Intermediate filament protein expression in early developmental stages of the mouse

A confocal scanning laser microscopy study of in vitro fertilized and in vitro cultured pre-implanation mouse embryos

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Abstract. Expression patterns of intermediate filament proteins have been studied during early mouse embryo development. For this purpose, pre-implantation embryos at different stages of development after in vitro fertilization were studied using antibodies to cytokeratins, vimentin and lamins, using the indirect immunofluorescence assay. The levels of expression were quantitatively and localization of the protein constituents was assessed by means of confocal scanning laser microscopy. Our studies showed that, although the embryos grew in culture, vimentin could not be detected in a filamentous organization. Immunofluorescence for cytokeratins was only positive from the 8-cell stage onwards. In the morula stage an increased level of cytokeratin expression was observed with a transitional staining pattern, combining a filamentous and diffuse occurrence. In the blastocyst stages profound cytokeratin filaments were seen in trophectoderm but not in the inner cell mass. When the cytokeratin subtypes were analysed separately, it became apparent that expression levels of cytokeratins 8 and 18 increased gradually up to a filamentous pattern in the blastocyst stage. Cytokeratins 7 and 19, although elevated in the latter stage and exhibiting a filamentous distribution, were not found as prominently as cytokeratins 8 and 18. A-type as well as B-type lamins could be detected in all developmental stages examined, as a faintly reactive nuclear lamina. In blastocysts both lamin types were detected in trophectoderm as well as in inner cell mass.

Introduction

The family of intermediate filament proteins (IFP) constitute cytoplasmic filaments as well as the nuclear lamina (for review see Skalli and Goldman 1991). Different types of cytoskeletal IFP have been identified, with a subfamily of cytokeratins specific for epithelial cells and vimentin, normally occurring in mesenchymal cell types but also often in cultured epithelial cells. Earlier reports indicated that the first type of IFP expressed during early mouse development comprises cytokeratins (Jackson et al. 1980; Paulin et al. 1980; Lehtonen et al. 1983). Findings differ with respect to the time of onset of cytokeratin expression: Lehtonen (1985) found non-filamentous cytokeratin-like proteins in the mouse oocyte, whereas Oshima et al. (1983) and Chisholm and Houlston (1987) detected mouse cytokeratin proteins (Endo A and Endo B) from the four-cell stage embryo on. Jackson et al. (1980) and Emerson (1988) did not detect cytokeratins until the late morula stage and compacted eight-cell stage, respectively.

The presence of distinct cytokeratin filaments in trophectoderm cells of the blastocyst has been described by several authors (Bruèlet et al. 1980; Paulin et al. 1980; Jackson et al. 1980; Lehtonen et al. 1983; Oshima et al. 1983; Lehtonen and Virtanen 1985; Emerson 1988). This filamentous organization of cytokeratins was evident in blastocyst outgrowths in which the trophectodermal cells flattened on a substratum (Lehtonen 1985). The results published to date on the presence of cytokeratins in the inner cell mass (ICM) are contradictory. Jackson et al. (1980), Paulin et al. (1980), Bruèlet et al. (1980) and Oshima et al. (1983) interpreted a weak fluorescence signal in the ICM as being non-specific. Lehtonen et al. (1983), however, detected a relatively strong (though diffuse) fluorescence signal in the cells belonging to the ICM when using a polyclonal antibody to cytokeratin in the immunofluorescence assay.

Contradictory data also have been published for the nuclear lamins with respect to their expression in mouse embryos. For example, an early report from Schatten et al. (1985) demonstrated A- and B-type lamins in nuclei of cleavage stage embryos, while strikingly the nuclei of morulae and blastocysts were negative for A-type lamins and positive only for a B-type lamin antibody. A similar result was obtained by Stewart and Burke (1987). Their studies showed that B-type lamins were present
in all pre-implantation stages whereas a low expression of A-type lamins was detected only in fertilized oocytes. Expression of A-type lamins gradually diminished during the first division cycles until no longer being detectable in blastocysts. Houliston et al. (1988), on the contrary, detected expression of both A- and B-type lamins throughout all stages of pre-implantation development. Röber et al. (1989) first detected expression of the A-type lamin in mouse embryos at 12 days of gestation in muscle cells of the trunk, head and appendages. B-type lamins were found in all developmental stages examined (10, 11, 13, 15 and 18 days of gestation).

