Disruption of vimentin intermediate filaments in transgenic mice by expression of a dominant negative mutant desmin subunit

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To investigate putative functions of vimentin intermediate filaments in the context of intact tissues and the developing organism, a construct (pVDV), driven by the vimentin promoter and encoding a truncated desmin subunit, was introduced into the murine germ line. The mutant desmin was assembly-incompetent and capable of disrupting preexisting vimentin filaments in a dominant negative fashion, both in transgenic mouse tissues and in fibroblast cultures derived from these mice. Mutant desmin expression strongly enhanced vimentin turnover. In tissues of some transgenic mouse lines, high level expression of pVDV occurred in 10 to 40 % of vimentin-containing cells and, surprisingly, in 1 to 10 % of the skeletal and tongue muscle cells. Immunohistochemical staining of muscle tissue showed a diffuse staining pattern instead of the punctuated aggregates into which mutant desmin typically accumulates in other cell types. The overexpression of pVDV and the concomitant disruption of the endogenous vimentin filament network and enhanced vimentin turnover in a significant percentage of cells did not cause detectable developmental abnormalities.

Introduction

The cytoskeleton of eukaryotic cells is a dynamic structure [28, 41] composed of microtubules, microfilaments, and intermediate filaments (IFs). The function of microtubules and microfilaments in cellular processes is well understood. In contrast, the function of IFs has for a long time remained elusive. The fact that several cell lines do not contain detectable amounts of cytoplasmic IFs and disruption of IF networks in cultured cells does not affect cell morphology, motility or division (reviewed in [21]), has led to the assumption that the function of IFs must be manifested at the level of the tissue or organ as a whole, possibly in providing mechanical strength, cellular organization and architecture [17, 27, 32]. In agreement with this notion is the observation that vimentin IFs display unique viscoelastic properties allowing them to resist breakage and become even stronger under mechanical stress conditions that would rupture other cytoskeletal networks [19].

To address the issue of IF function at this level, different experimental approaches can be made. These include ectopic production or overexpression of IFs in transgenic mice [1, 8, 9, 11, 15, 20, 22, 26, 31], specific interference with in vivo IF assembly by microinjection of IF-specific antibodies (reviewed in [21]) or anti-sense RNA into zygotes or developing embryos [46], disruption of IF networks in transgenic mice, embryonic cells or functional tissues by expression of dominant-negative mutant IF subunits [10, 11, 13, 17, 44, 47], and inactivation of IF genes via homologous recombination in embryo stem cells [4, 12, 29].

We [34–36] and others [2, 13, 18, 23, 26, 40, 47, 49] have demonstrated that in cultured cells IF networks can be disrupted in a dominant-negative fashion by expression of IF gene constructs containing modifications or deletions in the carboxy-terminal part of the central a-helical “rod” domain. Interaction between the mutant and wild-type IF subunits results in disassembly of preexisting IF networks and inhibits de novo IF formation.

Expression of such keratin gene mutants in skin of transgenic mice caused disruption of endogenous keratin IF assembly and resulted in a pathological condition equivalent to the human skin disease epidermolysis bullosa simplex [13, 17, 47].

As an approach to studying the in vivo functions of vimentin, we have generated an IF gene construct comprising the hamster vimentin promoter and a truncated desmin gene and introduced it into the murine germ line. We have shown previously that in transgenic mice, expressing wild-type desmin in vimentin-containing cells, no gross developmental abnormalities could be detected. However, non-lethal abnor-
milities, such as cataract formation and abnormal incisor tooth differentiation, did occur [5, 15, 31].

Vimentin displays a complex expression pattern and is tran-
siently coexpressed with other IF subunits during various
stages of development. When coexpressed with type III (des-
min, glial fibrillary acidic protein and peripherin) or type IV
(neurofilament) IF proteins, coassembly of vimentin and the
other IF subunits takes place [7, 16, 21, 42]. Hence, expression
of the construct described above is also expected to affect IF
networks containing other type III and IV IF subunits. More-
over, we [32] and others [50] have observed that in transfected
cells both intact and truncated vimentin and desmin can inter-
act with endogenous keratin and thereby divert these type I/II
IF proteins into precipitates. This precipitation process
eventually may lead to cell death [50].

