Cytoskeletal and contractile structures in bovine lens cell differentiation

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Summary. Bovine lenses from animals of different ages were separated into two epithelial sections, a cortical region and the lens nucleus. Both the 10,000 g supernatant fraction and pellet of these sections were analysed by electrophoresis in SDS-containing polyacrylamide gels. When comparing total protein patterns of the cytoskeletal preparations from the different parts of lenses of different ages a decrease in the amount of vimentin, the protein subunit of lens intermediate-sized filaments (IF), was observed upon lens cell differentiation and aging. Amounts of monomeric (G) and filamentous (F) actin in the different stages of lens cell differentiation were quantitated using the DNase I inhibition technique. A significant increase in the relative amount of F-actin was observed upon fibre cell formation. A slight, but significant increase in the total amount of actin relative to the total amount of cellular protein was observed when passing from the central part of the lens epithelium to the epithelial cells in the elongation zone. In the fibre cells the amount of total actin decreased from cortex to nucleus. A possible function of microfilament-assembly in the process of lens cell differentiation is suggested.

Terminal differentiation of lens cells involves a number of subsequent changes in morphology and biochemical parameters of the epithelial cells [1, 2]. During this process, long lens fibres are formed and the cell volume increases dramatically (see fig. 1). Furthermore there is an onset of synthesis of typical lens proteins, the crystallins. These proteins represent about 90% of the total protein in the lens [2, 3]. The remaining 10% are mainly composed of membrane and cytoskeletal proteins [4, 5]. Upon fibrogenesis, changes are observed in the protein patterns of the cellular membranes from epithelium and fibre cells [6, 7].

Recently, investigations in our laboratory have concentrated on a possible function of the lenticular cytoskeletons in the process of lens differentiation. The presence of microfilament-like structures in membrane preparations of bovine lenses has already been shown in the electron microscope by Bloemendal et al. in 1972 [8]. Thereafter various studies have confirmed these early observations (compare for example 4, 9–14). Kibbelaar et al. [9] extended the electron microscopic and immunofluorescence studies by chemical analysis of lenticular actin. Furthermore, it could be shown that also in lens, as in several other tissues, microfilaments interact with the cellular membrane [7, 15]. The functional aspects ascribed to the cytoskeleton, such as movement on a substrate, cytoplasmic streaming, saltatory movements, exocytosis and cell elongation [16, 17] may have their origin in this interaction. However, next to the formation of a plasma membrane-cytoskeleton complex, a dynamic equilibrium between G- and F-actin in the cell seems to be equally important for these processes [18]. Both the elongation of microfilaments by assembly of G-actin and the interaction of microfilament bundles (stress fibres) with other motility proteins such as myosin, tropomyosin and α-actinin may provide a molecular basis for the driving forces necessary in cell movements.

Microtubules and intermediate-sized filaments of the vimentin-type have also been shown to occur in the eye lens [4, 19]. On basis of experiments using colchicine [10] and studies in elongating chick epithelial lens cells Platisgorsky and coworkers [21, 22] proposed a role for microtubules in lens differentiation. In a recent paper, however, Beebe et al. [23] showed that these chicken epithelial lens cells elongate in culture despite the fact that their micro-


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tubule organization had been destroyed by nocodazole. Mousa & Trevithick [24] have
reported an inhibitory effect of cytochalasin B and D on rat lens cell differentiation
in vitro. These experiments, together with others [25] suggest an important role of
microfilaments in lens cell elongation (differentiation) in a way comparable to axon-
elongation [26]. Mousa & Trevithick [12] have quantitated the total amount of actin
in various in vivo stages of rat lens differentiation by means of gel electrophoretic
techniques. The highest amount of actin was found in the lens epithelial cells and
decreased when passing from periphery to the nucleus. Also the ratio between β- and
γ-actin appeared to change.

Here we show that differentiation of calf lens cells is accompanied by an increase in
microfilament assembly suggesting a shift of G-actin to F-actin during elongation,
rather than a change in the total amount of actin. Furthermore changes in the
amount of the intermediate-sized filament protein as a result of differentiation and lens
cell aging have been demonstrated.