Vimentin synthesis is initiated in some mesoderm cells of the primitive streak embryo (Jackson et al. 1981; Franke et al. 1982). These findings supported the results of Paulin et al. (1980) and were confirmed by Lehtonen et al. (1983), who showed that blastocystcs and cleavage stage embryos (including blastocysts) were negative for vimentin. The aim of this study was to survey the pattern of intermediate filament protein expression in mouse embryos resulting from in vitro fertilization during the different stages of their pre-implantation development.

Materials and methods

Embryos

Mouse oocytes were obtained from 7- to 11-week-old (C57Bl/6× DBA/2)F1 hybrid females (Charles River Wiga, Sulzfeld, Germany). Superoximation was induced by intraperitoneal injection of 10 IU pregnant mare serum gonadotropins (PMSG; Sigma, St. Louis, Mo., USA) and, 48 h later, 10 IU human chorionic gonadotrophin (HCG, Sigma). The females were sacrificed by cervical dislocation, 16 h after the HCG injection. Oviducts were dissected and placed in HEPES-buffered modified Tyrode’s medium (T6) according to Quinn et al. (1985). Oocytes were then released from the swollen ampulla of the oviducts, washed once with T6 and transferred to T6 medium containing 30 mg Fraction V bovine serum albumin/ml (BSA, Sigma).

Caudae epididymi from 12- to 18-week-old CD-1 mice (Charles River Wiga) were placed in T6 medium supplemented with 30 mg BSA/ml. The tissue was incised to allow the spermatozoa to disperse into the medium. After 30 min, the epididymal tissue was removed and the spermatozoa were left to capacitate for another 90 min at 37°C. The oocytes were inseminated with a final concentration of approximately 2 million motile spermatozoa/ml. At 4 h after insemination, the oocytes were rinsed and kept at 37°C in T6 medium containing 5 mg BSA/ml for further development (Dumoulin et al. 1992). In general, 72% of fertilized oocytes developed into two-cell embryos of which 69% developed into 4-cell embryos, 64% into morulae and 61% into blastocysts. For qualitative measurements ten or more embryos per developmental stage were used per antibody. Of those, at least three were used for intensity measurements by confocal scanning laser microscopy (CSLM).

Antibodies

The following antibodies were used in this study. RGE53: A mouse monoclonal antibody (IgG1 subclass), monospecific for keratin 18 (Ramaekers et al. 1983b). RCK105: A broadly cross-reacting mouse keratin antibody of the IgG1 subclass, which recognizes keratins 5 and 8 and as a result stains virtually all epithelial tissues (Ramaekers et al. 1987). RCK105: A mouse monoclonal antibody (IgG1 subclass), specific for cytokeratin 7, which has first been described by Ramaekers et al. (1987). LP2K: A mouse monoclonal antibody (IgG2b subclass), which stains most simple epithelia and basal cells of non-keratinizing epithelia; this antibody recognizes only keratin 19 in immunoblotting assays and was a generous gift of Dr. E.B. Lane, Dundee, UK (Lane et al. 1985). 41CC4: A mouse monoclonal antibody (IgM subclass), which has been described by Burke et al. (1983) to react with lamins A, B and C and was kindly provided by Dr. G. Warren, Heidelberg, Germany; in our hands it appeared to stain both A-type and B-type lamins in the immunofluorescence assays. R27: A mouse monoclonal antibody of the IgM subclass, reactive with A-type lamins; the antibody was used at a dilution of 1:500 and was a generous gift from Dr. G. Krohn, Heidelberg (unpublished). LN43: A mouse monoclonal antibody to lamin B (IgG1 subclass), kindly provided by Dr. E.B. Lane, Dundee. X223: A mouse monoclonal antibody to lamin B2 (IgG1 subclass), which was a generous gift from Dr. G. Krohn, Heidelberg (Höger et al. 1990). pVIM: An affinity-purified polyclonal antiserum to bovine lens vimentin; purification and characterization of this polyclonal rabbit antisera are described in detail elsewhere (Ramaekers et al. 1983a).

In order to rule out misinterpretations as a result of unspecific or background staining, controls were processed as described below and included (1) conjugated secondary antibody used without first antibodies (PBS was used instead) and (2) the non-relevant mouse monoclonal antibody RKS6 with fluorescein-conjugated secondary antibody. The RKS6 antibody is directed against cytokeratin 10 (specific for keratinizing epithelial cells; Ramaekers et al. 1983b) and is not expressed in the pre-implantation embryos used in this study.

