Endoglin is expressed in the chicken vasculature and is involved in angiogenesis

Ulla Raab[a,1], Pedro Lastres[a], Miguel A. Arévalo[b], Jose M. López-Novoa[b], Carlos Cabañas[c], Enrique J. de la Rosa[a], Carmelo Bernabéu[a,*]

[a] *Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas (CSIC), Velazquez 144, 28006 Madrid, Spain*
[b] *Facultad de Medicina, Universidad de Salamanca, 37007 Salamanca, Spain*
[c] *Facultad de Medicina, Universidad Complutense, 28040 Madrid, Spain*

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Abstract **Endoglin is a component of the transforming growth factor β (TGF-β) receptor complex, highly expressed by endothelial cells. Mutations in the endoglin gene are responsible for hereditary hemorrhagic telangiectasia type 1 (HHT1), an autosomal dominant vascular disorder caused by a haploinsufficiency mechanism. Vascular lesions (telangiectasia and arteriovenous malformations) in HHT1 are associated with loss of the capillary network, suggesting the involvement of endoglin in vascular repair processes. Using the chick chorioallantoic membrane (CAM) as an angiogenic model, we have analyzed the expression and function of chicken endoglin. A pan-specific polyclonal antibody (pAb) recognized chicken endoglin as demonstrated by immunostaining and Western blot analysis. In ovo treatment of chicken embryos with this pAb resulted in a significantly increased area of CAM. This effect was likely mediated by modulation of the ligand binding to endoglin as this pAb was able to inhibit TGF-β1 binding. These results support the involvement of endoglin in the angiogenic process.**

Key words: Endoglin; Angiogenesis; Chicken; Vasculature; Transforming growth factor β

1. Introduction

Endoglin is a component of the transforming growth factor β (TGF-β) receptor system able to modulate cellular responses to TGF-β [1–3]. Endoglin is a 180 kDa homodimeric membrane glycoprotein which binds at least TGF-β1, TGF-β3, activin-A, and BMP-7 [4–6]. This ligand binding requires the expression of signaling receptors [5,7], which form heteromeric complexes with endoglin [3,5,8]. To date, endoglin has been found to be highly expressed on endothelial cells of human [9], pig [8], and mouse [10] origin, but little is known about endoglin expression in the chicken. The high expression levels of endoglin in endothelial cells seem to be critical for the vascular physiology as mutations in the endoglin gene are responsible for hereditary hemorrhagic telangiectasia type 1 (HHT1), a dominant vascular disorder associated with frequent epistaxis, telangiectases, gastrointestinal bleedings, and arteriovenous malformations in brain, lung and liver [11–13]. It has been postulated that HHT1 is originated by a mechanism of endoglin haploinsufficiency [12–14]. This finding, together with the localized loss of capillaries found in the HHT lesions, suggests that endoglin might be involved in angiogenic repair processes. This hypothesis would be compatible with the crucial role that TGF-β plays in vascular development and remodeling [15]. Recently, Li et al. [16] have reported that mice lacking endoglin die at gestational day 11.5 with poor vascular smooth muscle development and arrested endothelial remodeling. In the current study we have identified chicken endoglin using a specific polyclonal antibody (pAb) and demonstrated that antibodies to endoglin are able to modulate the angiogenic process of the chick chorioallantoic membrane (CAM) in developing chicken embryos.

2. Materials and methods

2.1. Antibodies

The anti-endoglin pAb was obtained by infection of New Zealand rabbits with a recombinant vaccinia virus expressing human endoglin [17]. As a negative control, antisera from animals infected with a recombinant thymidine kinase-deficient vaccinia virus (anti-TK$^-$) were used. Rabbit immunoglobulins were purified from sera by affinity chromatography using protein G Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden). The mouse monclonal antibody (mAb) 44G4 (anti-endoglin) has been previously described [9].

