee et al., 1992). In the retina, in situ hybridization studies have shown that IGF-I mRNA is expressed in the ganglion cell layer at advanced stages of retina development in the rat (Lee et al., 1992) and chick (De la Rosa et al., 1994b). However, the expression (and hence function) of this factor in less mature retinas is not known.

In contrast, the ECM protein laminin-1 has been shown to affect early stages of retinal neuron development. Interestingly, a synergistic effect of laminin-1 and neurotrophin-3 (NT-3) results in a strong stimulation of neuron differentiation (De la Rosa et al., 1994a). This indicates that receptors for both laminin-1 and NT-3 are expressed by retina neuron precursor cells. Indeed, the α6-containing integrin receptor for laminin (Sonnenberg et al., 1988; Hall et al., 1990) is present both in the developing chick retina (De Curtis et al., 1991; De Curtis and Reichardt, 1993) and in other neuroepithelial cells of the CNS (Bronner-Fraser et al., 1992), suggesting that an α6-containing integrin receptor can mediate laminin-1-induced neuron differentiation. We have now investigated the role of IGF-I for early retina development at the stage (embryonic day 5, E5) when the vast majority of the neuroepithelial cells have been shown to still be proliferating (Prada et al., 1991). We demonstrate that IGF-I is necessary for neuron differentiation in vivo and in vitro at this stage. Furthermore, we show that IGF-I stimulates α6 integrin subunit expression and that the

effect of IGF-I is dependent on the presence of both this laminin-1 receptor subunit and laminin-1 substrata.

MATERIALS AND METHODS

Chick embryos

Fertilized White-Leghorn hens eggs were incubated at 38.5°C. Embryos were staged following the classification of Hamburger and Hamilton (1951).

Antibodies

The anti-IGF-I mAb used was the clone sm 1.2, described by Russell et al. (1984), which has been recently used for IGF-I neutralization experiments in ovo (Zakenfels et al., 1995). The antibody blocks the activity of IGF-I, although it shows some cross-reactivity with IGF-II. G4 mAb labels a glycoprotein located on neurites of both amacrine and retinal ganglion cells (RGCs) (Rathjen et al., 1987; see Fig. 8C). This mAb stains 10-12% of differentiated neurons in dissociated cultures from E12 retinas (data not shown). A recombinant fragment C of tetanus toxin and a mAb against this fragment were purchased from Boehringer Mannheim. An anti-α6 integrin subunit mAb (Bronner-Fraser et al., 1992), recognizing only the denatured protein, was obtained from the Developmental Studies Hybridoma Bank, University of Iowa (hybridoma P2C62C4). A polyclonal antibody recognizing the cytoplasmic domain of αv integrin subunit was purchased from Chemicon International Inc. (Temecula, California, USA).

Antibody treatment of chicken embryos

Embryos were treated with anti-IGF-I mAb by growing the hybridoma cells in ovo, as described by Rohrer et al. (1988). Eggs were incubated for 3 days (stage 14-15 of development), and were then opened at the blunt pole. The shell membrane was removed and a suspension of 2.5×10^6 hybridoma cells in 25 μl anti-IGF-I ascites fluid was laid onto the chorioallantoic membrane. Controls were made either by injecting 25 μl phosphate-buffered saline (PBS) or injecting 2.5×10^6 anti-α6 mAb-producing hybridoma cells suspended in 25 μl ascites fluid of anti-α6 integrin subunit mAb. This antibody is of the same class as the anti IGF-I antibody (IgG1); it was produced against a denatured form of α6 subunit and does not recognize the native form of this receptor, nor does it block the binding of laminin to cells (Bronner-Fraser et al., 1992; our unpublished results). Anti-α6 antibody did not produce any observable change in the development of the retina (data not shown). The levels of both antibodies produced by the two injected hybridomas within the embryonic neural retina were estimated by an ELISA assay and were found to range between 6 μg and 20 μg per gram of wet tissue (data not shown).

Immunohistochemistry

Immunohistochemistry was carried out on frozen tissue sections. Briefly, chicken embryo heads were fixed by immersion in 4% paraformaldehyde/PBS (w/v) for 5 hours at 4°C and then in 100 mM sodium phosphate, pH 7.3, containing 30% sucrose (w/v) for 36 hours. 12 μm cryostat sections were mounted on 2% 3-aminopropyltriethoxy-silane-coated slides. Sections were incubated in PTG buffer containing PBS with 0.1% Tween-20 (v/v) and 10% goat serum (v/v) for 1 hour. Immunostaining was performed using either the anti-IGF-I mAb (3 μg/ml) or G4 mAb (ascites fluid diluted 1:200) in PTG for 16 hours at 4°C. After rinsing, sections were incubated with biotinylated goat anti-rabbit IgG (Jackson Laboratories) diluted 1:300 in PTG. After 1 hour incubation at room temperature, slides were rinsed with PTG and further incubated with peroxidase-conjugated streptavidin (Jackson Laboratories), diluted 1:1000 in PTG, for 1 hour. Finally, the sites of peroxidase attachment were revealed with 0.05% 3-3′-diaminobenzidine tetrahydrochloride (w/v) solution.

Preparation of retinal cells and cell cultures

Retinas from either E5 or E9 embryos were dissected free from pigment epithelium and dissociated as described previously (Rodríguez-Tébar et al., 1989; De la Rosa et al., 1994a). Dissociated cells were suspended in culture medium and plated on 10-mm round glass coverslips into four-well dishes (Greiner). Coverslips were previously coated with polyornithine (Sigma)/laminin-1 (Gibco) (Collins, 1978). Cells were cultured in 50% DMEM/50% F12 HAM (Sigma) with the N2 supplements (Bottenstein and Sato, 1979) lacking insulin where indicated, but containing 0.5 μCi/ml [^{3}H]methylthymidine (25 Ci/mmol; Amersham). Cultures were maintained for 20 hours at 37°C in a water-saturated atmosphere containing 5% CO_2. Finally, cells were labelled with either G4 mAb or tetanus toxin fragment C (following the protocol of the maker, Boehringer Mannheim), and subjected to autoradiography.

Apoptosis assay of cultured cells

Cultured apoptotic cells were detected by using an in situ cell death detection kit (TUNEL assay; Boehringer Mannheim), following the manufacturer's instructions. Briefly, cells cultured for 16 hours were fixed in 4% paraformaldehyde in PBS (w/v). After permeabilizing the cells with 1% Triton X-100 (v/v), terminal deoxynucleotidyl transferase and a mixture of fluorescein-conjugated deoxynucleotides were added to the cells. After incubation for 1 hour at 37°C, cells were washed, fixed and mounted for fluorescence microscopy.