We show here that expression of a truncated desmin in
transgenic mice results in disruption of vimentin IFs in a signif-
icanct percentage of vimentin-containing cells without causing
detectable developmental abnormalities.

Materials and methods

Plasmid construction

For construction of pVDV, the 6.2 kb XbaI fragment from pVDEx [31]
was subcloned into the XbaI site of pUC19, yielding pVDEx-Xba. This
fragment comprises the 3.2 kb hamster vimentin promoter and 3 kb of
the hamster desmin gene. The upstream XbaI site is part of a poly-
linker, whereas the XbaI site in the desmin gene is located in intron 5.
A 3.4 kb HincII fragment containing the hamster vimentin exon 9, the
vimentin 3' untranslated region, the polyadenylation signal and 2.6 kb of
3' flanking sequences [33] was subcloned into the Smal polylinker
site of pVDEx-Xba immediately 5' of the XbaI site. Vimentin exon 9
contains 39 bp of coding sequence. As a result, pVDV encodes a pro-
tein composed of 353 amino acid (aa) residues: desmin aa 1-340 and
vimentin aa 452-462 (Fig. 1).

Generation and identification of transgenic mice

Transgenic mice were generated by pronuclear microinjection and
subsequently identified by Southern blotting as described previously [22, 31]. Plasmid sequences were removed by BamHI-EcoRI digestion leaving a 9.6 kb truncated IF gene. Isolation and purification of this
fragment were performed as described [22, 31].

Cell culture

Tissue culture and transfection of hamster lens cells, HeLa cells, BHK-
21 cells and C2C12 cells was carried out as described previously [6, 30,
32, 34]. Ear fibroblast cell lines derived from the transgenic mice, were
established and cultured as described by Bloemendal et al. [6].

Northern blot analysis

Northern blotting and hybridization were performed as described
previously [31]. A mixture of PslI cDNA fragments derived from
pVimi [14] was used as a vimentin probe. As a desmin probe we used
the 1.8 kb HindIII-KpnI fragment containing most of the hamster des-
min cDNA [35]. A 520 bp hamster vimentin Sau3A fragment (E46)
ranging from -150 to +370 relative to the transcription initiation site
was used as a probe to simultaneously detect vimentin and pVDV tran-
scripts. As an actin probe we used the 1.25 kb hamster actin cDNA
fragment isolated by Dodemont et al. [14], which hybridizes to α-, β-
and γ-actin.

Antibodies, immunohistochemical analysis and Western blotting

Immunofluorescence staining of frozen tissue sections and cultured
fibroblasts was carried out as described previously [22, 31]. The follow-
ing polyclonal and monoclonal antibodies were used in this study: 1) a
polyclonal rabbit antibody (poly-des, K5) against chicken gizzard
muscle desmin [37]; 2) an affinity-purified polyclonal antibody (poly-
vim, K36) against bovine lens vimentin [38]; 3) a monoclonal antibody
RV208 against bovine lens vimentin [8]; 4) an affinity-purified poly-
clonal antibody (poly-ker) against human skin keratin [38]; and 5) the
monoclonal antibodies RCK106 [39] and RCK103 [48] against human
keratins.

Western blotting and preparation of IF fractions from mouse tissues and
cultured fibroblasts were carried out as described previously [6, 32, 31, 34].

Determination of the solubility of intact and mutant desmin proteins
was carried out as described by Gill et al. [18]. Briefly, cells were lysed
in Triton X-100 buffer by sonication for 1 min, and the insoluble frac-
tion was removed by centrifugation and subsequently resuspended in
0.5% sodium dodecyl sulfate (SDS) buffer. Again, the insoluble frac-
tion was removed by centrifugation. To the Triton X-100 soluble as well
as the 0.5% SDS-soluble fraction 1 volume of 2 × SDS sample buffer was
added. Equal amounts of protein from both fractions were ana-
lized by Western blotting.

Electron microscopy

Electron microscopy, immunogold labeling and preparation of lens
ghosts were carried out as described previously [15].

Metabolic cell labeling, extraction and
immunoprecipitation of IF proteins

Metabolic labeling of cultured cells with [35S]methionine and prepara-
tion of IF fractions and total cell extracts were performed as described
previously [3, 34, 35].