2.2. Immunohistochemistry

Lung tissue sections were obtained by surgical procedures from White Leghorn adult chickens (Farm Rodríguez-Serrano, Salamanca, Spain). Tissue sections were fixed by immersion in 4% buffered formalin during 24 h. After dehydration, pieces were embedded in paraffin and 3 μm sections were cut and mounted on glass slides and stained with the rabbit pAb anti-endoglin. The presence of endoglin was revealed using an avidin-biotin-complex immunoperoxidase method (ABC Staining System, Santa Cruz Biotechnology, Santa Cruz, CA, USA) that uses 3,3′-diaminobenzidine (DAB) as a substrate. Samples were counterstained with hematoxylin. For CAM immunostaining, egg shells were excised and CAM were removed, fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.1, washed three times with phosphate buffered saline (PBS) and permeabilized with 1% Triton X-100 in PBS for 4 h. Membranes were then incubated with the rabbit pAb to endoglin, followed by a FITC-labeled anti-rabbit Ig. Endoglin labeling was revealed by fluorescence microscopy using a confocal scanner laser (MRC1024; Bio-Rad Laboratories, Hercules, CA, USA) mounted on a Zeiss Axiovert 135 microscope (Zeiss, Oberkochen, Germany).

2.3. Angiogenic assays in ovo

Fertilized White Leghorn eggs were incubated at 38.2°C and 60–90% relative humidity for 36 h, corresponding to stage HH8–HH10 of embryonic development [18]. After this period of time, windows were made in the shells and indicated treatments were applied directly over

*Corresponding author. Fax: (34) (91) 5627518.
E-mail: bernabeu.c@cib.csic.es

[1] Present address: Institut für Medizinische Mikrobiologie und Hygiene, Universität Regensburg, D-93053 Regensburg, Germany.

Abbreviations: CAM, chick chorioallantoic membrane; DAB, 3,3′-diaminobenzidine; HHT, hereditary hemorrhagic telangiectasia; mAb, monoclonal antibody; pAb, polyclonal antibody; PAGE, polyacrylamide gel electrophoresis; TGF-β, transforming growth factor β

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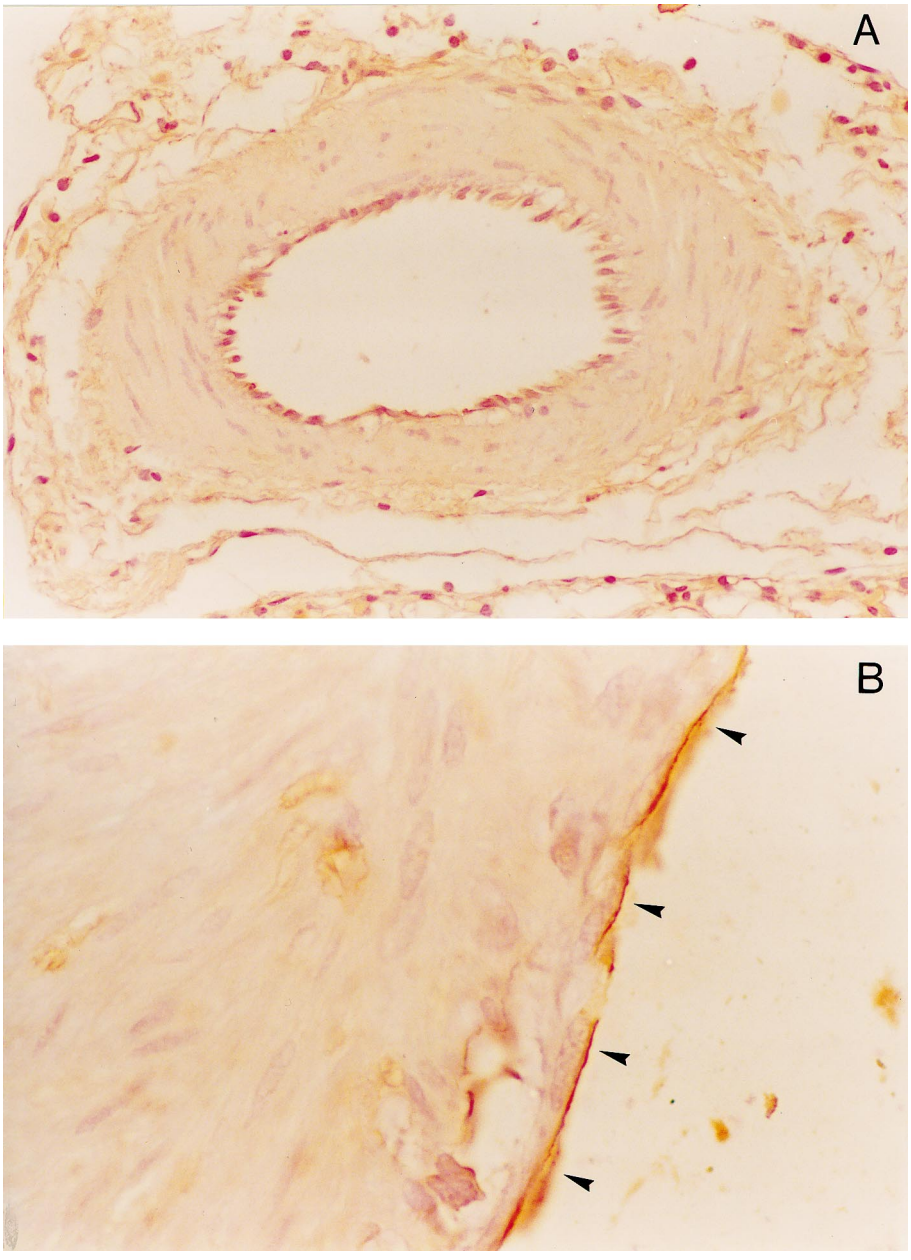