Methods
Cattle eyes from young (3–6 months) and old animals (about 4 years) were obtained fresh from the slaughter-
house. Within 4 h after dissection the lenses were iso-
lated from the eyes and separated into the four dif-
f erent cell lysate fractions (see below). The average
weights of the calf lenses and cow lenses were 1.5
and 2.6 g respectively. DNase I from bovine pancreas
(DN-100, 1,500 Kunitz units per mg solute) and the so-
dium salt of DNA from calf thymus (type I, D-1501)
were obtained from Sigma, St Louis, Mo. Guanidinium
hydrochloride was obtained from Merck, Darmstald.

Lenses from 3-month-old calves were sectioned at
room temperature into four fractions; the central re-

dion of the epithelial cells, the germinative region and the region of cellular elongation, the elongated fibre
cells in the superficial cortical region, and the fibre
cells in the deeper layers surrounding the nucleus. The
epithelial fractions were carefully excised from the
cortex using dissecting scissors. The cortical frac-
tions were obtained by using a metal tube (1.2 cm O) which separated young and old fibres. The subsequent
fractions were homogenized with a Dounce B homo-
genizer in lysis buffer, containing 2 mM phosphate
buffer (pH 7.6), 120 mM NaCl, 0.2 mM ATP, 2 mM
MgCl₂, 0.2 mM 1,4 dithioerythritol (DTE), 0.5%
Triton X-100 and 0.01 mM phenylmethylsulphonyl-
fluoride (PMSF).

Globular and filamentous actin concentrations were
estimated as described previously by Blikstad et al.
[27] with slight modifications. After centrifugation of
the tissue homogenate (5 min at 10000 g) the super-
natant fractions were used for the estimation of the
globular actin. The amounts of tissue and lysis buffer
were chosen in order to ensure a 30–70% inhibition
of the DNase I activity. In contrast to the method of
Blikstad et al. [27] no Ca²⁺ and Mg²⁺ ions were added
to the DNA stock solution, since we occasionally ob-
served DNA breakdown in the presence of bivalent
ions. This modification required adjustment of the
DNA-substrate buffer to 5 mM MgSO₄, 2 mM CaCl₂
and 0.1 M Tris/HCl (pH 7.5). In the actin assay,
300 μL DNA-substrate buffer and 50 μL DNA stock
solution (500 μg/ml) were mixed with 5 μL DNase I
stock solution (10 μg/ml). Hydrolysis of DNA was
followed by hyperchromicity measurement at 260
nm, using a Zeiss M400 111 spectrophotometer. The
slope of the linear part of the absorbance at 260 nm
is directly proportional to the amount of enzyme
added. For the determination of inhibitor (actin) ac-
tivity 5 μL DNase I stock solution is mixed with 1–5
μL cell lysate, chosen to give about 30% inhibition of
the DNase I. The standard amount of DNase I gave
an increase over the linear part of the hyperchromicity
curve of 0.012–0.014 absorbance units/min. The de-
crease in DNase activity is directly proportional to the
amount of monomeric actin added, at least in the
region between 30–70% inhibition. Phalloidin and G-
actin can be analysed by mixing an aliquot of the cell-
lysates with DNase I without GuHCl pretreatment,
whereas F-actin must be dissociated to monomeric
actin with GuHCl prior to the inhibition assay. Com-
plete inhibition of 1 μg of pure DNase I should
be obtained with 1.35 μg of pure actin. For calculation of the absolute amount of actin in the cell lysates
directly from the DNase I inhibition assay, the results
from Blikstad et al. were adopted [27]. These workers
estimated that the DNase I used contained about 0.7
μg of active DNase I per μg of protein.