Oligonucleotide treatment

E5 dissociated retinal cells were diluted in medium (see above) and plated on glass coverslips coated with polyornithine/laminin-1. After 30 minutes incubation, the medium was replaced with medium containing 0.5 μCi [^{3}H]thymidine/ml, alone or with the addition of either 5 μM sense or 5 μM antisense oligonucleotides. Synthetic sense and antisense oligonucleotides corresponded to bases 329-343 and 364-378 of the cDNA sequence of the α6 integrin subunit and did not display significant homology with other integrin subunits (De Curtis et al., 1991). After 5 hours incubation, a further 5 μM of concentrated sense or antisense oligonucleotides were added and incubation was continued for a further 15 hours. Then, cells were immunolabeled with G4 antibody and treated for autoradiography, as described previously. A total of three sets of triplicate experiments were carried out with each pair of sense/antisense oligonucleotides that were obtained from two different preparations. Results shown here were obtained with the oligonucleotides corresponding to bases 329-343.

Western blot analysis

To determine the effect of antisense oligonucleotides on levels of integrin protein and confirm that antisense treatment resulted in a selective decrease of α6 protein, 200,000 retinal cells were cultured in 10 cm^2 dishes with 100 μCi/ml [^{35}S]methionine (Amersham), in the presence of either sense or antisense oligonucleotides corresponding to bases 329-343 (De Curtis et al., 1991), or in the absence of any oligonucleotide. After a 20-hour culture, cells were washed with PBS and then lysed in 10 mM Tris/HCl, pH 7.4, containing 2 mM p-methylsulfonylfluoride. The lysates were collected and the particulate fraction was collected by centrifugation at 50,000 ***g*** for 30 minutes. Precipitates were dissolved in electrophoresis sample buffer and the radioactivity of each sample was measured. Samples containing identical amounts of radioactivity were subjected to SDS/PAGE under non-reducing conditions. After transfer to nitrocellulose membranes, blots were treated according to standard procedures. Pure mAb against the α6 integrin subunit was used at a concentration of 1 μg/ml. Anti-αv polyclonal antiserum was diluted 1:1000. After a second incubation with either an anti-mouse or an anti-rabbit peroxidase-conjugated antibody (Jackson), blots were developed by the chemiluminescence method (Amersham). Western blotting was also used for the determination of G4 antigen in the retina. To this end, retinas from either anti-IGF-I-treated or control

E9 embryos were homogenized in PBS. Extracts were briefly centrifuged for 5 minutes at 1000 ***g*** to remove nuclei and large debris and the supernatant were centrifuged at 100,000 ***g*** for 30 minutes. Precipitates were dissolved in denaturing buffer and 10 μg protein from each extract were subjected to SDS/PAGE. After blotting the gel, the antigen was detected by the G4 mAb, as described above.

Northern blot analysis and cDNA probes

Poly(A)$^+$ RNA from either pigment epithelium or neural retinas of several developmental stages was purified using oligo-d(T)cellulose, as described previously (Vennström and Bishop, 1982). Northern blots were performed according to standard protocols. cDNA probes were labeled by the random priming method using [α-^{32}P]dCTP (Amersham). Hybridizations were carried out overnight at 65°C in 7% SDS, 500 mM sodium phosphate buffer and 1 mM EDTA (Church and Gilbert, 1984). Filters were washed twice in 1% SDS, 40 mM sodium phosphate buffer, pH 7.2 at 65°C for 30 minutes. mRNA size was calculated using a RNA ladder as a marker (BRL). Membranes were exposed to Hyperfilms^{TM} MP films (Amersham). The cDNA probe for IGF-I mRNA was obtained as follows. Oligonucleotide primers derived from bp 97-118 and 744-765 of the cDNA sequence of chicken IGF-I (Kajimoto and Rotwein, 1989) were used to amplify a cDNA fragment derived from reversely transcribed RNA obtained from adult chicken liver. The fragment was cloned into the *Eco*RV site of the pBlueScript II SK+/– plasmid (Stratagene; pIGF-I). A similar procedure was used to clone a cDNA fragment corresponding to the cDNA sequence of the α6 integrin subunit (De Curtis et al., 1991). The primers used for amplification corresponded to base pairs 2239-2255 and 3139-3156 and the amplificate was cloned into the *Sal*I site of pBlueScript II SK+/– (pα6). pIGF-II was obtained from P. Brickell (Zakenfels et al., 1995). ^{32}P-labeled double-stranded cDNA probes were made by the random priming method.

In situ hybridization

Hybridization on retinal sections was carried out by the method of Wilkinson and Nieto (1993). Briefly, chicken embryos were fixed with paraformaldehyde/PBS as described above. Tissues were dehydrated in graded ethanol and treated for Paraplast (VWR) embedding. 10 μm sections were mounted on 2% 3-aminopropyltriethoxysilane-coated slides and dried at 37°C overnight. Slides were dewaxed, rehydrated, postfixed and treated with proteinase K followed by 0.3% (v/v) acetic anhydride. The hybridization mixture contained 2×10^6 cpm radioactive probe (see below) and was incubated for 16 hours at 60°C. Slides were then washed twice at high stringency (50% formamide (v/v), 60°C for 45 minutes) and treated with RNase. The location of the radioactive probe was revealed by autoradiography using NTB-2 emulsion (Kodak) after a 2-week exposure. The radioactive cRNA probes and their corresponding sense controls were prepared as follows: pα6 cDNA was digested with *Hin*cII and the two fragments coming from the insert were purified by agarose gel electrophoresis and cloned into *Hin*cII sites of the pBlueScript polylinker. The plasmid containing the fragment 2239-2675 bp of the α6 cDNA sequence (De Curtis et al., 1991) was treated with either *Kpn*I to produce the sense cRNA probe or *Hin*dIII for the corresponding antisense probe. ^{35}S-labeled probes were obtained by the procedure of Wilkinson and Nieto (1993).

Determination of α6 and αv integrin subunit mRNAs

pα6 plasmid was digested with *Bal*I and *Bsm*I, and the resulting 232-bp fragment was removed by agarose gel electrophoresis. The remaining plasmid was religated and transformed into *E. coli.* Clones yielding a 721-bp insert were selected. One of them was used for cRNA synthesis according to the procedure of Wilkinson and Nieto (1993). A quantified sample of this cRNA was added to the RNA extraction buffer (Chomczynsky and Sacchi, 1987) just before RNA extraction from cultured retinal cells. For semiquantitative PCR, a set of primers corresponding to bases 2467-2486 and complementary to bases 3077-3096 of the α6 integrin subunit (de Curtis et al., 1991), were used. PCR reaction mixtures contained 0.25 μCi [α^{32}P]dATP. 20-25 amplification cycles were performed, which in control experiments resulted in an exponential amplification under these conditions. After completion, samples were analyzed by agarose electrophoresis, gels were dried and exposed to X-ray films for 4 to 7 days. Only two bands were obtained in the autoradiograms: one 631-bp band corresponding to endogenous RNA and a 399-bp band from the cRNA control. The intensity of each band was measured with a General Dynamic densitometer.