Fig. 1. Endoglin expression in the chicken lung vasculature. Lung tissue sections were stained with a pAb anti-endoglin using an indirect immunoperoxidase method. Endoglin is present in the vessels as evidenced by the brown staining of the peroxidase reaction. Samples were counterstained with hematoxylin. Panel A shows expression of endoglin at the lumen of a small artery (magnification ×417). Arrowheads in panel B indicate expression of endoglin at the endothelial cell monolayer of an artery wall (magnification ×1038).

the embryos. All treatments were assessed in a final volume of 200 μl obtained by mixing equal volumes of stimuli and 1.4% methylcellulose (Serva) in PBS. Assays involving antibodies and TGF-β were carried out with either 50 μg of purified immunoglobulins and/or 25 ng of TGF-β (R&D Systems, Abingdon, UK) per embryo. These amounts were selected as optimal from a dose dependence curve. After addition of stimuli, eggs were sealed with cellotape and incubated for an additional period of 24 h. Then, egg shells were excised, the CAM were

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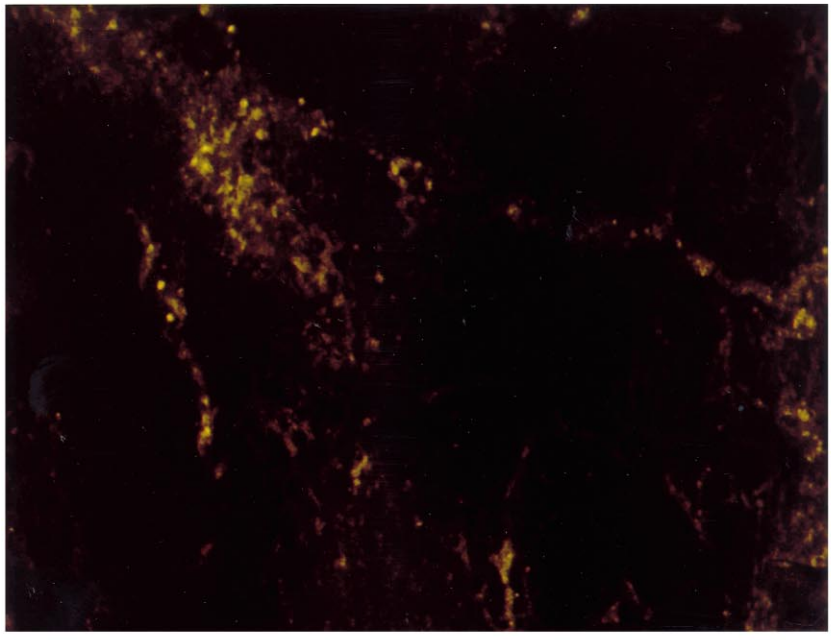
Fig. 2. Expression of endoglin in CAM. A,B: Immunostaining of CAM. Eggs with embryos were incubated for 2 days, shells were excised and CAM were removed, fixed, and permeabilized. Membranes were incubated with either the rabbit pAb to endoglin (A), or the anti-TK$^-$ pAb as a negative control (B), followed by a FITC-labeled anti-rabbit Ig. Samples were analyzed by confocal microscopy. Views of a total of 16 sequential layers at 3.6 μm intervals were collected, digitized and computerized to yield a three-dimensional composition. Fluorescein labeling reveals that endoglin is present in the branching capillary network (A). Magnification ×100. C: Biochemical characterization of chicken endoglin. CAM from embryos corresponding to 40 h were lysed and soluble extracts were electrophoresed on a 6% polyacrylamide gel under non-reducing conditions. After electrophoresis, samples were transferred to nitrocellulose and specific immunodetection was carried out by incubation with specific anti-endoglin or anti-TK$^-$ (negative control) purified immunoglobulins. The polypeptide corresponding to chick endoglin, indicated by an arrow, was visualized using a chemiluminescence assay.


