Expression of the homophilic adhesion molecule, Ep-CAM, in the mammalian germ line

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During normal embryonic development, mammalian germ cells use both cell migration and aggregation to form the primitive sex cords. Germ cells must be able to interact with their environment and each other to accomplish this; however, the molecular basis of early germ cell adhesion is not well characterized. Differential adhesion is also thought to occur in the adult seminiferous tubules, since germ cells move from the periphery to the lumen as they differentiate. In a screen for additional adhesion molecules expressed by the germ line, expression of the homophilic adhesion molecule, Ep-CAM, was identified in embryonic, neonatal and adult germ cells using immunocytochemistry and flow cytometry with an Ep-CAM-specific monoclonal antibody. At embryonic stages, germ cells were found to express Ep-CAM during migration at embryonic day 10.5 and early gonad assembly at embryonic day 12.5. Expression of Ep-CAM was also found on neonatal male and female germ cells. In the adult testis, Ep-CAM was detected only on spermatogonia, and was absent from more differentiated cells. Finally, embryonic stem cells were shown to express this receptor. It is proposed that Ep-CAM plays a role in the development of the germ line and the behaviour of totipotent cells.

Introduction

The germ line in mammals is established during the early stages of gastrulation. In mice, this corresponds to embryonic day 7.5. The precursors of the gametes are known as primordial germ cells (PGCs), and are morphologically similar in males and females. PGCs migrate from the hind-gut endoderm to the mesoderm of the urogenital ridges to be incorporated into the future gonads (for review, see Wylie, 1999). After this migration, germ cells of both sexes aggregate with each other and with epithelial somatic cells. The clusters formed are the progenitors of the ovarian follicles in females or the seminiferous tubules in males, and are sexually dimorphic by embryonic day 12.5 (for review, see Anderson *et al.*, 1998). In both adult males and females, germ cells are associated closely with a population of supporting somatic cells.

Cell-cell interactions are known to be required for germ cell survival in embryonic and adult mice (Nocka *et al.*, 1989). Mouse PGCs will not migrate on plastic or purified extracellular matrix proteins, but will migrate on fibroblast feeder layers (Donovan *et al.*, 1986). Although the cell-cell adhesion molecules necessary for germ cell development are not known, several of these types of receptor are known to be expressed in the germ line. Male germ cells have been shown to express E cadherin and N cadherin (Newton *et al.*,

1993; Wu *et al.*, 1993). Expression of E cadherin is detected on mouse oocyte surfaces after fertilization or parthenogenetic activation (Clayton *et al.*, 1995). mRNA encoding P cadherin has been detected in the testis of newborn rats (Lin and DePhilip, 1996), but is not required for fertility in mice (for review, see Hynes, 1996). N-CAM is not expressed in embryonic mouse gonads (Møller *et al.*, 1991), but it has been reported to mediate gonocyte-Sertoli cell adhesion in neonatal rat testis (Orth and Jester, 1995). Neural cell adhesion molecule (N-CAM) is not required for fertility in mice (Cremer *et al.*, 1994), so the role of this molecule in germ line development is not yet clear.

Recently, the pan-carcinoma marker known variously as EGP-40, KSA and ESA was shown to be a calcium-independent homophilic adhesion molecule, and was renamed Ep-CAM (Litvinov *et al.*, 1994). Ep-CAM is not related structurally to other cell-cell adhesion molecules, although it does contain a nidogen-like domain as well as thyroglobulin- and epidermal growth factor-like repeats (Strand *et al.*, 1989). Ep-CAM genes have been described in many vertebrate genomes (Linnenbach *et al.*, 1993). The human and mouse forms of Ep-CAM are 86% identical at the amino acid level (Bergsagel *et al.*, 1992). Ep-CAM expression was shown to be sufficient to induce clustering of L cells and segregation of transfectants in mixed-cell aggregates *in vitro* (Litvinov *et al.*, 1997). The role of Ep-CAM *in vivo* is not known, although experiments indicate that it plays a role in pancreatic islet morphoregulation and thymocyte function after stimulation (Nelson *et al.*, 1996; Cirulli *et al.*, 1998).

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Received 19 November 1998.

In the present study, the expression of Ep-CAM by embryonic, neonatal and adult germ cells was investigated.