A similar strategy was followed to determine levels of αv integrin subunit mRNA. A fragment corresponding to bases 2122-3053 of the cDNA sequence of αv (Bossy and Reichardt, 1990) was PCR-amplified with specific primers and cloned into the *Sal*I site of the pBlueScript II KS +/– (Stratagene) polylinker (pαv). The insert was sequenced and found to correspond to the published cDNA sequence of αv (Bossy and Reichardt, 1990). Plasmid pαv was excised with both *Bal*I and *Bsm*I to eliminate a fragment between bases 2741 and 2941. The religated plasmid (pαv′) was used for cRNA synthesis. For semiquantitative PCR, both endogenous αv mRNA and the αv′ cRNA were co-amplified with a set of primers derived from base pairs 2309-2329 and 2997-3016. Only two bands of 708 bp (endogenous) and 505 bp (exogenous) were obtained.

Control experiments performed in the absence of reverse transcriptase did not result in the amplification of any detectable cDNA bands after PCR (not shown). To determine retinal α6 and αv mRNA levels of either control or anti-IGF-I-treated embryos, retinas were dissected and total RNA was extracted (Chomczynski and Sacchi, 1987) and quantified. 400 ng RNA from each sample was reverse-transcribed and coamplified either with the α6 primers described above or with two primers corresponding to bp 2309-2329 (upstream) and complementary to bp 2997-3016 (downstream), respectively, of the cDNA sequence of the chick αv integrin subunit (Bossy and Reichardt, 1990). In the PCR mixtures, primers derived from bp 1250-1267 and 1548-1567 of the chicken β-actin sequence (Kost et al., 1983) were also added. PCR reaction mixtures contained 0.25 μCi [α^{32}P]dATP. After 25 cycles of amplification, the products were fractionated by agarose gel electrophoresis and subjected to autoradiography.

DNA fragmentation assay

To compare levels of apoptosis in retinas from either anti-IGF-I-treated or control E9 embryos, retinas were dissected and DNA extracted following the procedure of Huang and Plunkett (1992). 15 μg DNA were electrophoresed in 2% agarose gels and DNA ladders photographed after staining with ethidium bromide.

RESULTS

IGF-I is present in the early embryonic retina and induces conversion of cultured retinal neuroepithelial cells into neurons

Neuroepithelial cells dissociated from E5 chick retinas were cultured in chemically defined N2 medium lacking insulin (Bottenstein and Sato, 1979). When grown on a laminin-1 substrate, cells proliferated and after 20 hours in culture their number had multiplied 2.6-fold (Fig. 1). Under these conditions, about 4% of the cells were differentiated neurons, as identified by staining for the neuron-specific cell surface antigen G4 (De la Rosa et al., 1994a). A very small population of the G4-positive cells had proliferated in vitro, i.e. had incorporated [^{3}H]thymidine present in the culture medium and were also positive for G4. When the culture medium included IGF-I (10 nM), the number of G4-positive neurons was almost

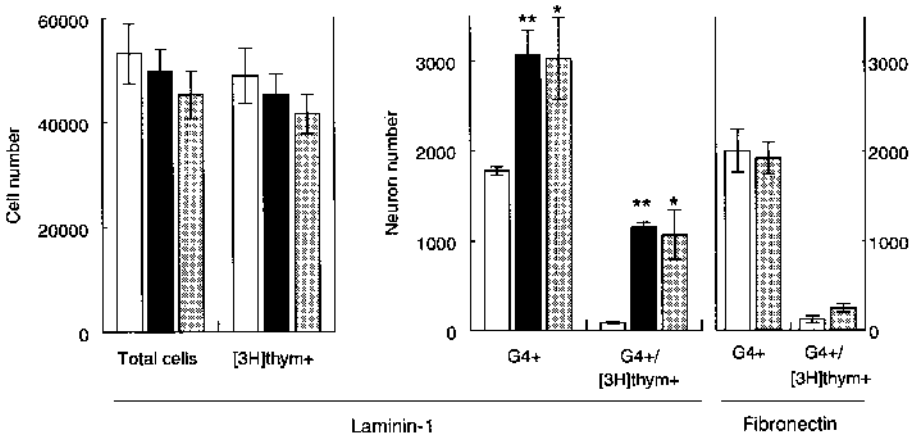

Fig. 1. Action of IGF-I on growth and differentiation of neuroepithelial cells from E5 retinas in vitro. Dissociated E5 retinal cells were plated on either laminin-1 or fibronectin at a density of 20,000 cells/cm^2 and cultured for 20 hours in the presence of [^{3}H]thymidine. Cells were then immunolabeled with the G4 mAb to specifically detect neurons and subsequently subjected to autoradiography to detect thymidine incorporation. After 2-3 days exposure, immunostained cells and labeling were observed by microscopy. On laminin-1, the presence of either IGF-I or insulin did not significantly affect either the total number of cells or the number of [^{3}H]thymidine positive ([^{3}H]thym+) cells, but the number of G4-positive (G4+)/[^{3}H]thymidine-positive cells, i.e. neurons generated in vitro, was significantly increased. On fibronectin, both the total number of cells and [^{3}H]thymidine-positive cells was about 80% of those plated on laminin-1. In contrast, the rate of neuron birth was much lower and unaffected by IGF-I. Values shown (± s.d.) are derived from triplicate cultures and are typical of at least 15 other independent experiments. $^{**}P<0.001$; $^{*}P<0.005$ Student's *t*-test; empty bars, control cultures; filled bars, cultures with 1 μM insulin; shaded bars, cultures with 10 nM IGF-I.

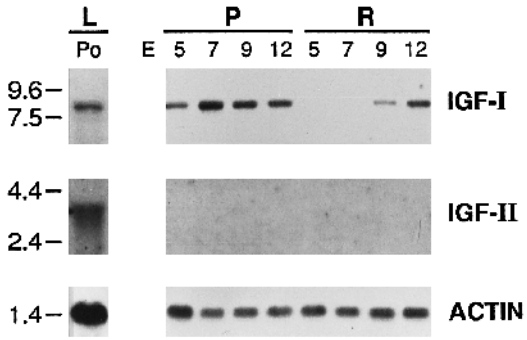

Fig. 2. IGF-I and IGF-II mRNA expression in the developing retina. Poly(A)$^+$-selected RNA from either pigment epithelium (P; 10 μg/lane) or neural retina (R; 10 μg/lane) from different embryonic ages (E) and from post-hatching (Po) liver (L) were hybridized on northern blots with specific ^{32}P-labeled cDNA probes for IGF-I, IGF-II and chick β-actin mRNA. The positions of marker standards (kb) are indicated on the left.