A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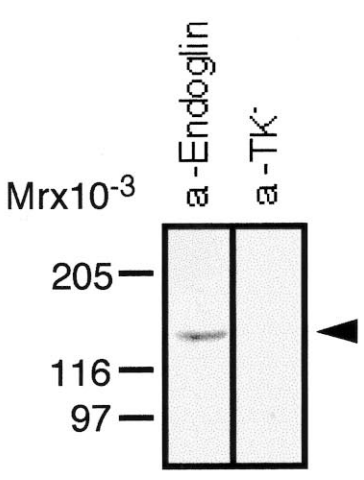
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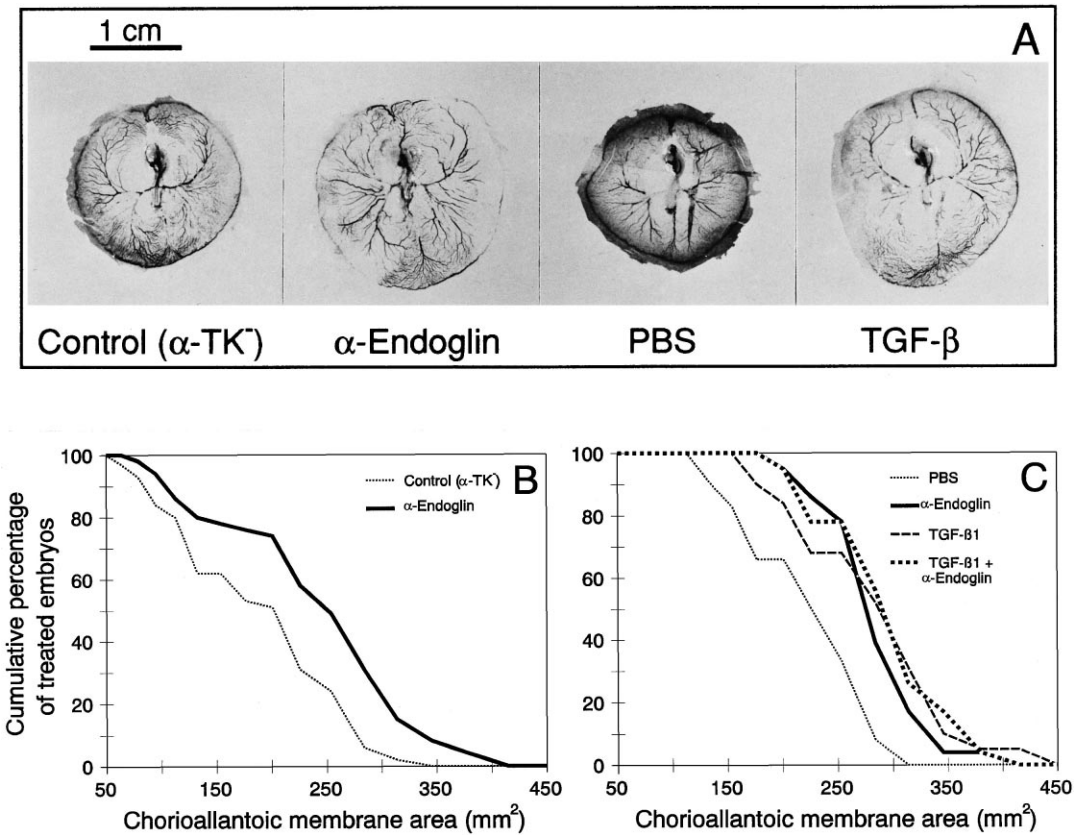