For pulse-chase labeling of cultured fibroblasts, cells were grown in
six-well dishes to 80% confluency, starved for 1 h in methionine-free
Eagle's minimal essential medium (EMEM), washed twice with
phosphate-buffered saline (PBS) and labeled for 30 min with 20 mCi
[35S]methionine per well (specific activity > 1 000 Ci/mmol; Amersham
Nederland B.V., Houten, The Netherlands) in methionine-free EMEM
containing 6% dialyzed fetal calf serum (FCS). After this 30-min pulse
cells were washed twice with PBS and grown in EMEM containing
10% FCS for different times (ranging from 0.5– 93 h). Cells were har-
vested after washing twice with PBS by addition of 70 µl of lysis buffer
(20 mM Tris-HCl, pH 7.5, 1.5 mM phenyImethylsulfonyl fluoride, 2% SDS
and 10 mM dithiothreitol).

Radiolabeled IF proteins were selectively immunoprecipitated with
the specific antibodies (poly-des: K5; poly-vim: K36) as described [3, 34].
Aliquots containing 5 to 50 × 10⁶ cpm of the labeled total cell
extract were immunoprecipitated. Equal amounts of protein were used
for each precipitation. The immunoprecipitates were analyzed on
one-dimensional 12% polyacrylamide gels containing SDS. After separa-
tion, the gels were fluorographed, dried and exposed to Kodak X-
OMAT AR-5 film.

Quantification was performed by densitometric scanning of autoradi-
ographs.

Results

Construction and characterization of pVDV

We have demonstrated previously that in transgenic mice expression of the muscle-specific IF subunit desmin can be tar-
targeted to vimentin expressing cells by using pVDVs consisting
of 3.2 kb of the hamster vimentin promoter region fused to the
structural hamster desmin gene [31]. In separate studies we have
shown that modifications of the carboxy-terminal part of the
desmin “rod” domain can severely affect the filament forming
capacity of the desmin subunits [35, 36]. Moreover, expression of the mutant desmin in vimentin-containing cells
caused complete disruption of the endogenous vimentin filament network [35, 36, 40].

A truncated version of the pVDes construct was generated by replacing pVDes desmin exons 6 to 9 by vimentin exon 9 (Fig. 1). Desmin exon 6 encodes a major part (64 of total 97 amino acids) of coil 2B of the central "rod" domain, exons 7 to 9 encode the desmin carboxy-terminal "tail". The resulting construct pVDV encodes a desmin protein which is truncated at amino acid residue 340, and contains 13 additional amino acid residues encoded by vimentin exon 9 (Fig. 1). The dominant-negative effects of pVDV expression on desmin and vimentin networks were confirmed by transiently transfecting four different cell types: hamster lens cells, HeLa cells, BHK-21 cells and undifferentiated C2C12 cells. Hamster lens cells express high levels of vimentin but no other cytoplasmic IF proteins. HeLa cells express both vimentin and keratin, BHK-21 cells and the myogenic cell line C2C12 express vimentin and desmin. In each cell type, expression of pVDV caused complete disruption of endogenous vimentin and/or desmin filament networks as determined by single- and double-label indirect immunofluorescence assays using antibodies against vimentin and desmin (Fig. 2). The mutant desmin colocalized with both the endogenous vimentin and desmin punctated aggregates. Incubation of pVDV transfected cells with a monoclonal antibody against vimentin (mono-vim) resulted in very weak immunofluorescence (Figs. 2b, c, f, j), while use of poly-vim yielded intense vimentin staining (not shown). This suggests that the disruption of vimentin filaments and the concomitant relocation of vimentin rendered the epitope, recognized by mono-vim, less accessible.

Shortly (6–8 h) after transfection, very small desmin aggregates were observed, indicative of low-level pVDV expression.

At this stage, complete disruption of endogenous vimentin/desmin networks was already observed, illustrating the dominant-negative effect of pVDV expression. Keratin filaments in HeLa cells remained undisturbed at this stage (not shown). Later after transfection (48 h), the majority of cells contained large clumps of mutant desmin, indicative of high levels of pVDV expression (Fig. 2). At this stage, not only endogenous vimentin but also the keratin filament network of HeLa cells appeared to be affected by the pVDV overexpression (not shown).

Using stably transfected C2C12 cells it was shown that the mutant desmin cannot be solubilized more readily than intact desmin and remains in the Triton X-100 insoluble fraction (Raats et al., manuscript in preparation).