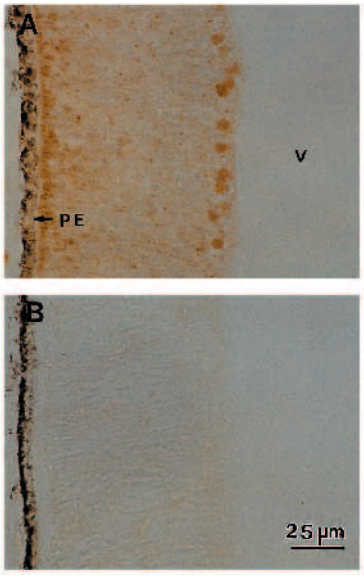

Fig. 3. Photomicrographs of immunoperoxidase staining of E5 retinal sections. 10 μm sections were stained with the anti-IGF-I mAb (3 μg/ml). Note the intense staining in the pigment epithelium and in its boundary with the neural retina. Diffuse immunoreactivity is found in the neural retina although it appears more concentrated on some cells at the vitreal surface (A). Pre-adsorption of the antibodies with IGF-I (0.3 μg/ml) resulted in inhibition of the immunostaining (B). PE, pigment epithelium; V, vitreous body. Scale bar, 25 μm.

double. However, the number of cells that had proliferated in culture and then became neurons (G4+/[^{3}H]thy+ cells) increased 13-fold (Fig. 1). Insulin at micromolar concentrations produced the same action as IGF-I but showed no effect when present in nanomolar concentrations (not shown). When neuroepithelial cells were grown on fibronectin, the level of neuron generation was fivefold lower than on laminin-1 and IGF-I did not increase the number of newly generated neurons (Fig. 1).

To determine if the increased number of neurons generated during culture was caused by an effect of IGF-I and laminin-1 on cell survival, dissociated E5 retinal cells were cultured for 16 hours and the TUNEL assay used to detect apoptotic cells (see Materials and Methods). We found that on laminin-1, 5.33±0.06% (±s.e.m., n=3) of the cells were apoptotic after 16 hours in culture, whereas in the presence of IGF-I 6.49±0.38 (±s.e.m., n=3) of the cells were apoptotic. These data indicate that IGF-I does not promote neuron generation in culture by preventing the death of newly differentiated or differentiating neurons.

To investigate the physiological significance of these results, northern blot analysis was used to measure the levels of IGF-I mRNA in the E5 eye. In the pigment epithelium, steady-state levels reached a maximum at E7 and subsequently decreased (Fig. 2). In contrast, in the neural retina, IGF-I mRNA only became detectable after E9. The 8.6 kb size of IGF-I mRNA detected in the retina corresponded to that of the IGF-I transcript detected in liver of hatched chicks (Zackenfels et al., 1995). IGF-II mRNA was neither detected in the pigment epithelium nor in the neural retina at any of the embryonic ages

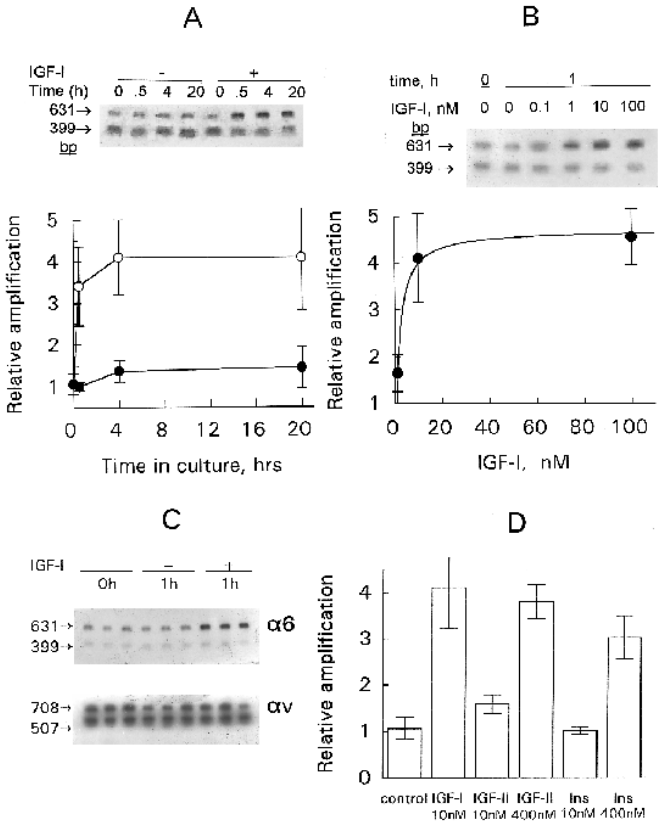

Fig. 4. IGF-I selectively increases integrin α6 subunit mRNA levels. Dissociated E5 retinal cells were plated at a density of 20,000 cells/cm^2 and allowed to attach to the laminin-1 substrate for 30 minutes. At this point an α6-derived control cRNA was added and total RNA was extracted from the cells (Time 0). Determination of α6 mRNA in the samples by semiquantitative PCR is described in Materials and Methods. After 25 cycles, the PCR reaction was still in the exponential phase of amplification and the signals obtained were proportional to the amounts of mRNA used (not shown). Products were separated by agarose gel electrophoresis, after which the gel was subjected to autoradiography. (A-C, top) Autoradiograms of ^{32}P-labeled amplified products after electrophoresis in the agarose gel. The upper 631 bp band corresponds to endogenous α6 mRNA, whereas the 399 bp band corresponds to exogenously added α6-modified cRNA in A-C. Additionally, in C (bottom), the upper 708-bp band corresponds to endogenous αv mRNA whereas the 507 bp band corresponds to exogenously added αv-modified cRNA. (A,B, bottom) The intensities of the bands were measured by densitometry and the optical density of the upper bands were normalised by dividing their densities by those of the respective lower bands. Values are expressed relative to those at time 0=1. The lower panels of A and B show a quantitative analysis of the data, ±s.d. (A) RNA was extracted from the cells after 30 minutes, 4 hours and 20 hours of incubation in the presence of 10 nM IGF-I. (B) Dissociated cells from E5 chick retinas were cultured for 1 hour in the presence of various concentrations of IGF-I, then RNA was extracted from the cells. Levels of IGF-I mRNA were plotted against the concentrations of IGF-I used. Half-maximal induction was accomplished with about 2 nM IGF-I. (C) Dissociated cells from E5 chick retinas were cultured either in the presence or in the absence of IGF-I (10 nM). Levels of both α6 and αv mRNA were measured by PCR amplification (see Materials and Methods for experimental details on αv). α6 mRNA is up-regulated in the presence of IGF-I, whereas αv mRNA levels remain unchanged. Each lane contains amplificates from independent cultures. (D) Dissociated neuroepithelial cells from E5 retinas were grown in the presence of either IGF-I, insulin or IGF-II at the concentrations indicated for 1 hour. Cells were processed for the determination of α6 mRNA amplification, as described. Values are the means of three independent determinations, ±s.d.

studied, although it was clearly detectable in the liver of hatched chick (Fig. 2).