Indirect immunofluorescence assays

Prior to fixation, embryos were incubated in serum-free medium for 1 h and washed several times in phosphate-buffered saline (PBS; 137 mM NaCl, 13 mM Na2HPO4, 2H2O, 3 mM KH2PO4 in demineralized water, pH 7.4). Fixation was done in methanol (−20°C) for 10 min followed by 10 min fixation in a 1:1 mixture of methanol/acetic acid. Embryos were then washed again in PBS, preincubated for 30 min in PBS containing 1% BSA (Sigma) and further incubated for 1 h with the primary antibodies, diluted in PBS containing 1% BSA. Before the secondary antibodies were applied, embryos were thoroughly washed for 45 min in several changes of PBS. Secondary antibodies were fluorescein-conjugated swine immunoglobulins directed against mouse Ig (Dako, Glostrup, Denmark) or, in the case of pVIM, fluorescein-conjugated swine immunoglobulins to rabbit Ig (Dako), diluted 1:100 in PBS containing 1% BSA.

After a final washing step in PBS the embryos were mounted in 90% glycerol, containing 2.3% 1,4-diazobicyclo(2.2.2)octane (DABCO, Sigma) to reduce photobleaching and 1.25 mg/ml 4',6-diamino-2-phenyl indole (DAPI, Sigma) or 0.5 μg/ml propidium iodide (PI, Sigma) to counterstain the nuclei. To retain the three-dimensional organization of the embryo, the supporting slide and the coverslide were separated by a ring of glue or vaseline, surrounding the embryo. The whole procedure was carried out at room temperature.

Microscopic analysis

Immunostained embryos were observed with a Biorad MRC600 confocal scanning laser microscope, equipped with a cryant argon mixed gas laser (Ion Laser Technology, Salt Lake City, USA) with two separate wavelengths for the excitation of fluorescein isothiocyanate (FITC, 488 nm) and Texas Red (TR, 568 nm) and a Zeiss Axioptot microscope. To quantitate fluorescence intensities, fluo-
resin-labelled embryos were excited with a single wavelength light beam of 488 nm, using a neutral density filter no. 1.3, showing 3% transmission and a fixed pinhole position. Intensities were scanned through the whole embryo (Z-series), accumulated via the linear projection procedure (Projection) and statistically analysed with the Area command. The expression levels are presented as the mean fluorescence intensity of the whole embryo, not corrected for the total surface area. In order to gain information on the specific staining patterns of the various antibodies used, image processing was performed to upscale the signal and obtain optimal contrast. Photographs were taken from the monitor with a Nikon camera using Kodak Tri-X-Pan 400 ASA film.

Results

Embryo culture

After approximately 24 h in culture medium, the fertilized oocytes had developed into 2-cell embryos (Fig. 1A). On day 2 (48 h of culture) 4-cell embryos were obtained (Fig. 1B, C). Figure 1D shows an embryo that has not yet completed the third division cycle. Morulae (8–16 cells; Fig. 1E) and blastocysts (Fig. 1F, G) could be harvested on day 3 and day 5, respectively. Embryos were staged according to their morphological appearance. In order to obtain plated blastocysts with outgrowing trophoectoderm, non-hatched day 4 blastocysts were transferred to gelatin-coated culture dishes with T6 medium, supplemented with 10% human serum.

On day 5, blastocysts had hatched and adherence to the bottom of the culture dish was followed by implantation in the gelatin layer during days 6 and 7, giving rise to trophoectoderm cells spreading on the substrate, leaving the ICM on top as an aggregate of cells (Fig. 1H).

Technical aspects

Since the fixation and immunocytochemical staining procedures involve several steps that may influence the morphology of the larger embryos in particular, their size and shape were estimated by means of CSLM. In general, no significant deformation of the embryonic structures was seen at the different stages of development. Blastocysts, for instance, had a diameter in the x,y-direction between 40 and 70 μm while the diameter in the z-direction varied between 30 and 60 μm.

Fig. 1. Different stages of pre-implantation mouse embryos after in vitro fertilization and growth in culture: a 2-cell stage; b, c 4-cell stage; d 6-cell stage; e morula; f early blastocyst; g hatched blastocyst; h hatched blastocyst with outgrowing trophoectoderm. Bar indicates 80 μm (a–f), 60 μm (g) and 100 μm (h)
Size and shape of embryos, which were fixed and incubated with the different primary and secondary antibodies, were similar to those that had not been subjected to this protocol. Particular care was taken in case of adhering blastocysts, since we anticipated that the exposed ICM could be lost during the staining procedure.