Materials and Methods

Antibodies

Purified G8.8, previously shown to bind murine Ep-CAM specifically (Nelson *et al.*, 1996), was obtained from the Developmental Studies Hybridoma Bank, Iowa City, IA. Clone TG-1, a monoclonal mouse anti-SSEA-1 hybridoma, was a kind gift of P. Beverley (Division of Hematology, Department of Medicine, Cornell Medical School). TG-1 specifically labels germ cells in the early embryo (Donovan *et al.*, 1986). Biotinylated anti-Forssman antigen (Fo) was purchased from Pharmingen, San Diego, CA. Fluorescein isothiocyanate (FITC)-anti-mouse IgM, FITC-anti-rat IgG, indocarbocyanine (Cy3)-anti-rat IgG, and phycoerythrin (PE)-streptavidin were purchased from Jackson Immuno-research (West Grove, PA). The Forssman antigen is a specific marker for germ cells in the early gonad (Stinnakre *et al.*, 1981). Polyclonal anti-laminin was purchased from Sigma (St Louis, MO), and used at a dilution of 1:500.

Tissue culture

STO cells (immortalized murine embryonic fibroblasts) were maintained as reported by Cooke (1993). The embryonic stem (ES) cell line, RA7 (50% FVB, 50% 129/J background; JAX mice) was derived and maintained in our laboratory using standard methods (Hogan *et al.*, 1994). ES cells were plated on γ -irradiated clone D9 Ep-CAM⁺ STO cells (see below).

Sub-cloning and transfections

The plasmid 5952, containing the full length Ep-CAM cDNA sequence, was a kind gift of L. Bergsagel (Department of Pathology, University College Hospital Medical School, London). The Ep-CAM coding sequence was removed from the vector by EcoRI digestion and subsequent gel purification, and was sub-cloned into the expression vector pCDNA3.1(-) (Invitrogen, Carlsbad, CA), which had been digested with EcoRI and treated with phosphatase. The resulting construct, pCDNA-EpCAM⁺, was linearized with BglII, extracted in phenol-chloroform and precipitated in ethanol. Next, 50 μ g of this construct was resuspended in H₂O and transfected into STO fibroblasts by electroporation using a GenePulser II (Bio-Rad). The electroporated cells were grown in 200 μ g G418 ml⁻¹ (GIBCO, Gaithersburg, MD) for 2 weeks, and resistant colonies were picked, expanded and analysed selectively for stably transfected STO cells. Several G418-resistant STO cell colonies were found to express Ep-CAM, including clone D9, which was used in this study.

Histology

Mouse embryos were obtained from CD1 mice (Charles River, Wilmington, MA) and the morning of vaginal plug

considered as day 0.5 of pregnancy. Sexing of embryos was performed by PCR (Hogan *et al.*, B. 1994). For frozen sections, tissue was embedded in OCT (Tissue-Tek, Torrance, CA), snap-frozen in isopentane, submerged in liquid N₂, and cut into 10 μ m sections. After brief fixation in acetone, slides were washed in PBS and blocked with PBS + 10% goat serum (PSA). G8.8 was diluted 1:1000 in PSA, and TG-1 was diluted 1:5 in PSA. After thorough washing in PBS and dilution in PSA (FIC 1:1000; Cy3 1:500), secondary antibodies were added. After several further washes in PBS, slides were mounted in 90% glycerol, 10% H₂O with 100 μ g 1,4-diazabicyclo-o[2.2.2]octane (DABCO) ml⁻¹ (Sigma). Sections were analysed with a Bio-Rad 1024 laser confocal microscope.

Flow cytometry

Cells were washed $\times 1$ with PBS, and subsequently incubated in PBS + 10 mmol EDTA l⁻¹ at 37°C for 15 min to assay parental and transfected STO fibroblasts for expression of Ep-CAM. Cells were then triturated into a single cell suspension, pelleted by centrifugation at 200 g for 5 min in a clinical centrifuge, and resuspended in 0.5 ml PBS + 1% fetal calf serum (FCS; Sigma). The gonads of male and female embryonic day 12.5 embryos were dissected free of adjacent mesonephric tissue, disaggregated, pelleted and resuspended as above to analyse embryonic day 12.5 germ cells. Cells were incubated at 4°C with primary antibodies for 30 min, pelleted as above and washed in 2 ml PBS + 1% FCS, incubated with secondary antibodies for 30 min, pelleted, and washed in 2 ml PBS + 1% FCS for immunolabelling. After the final wash, cells were pelleted as above and resuspended in PBS + 1% FCS at a concentration of 2×10^6 cells ml⁻¹. All antibodies used for flow cytometry were used at a dilution of 1:100 in PBS + 1% FCS. Flow cytometry was performed with a FACS Vantage (Becton Dickinson, San Diego, CA) and analysed with Cellquest (Becton Dickinson).