IGF-I immunoreactivity was detected in the pigment epithelium and in the interphase between pigment epithelium and neural retina in E5 eyes (Fig. 3A), retina, but it was also seen throughout the neural retina at somewhat lower levels at this stage. Some cells (probably young differentiated RGCs) near the vitreal surface were also labeled. Pre-adsorption controls using IGF-I abolished the signal (Fig. 3B).

Effects of IGF-I on expression of the α6 integrin subunit

Neuroepithelial cells from E5 retinas were cultured on laminin-1 either with or without IGF-I, and their α6 integrin subunit mRNA levels were measured by semiquantitative RT/PCR amplification. After plating, cells expressed comparatively small amounts of α6 mRNA, as shown in Fig. 4A,C. The addition of 10 nM IGF-I to the cultures produced a four- to fivefold increase of α6 mRNA that was apparent after 30 minutes. The IGF-I-induced increase of α6 mRNA levels takes place at the level of transcription, as it was shown to be fully prevented by prior addition of actinomycin D (data not shown). Fig. 4B shows that half-maximal induction of α6 mRNA was obtained with about 2 nM IGF-I. This concentration closely corresponds to the dissociation constant of ^{125}I-IGF-I binding to its receptor (Steele-Perkins et al., 1988). The levels of mRNA coding for the αv integrin receptor did not change in response to IGF-I treatment (Fig. 4C). Insulin or IGF-II induced a significant increase of α6 mRNA expression only when added at 400 nM (Fig. 4D).

α6 integrin expression during retinal development

α6 mRNA expression was measured by northern blot analysis in the developing retina from E5 to E13. Levels reached a maximum at E7 (Fig. 5, upper panel), a time coincident with the main onset of neuron differentiation in the retina (Prada et al., 1991). Using in situ hybridization (Fig. 5A-D), we observed α6 message in both undifferentiated (E5) and differentiated (E12) retinas. In the younger retinas, α6 mRNA expression was found evenly distributed throughout the layer of pseudostratified neuroepithelial cells (Fig. 5A,B). Later in development, α6 mRNA expression was largely confined to the retinal ganglion cell (RGC) layer and the inner part of the inner nuclear layer (INL) (Fig. 5C,D).

The role of the integrin α6 subunit in neuron differentiation in vitro

Dissociated neuroepithelial cells were cultured with IGF-I on laminin-1 in the presence of antisense oligonucleotides derived from the cDNA sequence of α6 subunit (De Curtis et al., 1991; see Materials and Methods). The data shown were obtained using antisense oligonucleotide derived from bp 329-343 of the cDNA sequence (De Curtis et al., 1991) and its complementary sense oligonucleotide as a control. A second antisense and complementary sense pair of oligonucleotides (derived from bp 364-378, see Materials and Methods) gave similar results (data not shown). After antisense oligonucleotide treatment,

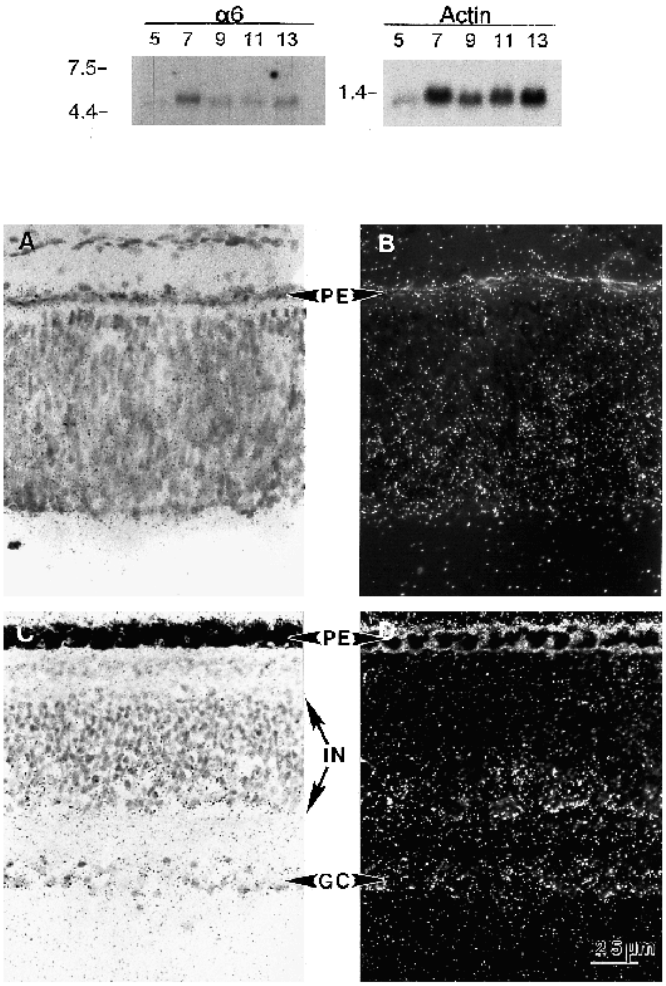

Fig. 5. Developmental expression of α6 integrin subunit mRNA in the developing retina. (Upper panels) Northern blot analyses showing α6 mRNA expression in chick neural retinas from various developmental ages. The right panel shows a control for β-actin mRNA levels. (A-D) α6 mRNA expression was studied by in situ hybridization at two representative ages of retinal development: E5 (A,B) and E12 (C,D). α6 mRNA localized to most cells of the undifferentiated retina, whereas in the stratified E12 retina, mRNA expression localized to the RGCs and inner cells of the INL. A,C, bright-field images; B,D, corresponding dark-field images. PE, pigment epithelium; GC, ganglion cell layer; IN, inner nuclear layer. Scale bar, 25 μm.

the number of newly differentiated cells (G4+/thy+) was significantly decreased (P<0.001) with respect to both sense-treated and untreated control cultures (Table 1). The total cell number was not significantly modified in the presence of the antisense oligonucleotide (Table 1). Furthermore, no effect was seen on differentiated neurons, since the number of G4-positive/thymidine-negative cells was not affected (data not shown) and incorporation of [^{35}S]methionine into proteins was not affected by the presence of oligonucleotides (Table 1). To assess if the α6 antisense oligonucleotide treatment affected apoptosis, we applied the TUNEL assay to control, sense- and antisense-treated cultures. No increase in apoptotic cell death upon antisense oligonucleotide treatment occurred (Table 1). These results show that antisense treatment does not involve cell death but rather that it impairs the differentiation of neuroepithelial into neurons.

