Differential expression of nuclear lamins in normal and diseased human cells

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Differential expression of nuclear lamins in normal and diseased human cells

Proefschrift

ter verkrijging van de graad van doctor
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volgens het besluit van het College van Decanen,
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Contents

Chapter 1	General Introduction	9
Chapter 2	A- and B-type lamins are differentially expressed in normal human tissues	19
Chapter 3	Nuclear lamin expression in normal testis and testicular germ cell tumours of adolescents and adults	41
Chapter 4	Comparison of A- and B-type lamin expression in nodular sclerosing Hodgkin's disease and reactive lymph nodes	57
Chapter 5	Abnormal A-type lamin organization in a human lung carcinoma cell line	71
Chapter 6	An alternative splicing product of the lamin A/C gene lacks exon 10	89
Chapter 7	Lamin A is not required for incorporation of lamin C in the nuclear lamina as demonstrated by GFP-tagged lamins	103
Chapter 8	Summary and General Discussion	117
Samenvatting		121
Curriculum Vitae		126
List of publications		127
Colour illustrations		130
Dankwoord		135

Chapter 1

General Introduction

General Introduction

Nuclear lamins form a network of intermediate-sized filaments at the nucleoplasmic site of the nuclear membrane. Two main subtypes of nuclear lamins can be distinguished, i.e. A-type lamins and B-type lamins. The A-type lamins comprise a set of three proteins arising from the same gene by alternative splicing, i.e. lamin A, lamin C [37] and lamin A Δ 10 [44], while the B-type lamins include two proteins arising from two distinct genes [28], i.e. lamin B1 and B2. Furthermore, several germ cell specific lamins have been detected in various species, but not in humans.

Lamin structure

The nuclear lamins comprise a unique subclass of the intermediate filament protein family. Lamins share their molecular domain organization with the other intermediate filament proteins in that they are fibrous molecules that have an aminoterminal globular head, a central rod of α -helices, and a carboxyterminal globular domain. The amino- and carboxyterminal domains are rather divergent among all intermediate filament subclasses, but the rod domains are highly conserved in length and sequence. The α -helical rod domain can be divided into three coiled regions termed 1a, 1b and 2 that are separated from each other by short linker sequences. In contrast to cytoplasmic intermediate filaments, the lamin rod domain contains an additional 42 amino acids in coil 1b, due to six additional heptad repeats. Identically increased rod lengths were seen in intermediate filament proteins of invertebrate cells [76], suggesting that lamins may be the evolutionary ancestors of the intermediate filament proteins. It is suggested that during evolution the invertebrate cytoplasmic intermediate filament genes arose by lamin gene duplication, followed by loss of the nuclear localization signal and the isoprenylation site [14]. During the transition from invertebrates to vertebrates the cytoplasmic intermediate filament genes lost six heptad repeats from the rod domain.

Figure 1. Schematic drawing of the primary structure of lamins, indicating the amino- (N) and carboxy- (C) termini, the phosphorylation sites (P) that play a role in lamina disassembly during mitosis, the α -helical rod domain, the nuclear localization signal (NLS) and the isoprenylation site (CaaX). Furthermore the open arrow indicates the site of lamin A where proteolytic cleavage takes place after farnesylation and carboxymethylation.

Although lamins share many characteristics with the other intermediate filament subclasses, they display several unique features [49]. For instance, lamins are localized in the nucleus instead of the cytoplasm. For transport into the nucleus the carboxyterminal tail contains a nuclear localization signal (Figure I). Furthermore, cytoplasmic intermediate filaments form fibrous structures often extending from the nucleus to the plasma membrane, whereas nuclear lamins form two-dimensional sheets underlying the nuclear membrane. Moreover, lamin expression is found to be widespread, occurring in virtually all nucleated cells, while other intermediate filament proteins show a more differentiation related expression pattern. Differentiation dependent expression of A-type lamins has been proven, but B-type lamins are still assumed to be constitutively expressed in all cells [8, 61].

Lamin polymerization and lamina assembly

Various approaches have been used to study lamin polymerization and lamina assembly. Expression of wild type and mutant lamin proteins in bacteria, followed by electron microscopic analysis of purified proteins, offers a powerful approach to investigate the structural determinants controlling lamin polymerization under defined *in vitro* conditions [20, 25, 26, 69]. The initial lateral interaction between single lamin molecules results in formation of parallel unstaggered homodimers, formed by a coiled-coil formation of the α -helical rod domains. *In vivo* studies using the yeast two hybrid system showed that A- and B-type lamins can form homodimers as well as heterodimers [77]. The next step is the formation of head-to-tail polymers. *In vitro* binding studies of mutant *Drosophila* lamins showed that the first 6-15 amino acids of coil 1a and the last 6-15 amino acids of coil 2 are essential for head-to-tail polymerization of lamin dimers [69]. These regions contain highly conserved sequences among all intermediate filament type proteins, and are also important for polymerization of cytoplasmic intermediate filaments. Lamin head-to-tail polymers may then form filaments by lateral association, but lamin filaments of a stable 10 nm width are difficult to obtain *in vitro*. Instead, head-to-tail polymers showed a pronounced tendency to form large paracrystals [25, 26].

In addition to the presence of the conserved rod sequences, phosphorylation of lamins is an important factor that determines whether lamins will assemble into dimers and further into head-to-tail polymers or whether they will remain unassembled. In assays employing purified nuclei [13, 57] or *in vitro* reconstituted head-to-tail polymers [56, 69], lamin phosphorylation by p34^{cdc2} kinase caused disassembly of the filaments. The phosphorylation sites have been mapped to residues flanking the conserved ends of the central rod domain [24]. Mutation of these sites, resulting in lamins that can not be phosphorylated, leads to formation of disassembly-resistant polymers [56].

The nuclear localization signal is responsible for the trafficking of lamins from the cytoplasm to the nucleus. Mutant lamins missing the nuclear localization signal assemble as cytoplasmic filamentous structures [38]. Furthermore, the lamins need to be targeted to the inner nuclear membrane. For this purpose B-type lamins and lamin A contain a carboxyterminal CaaX motif,

a site for isoprenylation [34, 42]. The cysteine residue of this motif will become farnesylated, and after proteolytic removal of the a-a-X motif this cysteine will be carboxy-methylated as well. These modifications of the carboxyterminus of the lamins provides them with a hydrophobic anchorage site for the nuclear membrane. B-type lamins will remain farnesylated throughout their lifetime, whereas lamin A is further processed. Another 15 carboxyterminal amino acids are proteolytically cleaved off the protein together with the farnesyl tail, resulting in lamin A without hydrophobic anchorage site [75]. It is not clear whether this cleavage takes place before or after incorporation of lamin A into the lamina.

Lamin C does not contain a CaaX motif [37], and is consequently not isoprenylated. Furthermore, blocking of isoprenylation of lamins did not affect lamina assembly [12], and the mechanism for targeting of non-isoprenylated lamins to the membrane remains to be established. Lamina-associated polypeptides (LAPs), a class of integral membrane proteins of the inner nuclear membrane that bind both A- and B-type lamins [18, 45, 65], may play an important role in membrane attachment and assembly of the nuclear lamina of non-isoprenylated lamins.

The fact that B-type lamins remain isoprenylated in contrast to A-type lamins, has an impact on lamin distribution during mitosis, when the nuclear membrane disintegrates into vesicles. At the same time the lamina disassembles as a result of hyperphosphorylation of the lamins by the p34^{cdc2}-cyclin B kinase complex that is active during this phase of the cell cycle [41]. During mitosis the B-type lamins remain attached to the membrane vesicles with their isoprenylated carboxyterminus and by attachment to the transmembrane lamin B receptor [46], while the A-type lamins are released as soluble dimers or tetramers [19]. After completion of mitosis the nuclear lamina reassembles in the daughter cells. Three alternative pathways have been proposed for this reassembly (for review [31]). In one model soluble A-type lamins first assemble on the surface of decondensing chromosomes, which allows vesicle bound B-type lamins to associate 'herewith and subsequently the nuclear envelope is formed' by fusion of B-type lamin associated vesicles. In another model the vesicle bound B-type lamins will first associate with the chromatin, and only after fusion of the vesicles the soluble A-type lamins are transported into the new nucleus and incorporated into the lamina. In the third type of model both A- and B-type lamins bind to the condensed chromatin cooperatively and allow optimal envelope assembly. Evidence for all three models has been reported in literature [7, 22, 31, 52].

Despite the relative stability of lamins *in vitro*, the lamina is not a rigid structure (for review [53, 54]). Phosphorylation of lamins in interphase nuclei has been reported [15, 33, 68]. Phosphorylation sites and kinases distinct from those that play a role in mitosis are responsible for these modifications. Phosphorylation of lamins in interphase cells may modulate lamin dynamics. It may, for instance, facilitate incorporation of new lamins when DNA is replicated during S-phase and consequently the nucleus increases in size. Since the lamina organization is maintained, it is likely that newly synthesized lamins are transported into the nucleus and incorporated into the lamina at this stage.

In apoptotic cells the lamin network is broken down. This is not due to phosphorylation and subsequent polymer dissociation, but the result of proteolytic lamin degradation [55]. Lamin cleavage during apoptosis requires the activation of ICE-like proteases, and lamin fragments of 46-48 kDa are generated [35, 74]. Lamin degradation has been reported to precede DNA fragmentation [51]. Expression of degradation resistant lamins delays the process of apoptosis by 12 hours, but does not block the process completely [60]. Therefore it was concluded that lamin breakdown during apoptosis facilitates the process but is not a prerequisite.

Differential lamin expression in cells and tissues

In general it is assumed that A-type lamins are expressed in well differentiated cells and tissues, whereas undifferentiated cells express B-type lamins [8, 9, 61]. Furthermore, inverse staining patterns of A-type lamins and the proliferation marker Ki-67 have been reported in lung tumour sections [9, 62], suggesting that A-type lamins represent a non-proliferation marker. Only recently it became clear that not only A-type lamin expression is differentially regulated, but that differential expression of distinct B-type lamins occurs as well in normal human tissues [5].

Contrasting results on A-type lamin expression in early mouse embryos have been reported. For instance, Schatten et al. [64] and Stewart and Burke [66] reported that A-type lamins were absent from several developmental stadia of mouse embryos, whereas others [10, 29] reported low, but detectable levels of A-type lamins throughout the whole preimplantation embryonal development.

In a study upon lamin expression during further embryonic development of mice [61] it has been reported that expression of A-type lamins was not seen until tissue differentiation takes place. Thereafter A-type lamin expression starts in some tissues and continues until well after birth. Expression of B-type lamins is seen throughout all developmental stages. Similar results have been described for chicken embryos [36], while development dependent expression has also been reported for *Xenopus* lamins [3, 67].

Many adult tissues express A-type lamins [5, 8], but in diseased cells the expression patterns may change. Downregulation of A-type lamin expression in chronic hibernating myocardium has been reported [1], and can be explained by the dedifferentiated state of these cells. Furthermore, differential expression of A-type lamins in human lung cancer subtypes [6, 62], and human testicular germ cell tumours [43] have been reported.

However, transfection of A-type lamins into undifferentiated cells that do not express endogenous levels of lamin A and C does not lead to differentiation of these cells, despite the fact that the lamins will be properly incorporated into the lamina [58]. Apparently, additional factors are required in the differentiation process.

Furthermore, cell type specific alternative splicing products of both A- and B-type lamins are found in specific cells. In mice, spermatocyte specific lamins B3 and C2 have been detected. Lamin B3 is an alternative splicing product of the lamin B2 gene [16]. Spermatocyte specific lamin C2 and somatic lamins A/C [17] arise from the same locus, but are under control of two

separate promoters, i.e. a somatic promoter for lamin A and C and a testis-specific promoter for lamin C2 [50]. The testis-specific promoter is located within the first intron of the lamin A/C gene. Also in rat lamins specific for male germ cells have been found [70, 71, 73]. In general, these germ cell specific lamins are shorter than the somatic lamins. Their expression is found during the process of spermatogenesis, in either pre- and postmeiotic germ cells. In pre-meiotic spermatogonia both germ cell specific and somatic lamins were detected, but the somatic lamins were down regulated upon further maturation of the germ cells.

Previously, in *Xenopus* lamin IV has been reported to be restricted to male germ cells [2], while lamin LIII (B3) was predominantly detected in *Xenopus* oocytes, but also in a few other highly differentiated cells such as muscle cells and neurons [3].

Possible functions of the nuclear lamina

Many biochemical and molecular features of lamins have been studied, but their functions remain still largely undetermined. The functions that have been proposed are based on ultrastructural observations (for review [48]).

Lamins form a stable polymer network adjacent to and interacting with the nuclear membrane. Therefore, one of the functions ascribed to the lamina is the maintenance of the structural integrity of the nucleus. Further indications for functions in maintaining structural integrity of the nucleus were obtained from experiments where mouse spermatocyte specific lamins were expressed in somatic cells [16]. This resulted in obvious changes in nuclear morphology of these cells. Additionally, interactions of nuclear lamins with cytoplasmic intermediate filaments may also be functional in the maintenance of cellular and nuclear morphology [59, 63]. Connections with cytoplasmic intermediate filaments may furthermore provide a mechanism for signal transduction and communication within the cell [11].

Besides interactions with the nuclear membrane and other intermediate filaments, lamins interact with the nuclear chromatin. Eukaryotic chromatin is organized into loops, which are attached to the nuclear matrix. This organization is thought to contribute to compaction of the chromatin and regulation of gene expression. Lamins, as a part of the nuclear matrix, may be involved in these processes since chromatin binding sites have been detected in both A- and B-type lamins [21, 27]. Several putative binding sites have been recognized in the α -helical rod domain, as well as on the carboxyterminal tail [21, 72]. Recently, it was shown that histones constitute a link between chromatin and the lamin C carboxyterminus. However, direct lamin-DNA interactions have been described to occur as well. Both A- and B-type lamins have been shown to interact with matrix attachment regions (MARs), defined DNA sequences that bind to the nuclear matrix [39, 40]. In concordance with these observations several reports describe intranuclear foci of A- and B-type lamins [4, 30, 47], suggesting a role for lamins in intranuclear chromatin organization. Recently, Moir et al. [47] provided evidence that intranuclear foci of B-type lamins coincided with intranuclear localization of sites of DNA replication in S-phase cells, suggesting a role for B-type lamins in reorganization of chromatin during DNA duplication. In contrast, A-

type lamins were not colocalized with sites of DNA replication. The function of intranuclear A-type lamins may be correlated with chromatin rearrangements in interphase nuclei during G1 phase, since the intranuclear structures were most obvious at this stage of the cell cycle [4]. Further evidence for the interactions of lamins with replicating DNA has been obtained from studies with *Xenopus* oocytes, in which the lamina was not assembled and DNA replication was not initiated [23, 32].

Scope of the thesis

Earlier studies in our research group have shown that lamin expression in lung tumours is dependent on the subtype of cancer [6], its aggressiveness, proliferative capacity and degree of differentiation. Furthermore, an aberrant cytoplasmic localization of nuclear lamins was noted, in particular in adenocarcinomas of the lung. The studies described in this thesis aim at establishing (1) the differential expression patterns of lamins in normal human tissues and cells as well as in several other human tumour types, and (2) the molecular background of aberrant lamin expression in cancer cells by means of *in vitro* studies.

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Chapter 2

A- and B-type lamins are differentially expressed in normal human tissues

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Summary

A selection of normal human tissues was investigated for the presence of lamins B1, B2 and A-type lamins, using a panel of antibodies specific for the individual lamin subtypes. By use of immunoprecipitation and two-dimensional immunoblotting techniques we demonstrated that these antibodies do not cross-react with other lamin subtypes and that a range of different phosphorylation isoforms is recognized by each antibody. The lamin B2 antibodies appeared to decorate the nuclear lamina in all tissues examined, except hepatocytes, in which very little lamin B2 expression was observed. In contrast to previous studies, which suggested the ubiquitous expression of lamin B1 in mammalian tissues, we show that lamin B1 is not as universally distributed throughout normal human tissues as was to be expected from previous studies. Muscle and connective tissues are negative, while in epithelial cells lamin B1 seemed to be preferentially detected in proliferating cells. These results correspond well with those obtained for lamin B1 in chicken tissues. The expression of A-type lamins is most prominent in well-differentiated epithelial cells. Relatively undifferentiated and proliferating cells in epithelia showed a clearly reduced expression of A-type lamins. Furthermore, most cells of neuroendocrine origin as well as most hematopoietic cells were negative for A-type lamin antibodies.

Introduction

The nuclear lamina is a polymeric meshwork of proteins lining the inner nuclear membrane. The most prominent proteins within this structure are the nuclear lamins, which in mammals are subdivided into A- and B-type lamins. A-type lamins consist of lamin A, lamin A Δ 10, lamin C and lamin C2, arising by alternative splicing from the lamin A/C gene [17]. Lamins A and C share the first 566 amino acids and differ in their carboxy-terminal tail regions, which consist of 98 and 6 unique amino acids, respectively [9]. Lamin C2 is identical to lamin C except for the N-terminal segment, containing the head and the α -helical coil 1A domain, which is replaced by a short non- α -helical stretch of amino acids [11]. Lamin A Δ 10 is identical to lamin A, except for a stretch of 30 amino acids, encoded by exon 10 of the lamin A/C gene, which is absent [19]. In general it is assumed that lamins A and C are expressed in well-differentiated cells and tissues and can be absent in highly proliferating and/or poorly differentiated cells such as embryonic cells [8, 12, 23, 25, 26]. The presence of lamin C2 mRNA has only been demonstrated in (mouse) spermatocytes [11]. Lamin A Δ 10 mRNA is present in minor amounts in a variety of human tissues [19]. B-type lamins B1 and B2 are encoded by two different genes [2, 16, 24]. Lamin B3 occurs only in (mouse) spermatocytes and is an alternative splicing product of lamin B2 [10]. While it is generally accepted that lamin B1 is present in all somatic cells [13, 24], there is only little evidence to support this assumption, since most studies were performed with antibodies which do not differentiate between lamins B1 and B2 (see also [7]).

In this study we have investigated the tissue distribution of lamins using a panel of antibodies which can differentiate between lamin B1, lamin B2, lamin A and lamin A/C protein expression. We show that in particular the expression level of lamin B1 is highly variable and even absent in many tissues examined. Furthermore, lamin B1 expression seems to be inversely related to the expression of A-type lamins in epithelia, while lamin B2 shows a universal distribution pattern.

Materials and Methods

Tissues

The tissue samples used in this study were obtained at autopsies with post mortum delay of less than four hours. Specimens were snap-frozen in liquid nitrogen and stored until use.

Frozen 5-10 μm thick sections were cut, air-dried and fixed in methanol (-20°C for 1 minute) followed by acetone (4°C , 3 dips 5 seconds each).

Immunoperoxidase technique

The immunoperoxidase staining procedure has been described before [4, 5]. The following primary antibodies were used in this study:

1) monoclonal antibody LN43 (IgG1), directed against lamin B2 [3], kindly provided by Dr. E.B. Lane (Dundee, U.K.); 2) monoclonal antibody X223 (IgG1) directed against lamin B2 [13], kindly provided by Dr. G. Krohne (Würzburg, Germany); 3) monoclonal antibody 119D5F1 (IgG1) directed against lamin B1 [18]; 4) monoclonal antibody 41CC4 (IgM) kindly provided by Dr. G. Warren (Heidelberg, Germany) and recognizing human lamins A and C by immunoblotting [6]; 5) monoclonal antibody R27 (IgM), a gift from Dr. G. Krohne (Würzburg, Germany), recognizing only lamins A and C and not reacting with lamin B [31]; 6) mouse monoclonal antibody 133A2 recognizing lamin A and not lamin C [14]. In immunoperoxidase staining procedures LN43, X223, 41CC4 and R27 were used as undiluted culturing supernatant, while 119D5F1 (mouse ascites) was used 1:250 and 133A2 (mouse ascites) 1:1000. As secondary antibody rabbit anti mouse immunoglobulins conjugated to peroxidase (DAKO A/S, Glostrup, Denmark), diluted 1:100 was used. Peroxidase activity was detected with 3-amino-9-ethylcarbazole (Sigma, St. Louis, MO, USA) as a substrate. Nuclei were counterstained with hematoxylin and preparations were mounted in Kaiser's glycerol gelatin (Merck, Darmstadt, Germany).

Immunoprecipitation technique

Cells from the adenocarcinoma cell line GLC-A2, previously shown to abundantly express both A- and B-type lamins [18], were grown in 162 cm^2 flasks, washed twice with PBS, followed by the addition of 2 ml of lysis buffer, containing 140 mM NaCl, 20 mM Tris-HCl pH 8.0, 0.75% (w/v) sodium dodecyl sulphate (SDS, Merck, Darmstadt, Germany), 0.5% (w/v) sodium deoxycholate (Merck), 1% (v/v) Triton X-100 (BDH, Poole, England), 1 mM

phenylmethylsulfonyl fluoride (Aldrich, Steinheim, Germany), 10 µg/ml aprotinin (Sigma) and 12.5 µg/ml leupeptin (Sigma). In this solution cells were scraped from the bottom of the culture flask using a rubber policeman. The cell suspension was collected and put on ice for 30 minutes, while vortexing at regular intervals. The lysate was centrifuged for 10 minutes at 13000 g at 4°C and the supernatant was divided into 200 µl samples. To each of the samples one of the lamin antibodies was added (200 µl of culture supernatant or 5 µl ascites fluid) and the volume was adjusted to 1 ml with lysis buffer not containing Triton X-100. Antibodies were allowed to bind to lamins overnight at 4°C. Next, 5µl rabbit anti-mouse immunoglobulins (DAKO A/S, Glostrup, Denmark) was added and the mixture was incubated for 3 hours at 4°C. Thereafter, 100 µl of protein A Sepharose (30 µg/ml, Pharmacia, Uppsala, Sweden) was added and the suspension was incubated while rotating for 3 hours at 4°C. The complexes were pelleted at 13000 g for 10 minutes, washed with a buffer containing 140 mM NaCl, 20 mM Tris-HCl pH 8.0, 0.75% SDS and 1% Triton X-100, and solubilized in 30 µl of SDS-sample buffer by boiling for 5 minutes. Samples were analysed by gel electrophoresis followed by immunoblotting.