**Generation of transgenic mice**

After removal of plasmid sequences the pVDV construct was introduced into the mouse germ line via pronuclear microinjection. Southern blot analysis of tail DNA of mice born from microinjected zygotes showed that 9 of 61 mice had incorporated copies of the construct into their genome. All transgenic mice produced offspring, 7 mice transmitted the transgene (yielding strains no. 5, 7, 9, 33, 47, 50, and 55). Southern blot analysis revealed no rearrangements in the pVDV transgenes (not shown). Copy numbers varied from 2 to ~50. All transgenic mice appeared healthy.

**pVDV expression in cultured fibroblasts: expression levels, stability of pVDV encoded protein and disruption of endogenous vimentin filaments**

Fibroblast cell lines were derived from 5 founder mice (no. 5, 7, 33, 50, and 55) by immortalization of ear-shell fibroblasts with SV40 virus. Northern and Western blotting showed that pVDV was expressed in all cell lines.

Expression levels as estimated by Western blotting varied widely between cell lines (data not shown) and, to a lesser extent, also per cell line between experiments. Generally, pVDV desmin levels ranged from low (lines 5, 7, 33: 0.1-1% of BHK-21 desmin) to relatively high (line 50: 5-10% of BHK-21 desmin and line 55: 20-30% of BHK-21 desmin). Northern blot analysis using a probe which recognizes both endogenous vimentin and pVDV transcripts (E49) confirmed that lines 50 and 55 expressed the highest levels of pVDV, but also revealed that pVDV transcript levels were in the same range as endogenous vimentin levels (not shown). In fibroblast cell lines derived from pVDes transgenic mice, which express intact desmin, transcript levels similar to those of pVDV lines 50 and 55 resulted in much higher expression levels of transgene-encoded protein [31]. Taken together, these data suggest that the turnover of the pVDV encoded truncated desmin protein takes place at a higher pace than intact desmin.

To determine the stability of the pVDV-encoded protein and the effects of pVDV expression on endogenous vimentin turnover, the fibroblast cell line 55 and a control fibroblast cell line derived from a non-transgenic mouse were subjected to pulse-chase labelling with [35]methionine, while total cell extracts were analyzed by immunoprecipitation with polyclonal desmin (poly-des) and vimentin (poly-vim) antibodies (Fig. 3). Measured over a 93 h period, labeled endogenous vimentin levels of the control cell line decreased 1.6-fold. A
Fig. 2. Single- and double-label indirect immunofluorescence assay of HeLa cells (a-d, g, h) and hamster lens cells (e, f, i, j) transfected with pVDV. Cells were incubated with poly-des and mono-vim. Double-label staining with poly-des (a, d, e, i) and mono-vim (b, c, f, j) of pVDV-transfected HeLa cells (a-d) and pVDV-transfected hamster cells (e, f, i, j) showing punctated staining pattern. Note complete disruption of vimentin filaments, absence of intact mutant desmin filaments, and colocalization of mutant desmin and vimentin. Vimentin staining of transfected cells was very weak. Double staining with poly-des and mono-vim of HeLa cells, expressing pVDV at relatively low (punctated staining; g) or very high levels (cells filled with mutant desmin; g, h). - Bar 10 μm.
similar turnover rate has been reported for intact desmin in vimentin-expressing cells [36]. In contrast, pVDV mutant desmin as well as the endogenous vimentin level in fibroblast cell line 55 decreased 12-fold in the same period (Figs. 3a, b). This indicates that pVDV expression increased the endogenous vimentin turnover 7.5-fold.

Fibroblast vimentin transcript levels were not influenced by pVDV expression (Fig. 3c). Similarly, vimentin and desmin transcript levels remained unchanged in C2C12 cells expressing the mutant desmin (Fig. 3c).

Indirect immunofluorescence assays on the various fibroblast cell lines using desmin and vimentin antibodies revealed that pVDV expression resulted in disruption of the endogenous vimentin filament network, even in lines 5 and 7 which express the mutant desmin at relatively low levels (Fig. 4). However, cells in which the vimentin filaments had not been completely disrupted (in spite of detectable levels of pVDV expression) were also observed in each cell line suggesting that pVDV expression levels varied between individual cells. Cell lines 50 and 55, which express relatively high levels of mutant desmin, contained only a small number of cells (1–10%) with undisturbed vimentin filaments.