Fig. 3. Effects of anti-endoglin antibodies or TGF-β1 on CAM development. A: Analysis of CAM vascularization. CAM were excised, fixed, permeabilized and stained with DAB in the presence of hydrogen peroxide. Endogenous peroxidase activity catalyzes precipitation of oxidized substrate, rendering staining of blood vessels with a brown color. The ratio of blood vessels/area is not altered by treatments, but the total area of CAM is larger in embryos treated with anti-endoglin antibodies or TGF-β1 as compared with controls (PBS or anti-TK$^-$ antibodies). B,C: Quantitation of CAM growth. The number of cumulative embryos, expressed as percentage, showing an area scoring equal to or higher than the *x*-axis value was plotted. The area corresponding to the *y*-axis value of 50% represents the median of the distribution. Panel B represents data from embryos treated with anti-endoglin or anti-TK- antibodies, as indicated. Panel C represents data from embryos treated with PBS, TGF-β1, or anti-endoglin pAb, as indicated.

removed and fixed on 4% paraformaldehyde buffered in 0.1 M phosphate buffer, pH 7.1. After fixation, membranes were washed three times with PBS and permeabilized with 1% Triton X-100 in PBS for 4 h. Finally, membranes were stained with DAB, making use of the endogenous peroxidase, and photographed. Quantitative measurements of the CAM areas were performed with a ruled square mask under a binocular lens estimating horizontal diameters between CAM growth edges. Areas were calculated assuming a circular shape of the CAM. Results showing CAM growth were plotted as cumulative percentage of embryos with equal or larger areas compared to their corresponding values. A total of nine experiments were carried out and on average, 10 embryos were tested for each treatment, discarding non-viable embryos.

2.4. Western blot analysis

Isolated embryos corresponding to 2 days of development were excised and minced in PBS containing a protease inhibitor cocktail with a glass potter in microcentrifuge plastic tubes. Approximately 500 mg of wet crude tissue was used as starting material, and all manipulations were carried out at 4°C. After mincing, non-disrupted tissues were discarded by spinning down at $600\times g$ for 10 min. Disrupted materials were pelleted and washed twice by centrifugation at $20\,000\times g$ for 30 min and finally resuspended at 1.5 mg/ml of total protein concentration (recovery as wet weight was estimated as 10% of the starting material) for 20 h in solubilization buffer (2% Triton X-100, 10 mM Tris, 150 M NaCl, 1 mM EDTA pH 7.4, and a protease inhibitor cocktail). After solubilization, samples were centrifuged as described above and soluble fractions were used in Western blot analysis. For this purpose, equal amounts of total protein were subjected to SDS-PAGE on 6% polyacrylamide gels under non-reducing conditions. Proteins were electrophoretically transferred to nitrocellulose membranes (Millipore Corp., Bedford, MA, USA). Filters were blocked with PBS containing 5% milk powder for 1 h. Specific immunodetection was carried out by incubation overnight at room temperature with anti-endoglin or anti-TK$^-$ rabbit pAb, followed by peroxidase-conjugated goat anti-rabbit Ig. The presence of endoglin was revealed using a chemiluminescence assay (ECL detection kit, Amersham Ibérica, Madrid, Spain).