Gel electrophoresis and immunoblotting

One-dimensional SDS polyacrylamide-gel electrophoresis and two-dimensional gel electrophoresis were essentially performed as described [21]. Samples from cell lines were Triton X-100 extracted and treated with RNase\DNase as described for two-dimensional gel electrophoresis [18]. For the analysis of tissue samples twenty to thirty frozen sections (20 µm thick) were collected and extracted using the same procedure as used for the cell lines. Gels containing 10% acrylamide and 0.1% SDS were used for one-dimensional gel electrophoresis, while isoelectric focusing for two-dimensional gels was performed in 4% polyacrylamide gels containing 8M ureum, 2% Bio-Lyte 3/10 carrier ampholytes (Bio-Rad) and 25 mM 3-[(3-Cholamido-propyl)-dimethylammonio]-1-propanesulfonate (CHAPS, Bio-Rad). Immunoblotting was performed essentially according to Towbin [28], using the Mini Trans-Blot Cell (Bio-Rad) at 100 V for 1 hour. Proteins were blotted onto nitrocellulose sheets (BA85, Schleicher and Schüll, Dassel, Germany). Immunochemical detection was performed as described [18] using an enhanced chemiluminescence western blotting detection kit (ECL, Amersham, Buckinghamshire, England).

Results

Lamin subtype specificity of the antibodies

To confirm the immunospecificity and to exclude mutual cross-reactivity of the antibodies used in this study, immunoprecipitation assays were performed with extracts of cell line GLC-A2, which was previously shown to contain lamins B1, B2, and lamins A and C [18]. Figure 1, Panel A shows that when lamin B1 was immunoprecipitated with antibody 119D5F1, this protein was only recognized by this antibody in immunoblots of these preparations, and not by the

antibodies to lamin B2, lamin A or antibodies to lamins A/C (panel A). Similarly, when lamin B2 was immunoprecipitated from this cell line with LN43 (panel B) or X223 (not shown), only the antibodies to lamin B2 and not the antibodies to lamin B1, A, or A/C were able to recognize the precipitated lamin B2. After immunoprecipitation with the lamin A antibody 133A2 (panel C), the captured lamins showed no cross-reactivity with the lamin B1 or lamin B2 antibodies. When the lamin A antibody or the lamin A/C antibodies were applied to the immunoblot, a single band was observed at the lamin A level. These results confirm that the lamin antibodies used in this study do exhibit the expected lamin subtype specificity.

Figure 1. Lysates of cell line GLC-A2 were immunoprecipitated with the lamin B1 antibody 119D5F1 (panel A), the lamin B2 antibody LN43 (panel B) and the lamin A antibody 133A2 (panel C). The blots were incubated with antibodies 119D5F1 (lanes 1), the lamin B2 antibody LN43 (lanes 2), the antibody 133A2 directed against lamin A (lanes 3), the lamin A/C antibody 41CC4 (lanes 4), or the lamin A/C antibody R27 (lanes 5). The immunoprecipitated heavy and light chains of immunoglobulins recognized by the secondary antibody are indicated by h and l, respectively.

Since only one lamin B1 antibody was available for our immunohistochemical studies it was of importance to show that this reagent recognizes the non-phosphorylated as well as different phosphorylated states of lamin B1. Two-dimensional immunoblotting shows that at least four different phosphorylation isoforms of lamin B1 are recognized by this antibody (Fig. 2). In addition, different phosphorylation states of the 45 kDa fragment, a well-recognized degradation fragment of lamin B1 [22], are observed (asterisk). Moreover, we observed a prominent staining of the lamin B1 antibody with lamina aggregates in mitotic figures of cultured cells (not shown)

indicating that hyperphosphorylated lamins are recognized by this antibody. Conversely, bacterially expressed recombinant lamins B1, which are unphosphorylated, are also positive with the antibody used (data not shown).

Figure 2. Two-dimensional immunoblots of an extract from cell line GLC-A2 using the lamin B1 antibody 119D5F1. Note that at least four different phosphorylation isoforms of both lamin B1 and its 45 kDa breakdown product (asterisk) are recognized.

Distribution of lamin subtypes in tissue and cell types

The results of the immunostaining reactions in normal human tissues are outlined in Table 1 and illustrated in figures 3 to 5. The subcellular localization of the staining reactions observed with all lamin antibodies was in general restricted to the nuclear periphery.

Skin

The basal cells of skin epidermis show a strong expression of lamin B2, while the expression of A-type lamins and lamin B1 is reduced or absent. In the parabasal cells A-type lamins are rather weakly expressed, while lamin B1 shows a strong staining. In the prickle cell layer the expression of lamin B1 decreases, while the expression level of the A-type lamins increases considerably. Finally, in the granular layer expression of all lamins appears to be lost in many cells. In the underlying connective tissue lamin B2 is recognized in all cells, while stromal fibroblasts, but not lymphoid infiltrates in the stroma are positive with the A-type lamin antibodies. In contrast, the lamin B1 antibody reacts only with some lymphoid cells but not with stromal fibroblasts. Endothelial cells present in the stromal layer are strongly positive for lamin B2, lamins A and C, but negative with the lamin B1 antibody. Since this staining pattern was observed in all endothelial cells, we will not further mention the staining of these cells in other tissues.

Digestive tract

In tongue (Fig. 3a-c) and epiglottis (not shown) similar staining patterns were observed. In tongue all epithelial and stromal cells are positive with the lamin B2 antibody (Fig. 3a), while the lamin B1 antibody predominantly decorates parabasal cells and the lower layers of prickle cells (Fig. 3b). In the underlying connective tissue only occasional lymphoid cells are positive.

A-type lamins are only very weakly expressed in the basal and parabasal epithelial layers, while the prickle cell layer is strongly positive (Fig. 3c). In epithelium of the submandibular gland all acinar and ductal cells are positive for all lamin antibodies. In stomach the epithelial lining is positive with all lamin antibodies, except for gastric gland cells, which appear to be negative for lamin B1.

In small intestine all cells are recognized by the lamin B2 antibody, while the antibody to lamin B1 is positive in most epithelial cells and negative in most stromal cells. A-type lamins are present in all epithelial as well as most of the stromal cells. In colon and appendix all crypt cells are strongly positive with the lamin B1 and B2 antibodies, while the lamin B1 antibody is negative in fibroblasts. Lymphocytes in connective tissue are clearly positive. A-type lamins are present in all epithelial cells as well as most stromal cells. However, lymphoid cells of the B-cell lineage such as plasma cells appear to be negative for A-type lamin antibodies.

In liver the reduced expression of lamin B2 (Fig. 4a) and lamin B1 (not shown) in hepatocytes is very striking. Both B-type lamins are only weakly expressed in some of the cells. In contrast, Kupffer cells and bile duct epithelium are positive with B-type lamin antibodies. The lamin A/C antibodies react weaker with hepatocytes than the lamin A antibody, while the reactivity with bile duct epithelial cells is similar.

In pancreas acini both lamin B2 and A-type lamins are present, while the expression of lamin B1 has a rather heterogeneous appearance (Fig. 4b).

Respiratory tract

In non-neoplastic bronchi lamin B2 appeared to be present in all cases in virtually all epithelial and non-epithelial cells in a rather homogeneous fashion (Fig. 3d). The localization and expression level of lamin B1, however, varied considerably. The most commonly observed pattern was staining of the bronchial basal cells and not of the columnar cells (Fig. 3e). A similar heterogeneity was observed with the A-type lamin antibodies. In general, the antibodies to lamins A/C showed a predominant staining in columnar cells and not in basal cells of the bronchus (Fig. 3f). However, in several sections lamins A/C were observed throughout the whole bronchial epithelium. Alveolar pneumocytes were positive for the lamin B2 and the A-type lamin antibodies, while lamin B1 seems to be very heterogeneously expressed. The epithelium of sero-mucous glands is positive for the lamin B2 and the A-type lamin antibodies, and only partly positive for the lamin B1 antibody.

Urinary tract

In human kidney the lamin B2 antibodies stain all epithelial and connective tissue cells, with the epithelium of proximal and distal tubules showing weaker reactivity, as compared to the surrounding tissues (Fig. 3g). Lamin B1 is detected as a faint staining reaction in most cells of the proximal and distal tubules (Fig. 3h), and more prominently expressed in Henle's loops. Strikingly, lamin B1 is completely absent in both the epithelial and endothelial cells of the

Figure 3. Immunoperoxidase staining of tongue (A-C), bronchus epithelium (D-F), kidney (G-I), and prostate (J-L) using antibodies to lamin B2 (A,D,G,J), lamin B1 (B,E, H,K), lamins A/C (C,F, I) and lamin A (L). Note immunoreactivity in all nuclei with the lamin B2 antibody LN43 (A,G) and X223 (D,J). In tongue epithelium staining of lamin B1 is most pronounced in the parabasal cell layer (B), while lamins A/C, stained with antibody 41CC4, are most clearly present in the prickle cell layer and much weaker in the (para)basal cells (C). In bronchial epithelium the lamin B1 antibody reacts with most basal cells and only few columnar cells (E), while the lamin A/C antibody R27 reacts predominantly with columnar cells and much weaker with basal cells (F). In kidney, both the lamin B2 antibody LN43 (G) and the lamin B1 (H) antibody react rather weakly with some nuclei in the proximal tubules, while nuclei of distal tubules are clearly positive. The lamin A antibody 133A2 strongly stains all tubular epithelium cells of kidney (I). In prostate the lamins B1 antibody stains only part of the basal epithelial cells and not any luminal cells nor surrounding muscle cells (K). In contrast the lamin A antibody stains all epithelial cells as well as surrounding muscle cell nuclei (L). Bar represents 80 μ m in A, B and C; 50 μ m in D,F-L; and 70 μ m in E.

Figure 4. Immunoperoxidase staining of liver (A), pancreas (B), parathyroid (C) and vagina epithelium (D-F), using the lamin B2 antibody LN43 (A, D), the lamin B1 antibody (B,E) the lamin A antibody (C) and the lamin A/C antibody 41CC4 (F). Note the very weak staining of hepatocytes as compared to bile duct cells using the lamin B2 antibody (A). In pancreas lamin B1 staining shows large variation in intensities between different acinar cells (B). Parathyroid cells are only very weakly stained with the lamin A antibody, while surrounding stromal and vascular cells are strongly positive (C). In vagina (D-F), all epithelial and stromal nuclei are stained with the lamin B2 antibody (D). In contrast, the lamin B1 antibody stains few basal cells and all parabasal cells. Staining intensity is clearly decreased in the upper intermediate layer and superficial layer of epithelium (E). Lamins A/C are (very) weakly expressed in basal cells and staining intensity increases with level of differentiation (F). Bar represents 50 μm. For colour illustrations see page 130.

glomeruli. Lamins A/C are present in all cells of the kidney, with a pronounced staining in distal and collecting tubules (Fig. 3i), and a somewhat reduced expression in the proximal tubules. In the urinary bladder the transitional epithelium is negative with the lamin B1 antibody. All other antibodies show a clear staining reaction in all epithelial cell layers.

Male reproductive tract

In the prostate antibodies to lamin B2 react positively in all epithelial as well as non-epithelial cells (Fig. 3j). Using the lamin B1 antibody the absence of staining in the luminal cells of the prostate gland is remarkable (Fig. 3k). In addition, not all of the basal cells seem to be positive. The antibodies to lamins A/C show a heterogeneous reactivity. While both basal and luminal cells are positive, the intensity of staining seems to be weaker than in surrounding smooth muscle cells (Fig. 3l).

Female reproductive tract tissues.

Vagina epithelium show staining patterns identical to the other non-keratinizing squamous cell epithelia, i.e. the presence of lamin B2 in all epithelial layers (Fig. 4d) and the most prominent staining of lamin B1 in the proliferative parabasal cell layer (Fig. 4e). Lamin A/C antibodies are most prominently expressed in layers of highly differentiated cells of the prickle cell layer (Fig. 4f). Interestingly, in the superficial layer, containing many apoptotic cells, the detectability of all lamins decreases considerably. In the vaginal connective tissue cells expression of lamin B2 is constantly high, while lamin B1 is absent in fibroblasts. The expression of lamins A/C in connective tissue cells is variable.

In normal ectocervical stratified epithelium lamin B2 is expressed in all cell layers (Fig. 5a). In contrast to other non-keratinizing stratified squamous cell epithelia, lamin B1 is most prominently expressed both in the basal and in the parabasal epithelial cells (Fig. 5b), with a reduction of staining in the upper cell layers. Also in contrast to other stratified epithelia, lamins A/C antibodies show prominent staining of the entire epithelium (Fig. 5c).

More than 30 cases of cervical intra-epithelial neoplasia (CIN) were examined for their lamin expression patterns. In both low and high grade CIN lesions the normal differentiation of the squamous epithelium is largely lost. While lamin staining patterns are similar in either lesion type, a general decrease in lamin expression is noticeable in high grade CIN lesions. Lamin B2 is present in most epithelial cells (Fig. 5d). Lamin B1 expression seems to remain confined to the lower layers of this epithelium (Fig. 5e), while A-type lamins are expressed heterogeneously throughout all layers of the epithelium (Fig. 5f).

In endocervical columnar epithelium most cells are positive with the antibodies examined, except for the reserve cells which are largely negative for lamin B1. In connective tissue cells of the endocervix all antibodies are positive except for the lamin B1 antibody, which is negative in the fibroblasts, but positive with most lymphoid cells.

Figure 5. Immunoperoxidase staining of ectocervix (A-C), cervical intra-epithelial neoplasia (CIN, D-F), endometrium (G-I), and fallopian tube (J-L), using antibody LN43 to lamin B2 (A,D,J), the antibody to lamin B1 (B,E, G,H, K), lamins A/C antibody R27 (C,F) and the lamin A antibody (I,L). Normal ectocervix is positive in all cells with the lamin B2 antibody (A), while lamin B1 expression is confined to the lower layers of the epithelial cells (B). Lamins A/C are expressed throughout the ectocervical epithelium (C). In a high grade CIN lesion lamin B2 shows expression in all epithelia cells (D), while lamin B1 is mainly expressed in lower epithelial compartment (E). Lamins A/C are only present in scattered cells throughout this epithelium (F). In endometrium lamin B1 is expressed in cells of both proliferating (G) and secretory (H) epithelial cells. Strikingly, stromal cells of proliferating endometrium are strongly positive, while these cells are barely stained in secretory endometrium (compare G and H). Lamin A can be stained in all nuclei of stromal and epithelial endometrium cells (I). In Fallopian tube all nuclei are stained with the lamin B2 antibody (J), while lamin B1 is only present in part of the columnar epithelial cells (K). The lamin A antibody stains most, but not all columnar cells next to all stromal cells (L). Bar represents 110 μ m in A; 100 μ m in B, D and E; 90 μ m in C and F; 70 μ m in G, H, J, K and L; and 50 μ m in I.

We have examined endometrium tissue specimens from different stages of the menstrual cycle. In all cases examined the lamin B2 antibodies recognize virtually all nuclei. In myometrium most cells are strongly positive for lamin B2. Lamin B1 appears to be present in all epithelial cells, but not in all stromal cells of endometrium. The stromal cells are mostly positive in the proliferation phase (Fig. 5g). In contrast, in late secretory phase and in inactive endometrium there is barely any staining with the lamin B1 antibody in the stromal cells (Fig. 5h). In myometrium no staining of lamin B1 is observed. In the basal layer of endometrium the expression of A-type lamins is generally stronger than in the functional layer, especially in endometrium in the proliferative phase. However, expression of lamins A/C and of lamin A is visible in epithelial cells of both proliferative and secretory endometrium (Fig. 5i). In proliferating endometrium the expression of A-type lamins, especially using the lamin A antibody, appears to be reduced in the epithelial cells as compared to the surrounding stromal tissues.

In the fallopian tube the lamin B2 antibodies react strongly with the epithelial lining (Fig. 5j). The antibody to lamin B1 appears to recognize the intercalated cells and only a part of the ciliated and secretory cells (Fig. 5k). In contrast, the lamins A/C antibodies do not recognize the intercalated cells while most ciliated and secretory cells are positive . The antibody to lamin A seems also to recognize some intercalated cells next to the ciliated/secretory cells (Fig. 5l). In ovary both lamin B2 and A-type lamins are expressed in all stromal cells. No staining is observed with the lamin B1 antibody in stromal cells of the ovary.

Endocrine tissues

We investigated some endocrine tissues which were previously reported to be negative for A-type lamin antibodies. In thyroid lamin B2 and A-type lamins are present in all cells, while lamin B1 is absent. The staining of lymphocytes in these samples is however very obvious using the lamin B1 antibody. In parathyroid lamin B2 is again clearly positive, while both lamin B1 and A-type lamins (Fig. 4c) are negative in the endocrine cells of this organ. Also in adrenal gland both lamin B1 and A-type lamins are negative in most endocrine cells.

Muscle tissues

In striated skeletal muscle and in myocard lamin B2 and A-type lamins were expressed in most or all cells. In contrast, the expression of lamin B1 seems to be absent in both types of muscle cells (see also [1]). Also in several smooth muscle cells studied in several organs lamin B1 seems to absent.

Lymphoid tissues

In spleen lamins B2 and B1 are evident in all cells, while B-type lymphocytes in the white pulp are negative for A-type lamins. The expression of lamins in lymph nodes will be described in chapter 4 of this thesis.

Absence of lamin B1 staining in several tissues is not caused by epitope masking

We have performed immunoblotting experiments of myometrium and endometrium in order to confirm our immunoperoxidase staining data. These tissues were chosen because of the observation that in the immunoperoxidase technique myometrial cells are not stained, while in endometrium all epithelial cells are positive with the lamin B1 antibody. Figure 6 shows immunoblots incubated with the lamin B1 antibody (lanes 1 and 2). In endometrium a discrete band at the lamin B1 level is present (arrow), while in myometrium no band is visible. Only after prolonged exposure of the immunoblot a very faint band became visible in myometrium (not shown). For comparison of the amounts of protein loaded onto the gel lanes, a parallel blot was incubated with a lamin B2 antibody, showing no obvious differences in staining intensities (lanes 3 and 4).

Figure 6. Immunoblotting of an extract of myometrium (lanes 1 and 3) and endometrium (lanes 2 and 4) using the lamin B1 antibody 119D5F1 (lanes 1 and 2) and the lamin B2 antibody LN43 (lanes 3 and 4). Note the presence of lamin B1 (arrow) in endometrium and not in myometrium. Based on the similar reactivity of the lamin B2 antibody (compare lanes 3 and 4) and the accompanying Coomassie stained gel (not shown) we conclude that the protein amounts loaded onto the lanes were similar.