Cells containing large, strongly fluorescent aggregates of mutant desmin, typical for transiently transfected cells, were not observed. Instead, relatively small fluorescent dots were detected in the fibroblast cells. This suggests that pVDV expression per cell is lower in the fibroblasts than in the transiently transfected cells.

We conclude that the mutant desmin encoded by the pVDV transgene is capable of interacting with mouse vimentin and thereby disrupts the vimentin IF network. As a result, the vimentin turnover rate is greatly increased. This is not compensated for by increased vimentin transcription.

Expression of pVDV in mouse tissues

The expression of the pVDV-encoded protein in transgenic mice was analyzed by indirect immunofluorescence on tail sections and blood cells from all founder mice and their offspring, using poly-des.

Strongly fluorescent aggregates were detected in stromal cells in tail sections of strains 47, 50 and 55 (Figs. 5a–f), whereas in strains no. 7, 9, and 57 a weakly fluorescent, punctated staining pattern was observed (not shown). Strain 33 displayed an intermediate staining pattern with regard to aggregate size and fluorescence intensity. Expression levels in fibroblast cell lines and the corresponding mouse tissues appeared to correlate. There was no relationship between transgene copy number and expression levels (data not shown). pVDV expression could not be detected in all vimentin expressing cells of strains no. 47, 50 and 55. Only 10 to 40% of the stromal cells were stained with poly-des (Figs. 5a–c). For strain 33 this was 1 to 5%. The percentage of cells containing detectable levels of mutant desmin varied between litter mates and even between tissue sections from the same individual mouse taken from different parts of the tail or at different time points. Although the much lower expression levels of strains no. 5, 7, 9 and 57 made it difficult to determine the percentage of mutant desmin expressing cells for these mice, it was clear that not all stromal cells expressed pVDV at detectable levels.

Staining of tail sections from strains no. 47, 50 and 55 with antibodies against vimentin revealed that the endogenous mouse vimentin filament network was severely affected by pVDV expression (Figs. 5d–f).
Immunostaining of blood cells using poly-des confirmed the findings described above. Intensive staining of mutant desmin aggregates was observed in blood cells from strains no. 47, 50 and 55 (Figs. 5g, h). All other strains displayed much weaker staining (not shown).

Immunofluorescent staining of blood cells from strains 47, 50 and 55 with poly-vim revealed that expression of the truncated desmin caused disruption of the vimentin filaments in these cells (Figs. 5i, j). This was confirmed at the ultrastructural level by immunogold labeling of lens fiber ghosts (Fig. 6).

The overexpression of pVDV and the concomitant disruption of the endogenous vimentin filament network in a significant percentage of stromal and blood cells did not seem to cause detectable developmental or physiological abnormalities.

### Tissue specificity and levels of pVDV expression
Since mouse strains 47, 50 and 55 exhibited the highest pVDV expression levels, they were used for a more detailed analysis of the tissue specificity and levels of transgene expression (Fig. 7).

Mouse tissues were split into two halves. One part was used for RNA analysis, the other part was cryosectioned and used for immunostaining.

Total RNA samples from various tissues including heart, skeletal muscle, oesophagus, spleen, liver, testis (or ovary), and kidney were analyzed by Northern blotting (Figs. 7a, b). The three independent strains displayed similar expression patterns of the correctly sized (1.7 kb) pVDV transcript. High levels of pVDV expression, in the range of endogenous desmin, were detected in skeletal muscle and tongue tissue. In contrast, heart pVDV transcript levels were at the threshold of detection. Low level pVDV expression was detected in oesophagus and testis.

Apparently, pVDV expression did not follow the endogenous vimentin expression pattern. Skeletal muscle tissue expresses only very low levels of vimentin, while in heart tissue higher levels of vimentin have been found (Fig. 7a).

Western blotting of desmin confirmed these observations. Correctly sized (40 kDa) pVDV encoded protein was detected in skeletal muscle and tongue (Fig. 8) and, at the threshold of detection, in eye lens (not shown).