Expression of cytokeratins during pre-implantation development

The staining patterns of the different stages of pre-implantation embryos with the different anti-cytokeratin antibodies are shown in Fig. 2. Their staining intensities, determined as described in the Materials and methods, are schematically represented in Fig. 3. From the 2-cell stage up to the 8-cell stage, antibodies against cytokeratins 7, 8, 18 and 19 showed a diffuse and weak fluores-
cent signal in the indirect immunofluorescence assays (Figs. 2a–d). Removal of the zona pellucida before incubation with the primary antibody did not result in higher staining intensities. In morulae slightly increased levels of expression were seen for all the cytokeratin types mentioned above, while the staining pattern changed from completely diffuse to a pattern in which faintly fluorescent filament bundles appeared (Fig. 2e, f). In the blastocyst stage antibodies against cytokeratins 7, 8, 18 and 19 revealed a profound filamentous network in the trophectoderm cells, but no staining of the cells comprising the ICM was seen (Figs. 2g, h and 4). At this stage differences in expression levels became apparent, as detected by the individual anti-cytokeratin antibodies (Fig. 3). The expression levels of cytokeratin 7 and 19 in blastocysts remained similar to those in morulae, but the expression levels of cytokeratins 8 and 18 in blastocysts increased three- to fourfold, compared to the morula stage. In plated blastocysts all antibodies revealed distinct cytokeratin filaments in the outgrowing trophoderm cells (Fig. 5a).

Expression of vimentin during pre-implantation development

Although we expected vimentin to be expressed in the in vitro cultured embryos, no significant staining pattern for filamentous vimentin was observed in any of the developmental stages studied. Also the staining intensities as measured by CSLM remained low (data not shown).

Expression of lamins during pre-implantation development

A-type as well as B-type lamins were detected in 2-cell stage embryos and at all subsequent stages of pre-implantation development that were examined. The fluorescence intensities as estimated by CSLM seemed to be more or less constant during embryonic development from the 2-cell stage up to the blastocyst stage (Fig. 3). Even the 2-cell stage embryo showed, adjacent to a diffuse cytoplasmic staining reaction, a specific staining pattern at the nuclear periphery following the indirect immunofluorescence assay with antibodies to A-type and B-type lamins (Fig. 6a, b), despite the
low fluorescence levels. Also at the 4-cell stage (Fig. 6c, d), the morula (Fig. 6e, f) and the blastocyst stage (Fig. 6g, h) antibodies to A-type and B-type lamins gave a positive staining of the nuclear periphery. The diffuse cytoplasmic staining reaction gradually diminished with increasing cell number. In blastocysts no difference was seen with respect to staining intensity or localization when ICM and trophoderm were compared. In the hatched blastocyst (Fig. 5b), the trophoderm cells were strongly positive for both A- and B-type lamins.

Discussion

This study reports an inventory of IFP expression in mouse embryos resulting from in vitro fertilization. To date, only naturally developed stages resulting from in vivo fertilized zygotes have been used for such studies. A delay was noted in the development of in vitro cultured embryos compared to the in vivo situation. For this reason morphological criteria, rather than the gestation period, were applied when determining the developmental stages. In addition, we used a panel of well-defined, chain-specific cytokeratin monoclonal antibodies, in combination with quantitative analysis of the immunocytochemically detected expression levels. When interpreting the data described one should keep in mind the fact that the monoclonal cytokeratin antibodies used in this study have mainly been characterized in human tis-
sues and shown to be monospecific for a subtype of the human cytokeratins. Therefore, in mouse they are supposed to recognize the murine equivalent of this human cytokeratin. As mentioned before, discrepancies exist in the literature with respect to the expression of IFP in mouse embryonic development (see the introduction and references cited therein). In this study the localization of cytokeratins, lamins and vimentin was assessed and expression levels of the different IFPs were quantitated using confocal scanning laser microscopy (CSLM).

During the experiments three advantages of CSLM became apparent. Firstly, in the earlier stages CSLM allowed a quantitative analysis of diffuse staining patterns. Secondly, optical sectioning particularly of the blastocyst stage provided the possibility to obtain information on the localization of the various compartments of the embryo, i.e. the ICM and the trophoderm, and thus allowed a better interpretation of IFP expression in these distinct embryonic cell types. Thirdly, optical sectioning of the embryos also allowed a better evaluation of the specific staining patterns at the cellular level, particularly in cases where expression of certain constituents was low. A minor disadvantage of CSLM might be the risk of photobleaching by laser illumination during repeated scans of an embryo in different optical planes. Therefore, the intensity of the laser light was kept low to minimise photobleaching. Bleaching was also prevented by adding DABCO anti-bleach reagent to the mounting medium. Performing an indirect immunofluorescence assay with Fast Red, which is known to show minimal quenching upon illumination (Speel et al. 1992), could be used in future studies to overcome this problem.