Table 1: Immunoperoxidase staining patterns of human tissues with lamin antibodies

	Lamin B2	Lamin B1	Lamin A/C	Lamin A
<i>Skin</i>				
Epidermis				
Basal cells	++	±	±	+/-
Parabasal cells	++	++	±	+/-
Prickle cell layer	++	+	++	++
Granular layer	++/-	+/-	++/-	++/-
Stromal fibroblasts	++	-	++	++
Endothelial cells	++	-	+	+
<i>Digestive tract</i>				
Tongue and epiglottis				
Basal cells	++	-	±	+/-
Parabasal cells	++	++	±	+/-
Prickle cell layer	++	++	++	++
Granular layer	++	+/-	++	++
Submandibular glands				
Acini	+	+	+	+
Ducts	++	+	+	+
Stomach corpus				
Surface epithelium	++	++	++	++
Gastric gland cells	++	-	++	++
Small intestine				
Crypt cells	+	++	++/-	+/-
Colon				
Crypt cells	++	++	+/-	+/-
Fibroblast	++	-	++	++
Stromal lymphoid cells	++	++	+/-	+/-
Liver				
Hepatocytes	±/-	+/-	+/-	+
Kupffer cells	+	+	+	+
Sinusoidal endoth.	+	+	+	+
cells	++	+/±	+	+
Bile duct epithelium				
Pancreas				
Acinar cells	++	++/±	++	++

Table 1 (continued)

	Lamin B2	Lamin B1	Lamin A/C	Lamin A
<i>Respiratory tract</i>				
Bronchus				
Basal cells	++	++	+/-	+/-
Columnar cells	++	+/-	++	++
Lung alveoli				
Pneumocytes	++	+/-	++	++
Stromal cells	++	+/-	+	+
<i>Urinary tract</i>				
Kidney				
Prox. tubules	+	+/-	+	+
Distal tubules	+	+/-	++	+
Loops of Henle	++	+	+	+
Glomeruli				
Epithelium	++	-	++	++
Endothelium	++	-	++	++
Collecting tubules	++	+/-	+	+
Juxtaglom. apparatus	++	N.D.	+	+
Urinary bladder				
Urothelium	++	-	+	++
Fibroblasts	++	-	++	++
Smooth muscle	++/-	-	+/-	+/-
<i>Male reproduct. tract</i>				
Prostate				
Basal ep. cells	++	++/-	++/-	++/-
Luminal cells	++	-	+/-	+/-

Table 1 (continued)

	Lamin B2	Lamin B1	Lamin A/C	Lamin A
<i>Female reproduct.tissue</i>				
Vagina				
Epithelium				
Basal layer	++	±	-	±
Parabasal layer	++	++	+/-	+
Intermediate layer	++	++	++	++
Superficial layer	+/-	+/-	+/-	+/-
Fibroblasts	++	-	+/-	+/-
Endocervix				
Columnar epithelium	++	++	++	++
Reserve cells	+	+/-	+	+
Fibroblasts	++	-	++	++
Endothelial cells	++	-	+	++
Ectocervix				
Normal epithelium				
Basal layer	++	++	+	+
Parabasal layer	++	+	+	+
Intermediate layer	++	+/-	++	++
Superficial layer	+/-	-	+/-	++
Low-grade CIN				
Basal compartment	++	++	+	++
Intermediate comp.	++	-	+	++
Superficial comp.	++	-	+	++
High-grade CIN				
Basal compartment	++	++	+	+
Intermediate comp.	+	+	±	±
Superficial comp.	±	±	±	±
Uterus				
Endometrium				
Basal part				
Column. epithelium	++	++	++	+
Stroma	++	±/-	++	++
Functional layer				
Column. epithelium	++	++	+	+/-
Stroma	++	++/-	++/±	++/±
Myometrium	++/-	-	+	+

Table 1 (continued)

	Lamin B2	Lamin B1	Lamin A/C	Lamin A
Fallopian tube				
Epithelium				
Ciliated/secr cells	++	+/-	++	+
Intercalated cells	++	++	-	+/-
Fibroblasts	++	-	++	++
Smooth muscle	++	-	+/++	++
Ovary				
Stromal cells	++	-	+	+
Smooth muscle	++	-	±	+
Muscle				
Skeletal muscle	++	-	+	+
Heart				
Muscle cells	+	-	+/-	±
Interstitial fibroblasts	+	-	+	+
Smooth muscle	++	-	+	+
Immune system				
Spleen				
White pulp	++	++	-	-
Red pulp	++	++	++	++
Neuroendocrine tissues				
Thyroid foll. epithelium.	++	-	++	++
Parathyroid gland cells	++	-	+/-	+/-
Adrenal gland cells	++	-	±	+/-

++ strong reactivity in all cell
 + positive staining in most cells
 ++/- or +/- positive staining in part of the cells
 ± weak staining in part of the cells
 - no staining in any cells
 N.D. not determined

Discussion

In this paper we describe the differential expression of the nuclear lamins in a variety of normal human tissues. The study was performed using a panel of well-defined monoclonal antibodies, which not only differentiate between lamins B1 and B2, but can also recognize specifically lamin A, in addition to antibodies which recognize both lamins A and C. Most of the previous studies to categorize the expression levels of lamins in normal tissues have been performed in animal tissues of several species [15, 25, 29] or used antibodies with unknown specificity for the B-type lamins (see e.g. [7]). As a result, the exact tissue distribution of lamins in human tissues remained to be clarified.

Our immunoprecipitation studies combined with the two-dimensional gel electrophoresis experiments show that antibodies used in this study are indeed specific for the individual lamin subtypes.

Differential distribution of nuclear lamins in human tissues

To generalize the lamin subtype distribution patterns, we have summarized our findings according to cell type (Table 2).

The lamin B2 antibodies were positive in all cell types in all tissues examined, except for hepatocytes, which were partly negative.

Lamin B1 expression was much less ubiquitous as compared to lamin B2. In non-keratinizing squamous epithelia lamin B1 was mainly present in parabasal cells, while the staining intensity decreased in more differentiated epithelial layers. In pseudostratified epithelia basal cells were positive, while columnar cells were only partly positive. In simple columnar epithelia most cells were positive, while in columnar glands the absence of lamin B1 in most cells was striking. Lamin B1 appeared to be absent in almost all non-epithelial cell types, except for part of the hematopoietic cells types and stromal cells of proliferating endometrium, which were strongly positive.

The expression of A-type lamins in stratified squamous epithelia was most abundant in layers of highly differentiated cells, while basal as well as parabasal cells were in general negative to weakly positive. In pseudostratified columnar epithelia in particular antibodies recognizing both lamin A/C showed a reduced staining reaction in the basal cells. This reduction was less pronounced with the lamin A antibody. Most columnar cells were positive. In other columnar epithelia A-type lamins were positive in almost all epithelial cells, with the exception of reserve cells which were occasionally negative. In other tissues A-type lamins were positive in connective tissue fibroblasts and muscle cells, although the staining intensity in cardiac muscle cells was relatively weak. Several types of blood cells, including lymphocytes in inflammatory infiltrates and cells of white pulp in spleen, were negative. In addition, A-type lamins were absent in most neuroendocrine cells examined.

Table 2: Lamin subtype distribution according to cell type

	Lamin B2	Lamin B1	Lamins A/C
Stratified squamous epithelia			
basal cells	++	-	-
parabasal cells	++	++	+
intermediate cells	++	+	++
superficial cells	+/-	+/-	+/-
Pseudostratified columnar epithelia			
basal cells	++	+	+/-
columnar cells	++	+/-	+
Simple columnar epithelial cells	++	+/-	+/-
Columnar glands			
basal cells	++	++/-	++/-
columnar cells	++	-	+/-
Transitional epithelia	++	+	+
Hepatocytes	+/-	+/-	+
Connective tissues fibroblasts	++	-	++
Smooth muscle	++	-	++
Skeletal/cardiac muscle	++	-	+
Neuroendocrine cells	++	-	±
Endothelial cells	++	-	++
Inflammatory cells	++	++	-
++	strong reactivity in all cell		
+	positive staining in most cells		
++/-			
or +/-	positive staining in part of the cells		
±	weak staining in part of the cells		
-	no staining in any cells		

Expression of lamin B1 is much more restricted than lamin B2

Our study shows that of the B-type lamins, lamin B2 and not lamin B1 is extensively expressed in human tissues. The presence of two distinct B-types lamins was not generally recognized until 1990 [13, 25]. Later studies have shown that both B-type lamins are products of different genes [2, 24] located on different chromosomes [30]. Previous studies in chicken tissues have shown that lamin B2 and not lamin B1 is the main lamin in this species [15]. Studies in other animals also indicate differences between B-type lamin isoform expression (see e.g. [25]), but

from these studies it is not clear whether the antibodies used differentiate between lamins B1 and B2, or between different phosphorylation isoforms of either lamin. The underlying study revealed similar lamin distribution patterns in human tissues as compared to chicken, in that lamin B1 is absent in human muscle cells (see also [1]), fibroblasts and endothelial cells. Moreover our study shows that lamin B1 expression is reduced or absent in epithelial cells with a high level of differentiation.

Our observation that basal cells of squamous epithelia, which in general serve as stem cells and do not proliferate, are negative for lamin B1 and the observation that parabasal cells, which show proliferation as based on Ki-67 expression [27], are strongly positive with the lamin B1 antibody, suggest that lamin B1 expression is correlated with proliferation in some cell types. This correlation has also been suggested in a study, which showed that reactive regions of lymph nodules express more lamin B1 than lamin B2. Furthermore, when normal peripheral blood mononuclear cells were stimulated to proliferate a dramatic increase in lamin B1 mRNA expression was noticed [20]. However, such a correlation does not hold true for the CIN lesions examined. In high grade CIN proliferation is higher than in low grade CIN [27] and is not confined to the lower compartment of the epithelial cells. Surprisingly, lamin B1 expression does remain confined to this lower compartment. Possibly the high incidence of human papilloma virus (HPV) infection in especially high grade CIN lesions can cause alterations in lamin expression. This suggestion is currently under investigation. Another indication that lamin B1 expression is not a universal proliferation marker is found in cells which have ceased to proliferate, such as secretory phase endometrium cells, in which the expression of lamin B1 seems to be maintained. The negative reaction of the lamin B1 antibody in several tissues examined is not due to the lack of cross-reactivity of this antibody with different phosphorylation states of its antigen, since our studies have shown that the complete scale from unphosphorylated up to hyperphosphorylated lamin B1 is recognized by the antibody used. Therefore, we cannot sustain previous findings which suggest that observed differences in B-type lamin expression are due to differences in cross-reactivity of antibodies with different phosphorylation states of these proteins [29]. Also masking of the lamin B1 epitope in tissues sections has been excluded since denatured protein samples of myometrium, showing no staining in frozen tissue sections, also showed no immunoreactivity after immunoblotting. The general assumption that lamin B1 is present in all tissues (see also [16]) may be due to the use of antibodies that do not completely differentiate between lamin B1 and lamin B2. Our two-dimensional immunoblots have revealed that the different phosphorylation forms of (human) lamin B1 do not completely separate from lamin B2 isoforms (not shown). Therefore, if conclusions are to be drawn about the specificity of lamin antibodies next to two-dimensional gel electrophoresis additional experiments, such as immunoprecipitation should always be performed to exclude cross-reactivity. Using two different antibodies to lamin B2 we have shown that lamin B2 is expressed in large amounts in nearly all cells examined.

A-type lamins are related with the commitment to a differentiation lineage

There is general consensus regarding the tissue distribution of A-type lamins. Several studies reported the absence or reduced expression of A-type lamins in developing embryonic tissues of mouse [26], and chicken [15]. In adult mouse tissue A-type lamins are absent from spleen, bone marrow and thymus [26], while in adult human tissues a variety of neural and neuroendocrine cells showed no A-type lamins expression [7]. These findings were confirmed in our study. The reduced expression of A-type lamins in proliferating cells as previously suggested [7, 8] was confirmed using three different A-type lamin antibodies. However, a general correlation between absence of cellular proliferation and increased A-type lamin expression can not be sustained, since cells from many tissues, known to be quiescent, do not stain with any A-type lamin antibody examined.

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Chapter 3

Nuclear lamin expression in normal testis and testicular germ cell tumours of adolescents and adults

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Summary

Nuclear A- and B-type lamins are differentially expressed in tissues, depending on the degree of cellular differentiation and proliferative status. By studying lamin expression in testis parenchyma and testicular germ cell tumours, further insight may be gained into the degree of cellular differentiation in normal testis and into the whole spectrum of differentiation lineages found in testicular germ cell tumours. Frozen tissue sections of normal testis and the different types of testicular germ cell tumours were immunostained with monoclonal antibodies to distinct lamin subtypes. Lamin reactivity was evaluated in relation to the lineage and degree of cellular differentiation and the reactivity patterns were compared to each other and to those in normal testis. In normal testis, both A- and B-type lamins were expressed in Sertoli, Leydig and peritubular cells, while in spermatogonia only B-type lamins were found and spermatocytes showed weak reactivity with the A-type lamin antibodies. Carcinoma in situ was most often positive for both of the B-type lamins and negative for the A-type lamins (lamins A and C). In testicular germ cell tumours, B-type lamins were always expressed, while A-type lamins were differentially expressed. Differentiated nonseminomas were positive for both of the A-type lamins, whereas embryonal carcinomas were positive for lamin C and negative for lamin A. Seminomas were negative for both of the A-type lamins, with the exception of seminomas containing a Ras mutation. Spermatogonia and seminoma cells, which follow a differentiation pathway along the spermatogenic lineage and show characteristics of germ cells, do not express A-type lamins. Nonseminomas, showing embryonal or extra-embryonal differentiation, express A-type lamins to varying degrees, distinguishing embryonal carcinoma cells from other nonseminomatous components. This may aid in the evaluation of the percentage of embryonal carcinoma in nonseminomatous testicular germ cell tumours as a prognostic parameter.

Introduction

The incidence of human testicular germ cell tumours (TGCT) of adolescents and adults, the most common type of cancer in young men [38], has more than doubled in most western countries over the last forty years [5]. TGCT present a complex histopathology, in which two major, clinically relevant entities can be distinguished; seminomas (SE) and nonseminomas (NS) [23]. The SE are composed of malignant counterparts of early (primordial) germ cells. The NS are histologically subdivided into embryonal carcinoma (EC), teratoma (TE), yolk sac tumour (YS) and choriocarcinoma (CH), occurring either in a pure form or intermixed. The EC cells are now considered to be the stem cells from which the other NS-components originate [1]. Histogenesis within TGCT thus represents a caricature of cell differentiation during early embryogenesis [31]. In spite of the histological heterogeneity of TGCT, they all develop via carcinoma in situ (CIS) [36], which is most probably initiated during early embryonic life [16].

No clear cut differences between CIS and SE have been reported, except for the invasive behaviour of the latter [31].

Studying differentiation antigens may provide further insight into normal germ cell development, as well as into different derivatives of malignant transformed early germ cells, as represented by the whole spectrum of differentiation lineages found in testicular germ cell tumours. For example, intermediate filament proteins are markers for distinct tissue types and the tumours originating therefrom [34]. Lamins are intermediate filament-type proteins that form a major component of the nuclear lamina, a filamentous meshwork associated with the inner nuclear membrane [22, 26]. Two main types of lamins can be distinguished, A-type and B-type lamins. A-type lamins consist of lamins A and C, two products of the same gene, generated by alternative splicing [19]. A-type lamins are differentially expressed in mammalian tissues, depending on the degree of differentiation or proliferative activity of the cells. Undifferentiated and highly proliferative cells usually lack A-type lamin expression. B-type lamins consist of lamins B1 and B2, products of two distinct genes [3, 32]. B-type lamins are considered to be expressed constitutively and independently of cellular differentiation.

The lamin expression patterns in TGCT were compared to the expression patterns in normal testis. Furthermore, by studying nuclear lamin expression in preinvasive and invasive TGCT, we have gained further insight into the relationship between the different histological components of this tumour type.

Materials and Methods

Antibodies

The following mouse monoclonal antibodies were used in this study:

- LN43 (IgG1), directed against lamin B2 and not cross-reacting with lamin B1 [6], kindly provided by Dr. E.B. Lane (Dundee, U.K.).
- X223 (IgG1), directed against lamin B2 and not cross-reacting with lamin B1 [13], kindly provided by Dr. G. Krohne (Heidelberg, Germany).
- 119D5 (IgG1), directed against lamin B1 and not cross-reacting with lamin B2 [20], kindly provided by Dr. Y. Raymond (Montréal, Canada).
- 41CC4 (IgM), recognizing human lamins A and C in immunoblotting [7], kindly provided by Dr. G. Warren (Heidelberg, Germany).
- R27 (IgM), recognizing both lamins A and C and not reacting with human B-type lamins [40], a gift from Dr. G. Krohne (Heidelberg, Germany).
- 133A2 (IgG3), raised against the carboxy-terminus of 98 amino acids present in lamin A and absent from lamin C. As a result this antibody recognizes lamin A but not lamin C. Epitope mapping using several deletion mutants of lamin A showed that the amino acids 598-611 form the epitope recognized by 133A2 [15]. The antibody was kindly provided by Dr. Y. Raymond (Montréal, Canada).

In immunostaining and immunoblotting studies LN43, X223, 41CC4 and R27 were used as undiluted culturing supernatant, while 119D5 (mouse ascites) was used in a 1:750 dilution for immunostaining of sections and 1:250 in immunoblotting. Antibody 133A2 (mouse ascites) was diluted 1:1000 for immunostaining of sections and for immunoblotting.

The antibodies 41CC4 and R27 both recognize lamin A and C and will be referred to as lamin A/C antibodies unless otherwise specified. Since the antibodies LN43 and X223 both recognize lamin B2 they will be referred to as lamin B2 antibodies unless otherwise specified. 133A2 will be referred to as lamin A antibody and 119D5 will be referred to as lamin B1 antibody.

Testicular germ cell tumours

Thirty-two orchidectomy specimens for germ cell tumour were collected and representative parts of both the tumour and the adjacent parenchyma (when available) were snap frozen in liquid nitrogen. Tumours were classified according to the World Health Organization recommendations [23] as described [30]. Sixteen cases were diagnosed as SE and another 16 as NS (seven pure and nine mixed). Six of 16 SE contained a mutated Ras gene [28]. Four samples of normal testicular parenchyma (two cases unrelated to TGCT and two orchidectomy specimens because of prostatic carcinoma) were included in this survey, as well as 10 samples of parenchyma adjacent to SE and 12 adjacent to NS.

Immunohistochemistry

Frozen tissue sections (5 μ m) were air dried at room temperature (RT) for 1 hour. Subsequently they were fixed in cold (-20°C) methanol for 15 seconds followed by three dips in acetone (RT) and air dried for 5 minutes. Endogenous peroxidase activity was inactivated by incubation for 10 minutes with 0.15 M phosphate buffered saline (PBS; containing 8 g/l NaCl, 0.2 g/l KCl, 1.15 g/l Na_2HPO_2 and 0.2 g/l KH_2PO_4 at pH 7.4) containing 0.2% (w/v) bovine serum albumin (BSA; Sigma, St Louis, MO/USA) and 0.3% H_2O_2 . Then the sections were washed twice with PBS for 5 minutes and subsequently pre-absorbed for 10 minutes with PBS containing 1% BSA. The sections were washed with PBS and incubated for 1 hour with the lamin antibodies, appropriately diluted in PBS containing 1% BSA. Thereafter, the sections were washed three times for 10 minutes with PBS and incubated for 1 hour with a rabbit anti-mouse Ig antibody conjugated to peroxidase (DAKO A/S, Glostrup, Denmark) diluted 1:100 in PBS containing 1% BSA. The sections were washed three times for 10 minutes with PBS and peroxidase reactivity was visualized by incubation with 1 mg/ml 3,3' diaminobenzidine-HCl (DAB, Sigma) and 0.01% H_2O_2 in 5mM Tris-HCl pH 7.7. The sections were washed with water for 5 minutes, stained with haematoxylin (haematoxylin solution, Gill no. 3, Sigma) diluted 1:20 in water for 30 seconds, washed with water for 10 minutes, dehydrated and mounted in Entellan (Merck, Darmstadt, Germany). Immunostained preparations were evaluated for lamin reactivity by two individuals experienced in the histology of TGCT. In a number of cases, data from immunostaining of tissue sections were confirmed by immunoblotting.

Tissue sample preparation for gel electrophoresis

Ten 20 µm thick frozen sections were suspended in buffer containing 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 1.5 mM MgCl₂, 0.5 mM phenylmethylsulphonyl chloride (PMSC, Merck) and 0.5% Triton X-100 (BDH, Poole England), and stirred vigorously for 10 minutes at 0°C. The tissue remnants were collected by centrifugation at 1500 x g at 4°C and the pellet was washed with buffer containing 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 1.5 mM MgCl₂, 0.5 mM PMSC (Merck). Thereafter the tissue remnants were collected by centrifugation at 1500 x g at 4°C, resuspended in buffer containing 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 1.5 mM MgCl₂, 0.5 mM PMSC (Merck), 1 mg/ml DNase 1 (Sigma) and 50 µg/ml RNase A (Sigma), and incubated for 20 minutes at RT. Then the pellet was washed with 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 1.5 mM MgCl₂, 0.5 mM PMSC (Merck). After centrifugation and discarding of the buffer the tissue pellet was resuspended in approximately 1 volume of SDS-sample buffer and boiled for 5 minutes.

For two-dimensional gel electrophoresis Triton X-100 extracted NCI-H125 cell pellets were used, prepared as described before [20].

Gel electrophoresis and immunoblotting

One-dimensional SDS-gel electrophoresis in 10% polyacrylamide (Bio-Rad Laboratories, Hercules, California) slab gels containing 0.1% SDS (Merck), was performed according to Laemmli [18]. Gels were run on the Mini-Protein II system (Bio-Rad Laboratories) for approximately 45 minutes at 200 V. Two-dimensional electrophoresis gels were run essentially as described by O'Farrell [27], with minor modifications as described by Machiels et al. [20].

Western blotting was performed essentially according to Towbin et al. [39] using the Mini Trans-Blot Cell (Bio-Rad Laboratories) at 100 V for 1 hour. Proteins were blotted onto nitrocellulose (BA85, Schleicher and Schüll, Dassel, Germany), the sheets were air-dried and blocked for 1 hour at RT in buffer containing 10 mM Tris-HCl pH 7.6, 0.35 M NaCl and 3% BSA (Sigma). Thereafter the blots were incubated with the primary antibodies for 30-60 minutes at RT in appropriate dilutions in buffer containing 10 mM TRIS-HCl pH 7.6, 0.15 M NaCl, 0.3% BSA, 1% Triton X-100 (BDH), 0.5% DOC (Merck) and 0.1% SDS (Merck). Then the blots were washed three times with PBS containing 0.5% Triton X-100 (BDH), and incubated for 30-60 minutes at RT with the secondary peroxidase conjugated rabbit anti-mouse Ig antibody (DAKO A/S), diluted 1:1000 in PBS containing 0.5% Triton X-100 (BDH) and 0.5% BSA (Sigma). Then the blots were extensively washed with PBS containing 0.5% Triton X-100 (BDH) and peroxidase activity was detected by chemiluminescence (ECL-kit, Amersham, Buckinghamshire, UK). RX Fuji medical X ray films (Fuji, Tokyo, Japan) were used for the visualisation of the luminescent signals.