2.5. Receptor affinity labeling

For affinity labeling, 5×10^6 stable transfectants of rat myoblasts overexpressing human endoglin [7] were subjected to specific affinity labeling with 50 pM of [^{125}I]TGF-β1 (specific activity 1200–2000Ci/mmol; Amersham Ibérica) for 4 h in the absence or presence of 5–50 μg of purified anti-endoglin or anti-TK$^-$ immunoglobulins in HEPES buffer containing 0.1% bovine serum albumin. After affinity labeling, cells were washed and radiolabeled TGF-β1 was cross-linked to specific cellular receptors with 0.30 mM disuccinimidyl suberate (Pierce Chemical Co., Rockville, IL, USA) in HEPES buffer for 15 min at 4°C. After chemical cross-linking cell monolayers were washed three times with HEPES buffer. Then, cells were detached by scraping and soluble extracts were obtained by incubation with lysis buffer (1% Triton X-100, 10 mM Tris, 150 mM NaCl, 1 mM EDTA, and a protease inhibitor cocktail) for 1 h at 4°C. Soluble extracts were subjected to specific immunoprecipitation with mAb 44G4 (anti-endoglin) and SDS-PAGE. Detection of the ^{125}I-labeled endoglin was revealed with a PhosphorImager 410A and ImageQuant software (Molecular Dynamics).

3. Results

3.1. Expression of endoglin in the chicken vasculature

Mammalian endothelial cells express high levels of endoglin [8–10]. Thus, we analyzed whether endoglin was expressed in the chicken vasculature using a rabbit pAb to human endoglin [17]. First, a section of adult chicken lung was stained with the pAb anti-endoglin. As shown in Fig. 1A, the pAb specifically recognized the endothelium of a large pulmonary artery, being unreactive with its tunica media. At a higher magnification, the expression of endoglin was clearly located in the endothelial cell monolayer of the vessels (Fig. 1B). Next, anti-endoglin antibodies were used to stain the chicken CAM. As evidenced by the immunofluorescence assay of Fig. 2A, the capillary network was also recognized by the anti-endoglin antibodies. As expected, a control pAb (anti-TK$^-$) was unreactive with the CAM vessels (Fig. 2B). Furthermore, we analyzed the molecular weight of the chicken antigen cross-reactive with the pAb. Total extracts from CAM were analyzed by Western blot under non-reducing conditions (Fig. 2C). A specific band with an apparent molecular weight of 160 kDa corresponding to the dimeric form of endoglin was detected, in agreement with a recent report [19]. Taken together, the tissue distribution and biochemical analyses indicate that endoglin is expressed on the chicken vasculature.

3.2. Involvement of endoglin in the angiogenic process

Endoglin has been described as an endothelial marker of neovascularization in humans [20]. To determine whether endoglin has an active role in angiogenesis, we used the well established chicken CAM in vivo model [21]. Basically, eggs containing developing embryos are injected with the test reagents, incubated, and vascularization of the CAM is determined. We examined the effect of anti-endoglin antibodies on CAM from young embryos (approximately 40 h), a stage marked by active neovascularization. Fig. 3A shows representative CAM subjected to different treatments, the ratio of blood vessels/area not being altered by the treatments. Quantitative analysis of these experiments revealed that the total area of CAM was larger in embryos treated with anti-endoglin antibodies than that of control samples treated with anti-TK$^-$ antibodies (Fig. 3B). This increase was similar to that found in the presence of TGF-β1, included as a positive control (Fig. 3C). On the other hand, simultaneous addition of anti-endoglin antibodies and TGF-β1 did not result in an additive effect. These results suggest an important role of endoglin in the angiogenic process.

As TGF-β plays an important role in vascular development and remodeling [15], and endoglin is a component of the TGF-β receptor complex, we sought to assess whether the anti-endoglin pAb used was able to affect ligand binding to endoglin. TGF-β1 binding of cell transfectants overexpressing endoglin was analyzed by affinity labeling followed by cross-linking. Fig. 4 shows that a band of approximately 200 kDa, corresponding to the endoglin dimer, and the putative TGF-β receptor types I and II were specifically immunoprecipitated. Addition of the pAb to the affinity labeling assay prevented endoglin from binding ligand, whereas control IgG had no significant effect. This finding indicates that the pAb to endoglin is able to modulate ligand binding to endoglin.