The total amount of protein in the cell lysate was
determined according to Lowry et al. [28] with the
modification that 0.5% SDS was used to prevent pre-
paration after addition of the Folin reagent due to
the presence of Triton X-100 in the cell lysate [29].
For polyacrylamide gel electrophoresis lenses were
sectioned as described for the actin assay. From the
fetal lens (6 months) first the whole epithelium was
dissected and the cortex fractionated as follows. The
lens was first frozen to -70°C and after thawing the
outer layers of the lens were scraped off, thus giving
four fractions. Fraction I represents the outermost,
fraction IV the innermost fibre cells. The cell lysates
used for gel electrophoresis were prepared with the
same lysis buffer as used in the actin assay. After
homogenizing and centrifugation (5 min 10000 g) the
supernatant and the pellet fractions were used sep-
arately for protein analysis. Electrophoresis in sodium dodecyl sulphate (SDS)-
containing polyacrylamide gels was performed accord-
ing to Laemmli [30] with the modification that a slab
gel instead of gel rods was used. The gel was 12 cm
long and contained 13% acrylamide, 0.4% methylene-bisacrylamide and 0.1% SDS. In this method a stacking gel was applied. Staining and destaining were performed as described by Weber & Osborn [31]. Two-dimensional gel electrophoresis was performed according to O’Farrell [32].

Results
In our experiments the lens was sectioned into four fractions (see fig. 1a, b): (1) Central region of the epithelial cells; (2) germinative region and the region of cellular elongation; (3) young fibre cells; (4) old fibre cells and the nucleus. In fig. 1c, cells in the central epithelial region are depicted. Fig. 1d shows a detail of a section through a calf lens in the equatorial zone.

The ratio between filamentous and monomeric actin (F/G ratio) in each section was measured as described under Methods. The amount of G-actin is calculated from the inhibition of the DNase I activity after addition of an aliquot of the cell lysate. To
determine the amount of total actin, GuHCl is mixed with the cell lysate prior to the actin assay. The increased inhibition (as compared with the DNase I inhibition obtained without GuHCl addition) gives the amount of F-actin in the cell lysate. In interpreting these results one can not, however, be certain that the amount of 'F-actin' measured in this way might include complexes of actin with other actin-binding proteins.

To determine the F/G ratio in a cell lysate, at least two different volumes of cell lysate were used. Only those volumes which showed DNase I inhibition of 30–70% were used to establish the mean F/G value of the cell lysate. The F/G ratio of a particular lens fraction and the standard deviation were determined by using the mean F/G values of at least four different cell lysates from this particular fraction.

The results of actin assays for the various lens sections presented in table 1 and fig. 2 indicate a significant increase in the F/G ratio during the process of lens differentiation. The increase is most pronounced in those regions where elongation takes place (0.22–0.66 in going from the germinative region to the young fibre cells).

Table 2 shows the amounts of actin as compared to the total protein amounts in the different lens sections. It can be seen that the amount of actin is maximal in the germinative region and the region of cellular elongation. In the elongation process thereafter the amount of actin decreases.

In order to visualize changes in the protein pattern of the lenticular cytoskeleton during differentiation and/or aging cell lysates were centrifuged and separated into a 10,000 g supernatant and pellet fraction. Both fractions from all lens sections were analysed by gel electrophoresis (fig. 3).

The increase of actin filaments during differentiation of calf lens epithelium, as determined by the actin assay, is substantiated by the protein patterns of the sections after electrophoresis. The actin band in the insoluble cortical sections (fig. 3, lanes 8 and 9) is stronger than in the insoluble epithelium sections (fig. 3, lanes 6 and 7), indicating that a larger amount of actin, insoluble in lysis buffer, is present in the cortex. The main cytoskeletal constituents (vimentin and actin) were identified and characterized by 2D-gel electrophoresis. Fig. 4 depicts a detail of a 2D-gel, showing the relative amounts of vimentin (V), β- and γ-actin (A) and a 100 kD protein. This latter protein is only observed in the insoluble fractions of the fibres (see fig. 3, lane 8 and 9). The intermediate-sized filament protein represents the main constituent in this fraction. Furthermore it can be seen that the ratio of β/γ-actin is in favour of γ-actin.