Results

Antibody specificity

Two-dimensional immunoblotting studies were performed to examine cross-reactivity of the lamin antibodies. The results of these assays on Triton X-100 extracted cell pellets of NCI-H125 cells are shown in figure 1. The arrays of spots with identical molecular weights are the distinct phosphorylation isoforms of the individual lamins. From these experiments, it was obvious that the lamin antibodies used in this study exhibited the reported specificity for lamin subtypes (see Materials and Methods). Strikingly, antibody 41CC4 shows a relatively stronger reactivity with lamin C than with lamin A.

Figure 1. Two-dimensional immunoblot analysis confirmed the specificity of the lamin antibodies used in this study. Triton X-100 extracted cell pellets of NCI-H125 cells were used for two-dimensional gel electrophoresis and blotted. The blots were incubated with (a) lamin B1 antibody 119D5, (b) lamin B2 antibody LN43, (c) lamin B2 antibody X223, (d) lamin A antibody 133A2, (e) lamin A/C antibody R27, (f) lamin A/C antibody 41CC4 and (g) a mixture of the antibodies to lamin B1 (119D5), lamin B2 (LN43), lamin A (133A2) and lamin A and C (41CC4).

Normal testis

A summary of the results of lamin immunostaining reactions in tissue sections of normal testis and testicular parenchyma adjacent to TGCT is shown in table I and depicted in figure 2a-c. Sertoli, Leydig and peritubular cells, all express both A- and B-type lamins. However, it should

be noted that Sertoli and Leydig cells expressed lamin B2 (Fig. 2b), but showed in general no reaction with the lamin B1 antibody (Fig. 2a). Spermatogonia were mostly positive for lamins B1 and B2, but no A-type lamins were detected in these cells (Fig. 2c), despite their highly specialized commitment to differentiate into spermatocytes. In contrast, spermatocytes showed no reactivity at all with the B-type lamin antibodies. In some cases, a reactivity with the A-type lamin antibodies was seen, but this reaction was weak and only detectable in a part of the spermatocyte population.

Table I Results of immunostaining reactions with lamin antibodies in normal testis and parenchyma adjacent to TGCT
(positive cases / total number of cases tested)

	lamin A 133A2	lamin A/C R27	lamin A/C 41CC4	lamin B1 119D5	lamin B2 LN43	lamin B2 X223
Sertoli cells	4/4	4/4	4/4	0/4	4/4	4/4
Leydig cells	4/4	4/4	4/4	0/4	4/4	4/4
Peritubular cells	4/4	4/4	4/4	4/4	4/4	4/4
Spermatogonia	0/4	0/4	0/4	4/4	4/4 W	4/4 W
Spermatocytes	4/4 W	4/4 W	0/4	0/4	0/4	0/4
Adjacent to SE						
Sertoli cells	4/4	6/6	5/5	0/8	9/10	9/10
Leydig cells	4/4	6/6	6/6	0/8	10/10	9/10
Peritubular cells	4/4	6/6	6/6	6/8	10/10	10/10
Spermatogonia	0/4	0/6	0/6	8/8	8/10	7/10
Spermatocytes	1/4 **	2/6 **	0/6	0/8	0/10	0/10
Adjacent to NS						
Sertoli cells	5/6	8/8	8/8	4/10	12/12	11/11
Leydig cells	6/6	8/8	8/8	2/10	12/12	11/11
Peritubular cells	6/6	8/8	8/8	8/10	12/12	11/11
Spermatogonia	0/6	1/8	1/8	6/10	11/12	6/11
Spermatocytes	1/6 *	1/8 *	1/8 *	0/10	1/12	0/11

* One case was partially positive for all A-type lamin antibodies

** Two cases were partially positive with antibody R27, and one of those was also positive with antibody 133A2

W: Weak positive reaction in a fraction of the cells

Testicular germ cell tumours

The results of the immunohistochemical staining reactions of the different subtypes of TGCT are summarized in table II and depicted in figure 2d-h. It was obvious that all tested TGCT gave perinuclear staining with the B-type lamin antibodies for both the B1 and B2 subtypes (illustrated for SE in Fig. 2d).

A-type lamin expression patterns were heterogeneous. The SE used in this study could be divided into two groups, a group of 6 selected SE that contained a mutation in K- or N-Ras (Ras+, see reference [28]), and a group of 10 SE without any detectable mutation in the Ras genes (Ras-). The overall incidence of Ras mutations in TGCT is low, therefore the presence of six SE with such a mutation in our series of 16 does not reflect the actual incidence (being approximately 15%, see reference [28]). The difference in the staining pattern of antibody 41CC4 when comparing Ras+ and Ras- SE cases was striking: the Ras- SE were negative and the Ras+ SE were positive, although sometimes weakly. Interestingly, one case was known to contain a heterogeneous population of tumour cells with and without Ras mutation [28]. This tumour also showed a heterogeneous staining pattern with antibody 41CC4 (not shown). Most SE were negative with the lamin A/C antibody R27, with only one Ras+ SE case being positive, while the lamin A antibody 133A2 showed partial reactivity with only two Ras+ SE. None of the Ras- SE gave a staining reaction with the A-type lamin antibodies (Fig. 2e).

Most NS-components were positive with lamin A/C antibodies R27 and 41CC4. Strikingly, EC was found to be negative for lamin A using antibody 133A2, but positive for lamin A/C using antibody R27 and 41CC4 (Fig. 2f,g). A low expression of lamin A together with a normal or high expression of lamin C may explain this reactivity pattern. Normally lamins A and C are expressed to comparable degrees, although an imbalance in the expression ratio of lamin A over C may occur, and has already been reported for a lung adenocarcinoma cell line [20]. To examine this phenomenon further, samples of three EC were used for immunoblotting. When these blots were stained with the lamin A/C antibodies, two bands were detected for lamin C and a single band was found for lamin A (Fig. 3). It was obvious that the reactivity level of the lamin C bands was much stronger than that of lamin A, which confirmed the immunohistochemical observations. The presence of lamin A in the blots was confirmed by reactivity with the lamin A antibody 133A2 (Fig. 3). Lamin A reactivity on blots of EC samples may be overrepresented as a result of non-EC components that are present in the tumour tissue, such as small blood vessels, and that do express A-type lamins. The very low expression level of lamin A and the imbalanced expression of lamin A and lamin C may account for our interpretation that the sections of EC are negative for lamin A.

YS, CH and TE could not be distinguished by A-type lamin expression. These three histologically distinct tumour types gave perinuclear staining with the lamin A antibody as well as the lamin A/C antibodies (illustrated for TE in Fig 2h).

Figure 2. Representative illustrations of immunostainings of parenchyma adjacent to testicular germ cell tumours (a-c) and testicular germ cell tumours (d-h). (a) Testicular parenchyma adjacent to testicular germ cell tumours containing carcinoma in situ, stained with lamin B1 antibody 119D5; Sertoli (arrowhead) and Leydig (arrow) cells are negative for lamin B1. (b) Testicular parenchyma adjacent to embryonal carcinoma containing carcinoma in situ, stained with lamin B2 antibody X223; Sertoli cells (arrows) are positive for lamin B2. (c) Testicular parenchyma adjacent to a testicular germ cell tumour containing carcinoma in situ, stained with lamin A/C antibody R27; spermatogonia (arrowhead) and carcinoma in situ (arrows) are negative for A-type lamins. (d) Ras- seminoma stained with lamin B2 antibody X223; both normal (lymphocytes, arrow) and tumour cells are positive for lamin B2. (e) Ras- seminoma stained with lamin A/C antibody R27; tumour cells are negative for lamin A and C, while focal spots of normal tissue (lymphocytes, arrow) are positive for lamin A and C. (f) Embryonal carcinoma stained with lamin A antibody 133A2; tumour cells are negative for lamin A. (g) Embryonal carcinoma stained with lamin A/C antibody R27; both tumour cells and normal tissues are positive. (h) Teratoma stained with lamin A/C antibody R27; the inset shows a higher magnification of the teratoma illustrating the lamina staining. For colour illustration see page 131.

Table II Results of immunostaining reactions of TGCTs with lamin antibodies
(positive cases / total number of cases tested)

	lamin A 133A2	lamin A/C R27	lamin A/C 41CC4	lamin B1 119D5	lamin B2 LN43	lamin B2 X223
SE	2/16 *	1/16 *	6/16 *	16/16	14/15	16/16
EC	0/7	9/10	8/10	7/7	10/10	10/10
YS	5/5	5/5	6/6	5/5	6/6	6/6
CH	5/5	5/5	4/5	5/5	5/5	5/5
TE	4/4	4/5	4/4	4/4	5/5	6/6
CIS (SE)	0/4	0/6	2/6 *	8/8	7/10	6/9
CIS (NS)	0/5	0/8	0/7	6/9	11/11	8/9

* Positive cells were detected only in SE containing a Ras mutation and its adjacent CIS
SE: seminoma; EC: embryonal carcinoma; YS: yolk sac tumour; CH: choriocarcinoma; TE: teratoma; CIS (SE): carcinoma in situ adjacent to seminoma; CIS (NS): carcinoma in situ adjacent to nonseminoma.

**Table III Lamin B1 expression in Sertoli cells,
spermatogonia and CIS adjacent to NS**

Adjacent to NS	Sertoli	Spermatogonia	CIS
4 cases	+	-	-
5 cases	-	+	+
normal testis	-	+	

- all cells negative
+ all cells positive

Figure 3. Immunoblotting results of three EC samples. The blots were incubated with lamin A/C antibody R27 (lanes 1,2,3) and lamin A antibody 133A2 (lanes 4,5,6). Note the low expression level of lamin A compared to lamin C in EC. For comparison, the lamin A over lamin C expression ratio in cell line NCI-H125 is shown (lane 7).

Carcinoma in situ

Lamin expression in carcinoma in situ (CIS), the precursor of all TGCT [36], resembled in general that of SE (table II). Although in most of the cases both B-type lamins were seen, CIS adjacent to SE may lack lamin B2 and CIS adjacent to NS may lack lamin B1. Furthermore the A-type lamins were absent, except in two cases of CIS adjacent to Ras+ SE, which were partially positive with antibody 41CC4.

Comparison of B-type lamin expression patterns in CIS with those in spermatogonia at the level of individual cases resulted in some remarkable observations. The B-type lamin expression in CIS resembled that in spermatogonia. When CIS adjacent to SE was positive with a lamin B2 antibody, spermatogonia were also positive (with one exception); when CIS was found to be negative with a lamin B2 antibody, the spermatogonia were negative as well. Spermatogonia in normal testis were only partially and weakly positive for lamin B2, while in parenchyma adjacent to SE all spermatogonia were clearly positive. Lamin B2 expression in spermatogonia adjacent to SE seems therefore slightly increased. When CIS adjacent to NS (table III) was negative for lamin B1, spermatogonia were also negative; when CIS was positive for lamin B1, spermatogonia were also positive. In addition, the reaction of the Sertoli cells in these sections was always opposite to that of spermatogonia and CIS. In normal testis Sertoli cells were negative and spermatogonia were positive for lamin B1, being the physiological expression pattern. In four cases of parenchyma adjacent to NS, completely inverse reaction patterns were found in Sertoli cells, spermatogonia and CIS. Furthermore, it was remarkable that such inverse lamin expression patterns were detected in parenchyma adjacent to NS with a large component of EC or pure EC.

Discussion

Normal testis

B-type lamins have been found to be expressed almost ubiquitously in all somatic cell types, regardless of their degree of differentiation or proliferative activity. However, differential expression patterns of lamins B1 and B2 have been described occasionally, with lamin B1 not being expressed, in general, in differentiated cells which lack proliferative activity. For instance, heart muscle cells, which are fully differentiated and non-proliferative cells, were negative for lamin B1 and positive for lamin B2 [2], while centroblasts, which are proliferating cells in the follicle centre of reactive lymph nodes, were positive for lamin B1 and negative for lamin B2 (unpublished results). Absence of lamin B1 in Sertoli and Leydig cells, which are fully differentiated cells, is in concordance with the previously reported differential expression of B-type lamins.

Although A-type lamin expression is restricted to differentiated cells, no A-type lamins were detected in spermatogonia, despite their highly specialized commitment to differentiate into spermatocytes. Furthermore, spermatocytes were found to be negative for B-type lamins and

only weakly positive for A-type lamins. Apparently, the spermatogonia lose their B-type lamin expression in the process of differentiation towards spermatocytes. A similar phenomenon has been found in rats, in which the somatic B-type lamins disappear during differentiation of spermatogonia into spermatocytes [21]. At the same time, a germ cell specific B-type lamin becomes expressed in these cells. Also in mice two distinct germ cell specific lamins have been identified, lamin B3 [12] and lamin C2 [11]. So far such a germ cell specific lamin protein has not been identified in humans, but its existence is likely. The weak reactivity of the A-type lamin antibodies in spermatocytes may on the one hand be due to a low A-type lamin expression in these cells, but might on the other hand be due to cross-reactivity of the antibodies with an as yet unidentified human counterpart of the germ cell specific lamins identified in other species.

Testicular germ cell tumours

Ras-induced changes in A-type lamin expression have been described before in a human lung cancer cell line by Kaufmann et al. [17]. Elevated levels of Ras expression resulted in elevated levels of A-type lamins. The possibility of a Ras-responsive element in the promoter region of the lamin A/C gene was proposed. The results with Ras+ SE, in which the Ras gene is constitutively activated [28] and A-type lamins were detected with antibody 41CC4, are in concordance herewith.

Based on the lamin expression patterns in TGCT, it became apparent that NS, in particular YS, CH and TE, should be considered as the most differentiated of the spectrum, and SE as the least differentiated TGCT type. These results are in favour of the "linear progression model" as originally proposed by Ewing [8]), and further developed by others [9, 25, 29, 30, 33]. In this model, EC is an intermediate between SE and NS. When this model is translated to lamin expression in TGCT, this would imply that expression of only lamin C, as seen in EC, is an intermediate state between absence of A-type lamins in undifferentiated SE and expression of lamins A and C in differentiated TGCT. This would mean that during the process of differentiation, expression of lamins A and C is not started simultaneously, but that expression of only lamin C precedes expression of both A-type lamins. In a slightly distinct approach, SE may be considered a TGCT that follows a differentiation pathway along the spermatogenic lineage; their lamin expression pattern therefore resembles that of spermatogonia. In contrast, NS follow an embryonal or extra-embryonal differentiation, in which CIS is the most undifferentiated component, EC an intermediate component expressing lamin C but not lamin A, and YS, CH and TE fully differentiated components expressing lamins A and C. Identification of human germ cell specific lamins and immunostaining of TGCT sections with antibodies to such lamins may give insight into this aspect of differentiation.

Regarding A-type lamin expression in SE, it should be kept in mind that the number of Ras+ SE in this study is much higher than the actual incidence. Although Ras+ SE show reactivity with one of the A-type lamin antibodies and may therefore be considered as being more differentiated than Ras- SE, the transition from CIS to NS apparently does not occur via Ras+

SE as an intermediate, since the Ras+ NS incidence is much lower than the Ras+ SE incidence [28].

In TGCT, apparently, expression of A-type lamins is dependent on the degree of differentiation, illustrated by the presence of lamin A and lamin C in the differentiated NS components, and the lack of one or both A-type lamins in EC and SE, respectively. A correlation with proliferative activity is less obvious, since a similar expression was found in YS, CH and TE, while differences in proliferative activities have been found (unpublished observations).

Since lamin A detection in NS discriminates between EC on the one hand and YS, CH and TE on the other hand, this may provide a useful parameter of clinical relevance, since the percentage of EC cells together with the degree of vascular invasion have been described as a prognostic factor [10, 24, 35].

Carcinoma in situ

The absence of A-type lamins in CIS can be explained by the highly proliferative character of these cells. Furthermore, in spite of the fact that CIS resembles early (primordial) germ cells [16], lamin expression in CIS was similar to lamin expression in more differentiated germ cells, i.e. spermatogonia. This indicates that early germ cells probably show a lamin expression pattern that is identical to that of the more differentiated spermatogonia.

The findings shown in table III, support a close interaction between normal cells, malignant germ cells and their nursing cells within the testis [14, 37]. It has been claimed that the extracellular matrix plays an important role in the communication between cells, possibly resulting in differences in expression of nuclear lamins [4]. Whether certain factors of EC play a role in this phenomenon in the testis remains to be established.

In conclusion, lamin expression in CIS resembles, on the one hand, lamin expression in spermatogonia and on the other hand, lamin expression in SE, indicating a close relationship between these three cell types, which is confirmed by their morphological similarity. These cell types may follow a differentiation pathway along the spermatogenic lineage, and therefore do not express A-type lamins, but they might express an as yet unidentified germ cell specific lamin. In contrast, NS follow an embryonal or extra-embryonal differentiation pathway, resulting in A-type lamin expression in these cells. EC, the most undifferentiated NS component, expressed only lamin C and not lamin A. From a cell biological point of view, this would mean that lamin C is expressed earlier in the process of differentiation than lamin A. From a clinical point of view, lamin immunostaining of NS sections may be used to identify clearly the EC component in NS, which may aid in the use of the percentage of EC in NS as a prognostic marker.

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Chapter 4

Comparison of A- and B-type lamin expression in nodular sclerosing Hodgkin's disease and reactive lymph nodes

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Summary

Lamins are a family of nuclear membrane-associated intermediate filament proteins, of which the A-type lamins are preferentially expressed in differentiated cells. The B-type lamins B1 and B2 have long been considered to be constitutively expressed, but only recently we have come across several examples of a differential expression of these two latter lamin subtypes, with lamin B1 being related to proliferation.

In order to clarify the differentiation and proliferation status of the Reed-Sternberg and Hodgkin cells we studied A- and B-type lamin expression with specific monoclonal antibodies in Hodgkin's disease. Its normal counterpart, the reactive lymph node, was also examined for lamin subtype expression.

The CD20 positive centrocytes and centroblasts of the follicle centre in the reactive lymph nodes expressed lamin B1, but were not or only very weakly positive for lamin B2 or A-type lamin antibodies. Mantle zone lymphocytes displayed lamins B1 and B2, but were negative for A-type lamins. Furthermore, CD3- and CD20-positive lymphocytes in the medulla and paracortex lacked A-type lamins, but were positive for both B-type lamins. Finally, the proliferation marker Ki-67 was mainly detected in the centroblasts, but also in a fraction of the A-type lamin negative cells in the paracortex and medulla.

In Hodgkin's disease, all cells expressed lamins B1 and B2, whereas a small subpopulation of cells had A-type lamins. CD30-positive Reed-Sternberg and Hodgkin cells were primarily positive for A-type lamins and about 20% of the Reed-Sternberg and Hodgkin cells expressed Ki-67, with co-expression of lamin A in most of these cells.

All in all, Ki-67 and A-type lamin positivity in general were mutually exclusive in lymph nodes, indicating that A-type lamin positive cells are not proliferative. This suggests also that the A-type lamin expression in Reed-Sternberg and Hodgkin cells is correlated with a relatively mature phenotype of these malignant cells. However, part of these differentiated malignant cells still have a capacity to proliferate as indicated by Ki-67 positivity. Our observation that lamin B2 expression in the follicle centre cells of the reactive lymph node is low or absent indicates that this lamin subtype is not always expressed in nucleated cells, which is in clear contrast to the results obtained in previous studies in other diseases and in normal tissues. Absence of lamin B2 expression may be associated with the follicle centre stage of B-cells.

Introduction

Lamins belong to the intermediate filament protein family and are constituents of the nuclear lamina. In vertebrates two types of lamins have been recognized, i.e. lamins of the neutral A-subtype and lamins of the acidic B-subtype [11, 18, 23]. These subtypes differ in their primary sequences and pI, and each subtype consists of several distinct phosphorylation isoforms [2, 15]. The B-type lamins B1 and B2 (Mw 65-67 kDa) seem to be involved in the

association of the nuclear lamina to the inner nuclear membrane, which may result in stabilisation of the nuclear envelope [26]. B-type lamins have long been thought to be constitutively expressed in all somatic nucleated cells of vertebrates [6, 22]. During mitosis, the nuclear lamina disintegrates due to phosphorylation of the lamins, but B-type lamins remain associated with vesicles originating from the nuclear membrane [11].

Expression of the A-type lamins, consisting of lamin A and lamin C, is largely restricted to differentiated cells and not extensively found in proliferating cells [6, 23]. Recent results indicate that statin, an antigen associated with non-proliferative cells, is identical to A-type lamins [7]. Statin and the proliferation marker Ki-67 segregate to distinct populations in cytopspins of lymphoid cell suspensions, confirming the anti-proliferative expression of statin or A-type lamins [30]. A-type lamins have intrinsic chromatin-binding capacity, and as a result may be involved in a topological organization of chromatin and in the regulation of replication and transcription [10-12, 24]. During mitosis the phosphorylated A-type lamins, in contrast to the B-type lamins, become soluble and disperse throughout the cytoplasm.

Human lymphocytes of the early differentiation stages, as well as the neoplastic cells in acute lymphoblastic leukemia (ALL) and non-Hodgkin's lymphomas (NHL), are known to lack A-type lamins [13, 25, 29]. Lamin expression in Hodgkin's disease has until now not been characterized. Next to lymphocytes, this latter tumor type contains macrophages and histiocytes, as well as the morphologically distinguishable Reed-Sternberg and Hodgkin cells. The multinucleated Reed-Sternberg cells and mononucleated Hodgkin cells are considered to represent the malignant fraction in Hodgkin's disease, although they amount to only 2-3% of the total cell population. So far, the origin and stage of differentiation of the Reed-Sternberg cells have not been completely elucidated [14, 19]. A recent study, however, suggests on base of rearranged V_H and V_L genes that the malignant cells in Hodgkin's disease originate from germinal centre B-cells [20].