Results

The aim of this study was to determine whether cells of the mammalian germ line express the homophilic adhesion molecule, Ep-CAM. For this purpose, G8.8, a monoclonal antibody shown to detect Ep-CAM on thymocytes and thymic epithelial cells (Nelson *et al.*, 1996), was used. Studies with this antibody showed that STO fibroblasts were Ep-CAM⁻ by flow cytometry and immunofluorescent staining (Fig. 1; data not shown). However, upon stable transfection with a mouse Ep-CAM construct, several STO cell clones were found to be Ep-CAM⁺ by flow cytometry and immunofluorescent staining (Fig. 1; data not shown). These experiments confirmed the use of G8.8 as a suitable marker for identification of Ep-CAM⁺ cells by flow cytometry and immunofluorescent staining in the system used.

Frozen sections of mouse embryos at stages of PGC migration (embryonic day 10.5) and germ cell coalescence (embryonic day 12.5) were double-stained with Ep-CAM-

Fig. 1. Expression of the homophilic adhesion molecule, Ep-CAM, by transfected STO cell line D9. Flow cytometry scan of untransfected (grey curve) and clone D9 STO cells stably transfected with a mouse Ep-CAM expression construct (dark curve). G8.8 is a monoclonal antibody shown to detect Ep-CAM.

and germ cell-specific monoclonal antibodies to determine whether immunoreactive Ep-CAM is expressed in the early germ line. Expression of Ep-CAM was detected on the membranes of migratory PGCs (Fig. 2). In particular, anti-Ep-CAM heavily labelled dendrite-like processes of migratory PGCs (Fig. 2a,c). Ep-CAM⁺ PGC processes often extended > 15 μ m beyond the margins of the cell bodies (Fig. 2c). Ep-CAM expression was detected in the sex cords of embryonic day 12.5 male and female embryos (Fig. 2e-h), although expression was less prominent than at embryonic day 10.5. At embryonic day 12.5, mesonephric tubules were heavily labelled with the anti-Ep-CAM antibody (Fig. 2e,g).

Two-colour flow cytometry of embryonic day 12.5 male and female gonads was performed with germ cell- and Ep-CAM-specific monoclonal antibodies to confirm that membrane-bound Ep-CAM was on the surfaces of germ cells and not on neighbouring somatic cells. PGCs expressed more immunoreactive Ep-CAM than the majority of somatic cells of the early gonad (Fig. 2).

Immunoreactive Ep-CAM on germ cells was found in the gonads of male embryos and fetuses. At birth, germ cell expression of Ep-CAM was detected in the testis cords (Fig. 3a). In the adult testis, Ep-CAM was restricted to small round cells adjacent to the basement membrane of the seminiferous tubules, the spermatogonia (Fig. 3c,e). Few Ep-CAM⁺ cells were detected that were not immediately adjacent to the basement membrane, suggesting down-regulation of Ep-CAM expression upon commitment to spermatogenic differentiation. In neonatal ovaries, immature oocytes expressed moderate amounts of Ep-CAM (Fig. 4a). However, by adulthood, expression of the molecule was not detectable in the ovary. Several phases of follicle development, with Ep-CAM below detectable amounts at all stages are shown (Fig. 4f).

In addition to germ cells, ES cells were examined for the expression of Ep-CAM by flow cytometry and were found to be Ep-CAM⁺ (Fig. 5a). When grown on untransfected (Ep-CAM⁻) fibroblast feeder layers, ES cells segregate into distinct colonies by preferentially adhering to each other.

Since Ep-CAM is reported to be a homophilic adhesion molecule (Litvinov *et al.*, 1994), it was investigated whether ectopic expression of Ep-CAM on feeder cells would disrupt ES cell morphology by increasing the adhesion between ES cells and the fibroblast monolayer. However, ES cell colony morphology was not affected by growth on Ep-CAM⁺ fibroblasts (Fig. 5b).