Vimentin is present in the epithelium and the peripheral fibres (see fig. 3, lanes 6–8).
Table 1. Distribution of filamentous and globular actin in the different regions of the calf lens

<table>
<thead>
<tr>
<th>Region</th>
<th>% F-actin</th>
<th>% G-actin</th>
<th>P/G ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central region of epithelial cells</td>
<td>13±8</td>
<td>87±8</td>
<td>0.15±0.10</td>
</tr>
<tr>
<td>Germintive region and region of cellular elongation</td>
<td>22±7</td>
<td>78±7</td>
<td>0.22±0.09</td>
</tr>
<tr>
<td>Young fibre cells</td>
<td>40±7</td>
<td>60±7</td>
<td>0.66±0.18</td>
</tr>
<tr>
<td>Old fibre cells and nucleus</td>
<td>46±5</td>
<td>54±5</td>
<td>0.86±0.18</td>
</tr>
</tbody>
</table>

The identity of this protein in the epithelium was also confirmed by 2D-gel electrophoresis (results not shown). When comparing the protein bands in the 57 kD-region of the nuclear fibres with the younger, less differentiated, fractions, it becomes obvious that the vimentin band, characteristic for the epithelial cells, is absent in the nuclear fibres.

In this context it should be mentioned that the changes in the crystallin pattern also occur during differentiation. The changes in the soluble fractions observed are mainly an increase in βB10 (the specific β11 subunit) when passing from the epithelium to the nucleus. Examination of the insoluble fractions reveals two features:

1. In the epithelium, next to vimentin, mainly two β-crystallin subunits remain attached to the membrane-cytoskeleton complex (see fig. 3, lanes 6 and 7), while almost no α-crystallin is present.
2. In contrast, β-crystallins are almost absent in the insoluble fractions of the fibres (see fig. 3, lanes 8 and 9). Here, however, α-crystallin becomes the main protein constituent.

Since lens cells are never shed, fibres in the nuclear region represent the oldest cells. We wondered whether changes in the patterns of subsequent stages of differentiation could be explained as an aging phenomenon. Therefore the protein patterns of the cell lysates from fetal calf lenses and 4-year-old cow lenses were also examined (see figs 5, 6). In the preparation of the fetal calf lens cell lysates, the epithelium could not further be sectioned and separated into a soluble and an insoluble part. The cortex was divided into four regions by scraping upon thawing of the frozen lens.

From the protein patterns, shown in fig. 5, it can be seen that in the epithelium and cortex fractions from fetal lenses, vimentin is present in a rather high concentration (lanes 7–10). The same holds true for actin, which is also present in the soluble fraction.

Table 2. Relative amounts of actin as compared with the total protein amounts in the different regions of the calf lens

<table>
<thead>
<tr>
<th>Region</th>
<th>% Total actin</th>
<th>% F-actin</th>
<th>% G-actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central region of epithelial cells</td>
<td>1.37±0.34</td>
<td>0.18±0.05</td>
<td>1.19±0.30</td>
</tr>
<tr>
<td>Germintive region and region of cellular elongation</td>
<td>1.98±0.49</td>
<td>0.34±0.09</td>
<td>1.54±0.36</td>
</tr>
<tr>
<td>Young fibre cells</td>
<td>1.30±0.32</td>
<td>0.52±0.13</td>
<td>0.78±0.20</td>
</tr>
<tr>
<td>Old fibre cells and nucleus</td>
<td>0.95±0.24</td>
<td>0.44±0.11</td>
<td>0.51±0.13</td>
</tr>
</tbody>
</table>

**Fig. 3.** SDS gel electrophoretic patterns of soluble proteins (crystallins) and insoluble fractions from the four different regions of 3–6-month-old calf lenses. 1, 10, Marker proteins: α-crystallin (20,000 D); ovalbumin (45,000 D); bovine serum albumin (68,000 D); phosphorylase A (93,000 D). 2–5, Soluble proteins from regions I–IV respectively; 6–10, The membrane–cytoskeleton complex of regions I–IV respectively. V, vimentin; A, actin.

**Fig. 4.** Two-dimensional gel electrophoretic analysis (detail) of main cytoskeletal proteins occurring in bovine lens fibres (3–6-month-old animals).
(lanes 3–6). When passing from the epithelium to the insides of the lens, two bands in the insoluble fractions with an MW slightly lower than vimentin occur and increase in intensity. Whether these are breakdown products of the intermediate filament protein remains to be established.