The aim of this study was to analyse lamin expression in cells of Hodgkin's disease in relation to their proliferative capacity and to compare these reaction patterns to those found in reactive lymph nodes, in an attempt to clarify the degree of differentiation of the malignant cells in Hodgkin's disease.

Materials and Methods

Tissue specimens

Frozen biopsy specimens of nine patients with Hodgkin's disease nodular sclerosis subtype were investigated. In addition, twelve frozen biopsy specimens of reactive lymph nodes were analysed.

Antibodies

The following mouse monoclonal antibodies against A- and B-type lamins were used in this study for immunocytochemical analyses:

1. 133A2 (IgG3) against lamin A [16], kindly provided by Dr. Y. Raymond (Montreal/Canada), mouse ascites diluted 1:1000;
2. 41CC4 (IgM) against lamin A/C [5], kindly provided by Dr. G. Warren (London/United Kingdom), culture supernatant used undiluted;
3. R27 (IgM) against lamins A/C [32], kindly provided by Dr. G. Krohne (Würzburg/Germany), culture supernatant used undiluted;
4. X223 (IgG1) against lamin B2 [15], kindly provided by Dr. G. Krohne, ascites diluted 1:100;
5. 101B7.2 (IgG1) against lamin B2 (Matritech Inc., Cambridge, MA), ascites diluted 1:100;
6. LN43 (IgG1) against lamin B2 [3] kindly provided by Dr. E.B. Lane (Dundee/UK), culture supernatant used undiluted;
7. 119D5 (IgG1) against lamin B1, kindly provided by Dr. Y. Raymond, ascites diluted 1:750.

The leucocyte common antigen antibody (LCA), diluted 1:200 was used as a positive control. Phosphate buffered saline (PBS) pH 7.2-7.4 without primary antibodies was used as a negative control. The secondary antibody for the immunocytochemical detection of antibody binding was a peroxidase conjugated rabbit anti mouse immunoglobulin (DAKO A/S, Glostrup, Denmark), diluted 1:100.

Primary antibodies recognizing lymphocyte subtypes and used for the double label immunofluorescence studies, are:

1. anti-CD3, a rabbit polyclonal antiserum recognizing T-lymphocytes (DAKO), diluted 1:100;
2. Anti-CD20, a mouse monoclonal IgG2a binding B-lymphocytes (L26; DAKO), diluted 1:1000;
3. Anti-CD30 (Ber-H2 clone; DAKO), a mouse monoclonal IgG1 detecting Reed-Sternberg cells, Hodgkin cells and activated lymphocytes [28], diluted 1:25.

Finally, a rabbit polyclonal antiserum recognizing the proliferation marker Ki-67 (DAKO) was used in combination with lamin antibodies and/or anti-CD30. The secondary antibodies used in the double-label immunofluorescence analyses were: fluorescein isothiocyanate (FITC) conjugated goat anti rabbit (Sigma, St. Louis, MO, USA), diluted 1:100, for the detection of anti-CD3; FITC labeled goat anti mouse IgG2a (Southern Biotechnology Associates, Inc., Birmingham, USA (SBA)), diluted 1:40, for the detection of anti-CD20; FITC labeled goat anti mouse IgG1 (SBA), diluted 1:40, for the detection of anti-CD30; biotin labeled horse anti mouse (DAKO), diluted 1:200, with AMCA conjugated avidin (Vector, Brunswick Chemie, Amsterdam, The Netherlands), diluted 1:100, for detection of CD30; Texas Red conjugated goat anti mouse IgM (SBA), diluted 1:40, for the detection of lamin A/C antibody R27. For signal enhancement FITC labeled antibodies were reincubated with rabbit anti FITC (DAKO), diluted 1:100 and FITC conjugated swine anti rabbit immunoglobulins (DAKO), diluted 1:100. Finally, Ki-67 antigen was detected with FITC conjugated swine anti rabbit antibody (DAKO).

Immunoperoxidase staining procedure

Five μm thick sections were cut with a cryostat (Cryocut 1800, Reichert-Jung, Leica) from all frozen biopsy specimens, brought onto poly-L-lysine coated glass slides and fixed in acetone for five minutes at 4 °C. After fixation, slides were washed three times five minutes in PBS at room temperature (RT). Slides were incubated with appropriately diluted mouse monoclonal antibodies for one hour at RT, followed by three times five minutes washing with PBS at RT. Thereafter, peroxidase conjugated secondary rabbit anti mouse Ig was incubated for one hour at RT. After another series of washing steps in PBS at RT (three times five minutes), peroxidase activity was visualized with a diaminobenzidine (DAB; Sigma) staining reaction (0.5mg/ml DAB in PBS/0.1M imidazol pH 7.6 and 0.03% H_2O_2), for four minutes at RT. Thereafter, slides were rinsed with MilliQ-water, counterstained, dehydrated and mounted in Entellan (Merck, Darmstadt, Germany). Control incubations included omission of the primary antibodies and incubation with all secondary antibodies.

Immunofluorescence procedure

For double-label immunofluorescence studies 5 μm thick frozen tissue sections from 3 cases, fixed for 5 minutes in acetone, were simultaneously incubated with mouse monoclonal IgM antibodies against lamin A/C, together with either anti-CD3, anti-CD20 or anti-CD30 antibodies for 45 minutes at 37 °C. Furthermore, monoclonal antibodies against lamin subtypes were also combined with the polyclonal antibody against the Ki-67 antigen. The slides were washed with PBS at RT and for detection of the CD-markers incubated with FITC conjugated goat anti rabbit IgG, FITC labeled goat anti mouse IgG2a, or FITC conjugated goat anti mouse IgG1 antibody, respectively, at 37 °C for 30 minutes. Ki-67 antigen was stained with FITC conjugated swine anti rabbit and the lamins were detected with Texas Red labeled goat anti mouse IgM. After each incubation step, slides were washed with PBS at RT. Finally, FITC was amplified with rabbit anti FITC and FITC conjugated swine anti rabbit Ig. Slides were dehydrated and nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Sigma; 1.25 ng/ml) in 90% glycerol, 0.02% NaN_3 , 10% 0.2M Tris-HCl (pH8.0), and 2.0% 1,4-diazobicyclo-(2.2.2)-octane (DABCO; Sigma) (pH8.0), to reduce photobleaching.

For triple-label immunofluorescence, anti-CD30, followed by biotin labeled horse anti mouse and AMCA conjugated avidin were first incubated, than the Ki-67 antigen was detected by the polyclonal antiserum, followed by FITC conjugated swine anti rabbit, and finally lamin A/C antibody R27 followed by Texas Red labeled goat anti mouse IgM were applied. Incubations were performed under similar conditions as described above for double-label immunofluorescence. Control incubations included omission of the primary antibodies and incubation with all secondary antibodies. The staining reactions were recorded with a CCD camera, using bright field and fluorescence filters.

Results

The immunophenotyping results obtained with the lamin antibodies, lymphocyte subtype markers, and Ki-67 as proliferation marker in reactive lymph nodes and of Hodgkin's disease are summarized in table 1 and illustrated in figures 1-8. Antibodies R27 and 41CC4, both recognizing lamin A and lamin C, showed similar reactivity patterns when compared to antibody 133A2, recognizing only lamin A. The results of the B-type lamins were extrapolated by combining results of the lamin immunoperoxidase staining reactions with those from immunofluorescence microscopy for lymphocyte markers and Ki-67.

Reactive lymph nodes

In the follicle centre few cells scattered through the follicle centre were positive for all three A-type lamin antibodies (fig. 1), while virtually all cells expressed lamin B1 as detected by 119D5 (fig. 2). The centrocytes and centroblasts of the follicle centre were not or very weakly positive for the lamin B2 antibodies (figs. 3 and 4). Mantle zone cells exhibited lamin B1 and B2 expression, but were completely negative for the lamin A and A/C antibodies. The paracortical and medullar cells of reactive lymph nodes showed similar lamin expression patterns, i.e. strong expression of B-type lamins in all cells and A-type lamins in a smaller population of cells (fig. 1). To further specify the A-type lamin expression patterns for the different types of lymphocytes, we performed double- and triple-label immunofluorescence microscopy on the tissue sections. All CD20-positive B-cells in the follicle centre, but also the CD20-positive B-cells outside the follicle, lacked A-type lamins. Additionally, CD3-positive T-lymphocytes in the paracortex and medulla were also negative for A-type lamins. However, activated lymphoid cells with CD30 expression, sparsely seen in the paracortex and medulla, displayed lamins A/C, although a small subpopulation ($\pm 10\%$) of CD30 positive cells lacked A-type lamins. Another fraction of the A-type lamin positive cells in the paracortex and medulla remained unidentified by the lymphocyte markers, and mainly represent histiocytes, reticulum, dendritic and endothelial cells but not plasma cells.

Proliferative cells were identified by staining with the Ki-67 antibody. Centroblasts in the follicle centre were positive for Ki-67, whereas centrocytes and the cells of the mantle zone, paracortex and medulla were predominantly negative (fig. 4). In general, A-type lamin expression was mutually exclusive with Ki-67 positivity.

Hodgkin's disease

The Reed-Sternberg and Hodgkin cells and all surrounding lymphocytes were positive for lamins B1 and B2 in all cases (fig. 5), although the staining intensity of the Reed-Sternberg and Hodgkin cells with the lamin B1 antibody seems lower than the staining intensity of the surrounding cells. A-type lamins were observed in most Reed-Sternberg and Hodgkin cells,

while also a small population of the cells in the background displayed this lamin subtype (fig. 6).

Again, double- and triple-label immunocytochemistry was used to correlate A-type lamin expression with the cellular subtype (Table 2). CD20 positive B-lymphocytes and CD3 positive T-lymphocytes in Hodgkin's disease lacked lamins A and C, which was comparable to the pattern observed in reactive lymph nodes. Again, A-type lamin expression commonly excluded Ki-67 positivity (fig. 7).

Furthermore, the majority of CD30 positive lymphoid cells and Reed-Sternberg or Hodgkin cells expressed A-type lamins (Table 2). However, a subpopulation of about 20% of the lamin A/C and CD30 positive malignant cells showed also Ki-67 expression (fig. 8).

Table 1 Summary of the immunophenotypes of the different cell types in reactive lymph nodes and Hodgkin's disease

	CD3	CD20	CD30	Ki-67	lamin A/C	lamin B2	lamin B1
Reactive lymph node:							
follicle centre:							
centroblasts	-	+	-	+	-	-	+
centrocytes	-	+	-	-	-	-	+
mantle zone cells	-	+	-	-	-	+	+
paracortex/medulla cells	+	-	-	-	-	+	+
	-	+	-	-	-	+	+
	-	-	-	-	+	+	+
	nd	nd	+	-	+	+	+
Hodgkin's disease:							
Hodgkin/Reed-Sternberg cells	nd	nd	+	-	+	+	+
	nd	nd	+	+	+	+	+
surrounding cells	nd	nd	-	-	-	+	+
	-	-	-	-	+	+	+

Results obtained after bright field microscopy with peroxidase staining (n=9), double and triple-label immunofluorescence microscopy (n=3), and extrapolation. Expression of antigen is indicated by +, whereas lack is represented by -, nd is not determined. When different expression patterns for a certain cell type occur, than the most common pattern is described first, in order of significance.

Figure 1. Reactive lymph node stained with antibody R27, showing that A-type lamins are present in a few cells of the follicle centre and a population of cells in the paracortex. For colour illustration see page 132-133.

Figure 2. Staining of the reactive lymph node with antibody 119D5 showing that cells of the follicle centre and the surrounding areas exhibit lamin B1. Colour illustration page 132-133.

Figure 3. The reactive lymph node after antibody LN43 staining, showing that lamin B2 is not or very weakly expressed in the cells of the follicle centre. For colour illustration see page 132-133.

Figure 4. Double-label fluorescence microscopy of a reactive lymph node showing that centroblasts of the follicle centre express the proliferation marker Ki-67 (in green) but not lamin B2 (in red), while the majority of the surrounding cells in the mantle zone, paracortex and medulla show the reverse staining pattern. Centrocytes were negative for both Ki-67 and lamin B2. cc: centrocyte region; cb: centroblast region. For colour illustration see page 132-133.

Figure 5. Reed-Sternberg/Hodgkin cells do express lamin B2, as shown after staining with LN43. Note that surrounding cells stain more strongly. For colour illustration see page 132-133.

Figure 6. Hodgkin lymphoma stained with antibody R27 shows that Reed-Sternberg and Hodgkin cells express A-type lamins. For colour illustration see page 132-133.

Figure 7. Double-label fluorescence microscopy of Hodgkin lymphoma with the proliferation marker Ki-67 (in green) and antibody R27 for A-type lamins (in red), showing that in general Ki-67 and A-type lamin staining are mutually exclusive. DNA was counterstained with DAPI (blue). For colour illustration see page 132-133.

Figure 8. Triple-label fluorescence microscopy of a CD30-positive Reed-Sternberg cell (in blue) expressing A-type lamins as detected with antibody R27 (in red) and the proliferation marker Ki-67 (in green). For colour illustration see page 132-133.

Table 2
Frequency distribution of cells in Hodgkin's disease expressing multiple phenotypic parameters. Results represented as the percentage of 1000 cells counted in double-label immunofluorescence samples and of 50 counted Reed-Sternberg and Hodgkin cells in the triple-label study of three cases.

Hodgkin's disease			
CD30	lamin A/C	Ki67	Frequency (in %)
	+	+	1.0
	+	-	15.3
	-	+	7.1
	-	-	76.5
+		+	0.3
+		-	1.0
-		+	7.6
-		-	91.1
+	+		0.8
+	-		0.2
-	+		10.5
-	-		89.6
Hodgkin and Reed-Sternberg cells			
+	+	+	18
+	+	-	74
+	-	+	4
+	-	-	4

Discussion

In this study nuclear lamin subtype expression was investigated in specific cell populations of reactive lymph nodes and Hodgkin's disease. Lamin expression of the malignant cells in Hodgkin's disease was compared to the expression in reactive lymph node, in an attempt to obtain further insight into the biology of malignant Reed-Sternberg and Hodgkin cells.

Differential expression of lamins B1 and B2 in reactive lymph nodes

Distinct areas of reactive lymph nodes exhibit a differential staining pattern for lamins B1 and B2. The CD20 positive centrocytes and centroblasts of the follicle centre, i.e. maturing B-lymphocytes, express high levels of lamin B1, but no or only very low levels of lamin B2, whereas cells of the mantle zone, the paracortex and medulla display both B-type lamins. Our observation implies that lamin B2 is not constitutively expressed in nucleated cells, in contrast to observations by other investigators [6, 22, 23]. The use of three different lamin B2 antibodies, recognizing both the hypo- and hyperphosphorylated forms of lamin B2 in immunoblotting [4], makes it very unlikely that epitope masking has prevented lamin B2 recognition in our study [3, 15]. Furthermore, the strong reactivity of the surrounding cells with these antibodies supports the suggestion that the centrocytes and centroblasts of the follicle centre do indeed not or only very weakly express lamin B2. This suggests that the absence of lamin B2 may be correlated with certain stages of B-cell maturation, because it is lacking only in centroblasts and centrocytes. In contrast, CD20 positive B-lymphocytes in the paracortex and medulla do express lamin B2.

Furthermore, it has been suggested that lamin B1 expression is correlated with proliferation [4]. Staining with the proliferation marker Ki-67 showed only positivity in the centroblasts and not in the centrocytes. However, lamin B1 was found in both cell types, which is in contrast with the assumed proliferation dependent expression of lamin B1. In conclusion, our observations with respect to B-type lamin expression in the reactive lymph nodes are striking in the light of recent observations in other normal human tissues [1, 4, 6, 21]. Lamin B1 is expressed constitutively in the lymphoid cell population, while lamin B2 expression seems correlated with lymphoid cell maturation of the B-cells.

A-type lamin expression in reactive lymph nodes

Several investigators have observed a lack of A-type lamins in early human hematopoietic cells, such as T-lymphoblasts and pre-B cells [13, 25, 29]. Cance et al. [6] also demonstrated that proliferating lymphocytes of the germinal centres of lymphoid tissue lacked lamins A and C. In our study A-type lamins were expressed in a small proportion of cells in reactive lymph nodes. Double-label fluorescence microscopy revealed that Ki-67 positive cells, CD20-positive B-lymphocytes and CD3-positive T-lymphocytes lack A-type lamins, confirming the earlier observations. A few of the lymphoid cells in reactive lymph nodes were CD30 positive, and

most of them expressed A-type lamins. The presence of A-type lamins in these CD30 positive cells suggests that this type of lamin expression may correlate with cell maturation or activation, because CD30 is expressed on mature activated lymphocytes. Only a minor fraction of CD30 positive lymphoid cells lacks A-type lamins. Moreover, next to these cells a population of histiocytes, reticulum, dendritic and endothelial cells exhibited A-type lamin expression.

Recent studies suggest that, in general, A-type lamin expression is restricted to differentiated cells [1, 4, 6, 8, 13]. Mature B- and T-lymphocytes of peripheral blood, for example, do display A-type lamins according to Guilly et al. [13]. Our Ki-67 study supported the above findings, indicating that A-type lamin expression is limited to the non-proliferative and more mature cell fraction in lymph nodes. Therefore, the presence of A-type lamins in the CD30 positive cells may correlate with the degree of differentiation rather than activation of the cells.

A- and B-type lamin expression in Hodgkin's disease

In general, Reed-Sternberg and Hodgkin cells contain lamins A, C, B1 and B2, while the surrounding B- and T-lymphocytes were only positive for both B-type lamins. Reed-Sternberg and Hodgkin cells may be derived from lymphocytes, since recent studies proof the presence of lymphoid markers, such as V_H and V_L rearrangements, in Reed-Sternberg and Hodgkin cells [9, 14, 19]. The presence of lamin B2, A-type lamins and CD30 in the Reed-Sternberg and Hodgkin cells suggests that they show a more differentiated phenotype than the centrocytes and centroblasts or the cells in the mantle zone of a reactive lymph node. Strikingly, Reed-Sternberg and Hodgkin cells resembled the lamin phenotype of CD30 positive cells of the paracortex or medulla. This may seem in contrast to the results of Kanzler et al. (1996) [20], who suggested that the malignant cells in Hodgkin's disease were derived from (crippled) germinal centre B-cells based on V_H and V_L gene rearrangements. We realize, however, that the Reed-Sternberg and Hodgkin cells may be derived from any lymphoid cell types by terminal differentiation, and hence show A-type lamin expression.

It should be noted that we did not detect centrocyte- and centroblast-like lamin B2 negative B-cells in Hodgkin lymph nodes. This population seems to be replaced by a population of relatively undifferentiated cells, which may arise from crippled follicle cells and possibly representing the precursors of the Reed-Sternberg and Hodgkin cells. Such crippled cells may initiate differentiation rapidly and aberrantly, resulting in the lack of centrocyte- and centroblast-like lamin B2 negative cells in Hodgkin's disease, which upon terminal differentiation result in Reed-Sternberg and Hodgkin cells.

The finding of Ki-67 positive Reed-Sternberg and Hodgkin cells confirms that a subpopulation of these malignant cells may proliferate [14, 27, 31]. However, most Ki-67 positive Reed-Sternberg and Hodgkin cells expressed next to CD30 also A-type lamins, indicating that these proliferating cells have a differentiated phenotype. Therefore, these results suggest that several populations of Reed-Sternberg and Hodgkin cells may exist [17] which differ in their degree of

differentiation, activation or proliferative status as shown by the absence or presence of A-type lamins and Ki-67 antigen.

In conclusion, Reed-Sternberg and Hodgkin cells express both A-type and B-type lamins in which they distinguish themselves from lamin B2 negative centroblasts and centrocytes and the A-type lamin negative mantle zone cells of the reactive lymph nodes. Furthermore, this staining pattern indicates that the malignant cells are more differentiated than these cell types although some of these cells still possess proliferative capacity.

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Chapter 5

Abnormal A-type lamin organization in a human lung carcinoma cell line

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Summary

We have studied the expression of lamins A and C (A-type lamins) in a lung carcinoma cell line using type-specific monoclonal antibodies. Using immunofluorescence and immunoblotting studies it was noted that several irregularities in lamin expression exist in the cell line GLC-A1, derived from an adenocarcinoma. First, the expression of the A-type lamins was lower than in other adenocarcinoma cell lines of the lung. Also the ratio between lamins A and C proteins was 1:8 instead of the 1:1 ratio seen in the other cell lines. Northern blotting confirmed the altered level of A-type lamin expression. Secondly, an abnormal localization of lamin A was observed. Intensely fluorescing lamin A aggregates were observed in the nucleus, rather than the typical perinuclear staining pattern. Confocal scanning laser microscopy revealed that the lamin A aggregates were indeed present throughout the internal nucleus. When these cells were extracted with Triton X-100 the nucleoplasmic aggregates disappeared, which indicates that the A-type lamins are not properly incorporated into the lamina. The A-type lamins in other cell lines derived from adenocarcinomas remained present in the nuclear periphery after extraction with the non-ionic detergent. Immunoblotting studies of the Triton X-100 soluble and insoluble fractions showed that lamin A and an apparently truncated product, which was detected with the lamin A antibody, were present in the insoluble fraction of GLC-A1. This truncated product is partly Triton X-100 soluble since it was also detected in the detergent soluble fraction. Thirdly, using an antibody to A-type lamins sporadic GLC-A1 cells showed a filamentous cytoplasmic staining pattern, which was Triton X-100 resistant. Double labelling immunofluorescence studies revealed that these cytoplasmic lamins colocalized with the vimentin cytoskeleton in this cell line.