To determine the effects of oligonucleotide treatment on integrin subunit expression, equal amounts of the cellular membrane fraction were subjected to SDS/PAGE, blotted and α6 and αv integrin subunit immunoreactivity was detected and quantified by densitometry. Treatment of the cultures with antisense oligonucleotides produced a significant decrease (P<0.001) in α6 immunoreactive protein with respect to the controls (Fig. 6 and Table 1). In contrast, αv integrin levels were not changed by the antisense tratment of the cultured (Fig. 6; Table 1). Thus, the decrease in levels of the α6 integrin subunit seen after antisense oligonucleotide treatment is specific for that integrin subunit.

Neutralisation of endogenous IGF-I by anti-IGF-I antibodies interferes with neuron differentiation in ovo

Anti-IGF-I-producing hybridoma cells were applied to the chorioallantoic membrane of E3 embryos, which were then killed at various developmental ages. E6 retinas from treated embryos contained significantly (P<0.001) reduced levels of α6 mRNA (Fig. 7), as measured by semi-quantitative RT/PCR. αv mRNA was also measured and showed similar levels in both control and treated retinas (Fig. 7). In addition to untreated embryos, parallel controls were performed with embryos that had been treated with hybridoma cells secreting an anti-α6 integrin subunit mAb, which does not recognize the native protein (see Materials and Methods). The latter embryos displayed retinal morphologies identical to those of untreated embryos (not shown). Together these results show that IGF-I specifically modulates both α6 integrin protein and mRNA expression in vivo.

Morphological analysis of retinas from antibody-treated E9 embryos revealed a number of distinct differences from controls (Fig. 8A,B). The outer plexiform layer was absent and the layer of developing photoreceptor neuroblasts did not present an ordered array. In addition, the inner plexiform layer was consistently thinner and contained a disorderly arrangement of migrating inverted amacrine cells. The inner nuclear layer appeared mostly to comprise tightly compacted neuroepithelial cells, whereas in control embryos, the inner third of this layer was apparently formed by loosely dispersed putative amacrine cells.

To delineate the morphological changes in more detail, sections were stained with the G4 antibody, specific for both RGCs and amacrine neurons at this developmental stage. In control E9 embryos, in addition to cells in the RGC layer, the cells in the inner third of the INL were labelled and are probably amacrine cells. In contrast, in treated embryos, labeling was restricted to RGCs, of which there appeared to be fewer than in the controls (Fig. 8C,D).

To quantitate the decrease of G4 immunoreactivity in treated retinas, equal amounts of protein from retinal membrane fractions of treated and control embryos were subjected to SDS/PAGE, the gels blotted, and G4 antigen was detected as described in Materials and Methods. As shown in Fig. 9A, anti-IGF-I treatment significantly (P<0.001) reduced G4 antigen levels.

To quantitate the effect of anti-IGF-I treatment on neuron differentiation, E9 retinas were dissociated to single cells and

Table 1. Effect of α6 integrin subunit sense and antisense oligonucleotides on cultured retinal neuroepithelial cells

Cell treatment	Radioactivity in membranes*	Cell number in the culture	Cell death (%)†	Levels of α6 integrin subunit‡	Levels of αv integrin subunit‡	Neuron generation (%)§
Control	25,882±4,619 (*n*=3)	38,628±1,357 (*n*=3)	5.33±0.06 (*n*=3)	1.00±0.00 (*n*=3)	1.00±0.00 (*n*=3)	100±5.72 (*n*=9)
Sense	22,884±1,936 (*n*=3)	37,148±5,336 (*n*=3)	4.90±0.23 (*n*=3)	1.19±0.19 (*n*=3)	1.24±0.37 (*n*=3)	94.50±3.63 (*n*=9)
Antisense	28,076±1,885 (*n*=3)	41,459±4,337 (*n*=3)	3.44±0.32 (*n*=3)	0.30±0.06* (*n*=3)	0.95±0.17 (*n*=3)	17.00±2.07* (*n*=9)

Sense and antisense oligonucleotides derived from the α6 cDNA sequence were added to E5 retina neuroepithelial cells cultured in the presence of [^{35}S]methionine and [^{3}H]thymidine. After 20 hours in culture, various parameters were determined.
*^{35}S-radioactivity incorporated into proteins of the membrane fractions.
†Percentage of apoptotic cells, estimated by the TUNEL assay.
‡Relative levels of a6 and av integrin subunit protein in the membrane fraction of the cultured cells.
§Relative level of neuron generation, as measured by the number of [^{3}H]thymidine-positive/G4-positive cells, with respect to the control.
Means ± s.e.m. are shown and the number of measurements (*n*) is given in parentheses. (*P<0.001; Student's *t*-test).

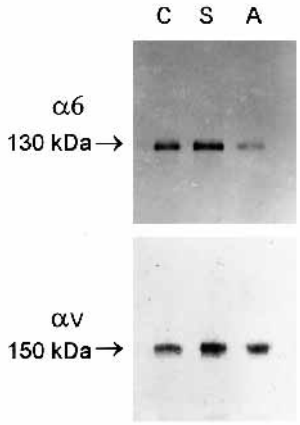

Fig. 6. Effects of α6 mRNA-derived oligonucleotides on cultures of retinal precursor cells. Dissociated neuroepithelial cells from E5 retinas were cultured on a laminin-1 substrate in the presence of 10 nM IGF-I and [^{35}S]methionine. The cells were treated with either antisense (A) or sense (S) oligonucleotides derived from bases 329-343 of the α6 cDNA sequence (see Materials and Methods). (C) Immunoblots of proteins from untreated cells. After 20 hours, membrane proteins containing equal amounts of [^{35}S]methionine were processed for western blot analysis to determine α6 and αv protein levels. The photographs of the western blots show that the α6 protein signal decreased in antisense-treated cells. In contrast, the presence of oligonucleotides did not affect the level of αv subunit. (See Table 1 for quantification of data, determination of [^{35}S]methionine incorporation and statistical analyses).