In lenses from 4-year-old cows a striking phenomenon can be observed. When comparing the protein patterns of the insoluble fractions of epithelium, cortex and nucleus (fig. 6, lanes 5–8) it is clear that in the old fibre fractions vimentin is absent. In the epithelial fraction an appreciable concentration of this intermediate filament protein is found, as is the case with two β-crystallin polypeptides, in the region of cellular elongation (lane 6). The latter protein pattern is comparable to that of 3-month-old calf lenses. In summary an active role of microfilaments in the process of lens cell elongation, which occurs in the equatorial zone, is suggested from the following results:

1. The ratio of F-actin and G-actin increases during the process of terminal differentiation (see table 1).

2. The amount of F-actin (as compared with the total amount of protein present in the cells) is almost doubled when epithelial cells from the central region and of the region of cellular elongation are compared (see table 2).

3. The total amount of actin seems to decrease already during the elongation process (see table 2), whereas the amount of F-actin still increases and diminishes only after the cells are differentiated.

In vitro studies (to be published elsewhere) give similar results. When the cells elongate in culture a gradual increase in filamentous actin in the cells takes place as demonstrated by several techniques including the actin assay, electron microscopy, in-direct immunofluorescence studies and gel electrophoretic procedures.

We wish to thank Mr F van Wingen from the Department of Cytochemistry and Histology, University of Nijmegen, for skilful technical assistance with the histological preparation.

The present investigations were carried out partly under the auspices of the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO).

References

The role of fibronectin in adhesion of metastatic melanoma cells to endothelial cells and their basal lamina

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Summary: Vascular endothelial cells synthesize an extracellular matrix or basal lamina composed of collagen, proteoglycans and glycoproteins such as fibronectin (FN). Using affinity-purified anti-FN, we have examined the role of FN in adherence of metastatic B16 melanoma cells to endothelial cell monolayers which lack FN on apical cell surfaces and to their basal lamina which contains FN. B16 melanoma cells, which do not contain significant amounts of FN, attached at much higher rates to endothelial basal lamina and polyvinyl-immobilized FN compared with intact endothelial cell monolayers. Anti-FN failed to inhibit attachment of melanoma sublines of low (B16-F1) or high (B16-F10) metastatic potential to intact endothelial cell monolayers, inhibited slightly B16 cell attachment to basal lamina and completely abolished attachment of B16 cells to polyvinyl-immobilized FN. The antibiotic tunicamycin which inhibits glycosylation of B16 cell surface glycoproteins and blocks experimental metastasis [18] inhibited B16 attachment to endothelial cells, basal lamina and immobilized FN. The results suggest that FN mediates, only in part, the adhesion of B16 melanoma cells to basal lamina through glycoprotein receptors on B16 cells.

Crucial steps in blood-borne tumor metastasis are the arrest of malignant cells in the microcirculation, often in specific organs [1], and subsequent extravasation or invasion of surrounding endothelium and escape to extravascular sites [2-4]. Metastatic tumor cells and invasive normal cells such as leukocytes attach to endothelial monolayers, cause rupture of endothelial cell--cell interactions and retraction of the endothelial cells from their underlying basal lamina, whereupon the invasive cells move from the endothelial cells and bind tightly to basal lamina [5-7]. Eventually the metastatic cells solubilize components such as fibronectin (FN) and sulfated proteoglycans in the endothelial cell basal lamina [8]. We have developed an in vitro model for extravasation [5, 6] that utilizes vascular endothelial cell monolayers which synthesize a basal lamina containing collagens [9, 10], sulfated proteoglycans and glycosaminoglycans [8, 11] and glycoproteins such as FN [9, 12] and laminin [13]. We report here that attachment of B16 melanoma cells to endothelial cell basal lamina (but not to endothelial cells) appears to be mediated, in part, by FN but also by other adhesive molecules in the extracellular matrix.

Materials and Methods

Cell cultures. Murine melanoma sublines selected once (B16-F1) or ten times (B16-F10) for blood-borne lung implantation, survival and growth [14] were obtained from Dr I. J. Pidler (NCI-Frederick Cancer Research Center, Frederick, Md) and were grown in Dulbecco-modified Eagle's minimum essential medium (Gibco, Grand Island, N.Y.) supplemented with 1% non-essential amino acids (Gibco) (complete medium), and 5% heat-inactivated fetal bovine serum (Flow Laboratories, Inc., Inglewood, Calif.). Low passage cell cul-