Introduction

Lamins are nuclear proteins, which belong to the family of intermediate filament proteins and consist of A-type lamins and B-type lamins. In mammalian cells, A-type lamins are represented by lamins A and C, which are different transcripts arising from the same gene [11, 29]. B-type lamins are represented by lamin B1 and B2 in mammalian cells and are encoded by different genes [17], while recently a third B-type lamin, denoted lamin B3, has been detected in mouse spermatocytes [13]. Lamins may have a significant impact on the expression of genes by interacting with the heterochromatin, directly or indirectly, by serving as a matrix for the attachment of nuclear matrix-associated proteins, which are thought to play important roles in gene regulation. In contrast to B-type lamins the expression of A-type lamins appears to be related to the state of cellular differentiation: well-differentiated cells do in general express A-type lamins, while relatively undifferentiated cells or cells at early stages of embryonic development do not express detectable levels of A-type lamins (for reviews see [16, 22, 31]). In addition, it was shown that A-type lamins were not expressed in proliferating cells of some adult

human tissues such as the basal keratinocytes of the skin [5] or certain cell lineages of the hematopoietic system [35].

Studies on the expression of nuclear lamins in neoplasms are relatively rare. In parallel to studies in embryonic cells, Lebel *et al.* [24] showed the absence of A-type lamins in undifferentiated teratocarcinoma cell cultures. In addition, Kaufmann *et al.* [21] have shown that A-type lamins were decreased by more than 80% in small cell lung cancer (SCLC) cell lines compared to non-SCLC cell lines. The findings were confirmed in our recent study on the expression of lamins in solid SCLC and non-SCLC [3], which showed the absence or reduction of A-type lamins in SCLC as compared to non-SCLC. In addition, striking aberrations in the organization patterns of A-type lamins were observed in some non-SCLC, in particular adenocarcinomas, which showed cytoplasmic rather than nuclear lamin staining patterns in a part of the tumour cells. In this study we have examined an adenocarcinoma cell line of the lung, which showed irregularities in A-type lamin expression.

Materials and Methods

Cell Lines

Three adenocarcinoma cell lines, i.e. NCI-H125 [6, 14], GLC-A1, GLC-A2 [9] were used. The characteristics and growth conditions of these cell lines have been described previously. All cell lines were maintained in a humidified incubator at 37°C in 5% CO₂ and were grown in Roswell Park Memorial Institute Medium 1640 (RPMI-1640, ICN Flow, Irvine, UK) containing 2 g/l NaHCO₃, supplemented with 10% newborn calf serum (ICN Flow). Cells were grown to almost confluency before harvesting.

Antibodies

The following mouse monoclonal antibodies were used in this study:

- LN43 (IgG1) directed against lamin B2 and not cross-reacting with lamin B1 [1], kindly provided by Dr. E.B. Lane (Dundee, U.K.).
- X223 (IgG1) directed against lamin B2 and not cross-reacting with lamin B1 [17], kindly provided by Dr. G. Krohne (Heidelberg, Germany).
- 119D5 (IgG1) directed against lamin B1 and not cross-reacting with lamin B2 (unpublished).
- 41CC4 (IgM) [4] kindly provided by Dr. G. Warren (Heidelberg, Germany). This antibody recognizes human lamins A and C in immunoblotting.
- R27 (IgM) was a gift from Dr. G. Krohne (Heidelberg, Germany). This antibody recognizes both lamins A and C and does not react with human B-type lamins [40].
- 133A2 (IgG3) raised against the carboxy-terminus of 98 amino acids present in lamin A and absent from lamin C. This antibody recognizes lamin A but not lamin C. Epitope

mapping using several deletion mutants of lamin A showed that the amino acids 598-611 form the epitope recognized by 133A2 [20]

- RV202 (IgG1) directed against vimentin [34].
- RCK102 (IgG1) directed against keratin 5 and 8 [34].
- pNF160, a rabbit polyclonal antibody, directed to neurofilaments, kindly provided by Dr. Y. Nakazato [30].

In immunofluorescence and immunoblotting studies LN43, X223, 41CC4, R27, RV202 and RCK102 were used as undiluted culturing supernatant, while 119D5 (mouse ascites) was used 1:750 in immunofluorescence and 1:250 in immunoblotting and 133A2 (mouse ascites) 1:2500 in immunofluorescence and 1:1000 in immunoblotting, pNF160 (rabbit serum) was used 1:600 in immunofluorescence.

The antibodies 41CC4 and R27 both recognize lamin A and C and will be referred to as lamin A/C antibodies unless otherwise specified. Since the antibodies LN43 and X223 both recognize lamin B2 they will be referred to as lamin B2 antibodies unless otherwise specified. 133A2 will be referred to as lamin A antibody and 119D5 will be referred to as lamin B1 antibody.

Indirect immunofluorescence technique

The indirect immunofluorescence technique that was applied to the cell lines has been previously described [2]. Briefly, cells were grown on glass coverslips for 1-2 days. After washing in phosphate buffered saline (PBS, containing 8 g/l NaCl, 0.2 g/l KCl, 1.15 g/l Na₂HPO₄ and 0.2 g/l KH₂PO₄ at pH 7.4) the cells were fixed in methanol (-20°C for 5 seconds) followed by acetone (room temperature, 3 times five seconds) and air dried. For single labelling immunofluorescence cells were then incubated with the primary antibody for 30-45 minutes, and after several washes with PBS, incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse Ig (1:100, DAKO A/S) for 30-45 min. For double labelling immunofluorescence the cells were incubated with a lamin A/C antibody of the IgM subclass for 30-45 minutes and after several washes with PBS incubated with FITC conjugated goat anti-mouse IgM (diluted 1/80, Southern Biotechnology associates (SBA), Birmingham, Alabama). Then after several washes with PBS, the antibody RV202 was applied for 5-10 minutes and after several washes with PBS the cells were incubated with Texas Red conjugated goat anti-mouse IgG1 (diluted 1/80, SBA). Thereafter, cells were washed again in PBS, mounted in 90% glycerol containing 0.02M Tris-HCl pH 8.0, 0.002% NaN₃, 2% 1,4 diazabicyclo (2,2,2)-octane (DABCO; Merck) and 0.5 µg/ml 4',6 diamino-2 phenylindole (DAPI; Merck) for DNA staining, and photographs were taken with an automatic camera using 400 ASA Tri-X pan film (Kodak, England).

Triton X-100 extraction of cells for immunofluorescence studies

Prior to Triton X-100 treatment the cells on the coverslips were rinsed with PBS at room temperature (RT). Then the coverslips were placed on ice for Triton X-100 extraction.

Precooled (4°C) PBS containing 1 mM EDTA (Titriplex III, Merck), 1 mM EGTA (Titriplex VI, Merck), 0.4 mM PMSF (Aldrich, Steinheim, Germany) and 0.1% Triton X-100 (BDH) was used for a 5 minute extraction. The coverslips were then washed three times with cold PBS containing 1 mM EDTA, 1 mM EGTA, 0.4 mM PMSF and the cells were fixed and immunostained as described above.

Sample preparation for one-dimensional gel electrophoresis

Samples of non-extracted cells:

The cells were rinsed with PBS at RT and the culture flask was placed on ice. Then 10 ml PBS containing 1 mM EDTA, 1 mM EGTA, 0.4 mM PMSF was added and the cells were collected by scraping with a rubber policeman from the bottom of the culture flask. The cell suspension was centrifuged at 4100 g and the pellet and the supernatant were separated. The proteins in the supernatant were precipitated with trichloroacetic acid (TCA, Merck), which was added to a final concentration of 10% (w/v) and allowed to precipitate on ice for 30-60 minutes. The precipitated proteins were pelleted by centrifugation at 2500 g for 10 minutes, washed twice with ice cold acetone (-20°C) and air dried. The 4100 g cell pellet and the TCA-pellet of the PBS soluble fractions were resuspended in 200 µl SDS-sample buffer and boiled for 5 minutes.

Samples of Triton X-100 extracted cells:

The cells were washed twice with PBS at RT. Then the flasks were placed on ice and the cells were extracted for 5 minutes with cold (4°C) PBS containing 1 mM EDTA, 1 mM EGTA, 0.4 mM PMSF and 0.5% Triton X-100 (BDH). The supernatant was discarded and the cell remainders were rinsed twice with cold PBS containing 1 mM EDTA, 1 mM EGTA and 0.4 mM PMSF. The cell remainders were collected by scraping in cold PBS containing 1 mM EDTA, 1 mM EGTA and 0.4 mM PMSF, the culture flasks were rinsed with the same buffer and the two fractions were pooled. These pooled fractions were centrifuged at 4100 g and the pellet and supernatant were separated. The proteins in the supernatant were TCA-precipitated as described above. All samples were dissolved by boiling for 5 minutes in 200 µl SDS-sample buffer.

As a result of this approach equal sample volumes of different cellular fractions correspond with equal cell numbers.

Sample preparation for two-dimensional gel electrophoresis

Cells were washed with cold PBS (4°C) containing 0.5 mM PMSC (phenyl methyl sulfonyl chloride, Merck, Hohenbrunn, Germany) and collected by scraping in cold PBS containing 0.5 mM PMSC. They were centrifuged for 5 minutes at 1500 g and the pellet was Triton X-100 extracted for 10 minutes by stirring the pellet in Tris-buffer containing 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 1.5 mM MgCl₂, 0.5 mM PMSF and 0.5% Triton X-100. The extracted cells were centrifuged at 1500 g and the pellet was treated with RNase/DNase mix (1 mg/ml DNase I, 0.5 mM PMSF, 0.05 mg/ml RNase A in 10 mM Tris-HCl pH 7.4, 110 mM NaCl and 1.5 mM

MgCl₂) for 20 minutes at RT. Then cold Tris-buffer was added and the cells were centrifuged at 1500 g. The supernatant was discarded and the pellet was boiled in SDS-sample buffer for 5 minutes. These samples were stored at -20°C. Before loading onto an isoelectric focusing (IEF) gel an equal volume of buffer containing 2% Nonidet P40 (Fluka, Buchs, Switzerland), 5% β -mercaptoethanol (Merck), 5% Bio-Lyte 3/10 carrier ampholytes (Bio-Rad Laboratories, Hercules, California) and 9 M urea (Merck) was added followed by addition of 1/5 volume 1 M 3-[(3-cholamidopropyl)dimethylammonio]1-propanesulfonate (CHAPS, Sigma) followed by addition of urea (Merck) until saturation.

Gel electrophoresis and immunoblotting

One-dimensional SDS-gel electrophoresis in polyacrylamide was performed according to Laemmli [23] using gels containing 10% polyacrylamide (Bio-Rad Laboratories, Hercules, California) and 0.1% SDS (Merck). Gels were run on the Mini-Protean II system (Bio-Rad Laboratories) for approximately 45 minutes at 200 V.

Two-dimensional gels were run essentially as described by O'Farrell [32], with the following modifications. Isoelectric focusing (IEF) was performed in 4% polyacrylamide gels containing 8M urea, 1.4% Bio-Lyte 3/10 carrier ampholytes (Bio-Rad) and 25 mM CHAPS (Bio-Rad Laboratories). Gels were run on the Mini-Protean II 2-D system (Bio-Rad Laboratories) and for IEF no pre-electrophoresis was performed. Gels were run at increasing voltages starting at 500 V for 10 minutes, followed by 1000 V for 20 minutes and 1500 V for 100 minutes.

In the second dimension, 10% polyacrylamide SDS gels were run similar to the one-dimensional gel electrophoresis as described above.

Western blotting was performed essentially according to Towbin [38] using the Mini Trans-Blot Cell (Bio-Rad) at 100 V for 1 hour. Proteins were blotted onto nitrocellulose (BA85, Schleicher and Schüll, Dassel, Germany). After blotting nitrocellulose sheets were air-dried and stored until immunochemical detection.

Prior to application of the primary antibodies the westernblots were blocked for 1 hour at RT in buffer containing 10 mM Tris-HCl pH 7.6, 0.35 M NaCl and 3% bovine serum albumin (BSA, Sigma, St. Louis, Missouri). Thereafter the blots were incubated with the primary antibodies for 30-60 minutes at RT in appropriate dilutions in buffer containing 10 mM TRIS-HCl pH 7.6, 0.15 M NaCl, 0.3% BSA, 1% Triton X-100, 0.5% DOC (Merck) and 0.1% SDS (Merck). Then the blots were washed three times with PBS containing 0.5% Triton X-100 (BDH). The secondary peroxidase conjugated rabbit anti-mouse Ig antibody (DAKO A/S) was diluted 1:1000 in PBS containing 0.5% Triton X-100 and 0.5% BSA and the blots were incubated with this antibody for 30-60 minutes at RT. Then the blots were extensively washed with PBS containing 0.5% Triton X-100 and peroxidase activity was detected by chemiluminescence (ECL-kit, Amersham, Buckinghamshire, UK). RX Fuji medical X ray films (Fuji, Tokyo, Japan) were used for the visualisation of the luminescent signals.

Messenger (m)RNA isolation and northern blotting analysis

Total RNA was isolated from the cell lines by the guanidine isothiocyanate procedure as described previously [7, 8]. The RNA was dissolved in 200-400 µl DEPC (Diethyl pyrocarbonate, Sigma) treated mQ water, two volumes of ethanol p.a. (Merck) was added and the samples were stored at -70°C.

Twenty µg of total RNA, as determined at OD 260, was size fractionated on a formaldehyde agarose gel [25] and blotted onto Hybond-N+ membrane (Amersham Life Science, Little Chalfont, United Kingdom). The blots were air dried for several hours and then the RNA was crosslinked with 120 Joules/cm² in an U.V. Stratalinker 1800 (Stratagene, La Jolla, California). The blots were prehybridized for 3 hours at 65 °C in a hybridization mix containing 7% SDS, 1% BSA, 2 mM EDTA, 0.5 M Na₂HPO₄ at pH 7.0 and an excess of single stranded sheared salmon sperm DNA. Then the [³²P] radioactive labeled probe (see below) was added to this mixture and hybridized overnight at 65 °C. After hybridization the blots were washed with saline citrate buffer (SSC, 0.15 M NaCl and 0.015 M sodium citrate pH 7.0) containing 0.1% SDS. Stringency conditions were optimized for each DNA probe. Autoradiography was done on RX Fuji medical X ray films (Fuji) at -70°C using an intensifying screen. Densitometry of the autoradiograms was performed using a Computing Densitometer 300 (Molecular Dynamics, Sunnyvale, California) with the Quantity one (PDI, Protein Databases Inc., Huntington Station, New York) software on a Sun Sparc 1+ station.

Probes

Hybridization was performed with 50-200 ng probe DNA. The lamin A/C probe, kindly provided by Dr. F McKeon [29], was labeled with [³²P] α-dATP by random priming labeling [10] and purified over a Sephadex G50 fine (Pharmacia) column. A GAPDH probe [12] was used as a control for the amount mRNA loaded onto the gel.

Results

Subcellular lamin localization as detected by immunofluorescence

Representative reactivity patterns of perinuclear lamin expression in the lung adenocarcinoma cell lines with the different lamin antibodies are shown in figure 1a,b,c.

The antibody to lamin A showed strong decoration of the nuclear lamina in the cell lines, but in GLC-A1 (Fig. 1e) an aberrant A-type lamin protein distribution pattern was observed in the immunofluorescence assay, in that intensely stained aggregates were detected in the nucleus. Confocal scanning microscopy revealed that the nuclear aggregates were localized throughout the whole nucleus and not only on the perinuclear lamina (Fig. 2). In addition to the clustered appearance, a normal lamina was stained in these cells with lamin A/C antibodies (Fig. 1f), but the fluorescence intensity was only very weak compared to the signal obtained in the other cell

Figure 1. Immunofluorescence micrographs of cell lines GLC-A2 (a,b,c) and GLC-A1 (d,e,f), stained with the lamin B1 antibody 119D5 (a), the lamin B2 antibody LN43 (d), the lamin A antibody 133A2 (b,e), the lamin A/C antibody 41CC4 (c) and the lamin A/C antibody R27 (f). Bar indicates approximately 12 μ m.

Figure 2. Z-series of confocal images of GLC-A1 cells, stained with the lamin A antibody 133A2. The series goes from the top (a) to the bottom (h) of the cells. Bar indicates 10 μ m.

lines. Also when compared to the reaction with B-type lamin antibodies in GLC-A1 (Fig. 1d) the perinuclear A-type lamin staining has to be interpreted as weak. The nuclear aggregates were dissolved when the cells were treated with 0.1% Triton X-100 prior to fixation (Fig. 3). However, the normal lamina staining with A-type and B-type lamin antibodies persisted treatment with Triton X-100 and was still detectable in the cells that had lost the aggregates. Furthermore in GLC-A1 another aberrant lamin distribution pattern was detected when stained with the lamin A/C antibody R27. In most cells the lamina was only very weakly stained. However, in some cells an apparent normal intensity of A-type lamin perinuclear staining was observed (Fig. 1f). Additionally, in a small fraction of the cells strongly staining extranuclear aggregates, next to a faint filamentous staining was observed with the antibody R27 (not shown). These A-type lamins appeared to be stably incorporated in cytoskeletal filaments, since they persisted treatment with 0.1% Triton X-100. Double labelling immunofluorescence studies with antibodies to cytoskeletal proteins revealed colocalization of the cytoplasmic A-type lamins with vimentin. Colocalization of A-type lamins with neurofilaments and cytokeratins did not seem to occur.

Figure 3. Immunofluorescence staining patterns of GLC-A1 cells with the lamin A antibody 133A2 before (a) and after (b) treatment with 0.1% Triton X-100. Bar indicates 10 μ m.

Solubility properties of lamins as detected by immunoblotting

In order to further characterize the Triton X-100 soluble and Triton X-100 insoluble lamin fractions in the cell lines, one-dimensional immunoblotting studies were performed of pellet and supernatant fractions after Triton X-100 extraction of the cells. Immunoblots of these fractions were tested with the six lamin antibodies.

After the Triton X-100 extraction step most of the cells were still attached to the bottom of the culture flask. These cells were scraped from the bottom and collected; this cell suspension was centrifuged and the Triton X-100 supernatant and Triton X-100 cell pellet were prepared for gel

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After the Triton X-100 extraction step most of the cells were still attached to the bottom of the culture flask. These cells were scraped from the bottom and collected; this cell suspension was centrifuged and the Triton X-100 supernatant and Triton X-100 cell pellet were prepared for gel

electrophoresis. A similar fractionation was done with cells that were not Triton X-100 extracted and the PBS supernatant and PBS cell pellet were prepared for gel electrophoresis.

When the fractions the cell lines were incubated with a lamin B1 or lamin B2 antibody, only one band was detected in the Triton X-100 cell pellets and PBS cell pellets (Fig. 4, lanes 1-4). These results matched with the results obtained in the immunofluorescence studies, in which no aberrant patterns for lamin B1 and B2 were detected in the cell lines.

Blots of the same samples were also incubated with antibody R27, reacting with both lamins A and C. The results of the cell lines NCI-H125 and GLC-A2 were comparable (illustrated for NCI-H125 in Fig. 4, lanes 5-8), in that the Triton X-100 cell pellets and PBS cell pellets of the cell lines showed both lamin A and lamin C as two strong bands in an approximate 1/1 ratio. The lamin A band is actually a doublet, which is seen as a single band after prolonged exposure in chemiluminescence.

In GLC-A1 the intensity of both lamin A and lamin C bands of the Triton X-100 cell pellet and PBS cell pellet was completely different from those seen in the other cell lines, while also the ratio of lamin A over lamin C was aberrant (Fig. 4, lanes 8-12). The lamin A band was hardly detectable in the GLC-A1 Triton X-100 cell pellet and PBS cell pellet, while the lamin C band was clearly visible, but weaker when compared to the other cell lines. In addition, when examined closer this lamin C band appeared to be a doublet. The overall weaker reaction of lamin A/C antibodies on blots of GLC A1 samples matched with the results of the

Figure 4. One-dimensional immunoblotting of lamin B2 and lamin A/C in the Triton X-100 supernatants (lanes 1,5,9), Triton X-100 cell pellets (lanes 2,6,10), PBS supernatants (lanes 3,7,11) and PBS cell pellets (lanes 4,8,12). Blots of these fractions of GLC-A1 (lanes 1-4) cells were incubated with the lamin B2 antibody LN43, blots of these fractions of NCI-H125 (lanes 5-8) and GLC-A1 (lanes 9-12) cells were incubated with the lamin A/C antibody R27.

immunofluorescence studies, in which the lamin staining with these antibodies was also weak and aberrant. In the soluble fractions of the cell lines no reactivity was detected with the lamin A/C antibodies (Fig. 4, odd numbered lanes).