Discussion

Immunohistochemical experiments presented here document, for the first time, the expression of the homophilic adhesion molecule Ep-CAM in the embryonic and postnatal germ line of both sexes in mice. In the embryo, Ep-CAM is expressed by migratory PGCs and post-migratory germ cells. Anti-Ep-CAM antibody was found to label a fine network of processes on migratory PGCs to a degree previously not seen with other antibodies. At birth, Ep-CAM is expressed by germ cells in both the testis and ovary. At sexual maturity, expression of Ep-CAM is confined to spermatogonial stem cells adjacent to the basement membrane of the seminiferous tubules in the testis, whereas no Ep-CAM was detected in the ovary. Undifferentiated ES cells also express large amounts of immunoreactive Ep-CAM.

PGCs interact with each during migration by means of long, filopodial processes (Gomperts *et al.*, 1994). The molecular basis of this interaction is not understood, but is presumably mediated by preferential PGC-PGC adhesion. Since PGC processes are Ep-CAM⁺, this molecule may play a role in this networking phenomena. Although it is well known that PGCs extend processes *in vivo* and *in vitro*, immunofluorescent staining with anti-Ep-CAM antibody revealed that these processes are much more extensive and complex than previously recognized. Although the function(s) of the filopodial networks made by PGCs are not yet known, it is possible that they play a role in migration, homing or aggregation.

After germ cells finish migration, they aggregate into tight clusters, an event hypothesized to be mediated by cell-cell adhesion molecule(s) on PGCs and somatic cells. In the present study, germ cell expression of Ep-CAM appeared to decrease from embryonic day 10.5 to day 12.5, at the same time that germ cells aggregate. This finding may seem paradoxical, given that Ep-CAM is a cell-cell adhesion molecule (Litvinov *et al.*, 1994). However, Ep-CAM expression has been shown to interfere with cadherin-based adhesion (Litvinov *et al.*, 1997). Thus, the downregulation of Ep-CAM expression may facilitate a cadherin-based compaction of germ cell clusters. In addition, embryonic and adult germ cells have intercellular channels that may facilitate cell-cell adhesion (De Felici *et al.*, 1989; Simon *et al.*, 1997; Pepling and Spradling, 1998).

At embryonic day 12.5, the mesonephric tubules of both sexes expressed large amounts of Ep-CAM; this was expected since Ep-CAM is expressed by most epithelia (Litvinov *et al.*, 1994). The mesonephros is adjacent to the early gonad. Despite the fact that the mesonephros frequently contains a large number of ectopic PGCs and that

Fig. 2. Expression of the homophilic adhesion molecule, Ep-CAM, by embryonic day 10.5 and day 12.5 mouse primordial germ cells. (a-d) Sections of embryonic day 10.5 urogenital ridge double-stained for Ep-CAM (a,c: mAb G8.8) and germ cells (b,d: mAb TG-1). Sections of embryonic day 12.5 female (e,f) and male (g,h) gonads stained for Ep-CAM (e,g: mAb G8.8) and germ cells (f,h: mAb TG-1). Asterisks indicate the location of the gonad; arrows indicate positive staining of mesonephric and metanephric tubules. (i-k) Flow cytometric analysis staining for germ cells (anti-Fo, y -axis) and Ep-CAM (G8.8, x -axis) of embryonic day 12.5 forelimb (i), female gonad (j) and male gonad (k). FITC, fluorescein isothiocyanate. Arrow denotes primordial germ cells. Scale bars represent 10 μ m (a-d) and 100 μ m (e-h)

the mesonephric tubules are Ep-CAM⁺, germ cells are rarely found in association with the tubular epithelium. Therefore, Ep-CAM expression does not appear to be sufficient to attract or anchor PGCs. The role of Ep-CAM in the networking and aggregation of the early germ line will require genetic analysis.