Sections from the same tissues used in Northern blot analysis were immunostained with poly-des and poly-vim. Significant expression was detected in skeletal muscle (Figs. 9a, b) and tongue only (Figs. 9c, d). In both tissues, most muscle fibers did not express detectable levels of pVDV-encoded protein. Expression of mutant desmin was observed in 1 to 10% of the fibers. Surprisingly, fluorescent desmin aggregates were not detected. Instead, the staining pattern was diffuse and much more intense than endogenous desmin staining, suggestive of high levels of mutant desmin expression (Fig. 9).

Non-transgenic mice and mice transgenic for pVDes [31] did not display such staining patterns (not shown). For a major part the diffuse desmin staining must be due to the mutant desmin and not to endogenous mouse desmin, since staining with polyclonal antibodies against vimentin (which in pVDV transfected cells and Western blotting had been shown to detect the pVDV encoded protein but do not react with mouse desmin), resulted in the same diffuse, strongly fluorescent staining pattern as staining with poly-des (not shown).

The striated pattern normally observed upon desmin staining of muscle fibers seemed to be present not only in fibers lacking pVDV expression, but also in pVDV expressing muscle fibers. This would indicate that sarcomeric desmin is not disturbed by pVDV overexpression.

### Discussion

**Disruption of mouse vimentin filaments**

As an approach to studying in vivo functions of vimentin IFs a construct (pVDV) encoding a truncated desmin protein was generated and introduced into the murine germ line. Using cell lines derived from mice transgenic for pVDV, it was shown that relatively low levels of mutant desmin expression are sufficient for disruption of endogenous vimentin filaments. This is in agreement with previous studies, describing dominant-negative effects of similarly truncated IF subunits on endogenous IF filament levels at levels of 1% of the total population of IF (e.g. [13, 18, 49]).

The stability of the pVDV-encoded mutant desmin appeared to be much lower than that of wild-type desmin and vimentin. Expression of pVDV caused a strongly increased (7.5-fold) turnover of endogenous vimentin, not compensated for by increased vimentin transcription.

In contrast, we previously demonstrated that different desmin mutants deleted in the C-terminal part of the central “rod” domain, capable of completely disrupting vimentin filaments, are protected from rapid turnover via interaction with wild-type vimentin [36]. However, the pVDV-encoded protein differs from the mutant desmin tested previously in two aspects. Most importantly, a larger part of cell 2B in the “rod” domain has been deleted from pVDV (64 instead of 39 amino acids). In addition, the pVDV C-terminal “tail” consists of 13 amino acid residues derived from vimentin, instead of 23 amino acids derived from the C-terminus of desmin.

We conclude that pVDV expression causes disruption of vimentin filaments and enhanced turnover of vimentin subunits.

The different strains of mice transgenic for pVDV displayed varying expression levels. Three independent mouse lines expressed pVDV at relatively high levels as determined by Northern blot analysis and indirect immunofluorescence. In these strains mutant desmin was detected in 10 to 40% of vimentin-containing stromal cells. Expression levels differed between individual cells, but were relatively high as judged from the size of the mutant desmin aggregates observed in the cytoplasm. As expected, also in vivo endogenous vimentin filament network was seriously affected by pVDV expression. Moreover, it may be assumed that coexpression of mutant desmin and other type III and IV IF proteins during vimentin transgenic.
Fig. 5. Indirect immunofluorescence assays of tail sections (a-f) and blood cells (g-j) from mice transgenic for pVDV, using poly-des and poly-vim. - a to e. Poly-des incubation of tail tissue sections from mouse strains 47 (a), 55 (b) and 50 (c). Note strongly fluorescent dots and clumps in stromal cells (not observed in control tissues). - d to f. Poly-vim incubation of tail sections from mouse strains 47 (d, e), and 55 (f). Vimentin expression was detected in virtually all stromal cells (d), while pVDV expression was detected in a minority of stromal cells (compare a-e to d). Identification of stromal cells containing completely disrupted vimentin filaments (using poly-vim) was hampered by the presence of many cells containing intact filaments. However, in areas less densely packed with stromal cells complete vimentin filament disruption could be observed (e, f). - g, h. Poly-des incubation of blood smears from strains 47 (g) and 55 (h, upper and lower panels). Note strongly fluorescent dots and clumps (not observed in control blood cells) in a small percentage of blood cells. - i, j. Poly-vim incubation of blood smears from strains 47 and 55. Note complete disruption of vimentin filaments in strain 47 (i, lower panel) and strain 55 (j). Partial disruption was also observed in strain 47 i, upper panel). - Bars 4 μm.