In this study we have used an extended panel of IFP antibodies, including chain-specific monoclonal antibodies directed against the cytokeratins that can reasonably be expected to occur in early mouse embryonic cells. The earlier studies with antibodies Endo A and Endo B (Oshima et al. 1983), shown to react with IFP corresponding to the human cytokeratins 8 and 18, respectively, and also studies with antibody TROMA-1 (Kemler et al. 1981), reactive with cytokeratin 8, demonstrated the presence of these IFP from the 4- to 8-cell stage onwards. In these cases the cytokeratins were evidently present as typical filaments in the 8-cell and subsequent stages. This finding was also reported by Jackson et al. (1980) on the basis of electron microscopic and immunofluorescence observations. Lehtonen (1985) was thus far the only author to have described a diffuse cytokeratin staining in the earlier stages of development. Our results support these previous findings and show that the earliest stages of in vitro fertilized and cultured mouse embryos express the equivalents of human cytokeratins 8 and 18 in a diffuse pattern. In fact, this can only be stated with certainty for the morula stage, since the intensity of the diffuse fluorescence patterns of the 2-cell and 4-cell stages did not exceed that obtained with the non-relevant, control antibody.

In the 8-cell (morula) stage, the fluorescence intensity was enhanced compared to the previous stages, but re-
mained diffusely distributed. The dramatic increase in fluorescence intensity during the transition from morula to blastocyst was accompanied by the appearance of typical cytokeratin filaments. The presence of cytokeratins 7 and/or 19 is less evident in the early stages. However in the blastocyst stage both cytokeratins were unequivocally detected, as was particularly apparent from the typical filamentous patterns revealed by the antibodies using indirect immunofluorescence.

The observation of the presence of cytokeratins 7 and 19 in pre-implantation embryos has not been demonstrated before, although Battifora (1987) has suggested that cytokeratin 19 is one of the first to be expressed in embryonic life. Jackson et al. (1980), when analysing pre-implantation embryos by two-dimensional gel electrophoresis, did not detect a cytokeratin 7 or cytokeratin 19-like cytoskeletal constituent. Therefore although the finding of cytokeratins 7 and 19 was unexpected, we feel that the (filamentous) staining patterns of RCK105 and LP2K are not the result of interaction with cytokeratins 8 and/or 18, based on the immunofluorescence patterns observed in mouse liver (unpublished results) (Coonen E, Kuypers H, and Ramaekers FCS).

The onset of vimentin synthesis in mouse embryos growing in situ has been found not to occur before the formation of mesoderm (Jackson et al. 1981; Franke et al. 1979) at day 8 or 9 of gestation. In principle, therefore, we would not expect vimentin intermediate filaments in our embryos; nevertheless, earlier reports by Franke et al. (1979) indicated that many cells, when brought into culture, start to synthesize vimentin. The lack of detection by indirect immunofluorescence of vimentin in cultured mouse embryos may be due to the fact that these cells grow in a tissue-like organization. These observations are in accordance with the suggestion made by several authors (Lane et al. 1983; Ramaekers et al. 1983c; Rheinwald et al. 1984; Czernobilsky et al. 1985) that expression of vimentin in epitheli-
cells or carcinoma cells may be related to the degree of dissociation of the cells.

With respect to the presence of the karyoskeletal IFP, i.e. the lamins, in different stages of early embryogenesis, our results are in accordance with those of Houliston et al. (1988). The presence of both A- and B-type lamins, although quantitatively hardly distinguishable from background levels, is evident in all stages as concluded from the perinuclear fluorescence seen in virtually all cells of the embryos. In the blastocyst both types of lamins occur in the ICM as well as in the trophoblast cells. In contrast to the suggestion of Schatten et al. (1985), we have no indications that expression of A-type lamins is lost as a result of dilution when going from the oocyte to the morula and blastocyst stage.

Mouse zygotes were obtained by in vitro fertilization.
and further cultured until reaching the appropriate developmental stages. This method enables a comparison between in vitro and in vivo mouse embryogenesis as far as the expression of IFP is concerned. From the foregoing discussion it is obvious that the in vivo and in vitro situation give comparable results, albeit that a slight delay in development of the embryos is seen in cultures compared to the in vivo situation. The techniques of in vitro fertilization and culture of mammalian embryos might therefore provide a future means to study normal and/or aberrant development.

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