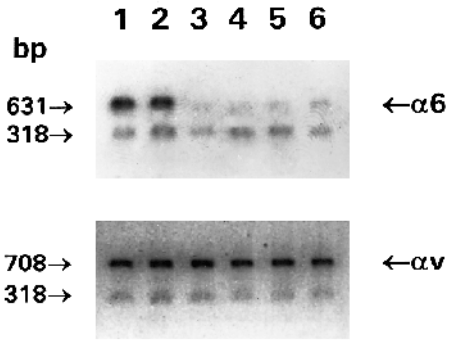

Fig. 7. Effect of antisense oligonucleotide treatment on the relative levels of α6 and αv mRNA in the neural retina. Total RNA was extracted from individual retinas and subjected to RT/PCR with specific primers for either α6 or αv integrin subunits and radioactive desoxynucleotides (see legend to Fig. 4 for details of the amplification). β-actin was co-amplified as an internal control, giving a single amplified band of 318 bp. The densitometric analysis of the autoradiogram of the α6 mRNA-derived product from treated retinas (lanes 3-6) showed that its levels had markedly decreased to some 25% of that of the controls (lanes 1,2), whereas those of αv and actin remained unchanged.

the proportion of differentiated neurons determined. To be able to identify the neurons immediately after dissociation of the retina, the trypsin-resistant marker tetanus toxin receptor was used. In freshly dissociated E9 retina of control embryos, 43.5±4.1% (±s.e.m., *n*=3) of the cells were tetanus toxin-positive, whereas only 27.9±0.57% (±s.e.m., *n*=9) of the cells from anti-IGF-I-treated embryos were labeled (P<0.001).

The rate of apoptotic cell death in control and treated embryos was studied by determining DNA fragments in the different retinas (see Materials and Methods). As shown in Fig. 9B, the levels of retinal DNA fragmentation in treated embryos were similar to those of controls, suggesting that, both in vivo and in vitro, IGF-I does not promote neuron survival, but rather affects their generation.

DISCUSSION

In the present study we demonstrate the involvement of IGF-I in the control of differentiation of retinal neuroepithelial cells, both in vitro and in vivo. We also show that IGF-I up-regulates a specific laminin-1 receptor component, the α6 integrin subunit, and provide evidence that this receptor mediates the stimulation of retinal neuron differentiation by IGF-I.

IGF-I is required for neuron differentiation in vitro and in vivo

In vitro studies suggest that IGF-I may be involved in the differentiation of a number of cell types including those of muscle (Florini et al., 1991) and mammary gland (Ruan et al., 1992). Here we have shown that cultured retinal neuroepithelial cells differentiate only if IGF-I at nanomolar concentrations was present and the cells were cultured on laminin-1. No effect of IGF-I was observed when the cells were plated on fibronectin. Because IGF-I did not decrease the numbers of

apoptotic cultured E5 retinal cells, we can discount IGF-I having a trophic effect on newly born neurons to support their survival.

The question therefore arises as to whether IGF-I is available to retinal precursor cells during normal development. Although we were able to demonstrate the presence of IGF-I immunoreactivity in the neural retina at E5, higher levels of immunoreactivity were observed in the pigment epithelium at that time.

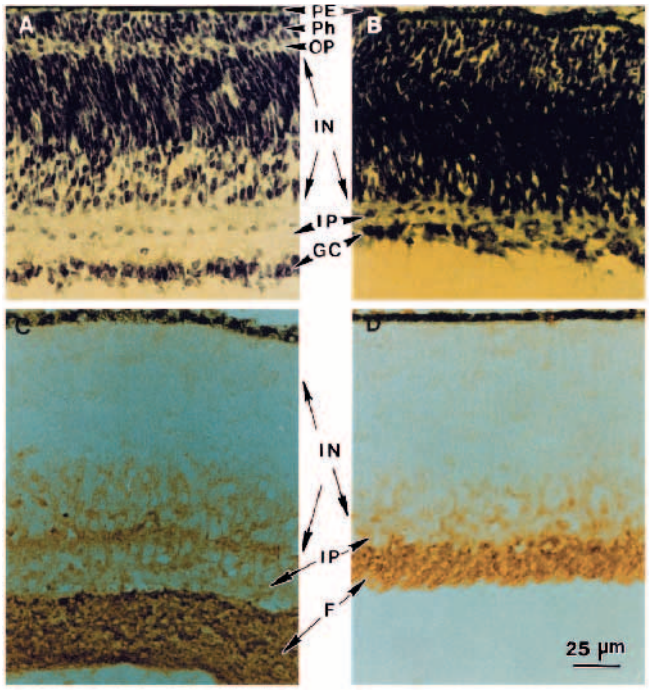

Fig. 8. Histological comparison of anti-IGF-I-treated and control E9 retinas. Micrographs of cresyl violet-stained sections (A,B) and G4 immunohistology (C,D) of retinas from E9 control (A,C) and anti-IGF-I-treated embryos (B,D). In the controls, the inner third of the inner plexiform layer is positive for G4 mAb, a marker of differentiated amacrine cells and RGCs, whereas in treated embryos cells in this area were negative for G4 staining. PE, pigment epithelium; Ph, photoreceptor layer; OP, outer plexiform layer; IN, inner nuclear layer; IP, inner plexiform layer; GC, ganglion cell layer; F, fiber layer. Scale bar, 25 μm.

Furthermore, although IGF-I mRNA was readily detected in the pigment epithelium at this stage, expression was undetectable in the neural retina prior to E9. In addition, we observed that the highest levels of IGF-I mRNA in the pigment epithelium occurred by E7, i.e. in temporal coincidence with the main onset of neuron differentiation in the neural retina (Prada et al., 1991). These data suggest that in the early embryogenesis of the retina, IGF-I present in the neural retina is likely to come from pigment epithelium. Further experimental evidence also points to pigment epithelium as the source of other diffusible factors that influence cells of the developing neural retina. Thus, pigment epithelium-derived factor, a protein related to serpins but devoid of protease inhibition capacity, induces differentiation and coronal organization of retinoblastoma cells into miniretina-like structures (Steele et al., 1992).

Our results support an action of IGF-I in vitro, by stimulating neuron differentiation of retinal neuroepithelial cells. Indeed, our embryo perturbation experiments indicate that IGF-I also influences cell differentiation in vivo. Retinal sections of E9 embryos previously treated with anti-IGF-I blocking antibodies contained a larger proportion of undifferentiated neurons, as assessed by the extent of G4 immunostaining and decreased levels of G4 antigen. Moreover, the fraction of differentiated neurons, i.e. tetanus toxin-positive cells, was significantly lower in antibody-treated E9 retinas compared to control embryos. These results are most easily explained by an effect of IGF-I on neuron differentiation in vivo, since a trophic effect of IGF-I on differentiated neurons was discounted by the demonstration that the level of apoptosis was not increased in anti-IGF-I-treated embryos. However, from these experiments, it is not clear whether IGF-I acts directly to induce neuron differentiation, or indirectly to induce receptors required for a response to other differentiation signals.