When stained with lamin A antibody 133A2 the blots of the soluble and insoluble fractions of the cell lines showed distinct differences between the cell lines (Fig. 5). The blotting results for

the cell lines NCI-H125 and GLC-A2 were identical (fig. 5, lanes 1-8). In the Triton X-100 cell pellet and PBS cell pellet of these two cell lines a lamin A doublet of approximately 72 kDa was detected (Fig. 5, lanes 2,4,6,8). Furthermore a relative faint additional band with a lower molecular mass of approximately 63 kDa was detected with the antibody to lamin A. In the Triton X-100 supernatant of NCI-H125 and GLC-A2 four bands were detected (Fig. 5, lanes 1 and 5). The upper two bands were at the same molecular weight levels as the bands detected in the Triton X-100 cell pellets (Fig. 5, lanes 2 and 6), while the two additional bands showed a lower molecular weight. In the PBS soluble fractions no bands were detected (Fig. 5, lanes 3 and 7).

The immunoblots with the GLC-A1 samples showed again that the lamin A expression level was much lower as compared to the other cell lines (Fig. 5, lanes 9-12). In the Triton X-100 cell pellet and PBS cell pellet of GLC-A1 identical bands were detected as in NCI-H125 and GLC-A2, but the intensity ratio between the two bands was changed (Fig. 5, lanes 2,4,6,8,10,12). Furthermore in the Triton X-100 supernatant of GLC-A1 cells only the three lower molecular weight bands, indicated as A' (Fig. 5, lane 9), were detected, while the band at the normal lamin A level was missing in this sample. Again no bands were detected in the PBS soluble fractions (Fig. 5, lane 11). It is remarkable that with the antibodies to lamins A and C (R27 and 41CC4) never any band was detected at the lamin A level in the Triton X-100 soluble fractions.

Figure 5. One-dimensional immunoblotting of lamin A in the Triton X-100 supernatants (lanes 1,5,9), Triton X-100 cell pellets (lanes 2,6,10), PBS supernatants (lanes 3,7,11) and PBS cell pellets (lanes 4,8,12). The fractions of the cell lines GLC-A2 (lanes 1-4), NCI-H125 (lanes 5-8) and GLC-A1 (lanes 9-12) were incubated with the lamin A antibody 133A2.

Immunostaining of two-dimensional blots of the insoluble fraction of GLC-A1, showed that after applying the lamin A antibody 133A2 and using an exposure time as used for the other cell lines and antibodies, only one very small spot was visible for lamin A. After relatively long exposure it became evident that two arrays of several isoforms of lamin A could be detected in GLC-A1 (Fig. 6a). However, the protein with the lower molecular weight, as detected in one-dimensional blotting (Fig. 5, lane 10 indicated as lamin A'), was not visible in this two-dimensional blotting. When the two-dimensional immunoblots of GLC-A1 were stained with

the lamin A/C antibody R27 a strong reaction was seen with lamin C and only a faint spot was detected for lamin A under these exposure conditions (fig. 6b). The signal for lamin C was a doublet of arrays with little difference in molecular masses and distinct phosphorylation isoforms. When the different lamins were detected simultaneously in one immunoblot, the relative abundance of each component became apparent. The relatively high abundance of B-type lamins is obvious from figure 6c, while also the extremely low concentration of A-type lamins is evident as compared to identical blots of the other cell lines (Fig. 6d).

The lamin A signals were seen as doublets, with only small differences in molecular masses. The isoelectric mobilities of both arrays of lamin A isoforms were identical. Several spots with lower molecular masses were consistently detected by the lamin antibodies. The most prominent of these have molecular masses of approximately 46 kDa, most likely representing degradation products of the lamins [33].

Figure 6. Two-dimensional immunoblotting of Triton X-100 extracted cells of GLC-A1 (a,b,c) and GLC-A2 (d). The immunoblots were incubated with the lamin A antibody 133A2 (a), the lamin A/C antibody R27 (b), or simultaneously with the lamin B1 antibody 119D5, the lamin B2 antibody LN43, the lamin A antibody 133A2 and the lamin A/C antibody 41CC4 (c,d).

Messenger RNA levels of lamins as detected by Northern blotting

RNA isolated from the three cell lines was subjected to Northern blotting analysis (Fig. 7). Hybridization with an A-type lamin probe showed major differences in lamin A and C mRNA expression between the cell lines. In the cell lines NCI-H125 and GLC-A2 both lamin A and Lamin C mRNA were detected. The lamin A over lamin C mRNA ratio varied from 1:1.5 to 1:2 as measured by densitometry. However in the mRNA samples isolated from GLC-A1 the total

A-type lamin mRNA expression was 5 to 10 fold decreased compared to the A-type lamin mRNA expression in the other cell lines. Moreover, the expression ratio of lamin A over lamin C was changed to 1:8. Consequently, the lamin A mRNA was hardly detectable. No abnormally sized mRNA was detected in the cell lines. The decreased A-type lamin mRNA level and altered ratio of lamin A over lamin C in GLC-A1 was in concordance with the immunoblotting results. The Northern blot was rehybridized with a GAPDH probe as a control for the amount of RNA blotted onto the membrane. This confirmed that the amount of mRNA from GLC-A1 loaded onto the gel was comparable to that of the other cell lines.

Figure 7. Northern blotting of mRNA isolated from cell lines GLC-A1 (lane 1), GLC-A2 (lane 2) and NCI-H125 (lane 3). The blot was hybridized with a lamin A/C probe (a) and a GAPDH probe (b).

Discussion

In this study the molecular basis of an aberrant expression pattern of lamins in a human lung carcinoma cell line has been investigated. Previous studies on lamin expression in normal lung tissues and lung tumours showed that B-type lamins seem to be ubiquitously expressed in cells of normal tissues and most lung cancers [36, 39], but that in adenocarcinomas of the lung they could not be detected in certain areas of these malignancies. Furthermore, cytoplasmic instead of nuclear localization of A-type lamins in adenocarcinomas has been reported [3]. In the study presented here the A-type lamin expression showed aberrancies at the protein and mRNA levels in an adenocarcinoma cell line.

In immunofluorescence studies with antibody 133A2, directed to the carboxy-terminal tail of lamin A, which is lacking in lamin C [20], an aberrant lamin A localization was seen in GLC-A1 cells. The perinuclear lamina was only slightly stained, while heavily stained aggregates

were detected throughout the nucleoplasm. Our experiments showed that the majority of the nuclear aggregates were removed upon Triton X-100 treatment. These results indicate that a soluble form of lamin A is present in these aggregates. It has been described that non-prenylated prelamins A accumulate as nucleoplasmic inclusions in the nuclei of cells that have been blocked in prenylation [28]. However, the possibility of accumulation of prelamins A in the nuclei of GLC-A1 could be excluded by using an antibody specific for prelamins A (kindly provided by Dr. M. Sinenski [37]), which did not show any nuclear reactivity in GLC-A1 (data not shown).

In order to identify the protein that was responsible for the aggregate-like staining pattern, immunoblotting studies were performed. In one-dimensional immunoblots of GLC-A1, that were incubated with the lamin A antibody 133A2, a protein band with a lower molecular weight than mature lamin A was detected. This lamin A' appeared to be preferentially extracted, as compared to lamin A. Probably this lamin A' is responsible for the Triton X-100 soluble nuclear aggregates that were detected in GLC-A1 cells. However, lamin A' was also present in the other cell lines, but the ratio of normal lamin A over the smaller lamin A' protein was completely different in these cell lines, in that the lamin A' was expressed at relatively very low levels. Therefore, the relative excess of normal lamin A might obscure the presence of the lamin A' protein in these cell lines.

Loewinger and McKeon [27] have described abnormal nuclear aggregation of lamin A lacking the carboxy terminal part distal to amino acid 444, indicating that this part of lamin A is necessary for correct incorporation into the lamina. Additional studies [18] have shown that not only the carboxy terminal CaaX motive is responsible for the incorporation, but that additional sequences of lamin A are required for proper incorporation. The fact that lamin A' is recognized by the antibody 133A2 indicates that at least part of the carboxy terminal region is intact, since the epitope for this antibody is localized between amino acid 598 and 611. However, the decreased molecular weight might predict that deletions within the molecule occur.

The observation that lamin A' is not detectable under our standard isoelectric focusing conditions, encompassing a pI range of pH 4-8, might indicate that such a deletion causes an extreme shift in the isoelectric point. The exact nature of lamin A' remains, however, to be established.

The absence of the typical aggregate-like reactivity with the lamin A/C antibodies may be explained by the observation that in two-dimensional blottings the lamin A/C antibodies have a stronger reactivity with lamin C than with lamin A, while the lamin A antibody showed a very strong reactivity with lamin A. Therefore preferential staining of lamin C with the lamin A/C antibodies might also occur in immunofluorescence assays, through which nucleoplasmic lamin A aggregates remain invisible.

Furthermore total A-type lamin expression levels were decreased in GLC-A1 and the ratio of lamin A over lamin C was altered, as detected in immunoblotting and northern blotting. Since lamins A and C arise from the same gene by alternative splicing [26] an explanation for the

altered ratio of lamin A over lamin C may be an altered relative preference for alternative splicing. However, the mechanism behind the regulation of the lamin A to C mRNA ratio is still unclear [26].

The shift in lamin A over lamin C protein ratio in the GLC-A1 cell line probably has consequences for lamina assembly, in particular for the incorporation of lamin C into the lamina, since it has been reported that the absence of lamin A disturbs proper assembly of lamin C into the lamina [19]. Most likely, in some cells the relative excess of lamin C becomes stably integrated in newly formed cytoplasmic filaments or into the pre-existing vimentin cytoskeleton, which leads to a Triton X-100 insoluble cytoplasmic localization. This would explain why the cytoplasmic filaments, that were seen in some GLC-A1 cells, were detected with an antibody directed to both lamin A and C and not with the antibody that recognized only lamin A. Unincorporated lamin C will not remain for long in the cytoplasm, since it has been found that unassembled lamins have a short half-life [15].

In summary, we have described an abnormal nuclear and cytoplasmic localization of A-type lamins in the adenocarcinoma cell line GLC-A1, and by additional immunoblotting and northern blotting studies we have tried to elucidate the nature of these aberrancies. To further validate and confirm our present results additional two-dimensional immunoblotting and molecular biology studies have been initiated.

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Chapter 6

An alternative splicing product of the lamin A/C gene lacks exon 10

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Summary

Expression of the A-type lamins was studied in the lung adenocarcinoma cell line GLC-A1. A-type lamins, consisting of lamin A and C, are two products arising from the same gene by alternative splicing. Northern blotting showed in GLC-A1 a relatively low expression level of lamin C and an even lower expression level of lamin A as compared to other adenocarcinoma cell lines. Immunofluorescence studies revealed highly irregular nuclear inclusions of lamin A, suggesting protein or gene expression abnormalities. Reverse transcriptase-PCR based cDNA analysis followed by sequencing indicated the presence of an as yet unidentified alternative splicing product of the lamin A/C gene. This product differs from lamin A by the absence of the 5' part of exon 10 (90 nucleotides). Therefore we propose to designate this product lamin A Δ 10. Deletion of the 30 amino acids encoded by exon 10 was predicted to result in a shift in pI of the protein from 7.4 to approximately 8.6, which was confirmed by two-dimensional immunoblotting. mRNA analysis in a variety of cell lines, normal colon tissue as well as carcinomas demonstrated the presence of lamina A Δ 10 in all samples examined, suggesting its presence in variety of cell types.

Introduction

Lamins are intermediate filament type proteins which form the major components of the nuclear lamina. Two main types of lamins are known in mammals; i.e. A-type lamins and B-type lamins. The B-type lamins, B1 and B2, are encoded by two distinct genes [21], while lamin B3 is a recently discovered alternative splicing product of the lamin B2 gene in embryonic cells [17]. At least one of the B-type lamins is ubiquitously expressed in mammalian cells and their expression appears to be independent of the state of cellular differentiation [38, 42]. The A-type lamins, represented by lamins A and C, are products arising from one gene by alternative splicing [14, 28, 32]. A-type lamin expression appears to be related to the state of cellular differentiation. In general, well-differentiated cells express A-type lamins, whereas undifferentiated cells synthesize low or undetectable levels of A-type lamins [6, 19, 25]. In addition it is shown that A-type lamins are not expressed in proliferating cells of some adult tissues such as basal cells of the skin [8] or certain lineages of the hematopoietic system [37]. The recent unravelling of the complete human lamin A/C gene [28] has provided a better insight into the mechanism by which lamins A and C are generated from the same gene. The site for alternative splicing has been demonstrated to be located within exon 10. While exon 1 through 9 of the lamin A and C mRNAs are identical, lamin A mRNA further contains the 5' 90 bases of exon 10, followed by exon 11 and 12. In contrast the lamin C messenger contains the complete 111 base sequence of exon 10, but not exon 11 and 12. In this paper we describe a third splicing product of the lamin A/C gene, identical to lamin A with the exception of the absence of exon 10.

Materials and Methods

Cell Lines

Adenocarcinoma cell lines NCI-H125, NCI-H23 [9, 18], GLC-A1, GLC-A2 [12] and NL-Ac1 [5], the neuroendocrine non-small cell lung carcinoma NCI-H460 [7], the large cell undifferentiated lung carcinoma cell line LCLC-103H [1], the breast carcinoma cell line T47D [16], and the (bladder) transitional carcinoma cell line T24 [36] were grown in Roswell Park Memorial Institute Medium 1640 (RPMI-1640, ICN Flow, Irvine, UK) containing 2 g/l NaHCO_3 , supplemented with 10% newborn calf serum (ICN Flow). The epitheloid lung carcinoma cell line MR65 [30] was grown in DMEM in 10% newborn calf serum, while the neuroblastoma cell line SK-N-SH [3] was grown in RPMI with 15% fetal bovine serum. All cell lines were maintained in a humidified incubator at 37°C in 5% CO_2 .

Human tissue specimens

Human lung adenocarcinoma specimens and a specimen of normal colon were obtained after surgery and snap frozen in liquid nitrogen. Samples were homogenized in ice-cold guanidine isothiocyanate buffer [11] using a Omni 1001 mixer (Omni, Waterbury, CT, USA) at 20,000 rpm for 15-60 seconds and stored at -70°C until use.

Immunofluorescence

The procedure for immunofluorescence has been described [31]. Primary antibodies used include 133A2 [23] recognizing lamin A and LN43 [4] recognizing lamin B2. As secondary antibody FITC-labeled rabbit anti-mouse immunoglobulins (DAKO, Glostrup, Denmark) was used.

RNA isolation and Northern blotting analysis

Total RNA was isolated from cell lines and tissue specimens by the guanidine isothiocyanate procedure [10, 11]. Twenty μg of total RNA was size-fractionated on a formaldehyde agarose gel [27], blotted onto Hybond-N+ membrane (Amersham Life Science, Little Chalfont, UK) and cross-linked with 120 Joules/cm² in an U.V. Stratalinker 1800 (Stratagene, La Jolla, CA/USA). Blots were hybridized overnight at 65 °C in a hybridization mix containing 7% sodium dodecyl sulphate (SDS, BRL Life Technologies, Gaithersburg, MD/USA), 1% bovine albumin (BSA, Sigma, St. Louis, MO/USA), 2 mM EDTA, 0.5 M Na_2HPO_4 (pH 7.0) and 0.1 mg/ml sheared salmon sperm DNA (Boehringer Mannheim GmbH, Mannheim, Germany), to which [³²P]dATP labeled probe (see below) was added. After hybridization the blots were washed with saline citrate buffer (SSC, 0.15 M NaCl and 0.015 M sodium citrate pH 7.0) containing 0.1% SDS. Stringency conditions were optimized for each DNA probe. Autoradiography was performed with RX Fuji medical X ray films (Fuji) at -70°C using intensifying screens.

Probes

The lamin A/C probe, kindly provided by Dr. F McKeon [32], and the lamin B1 probe, a kind gift of Dr. Pollard [35], were labeled with [32 P] α -dATP (Amersham) by random priming [13] and purified over a Sephadex G50 fine (Pharmacia, Uppsala, Sweden) column. Each hybridization was performed with 50-200 ng probe DNA. A GAPDH probe [15] was used as a control for the amount mRNA loaded onto the gel.

cDNA synthesis and PCR analysis

The reverse transcriptase (RT) reaction was performed with 25 μ g of total RNA or 5 μ g of poly A+ RNA. Samples were incubated for 1.5 hours at 37°C in a reaction mixture containing 10 μ g oligo dT primers (Pharmacia), 1 mM of each dNTP (Pharmacia), 40 units RNasin (Promega, Madison, WI, USA), 0.01 M dithiothreitol (Sigma) and 600 units MMV reverse transcriptase (Gibco/BRL) in a total volume of 50 μ l. After completion of the RT reaction 5 μ l of this reaction mixture was used for PCR in a total reaction volume of 50 μ l, containing 50 mM KCl (Merck), 10 mM Tris-HCl pH 8.3 (Merck), 4 mM MgCl₂ (Merck), 0.001% gelatin, 1 mM of each dNTP (Pharmacia), 1.5 μ g of primer 1 (sense, 5'-GCCTACCGCAAGCTCTTGGA-3' (Eurogentec, Seraing, Belgium), corresponding to lamin A codon 375-381 (nucleotides 1123-1142, see Fig. 2A) and 1.5 μ g of primer 2 (antisense, 5'-GGTGAGGAGGACGCAGGAAG-3', lamin A non coding region, nucleotides 2030-2049). This mixture was heated to 95°C for 5 minutes, cooled to 80°C for 5 minutes and thereafter 0.2 units of Taq polymerase (Super Taq, HT Biotechnology LTD, Cambridge, UK) was added. The mixture was overlaid with 60 μ l mineral oil (Sigma) heated at 94°C for 2 minutes and carried through 30 cycles of denaturation (94°C, 1 min.), annealing (54-58°C, 2 min.) and elongation (72°C, 2 min.), followed by a final extension step of 72°C for 10 minutes. PCR products were used for subcloning (see below) or for a second round of PCR. For this latter purpose, samples were first run on 1.5% agarose gel. Bands at the 800-900 bp level corresponding to the predicted size of products of lamin A and not lamin C cDNA were excised and (electro-)eluted. Next, PCR was performed under identical conditions as described above with primers 3 (sense, 5'-AGCCTGCGTACGGCTCTCAT-3', lamin A codon 525-531, nucleotides 1573-1592) and 4 (antisense, 5'-GCTCCTGAGCCGCTGGCAGA-3', lamin A codons 599-605, nucleotides 1795-1814).

Oligonucleotide hybridization

PCR products were run on a 2% alkaline agarose gel [39], blotted onto Hybond N+ (Amersham) and hybridized with 32 P- γ ATP end-labelled oligonucleotides. 5'-End labelling of 10 pmoles of oligonucleotides was performed using the USB T4 Polynucleotide Kinase system (USB, Cleveland, Ohio). Oligonucleotides comprised primer 3 (see above), hybridizing to a region within exon 9, and an oligonucleotide complementary to the last 11 bases of exon 9 and the first 11 bases of exon 11 (oligo 5: 5'-CAGTGGGAGCCTTCCCCAGTGG-3'), thus enabling a

specific hybridization to lamin A cDNA, in which exon 10 is lacking. Hybridization was performed at 46°C in 5xSSPE buffer containing 0.3% SDS. Washes were at 52°C in the same buffer. The same blot was first hybridized with primer 3, stripped and rehybridized with oligo 5.

Genomic DNA analysis

DNA was isolated from human leukocytes, human placenta and cell lines GLC-A1 and NL-Ac1 using standard procedures [39]. Purified genomic DNA was subjected to a single round of PCR using either primers 3 and 4 (see above) or primer 3 and primer 6, 5'-GAAAAGATTTTGGCACGG-3', complementary to the sequence in the untranslated region of exon 10. The use of primers 3 and 4 in PCR is supposed to produce a 1405 bp. fragment for the lamin A/C gene, while primers 3 and 6 will yield a 649 bp. fragment for this gene.

Plasmid subcloning, restriction analysis and sequencing

Two products of 927 and 837 bp resulting from PCR with primers 1 and 2 were subcloned into the pGEM-T vector (Promega) and used to transform E. Coli TG1 cells by electroporation. Cloned plasmids were analysed by restriction fragment analysis. The restriction digestions were performed with a combination of the endonucleases NcoI and SstII, or SstI and PstI (Promega). Relevant restriction sites are indicated in Fig. 2A. Cloned fragments of the PCR reaction with primers 1 and 2 were digested with PstI and the fragment from nucleotide 1496 to 1707 was subcloned in pUC 19. Subsequently these fragments were sequenced using the dideoxynucleotide chain termination method [40] with 1.5-2 µg template plasmid using a T7 sequencing kit (Pharmacia).

Two-dimensional gel electrophoresis and immunoblotting.

Cytoskeletal preparations of cell line GLC-A1 were prepared as described [31] and subjected to non-equilibrium pH gel electrophoresis [34]. Electrophoresis was performed for 1800Vh using 2% Bio-Lyte 3-10 carrier ampholytes (Bio-Rad Laboratories, Hercules, CA/USA). In the second dimension 10% SDS/polyacrylamide gels were used according to Laemmli [26].

Proteins were immunoblotted onto nitrocellulose and the presence of lamin A was detected by antibody 133A2 [23] using enhanced chemiluminescence (ECL kit, Amersham, Buckinghamshire, England) as described before [31].

Results

Immunofluorescence.