Fig. 6. Thin sections of lens fiber ghosts from the cortical region of lens from transgenic mouse strain 55 (a, b) and control lens (c). Immunogold labeling was carried out using mono-vim (a, c) and poly-des (b). Note disruption of vimentin network (a) and poor accessibility of accumulated mutant desmin to the antibody (b). - Gold particles (arrows) are 5 nm. - Bars 100 nm.
Fig. 7. Northern blot analysis of 5 to 20 μg of total RNA from pVDV mouse strains 47, 50 and 55. Panel a. Spleen, skeletal muscle, heart. Panel b. Liver, testis (lanes 1 and 4), ovary (lanes 2, 3); brain, tongue and oesophagus (from strain 55 only). Blots were subsequently hybridized to a desmin, a vimentin and an actin probe. The actin probe recognizes both α- (1.8 kb) and β-actin transcripts (2.1 kb). Between hybridizations, the blots were stripped. Total RNA (1–2 μg) from desmin- and vimentin-expressing BHK-21 cells (lanes BHK) and from pVDV-expressing fibroblast cell line 55 (lanes F55) was included as controls. – Lanes c: RNA from a non-transgenic mouse; lanes 1: founder mouse 55 (f1); lanes 2: strain 55 (f1); lanes 3: strain 47 (f1); lanes 4: strain 50 (f1). Note high levels of pVDV expression in skeletal muscle and tongue.

Fig. 8. Western blot analysis of cytoskeletal fractions of tissues derived from a strain 47 mouse (f1). The blot was incubated with poly-des and poly-vim, subsequently. Positions of desmin (des; 53 kDa) and the pVDV-encoded protein (Δdes; 40 kDa) are indicated. Mutant desmin expression was detected in muscle and tongue tissue. Lens also contained correctly sized mutant desmin (not shown).

Fig. 9. Indirect immunofluorescence assays of skeletal muscle (a, b) and tongue tissue sections (c, d) from pVDV transgenic mice strains 47 (a, c) and 50 (b, d), using poly-des. Note strongly fluorescent, diffuse staining pattern (not observed in non-transgenic or pVDVes transgenic mice) in a minority of muscle fibers. The striated pattern normally observed upon desmin staining also seemed to be present in the strongly fluorescent muscle fibers (not shown). Strongly fluorescent mutant desmin aggregates were not observed. – Bar 4 μm.

Stages of development affected IF formation in certain cell types. Previous work, describing disruption of keratin IF networks by intact and mutant type III IF subunits in transfected cells [32, 50], suggests that even keratin IF filaments may have been disorganized in cells normally coexpressing vimentin and keratin IFs.

However, neither the disruption of IF networks nor the concomitant enhanced turnover of vimentin subunits or the accumulation of mutant desmin resulted in detectable developmental or physiological abnormalities.

Expression of pVDV in muscle fibers

The pVDV-encoded mutant desmin also appeared to be expressed in tongue and skeletal muscle cells. Surprisingly, instead of immunofluorescent punctuated aggregates, a diffuse, intense staining pattern was observed. RNA analysis confirmed that pVDV was expressed at high levels in muscle tissue. pVDV transcript levels were equivalent to endogenous desmin transcript levels, while only 1 to 10% of the muscle fibers contained detectable amounts of mutant desmin, indicating that individual fibers overexpressed pVDV at least 10-fold. This did not seem to compromise the health of the transgenic animals.
During myogenesis, the longitudinally oriented IFS undergo phosphorylation-mediated disassembly and are redeployed to a transverse association along the Z bands [45]. Intact desmin filaments may be dispensable for in vitro myogenesis [40]. On the other hand, in vitro myoblast fusion is inhibited when the disassembly of longitudinal desmin filaments is blocked by the action of antibodies directed against the desmin phosphorylation site [43]. Moreover, disruption of IF organization in Xenopus tadpole myotomal dorsal muscle led to structural defects at the interomise function [10]. The diffuse staining pattern observed by us may indicate that the mutant desmin is not capable of interacting with endogenous desmin, leaving location and relocation, structure and function of endogenous desmin, and therefore the process of myogenesis, undisturbed.