IGF-I regulates levels of the α6 integrin subunit mRNA

Both induction of neuronal differentiation and rapid up-regulation of α6 mRNA levels in vitro were specifically triggered by IGF-I. The rapid increase of α6 integrin mRNA levels after IGF-I treatment (within 30 minutes) suggests that this effect is

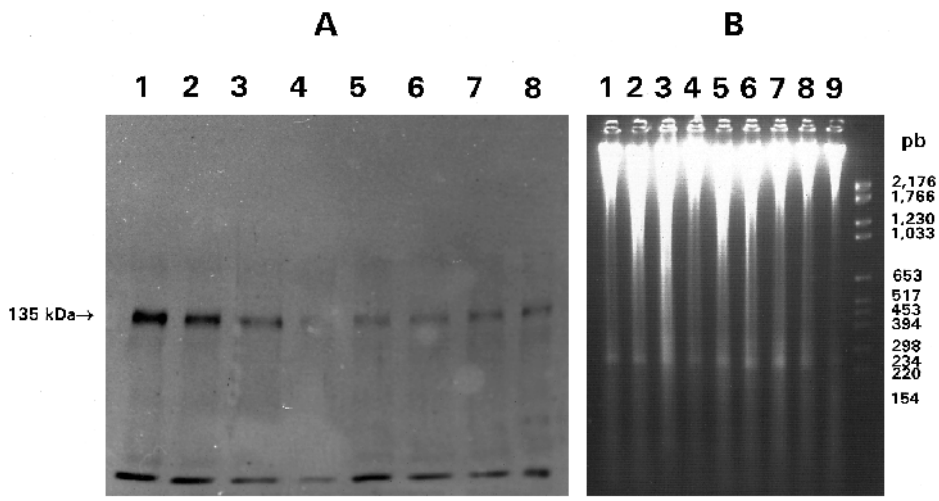

Fig. 9. Effect of IGF-I treatment on the amounts of G4 immunoreactive protein and DNA fragmentation in the retina. (A) Relative levels of G4 antigen in individual retinas of control (lanes 1-3) and anti-IGF-I-treated (lanes 4-8) E9 embryos. The membrane fraction from each retina was electrophoresed on a SDS/PAGE gel and then western blotted. Filters were probed with the G4 mAb. Densitometric analysis of the blots showed a decrease to 23±7% of G4 antigen in treated retinas with, respect to the controls (P<0.001; Student's t-test). (B) DNA fragmentation in retinas from either control (lanes 1-4) or anti-IGF-I-treated embryos (lanes 5-9). Densitometric quantitation of the ethidium bromide-stained gel did not show any significant difference between control and treated embryos. pb, base pairs.

direct. Experiments are in progress to see if there is a requirement for protein synthesis in this process. The dose-response relationship of α6 expression induced by IGF-I shows that half-maximal induction was obtained by a concentration of IGF-I very close to the dissociation constant of IGF-I binding to its receptor (Steele-Perkins et al., 1988), supporting the idea that the effects are mediated by that receptor. In this context, it should be noted that IGF-I receptors have previously been demonstrated in the E6 chick neural retina (Waldbillig et al., 1991).

In addition to these in vitro observations, we have also provided evidence that the expression levels of α6 mRNA are controlled by endogenous IGF-I in vivo: the use of antibodies blocking the biological activity of IGF-I resulted in a decrease of the levels of integrin α6 mRNA to about one quarter of the normal values. Although the antibody used here displays some cross-reactivity with IGF-II (Zackenfels et al., 1995), the retinal target of the antibody must be IGF-I, since IGF-II is produced neither in the neural retina nor in the pigment epithelium at the embryonic ages studied here.

Regulation of the expression of the α6 integrin subunit is necessary for the effects of IGF-I on neuronal differentiation

Reduction in the levels of the integrin α6 subunit mRNA and protein by the antisense oligonucleotides inhibited the action of IGF-I on neuron generation. The selectivity of this effect is evident because cell numbers, proliferation and protein synthesis were not affected. In addition, α6 protein levels were only reduced upon treatment with antisense oligonucleotide, and not with control (sense) oligonucleotide treatment. It remains to be shown, however, if the increase in levels of integrin α6, while being necessary, are sufficient to elicit the effects observed upon IGF-I treatment.

The developmental pattern of α6 integrin mRNA expression in the neural retina is also consistent with this integrin subunit being involved in neuron differentiation in vivo. The developmental profile of α6 expression in the chick retina revealed that the highest levels occurred at E7, coincident with the main onset of neuronal differentiation (Prada et al., 1991), and before most RGC axons reach the optic tectum (Rager, 1980). Furthermore, Bronner-Fraser et al. (1992) have shown that while α6 immunoreactivity is present in the neural tube of early chick embryos, at later stages immunoreactivity decreased, being limited to some differentiated neurons, including commissural and motoneurons. Similarly, we show here that in the early retina, integrin α6 mRNA is evenly expressed in undifferentiated neuroepithelial cells. When the differentiated retina is fully stratified later during development, α6 mRNA expression is restricted to the inner nuclear layer and the ganglion cell layer. The α6 subunit of RGCs probably interacts with laminin-1 in the inner limiting membrane and optic tract, thus contributing to the growth and guidance of RGC axons to the optic tectum at this later stage (Cohen et al., 1987). Taken together these observations are consistent with multiple roles for the α6 integrin subunit in neuronal differentiation and axonal growth.

In conclusion, our results show that IGF-I is a probable candidate to control early events of retinal morphogenesis by regulating neuron differentiation. IGF-I induces integrin α6 gene expression, and this subunit, in association with the β1 integrin subunit (de Curtis et al., 1991) may act as a specific receptor for laminin-1, which is also present in the early embryonic retina (Cohen et al., 1987; Frade et al., 1996). Thus the process of neuron differentiation in the retina may be regulated by the ability of IGF-I to modulate the response of developing neural cells to the extracellular matrix.

The monoclonal antibody against chick α6 integrin subunit, developed by Dr A. F. Horwitz, was obtained from the Developmental Studies Hybridoma Bank mantained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA and the Department of Biological Sciences, University of Iowa, Iowa City, IA 52242, USA under contract N01-HD-2-3144 from the NICHD. This research was financed by grants from the DGICYT (Ministry of Science of Spain, no. PB93-0124), Regional Governments of Madrid and Canary Islands, Spain and the European Union, Programme Science (no. SCC-CT90-0021).

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