The restriction of Ep-CAM expression to spermatogonia is reminiscent of E-cadherin expression in the testes of 8-day-old mice (Wu *et al.*, 1993). The significance of this expression pattern is unclear, but it indicates that spermatogonial differentiation is accompanied by the immediate loss of Ep-CAM expression. Ep-CAM⁺ E cadherin-transfected murine L (LEC) cells segregate to the outside of mixed cell clusters *in vitro* (Litvinov *et al.*, 1997). Therefore, it is possible that Ep-

CAM plays an analogous role in compartmentalizing the cells of the seminiferous tubules, with Ep-CAM⁺ spermatogonia segregating to the outside of the tubules. Alternatively, Ep-CAM expression may help to sustain the undifferentiated state of germ line stem cells by an adhesive or signalling mechanism. Regardless of the biological function of Ep-CAM in the adult testis, it should be considered a potentially useful immunocytochemical marker.

Embryonic stem cells are totipotent cells derived from cells of the inner cell mass. Embryonic day 11.5 PGCs are capable of forming ES-like cells (EG cells) in culture (Resnick *et al.*, 1992). ES cells share many of the properties of the early germ line, including expression of alkaline phosphatase, the tyrosine kinase, Kit, and the transcription factor, Oct-4 (for

Fig. 3. Expression of the homophilic adhesion molecule, Ep-CAM, in neonatal and adult mouse testis. Neonatal testis stained for Ep-CAM (mAb G8.8; a) or with omission of primary antibody (b). Arrows indicate staining of testis cord. Adult testis double stained for Ep-CAM (G8.8; c,e) and laminin as a basement membrane marker (anti-laminin; d,f). Scale bars represent 10 µm (a,b,e,f) and 50 µm (c,d).

review, see Yoem *et al.*, 1996; Gardner and Brook, 1997). Therefore, the observation that ES cells express Ep-CAM is not surprising, given the similarity of ES cells to the early germ line. In the present study, no differences in ES cell morphology were observed between plates of Ep-CAM⁺ and wild-type (Ep-CAM⁻) fibroblasts. This is probably because ES cells express additional adhesion molecules that favour self-adhesion (Tian *et al.*, 1997). It is possible that expression of Ep-CAM by the transfected fibroblast cell line (clone D9) used was not sufficient to induce changes in ES cell morphology. Similarly, differences in PGC behaviour have not been observed between plates of Ep-CAM⁺ and Ep-CAM⁻ fibroblasts (R. Anderson and C. Wylie, unpublished).

In conclusion, immunoreactive Ep-CAM is expressed in the mouse germ line throughout embryonic and fetal stages, indicating that it plays a morphogenetic or signalling role in the development of the gametes and totipotent cells. In addition, Ep-CAM is detectable on spermatogonia in the

Fig. 4. Expression of the homophilic adhesion molecule, Ep-CAM, in neonatal and adult mouse ovary. Neonatal ovary stained for Ep-CAM (mAb G8.8; a) or with omission of primary antibody (b). Adult ovary double stained for Ep-CAM (G8.8; c,e,g) and laminin as a basement membrane marker (anti-laminin; d,f,h). Arrow indicates positive staining of oviduct epithelium. Asterisk indicates location of developing oocytes (f) and Graafian follicle (h). Scale bars represent 10 µm (a,b), 200 µm (c,d) and 50 µm (e-h).

adult testis. Finally, Ep-CAM is expressed on the surface of ES cells. Given the widespread conservation of the Ep-CAM gene in vertebrates (Linnenbach *et al.*, 1993), it is an attractive candidate for genetic analysis by targeted deletion in mice. Experiments of this kind should provide insight into the function of Ep-CAM in the germ line and totipotent cells.

Fig. 5. Expression of the homophilic adhesion molecule, Ep-CAM, by mouse embryonic stem (ES) cells. (a) Flow cytometric analysis of ES cells stained for Ep-CAM (G8.8, dark curve) or with secondary antibody alone (grey curve). (b) ES cell colonies (arrows) have normal morphology when plated on Ep-CAM⁺ fibroblasts. Scale bar represents 30 µm.

The authors would like to thank the National Life and Health Insurance Medical Research Fund, the National Institute of Health (HD33440-01), the Harrison Fund and the Institute of Human Genetics for financial support of this work. The monoclonal antibody G8.8, developed by A. Farr, was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD, and maintained by the Department of Biological Sciences, University of Iowa, Iowa City, IA 52242. The authors would also like to thank P. Beverley for the gift of the TG-1 hybridoma, and Electra Coucouvanis for the gift of STO cells.

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