The diffuse staining pattern observed in this study differs from previous reports [10, 40] describing formation of mutant desmin punctated aggregates in muscle fibers upon expression of a truncated desmin. The reason for this difference is unclear. We consider it unlikely that the observed difference is caused by the size of the deletion in the desmin “rod” domain, since the construct of Schultheiss et al. [40] contains a larger deletion than pVDV but is still capable of interacting with endogenous desmin.

**Regulation of pVDV expression**

It remains to be elucidated why pVDV was expressed in a subset of vimentin-containing cells only. We have shown previously that in transgenic mice a vimentin-desmin hybrid gene (pVVim2; [22]) and complete desmin gene (pVDes; [31]), both under control of the vimentin promoter, were expressed in virtually all (pVVim2) or most (pVDes) vimentin-producing cells. The pVDes expression pattern was slightly more restricted than that of endogenous vimentin and pVVim2 (no pVDes expression in testis Sertoli and Leydig cells; expression in a subset of vimentin-positive cells in some tissues; [5, 15, 31]), and pVDes expression levels were lower. In turn, the expression pattern of pVDV appears to be more restricted than that of pVDes. In both cases, regulatory elements present in one construct may be missing in the other. For comparison these constructs are depicted in Figure 10.

Vimentin is not expressed in mature myotubes [21, 24, 25, 30, 45]. The expression of pVDV in myotubes (discussed above) may be caused by the lack of negative control elements in the vimentin gene suppressing expression in myotubes. However, we [30] and others [24] have shown previously that the vimentin promoter is sufficient for downregulation of vimentin expression during myogenesis. Alternatively, positive control elements stimulating muscle-specific expression, may be present in the sequence in pVDV derived from the desmin gene. This would imply that, in addition to the 5′ flanking region of the desmin gene, regulatory elements located in other parts of the gene are involved in vivo desmin expression.

**In vivo functions of vimentin**

Disruption of keratin filaments in the skin of transgenic mice [13, 17, 47], in Xenopus oocytes [46] and in F9 embryonal cells [44] by dominant negative mutation has revealed that keratins may have an important function in providing mechanical integrity to cells and/or tissues.

However, the pVDV transgenic mice described here did not display an obvious phenotype. Similarly, expression of truncated vimentin did not cause detectable abnormalities during early embryogenesis in Xenopus [11]. If vimentin functions at the level of tissues or organs [21], e.g. by imparting structural integrity to the cell in the context of its tissue, then the cells expressing the truncated desmin may have been “rescued” by non-expressing neighboring cells. However, it has been shown that mice lacking vimentin or glial fibrillary acidic genes also develop and reproduce normally [12, 29]. Since in these mice expression of IF proteins that are normally coexpressed and coassembled with vimentin/gial fibrillary acidic protein is not affected, it has been suggested that expression of vimentin is redundant in many cell types and that coexpressed IF proteins can take over the putative functions of vimentin. At present, neither rescue of function (in case of mutant desmin expression) nor functional redundancy (in case of inactivated vimentin gene) can be completely ruled out. Addressing the first issue would require generating transgenic mice expressing the truncated desmin in all vimentin-containing cells, while cross-breeding of mice carrying different IF genes inactivated via homologous recombination would be required to address the latter.

The possibility remains that we have not been able to detect phenotypic changes resulting from IF disruption, enhanced vimentin turnover or mutant desmin overexpression. We have previously shown that overexpression of desmin in vimentin-containing cells leads to non-lethal phenotypes which are not readily recognized, such as plasma membrane damage of lens fiber cells [15] or disturbed incisor tooth differentiation [5]. If in analogy with the keratins of squamous epithelia [17] IFS...
serve primarily structural requirements, physical stress or pathological situations may be necessary to discern a phenotype. For example, transgenic mice expressing dominantly negative keratin 14 mutants require mechanical trauma before the blistering phenotype becomes apparent. Even then, cytology remained dependent on factors additional to mutant keratin expression, such as cell shape [13]. More intense study of the transgenic mice described in the present study or of mice in which the vimentin gene has been knocked out, including culturing of cells or tissues from these animals and subjecting them to appropriate bioassays, may help in identifying subtle effects of vimentin IF disruption.

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