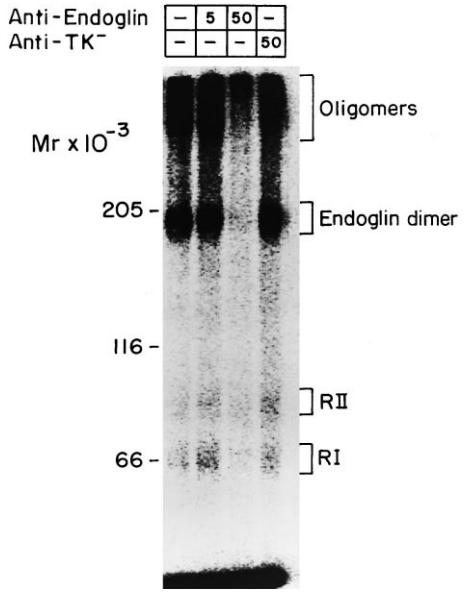

Fig. 4. Inhibition of TGF-β1 binding to endoglin in the presence of anti-endoglin antibodies. Myoblasts overexpressing endoglin were subjected to affinity labeling with [^{125}I]TGF-β1 in the presence of 5 or 50 μg purified anti-endoglin or anti-TK$^-$ immunoglobulins. Cells were washed to remove unbound ligand and chemical cross-linking with disuccinimidyl suberate was carried out. Cell extracts were subjected to specific immunoprecipitation with an anti-endoglin mAb. Samples were subjected to SDS-PAGE under non-reducing conditions. In the presence of 50 μg of purified anti-endoglin immunoglobulins the binding of [^{125}I]TGF-β1 to endoglin is blocked. Bands corresponding to oligomers, endoglin dimer, and putative receptors I (RI) and II (RII) are indicated.

4. Discussion

Endoglin has been cloned and sequenced in human [22,23], pig [8], and mouse [10,24], but the chicken endoglin sequence is not available yet. To the best of our knowledge, the only report about chicken endoglin describes its expression during heart development using a specific mAb [19]. Thus, during the formation of the primitive heart tube, endoglin is found at relatively high levels in both presumptive myocardium and endocardium; and when myocardium differentiates and development proceeds, endoglin expression is progressively reduced. However, little is known about endoglin expression in the endothelium of normal vessels. Here, we demonstrate the presence of chicken endoglin in the vasculature by immunohistochemical staining and Western blot analyses. The conserved expression from mammals to birds suggests an important function for endoglin in the vascular physiology. We demonstrate that antibodies to endoglin are able to modulate the angiogenic process in vivo, indicating an active role for endoglin in the neovascularization. This conclusion fully agrees with immunohistological data from three different types of pathologies. First, vascular lesions (telangiectasia and arteriovenous malformations) in endoglin haploinsufficient HHT1 patients are associated with a loss of the capillary network [11]; second, mice lacking endoglin die at gestational day 11.5, apparently from defective vascular development [16]; and third, augmented expression of endoglin has been detected in endothelia from neovascularized tissues such as tumors [20,25–28], Graves' disease and Hashimoto's thyroiditis [29], psoriasis [30], scleroderma [31], or ischemic stroke [20].

TGF-β has been reported to be angiogenic or anti-angiogenic in vivo, depending on the model system used [15]. In this sense, our data indicate that addition of TGF-β1 to the chick CAM has a potentiating angiogenic effect, in agreement with a previous report [32]. The molecular mechanism by which antibodies to endoglin modulate in vivo angiogenesis is not known. Since TGF-β1 shows an enhanced angiogenic effect similar to that of anti-endoglin, and the simultaneous addition of TGF-β1 and anti-endoglin does not result in an additive effect, it is tempting to speculate that both are using the same signaling pathway. We have shown that anti-endoglin antibodies are able to interfere with TGF-β1 binding, suggesting an epitope recognition at or near the endoglin ligand binding site. Thus, one possibility is that anti-endoglin antibodies are mimicking a ligand which leads to the release of endogenous TGF-β1 or to a signaling pathway common to that of TGF-β1. The facts that neovascularized tissues are associated with high expression levels of endoglin and, conversely, a deficient or null expression of endoglin is associated with a lack of vascular development and remodeling support an active role of endoglin in the angiogenic process. Whether the physiological ligand of endoglin in this process is TGF-β1, TGF-β3, activin, a yet unknown ligand [4–6], or a specific combination of them remains to be determined.

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