In cell line GLC-A1 an abnormal localization of lamin A has been detected using immunofluorescence microscopy (Fig. 1A, see also [31]). Instead of a perinuclear localization, as seen for the B-type lamins, nuclear aggregates of lamin A were seen in these cells, when stained with an antibody directed to lamin A. In contrast, lamin B2 appears to be organized in a

perinuclear lamina (Fig. 1B) in the same cell line. Another adenocarcinoma cell line GLC-A2 expressed a normal fluorescence staining pattern with the lamin A antibody (fig. 1C)

Figure 1. Immunofluorescence of cell line GLC-A1 (Panel A,B) and GLC-A2 (C), using an antibody to lamin A (Panel A,C) or lamin B2 (Panel B). Bar represents 20 μ m

Northern blotting.

Northern blotting studies, comparing lamin expression in several adenocarcinoma cell lines of the lung revealed that in the cell line GLC-A1 A-type lamin mRNA levels were largely reduced as compared to the other cell lines (Fig. 2). Lamin C mRNA levels in GLC-A1 were much lower than in the other cell lines and the lamin A messenger was barely detectable on Northern blot. No obvious abnormalities in the predicted sizes of the A-type lamin mRNAs were observed.

Figure 2. Northern blotting of cell lines GLC-A1, GLC-A2, NL-Ac1 and NCI-H125 (lanes 1-4), hybridized with the lamin A probe (Panel A). Note the reduced expression of A-type lamins in GLC-A1 (lane 1). Panel B: lamin B1 probe; Panel C: GAPDH probe.

Detection of a new type of lamin A/C mRNA

To examine whether minor mRNA abnormalities could be detected, RT-PCR was performed. Special emphasis was put in the region coding for the carboxy-terminus since the structure of the lamin tail is crucial for correct nuclear assembly. Therefore, we designed primers 1 and 2 (Fig. 3A), starting at nucleotide 1123 and terminating 55 bases into the 3' untranslated region of lamin A. Gel electrophoretic analysis of the PCR fragments of GLC-A1 showed two bands of about the predicted 927 bp (Fig. 3B, double arrows). Only one band was found in a control PCR using a lamin A cDNA clone (not shown). In addition, RT-PCR was performed with primer 1 and primer 6, complementary to part of the 3' untranslated region of lamin C mRNA, resulting in a single band around 630 bp, as predicted from the known cDNA sequence (data not shown). Subcloning of the two PCR-products using primers 1 and 2 showed that one band was of the correct size (927 nucleotides), whereas the other product was approximately 100 bp shorter (Fig. 3B). Restriction fragment length analyses of the latter product showed that fragments containing exons 9, 10 and 11 were reduced by approximately 100 bp (Fig. 3, Panel C, arrows). Sequencing of both the normal and the short products revealed that in both products the nuclear localization signal and the CaaX motif were present (not shown), but that the 90 bp of exon 10 were missing in the short product. The predicted mRNA structure of this shortened lamin A, which we suggest to designate lamin A Δ 10, is outlined in Fig. 3D. To map which region is missing from the normal lamin A, we have included a schematic drawing indicating the most important characteristics of lamin A (Figure 3E). The amino acid region encoded by exon 10 is located in the carboxy-terminal region, which forms the tail of the protein. This region contains a stretch of amino acids rich in aspartic acid and glutamic acid (E/D), followed by a sequence of four consecutive histidines (H4).

Presence of lamin A Δ 10 in other cell types

To determine whether lamin A Δ 10 was unique for a single cell line, nested PCR was performed using primers 1 and 2, followed by PCR with primers 3 and 4 (Fig. 3A) on cDNA of several lung cancer cell lines, other carcinoma cell lines and a neuroblastoma cell line. In addition, cDNA derived from tissues of normal colon and adenocarcinomas of the lung were examined (Figs. 4A and B). PCR of plasmid lamin A cDNA yielded the expected 242 bp product of lamin A, whereas the lamin A Δ 10 plasmid cDNA yielded a fragment of 152 bp (Fig. 4A, lanes 1 and 2). Analysis of cDNA from different sources on ethidium bromide stained agarose gels (A and B, upper panels) showed in addition to the expression of the 242 bp product a band of 152 bp of variable intensity in most samples. The identity of the stained bands was confirmed by an alkaline agarose gel, and PCR products were hybridized to the end-labelled primer 3 (Fig. 4A and B, middle panels) or oligo 5 (Fig. 4A and B, lower panels). Hybridization to primer 3 showed that lamin A and lamin A Δ 10 occur in all samples, except for the controls (lanes 1,2 and 7 of panel A). However, the ratio of lamin A/A Δ 10 expression showed a large variation amongst samples. Hybridization with oligo 5 allowed the positive identification of lamin A Δ 10 in all

samples tested, except for the two controls in panel A (lanes 2 and 7). No detectable cross-hybridization with normal lamin A was observed.

Figure 3. Panel A: Region of interest of the lamin A cDNA. Numbering started from nucleotide 1. Upper numbers indicate restriction sites and exon boundaries of interest. Lower numbers correspond to first nucleotide recognized by primers 1 through 4 (p1-p4). Numbers in boxes correspond to exon numbering according to Lin and Worman [28].

Panel B: Agarose gel electrophoretic analyses of PCR products resulting from primers 1 and 2 on cell line GLC-A1 (lane 1). Note the presence of two bands with sizes between 800 and 900 bp (double arrow). Excision and purification of each band, followed by electrophoresis, resulted in two bands with distinct molecular weights (lanes 2 and 3).

Panel C: Restriction fragment length analysis of the normal lamin A (lanes 1 and 3) and lamin AΔ10 (lanes 2 and 4) using SstI and NcoI (Lanes 1 and 2), or PstI and SstII (lanes 3 and 4). Note that after digestion with the first set of restriction enzymes the fragment of 704 bp (lane 1) is shortened in lamin AΔ10 (lane 2, arrow), and after digestion with the second set of restriction enzymes the expected fragment of 211 bp is shortened by approximately 100 bp (compare lanes 3 and 4, arrow). These results indicate a deletion between the PstI sites of exon 9 and 11.

Panel D: Structure of the three different mRNAs resulting from alternative splicing of the lamin A/C gene.

Panel E: Schematic diagram (adapted from [33]) showing the impact of the missing exon 10 in lamin A at the protein level for lamin AΔ10. P, phosphorylation site; NLS, nuclear localization signal; (E/D)₃, triple repeat of glu and asp; H₄, a sequence of four histidines; CAAX, motif for isoprenylation of lamin A.

Genomic DNA analysis

To investigate the possibility that lamin AΔ10 is a transcript from an as yet unidentified lamin A-like gene, PCR was performed on genomic DNA samples from carcinoma cell lines and normal tissues. Figure 5 shows that DNA from placenta, leukocytes, and from cell lines GLC-A1 and NL-Ac1 all show a single band at the predicted levels of 649 and 1409 bp.

Figure 4. Analysis of PCR products for the presence of lamin A and lamin AΔ10 cDNA. PCR using primers 1 and 2 was followed by a PCR using primers 3 and 4 in all cases, except for cell lines LCLC-103H and NCI-H125, which were subjected to a single round of PCR-reactions using primer 3 and 4.

A: cDNA from lamin AΔ10 (lane 1), normal lamin A (lane 2), GLC-A1 (lane 3), LCLC-103 (lane 4), NCI-H125 (lane 5), NCI-H23 (lane 6), normal lamin A (lane 7), GLC-A2 (lane 8), and NL-Ac1 (lane 9)

B: cDNA from cell line T24 (lane 1), T47D (lane 2), NCI-H460 (lane 3), MR65 (lane 4), SK-N-SH (lane 5), normal colon (lane 6), and four different adenocarcinomas of the lung (lanes 7-10).

The upper panel shows ethidium bromide stained agarose gels with height of the expected lamin A and lamin AΔ10 bands denoted by arrow.

Middle panel: hybridization with 32 P end-labeled primer 3, hybridizing to both lamin A and lamin AΔ10 cDNA.

Lower panel: hybridization with 32 P end-labeled oligonucleotide 5, specifically hybridizing to lamin AΔ10 cDNA only. m= 100 bp ladder markers

Figure 5. PCR-based analysis of genomic DNA of human placenta, human leukocytes, and cell lines NL-Ac1 and GLC-A1. In the first lane for each sample primers 3 and 6 were used and in the second lane primers 3 and 4. Arrows indicate the level of the 1404 bp band (upper) and 649 bp. fragment (lower arrow). Note that in 100 bp. ladder markers lane (m) bands below 500 bp are barely visible.

Two-dimensional immunoblotting

Two-dimensional immunoblotting of GLC-A1 using the antibody to lamin A showed the presence of a lamin A doublet at the predicted position (approximately 70 kDa, pI 7.0, Figure 6). In addition, however, a clear protein spot recognized by this antibody was observed with a molecular mass. of around 65 kDa and a pI value that was shifted approximately one pH unit to the basic site (arrow). No such protein spot was observed in immunoblots of cell line NCI-H125 (not shown).

Figure 6. Immunoblot detection of lamin A and lamin A Δ 10 after two-dimensional non-equilibrium gel electrophoresis. Note the presence of normal-sized lamin A with the expected pI of around 7.0, while an additional spot with lower molecular weight and more basic pI is also found (arrow).

Discussion

In this paper we describe the widespread occurrence of an as yet unidentified splicing product of the lamin A/C gene, which we designate lamin A Δ 10, since exon 10 is absent in this transcript. In a previous report we have demonstrated the presence of an A-type lamin protein, which forms intranuclear aggregates in cell line GLC-A1 [31]. In contrast to normal, perinuclear A-type lamins these intranuclear aggregates could be largely extracted by Triton X-100, indicating that these aggregates are not assembled into the nuclear matrix [2]. This abnormal nuclear localization of the protein suggested a distortion in the mRNA region coding for the carboxy-terminal part of the protein, since this part is known to govern targeting to the nucleus by the nuclear localization signal, and the CaaX-motif, the isoprenylation site of (pre)lamin A, which is essential for a proper incorporation into the nuclear lamina [20, 22, 29]. The structure of this region of the mRNA was examined by RT-PCR. Sequencing showed that the correct sequence for both motifs was present in the cDNA examined. However, gel analysis showed the presence of an additional shortened PCR product. Restriction fragment analysis, followed by sequencing showed that in the otherwise normal lamin A cDNA sequence exon 10 was lacking. Therefore we designated this novel protein lamin A Δ 10.

Hybridization with oligonucleotides hybridizing to either lamin A Δ 10 alone or both lamin A Δ 10 and lamin A showed that lamin A Δ 10 is extensively expressed in a variety of tissues, since all samples examined were positive. In addition, we found that the ratio of expression levels of

lamin A Δ 10 and lamin A varied significantly between samples. Especially in the lung cancer cell lines the relative concentration of the lamin A Δ 10 can be high. Similar differences in the expression ratio were observed between lamin A and lamin C at both protein and mRNA level [6, 31]. Which mechanism is involved in regulating the relative expression of the three alternative splicing products of the lamin A/C gene, remains to be elucidated. Preliminary studies show that different cultures from the same cell line can express different ratios of lamin A to lamin A Δ 10, which might be explained by differences in cell density.

A positive identification of the protein encoded by the lamin A Δ 10 mRNA is not yet possible since no antibody specific for this product is available. Therefore we cannot yet state that the abnormal lamin A expression patterns as seen in GLC-A1 are represented by the lamin A Δ 10 protein. However, evidence that indeed lamin A Δ 10 mRNA is translated into protein comes from one-dimensional [31] and two-dimensional gel electrophoresis followed by immunoblotting studies. Computer assisted calculation of the pI of the lamin A Δ 10 protein indicates a value of 8.58 as compared to a theoretical pI of 7.4 for normal lamin A (PepStats, Caos/CAMM, Nijmegen, The Netherlands). Furthermore, a deletion of 30 amino acids should give rise to an approximately 3.5 kDa smaller protein. In one dimensional gel electrophoresis a protein smaller than lamin A was detected with the lamin A antibody 133A2 [23, 31]. The additional protein spot detected in two-dimensional immunoblotting is significantly more basic (approx. 1 pI value) and about 5 kDa smaller than the normal lamin and fulfils the predicted electrophoretic characteristics of lamin A Δ 10. The possibility that the lamin A Δ 10 is a result from a translocation or deletion of lamina A/C, or is a transcript from a closely related as yet unknown gene has been examined. A previous study suggests that such a gene might exist [24]. Our PCR analysis within the region between exon 9 and 11 of genomic DNA provides no evidence for an additional gene. Thus, a single gene is responsible for lamins A, A Δ 10 and C. The possibility that lamin A Δ 10 is the result of a mutated lamin A/C gene has been eliminated by our finding that lamin A Δ 10 occurred in all samples examined.

It is feasible that the presence of lamin A Δ 10 mRNA has been overlooked in previous studies because of its relatively low abundance as compared to lamins A and C expression. A cell line with a low expression of normal lamins A and C has enabled us to identify this lamin A Δ 10 mRNA by RT-PCR. The same holds true for protein analyses, in which lamin A Δ 10 is easily overlooked, especially because of the relatively large pI shift. Furthermore, only in a cell expressing low levels of the normal A-type lamins an aberrant lamin can induce visible effects on the structure of the lamina. Apparently, in GLC-A1 the concentration of lamin A Δ 10 can reach relative high levels resulting in a distorted nuclear phenotype. The lamin A Δ 10 protein may be localized in the nuclear inclusions seen in this cell line [31]. This would be in agreement with transfection studies [29] which showed that different types of nuclear distortions can be induced with constructs of lamin A containing carboxy-terminal deletions either starting at codon 456 (within exon 7) or starting at codon 550 (within exon 10). An altered localization of the lamin A Δ 10 protein is likely, since the deletion of the first part of exon 10 results in loss of

an acidic domain (7 consecutive Glu or Asp residues) and a poly-histidine domain. This is bound to have impact on the interaction of lamin A Δ 10 with other nuclear components. Although suggested, it is not yet shown that this particular highly charged region is involved in chromatin binding [41]. If the protein extracted by Triton X-100 from GLC-A1 indeed represents lamin A Δ 10, then it is possible that this protein is not or only partially bound to the nuclear matrix and may be involved in other intranuclear interactions.

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Chapter 7

Lamin A is not required for incorporation of lamin C in the nuclear lamina as demonstrated by GFP-tagged lamins

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Summary

Chimeric proteins of A-type lamins and green fluorescent protein (GFP) were used to study the mutual dependence of A-type lamins for incorporation into the nuclear lamina, and to elucidate the localization of lamin A Δ 10, a recently described alternative splicing product of the lamin A/C gene. Two distinct cell lines, CHO and GLC-A1, were transfected with cDNA constructs encoding fusion proteins of lamin A-, lamin C- and laminA Δ 10-GFP. CHO cells express high levels of endogenous A-type lamins, while the GLC-A1 cells express low levels of A-type lamins of which lamin A does not seem to associate with the nuclear membrane, but assembles into intranuclear aggregates. Fusion of GFP to the amino-terminus of A-type lamins had no effect on their localization and incorporation into the lamina as proven by confocal immunofluorescence microscopy and resistance to extraction with detergents. None of the lamin-GFP proteins did incorporate into the intranuclear aggregates in GLC-A1, but all localized in the nuclear envelope, regardless of the expression levels. Also lamin C, which is not isoprenylated at its carboxy-terminus and therefore has no site for targetting and anchorage to the nuclear envelope, displays proper incorporation into the lamina. Contrary to previous suggestions (Loewinger and McKeon (1988) *EMBO J.*, 7: 2301-2309) we conclude that incorporation of lamin C into the nuclear lamina is not dependent on the presence of lamin A. Removal of exon 10 by alternative splicing has also no effect on the incorporation of A-type lamins in the nuclear lamina.

Introduction

Lamins form the major constituents of the nuclear lamina. Two main types of lamins can be distinguished, i.e. A-type lamins and B-type lamins. While lamins B1 and B2 are encoded by distinct genes [1, 37], A-type lamins, lamins A, A Δ 10 and C, arise from one gene by alternative splicing [11, 23, 29]. A-type lamin expression is differentiation dependent, with absence from for instance embryonic cells [12, 20, 34], cells of several epithelia [3] and spermatogonia [28], and presence in many other cell-types [3, 5]. Recently, we described a new splicing product of the lamin A/C gene, which is identical to lamin A except for exon 10, which is missing in the newly described product. The new protein was therefore designated lamin A Δ 10 and is present in many cell lines and human tissues [29].

A number of authors have indicated that isoprenylation of lamin A, and presumably also of lamin A Δ 10, is required for proper incorporation into the lamina [21, 26]. Lamin C does not contain a site for isoprenylation, and has consequently no site for targetting and anchorage to the nuclear envelope. It has been suggested that lamin C needs association with lamin A for proper incorporation into the lamina [24], and that lamins A and C are expressed in a 1/1 ratio [13]. CHO and GLC-A1 cells were used for transfection with cDNA fusion constructs of lamins and green fluorescent protein (GFP) to study the mutual dependence of A-type lamin expression on

their incorporation into the lamina. CHO cells express high levels of endogenous A-type lamins, but GLC-A1 cells express very low levels of lamin A and higher levels of lamin C, which are however still low as compared to other cell lines examined [27].

Wild type GFP and several mutants displaying brighter emissions [9, 14] have been expressed and detected by their fluorescent properties in bacteria [6], yeast [33], plants [8], and mammalian cells [7, 43]. GFP requires no substrates, cofactors or other proteins to fluoresce [6] and its cDNA is relatively short, making it a convenient reporter gene [7]. Chimeric proteins of GFP with several distinct target proteins have shown their usefulness for localization and trafficking studies in a variety living cells [10, 25, 35, 38], since GFP appears not to impair the function of GFP-tagged target proteins [30, 36].

Transfection assays of lamin A-GFP, lamin C-GFP and lamin AΔ10-GFP in GLC-A1 allowed us to address the following two questions: (1) do A-type lamins show mutual dependence for their incorporation into the nuclear lamina, and (2) does lamin AΔ10 localize at the nuclear membrane.

Materials and Methods

Plasmids

Lamin A-cDNA, kindly provided by Dr. F. McKeon [31], was cloned to the 3' end of GFP in the mammalian expression vector pS65T-C1 (Clontech Laboratories Inc., Palo Alto, CA/USA) using the SstII and BamHI sites, generating pS65T-lamA. Lamin AΔ10-cDNA was constructed by replacement of the 344 bp lamin A BsiWI/BstEII fragment by the 254 bp lamin A Δ10 BsiWI/BstEII fragment, obtained by BsiWI and BstEII digestion of a subcloned 837 bp RT-PCR fragment of lamin AΔ10 [29]. Lamin AΔ10-cDNA was cloned into pS65T-C1 using the SstII and BamHI sites, generating pS65T-lamAΔ10.

pS65T-lamC was generated by replacement of the 3' BsiWI/BamHI fragment of pS65T-lamA, encoding part of exon 9 and exon 10,11 and 12 of lamin A, by the 3' end of lamin C. The 3' end of lamin C was obtained by reverse transcription [29], followed by two rounds of PCR. In the first round of PCR primer A22 (sense, 5'-GCCTACCGCAAGCTCTTGGA-3', corresponding to nt 1123-1142 of lamin A and C; Eurogentec, Seraing, Belgium) and primer X37 (antisense, 5'-TGAAAAGATTTTGGCACGG-3', corresponding to nt 1779-1798 of the untranslated region of lamin C; Eurogentec), followed by a second round of PCR with primer Apr1 (sense, 5'-AGCCTGCGTACGGCTCTCAT-3'; BsiWI site underlined; corresponding to nt 1573-1592 of lamin A and C; Eurogentec) and primer Bar30 (antisense, 5'-TATAGGATCCCGGCCTCAGCGGCGGCTACC-3'; BamHI site underlined; the 3' 20 bases are complementary to nt 1705-1724 of lamin C, the 5' 10 bases are non-complementary, but contain a BamHI site for cloning; Gibco Life Technologies Ltd., Paisley, Scotland). The BsiWI and BamHI sites were used for cloning of the 3' lamin C PCR product into pS65T-lamA, resulting in pS65T-lamC.

Cell culture and transfections

CHO cells were grown on HAM'S F12 medium (ICN Biomedicals Inc., Costa Mesa, CA/USA) supplemented with 10% foetal calf serum (Gibco Life Technologies Ltd.) and 2mM L-glutamin. GLC-A1, a cell line derived from an adenocarcinoma of the lung, was grown on RPMI (ICN Biomedicals Inc.) containing 2 g/l NaHCO₃ supplemented with 10% newborn calf serum (Gibco Life Technologies Ltd.) and 2 mM L-glutamin. Cell cultures were maintained in an incubator at 37°C. For transfections cells were grown to 25-30% confluency in 6 well plates. A mixture of 2,5 µg plasmid DNA, Hepes 20 mM and DOTAP (Boehringer Mannheim GmbH, Mannheim, Germany) was added to 2.5 ml culture medium, according to the manufacturers specifications. The cells were grown overnight in the DOTAP/DNA containing culture medium. The medium was then replaced and after 6 hours geneticin (Gibco Life Technologies Ltd.) was added to a final concentration of 500 µg/ml. Transfected CHO cultures were subcloned and further maintained in geneticin containing medium in an incubator at 30°C. Transfected GLC-A1 cultures were not subcloned, and maintained on geneticin containing medium at 37°C, and were placed at 30°C 2 or 3 days before handling.

Antibodies

The following mouse monoclonal antibodies were used in this study:

- LN43 (IgG1), recognizing lamin B2 and not cross reacting with lamin B1 or A-type lamins [2]. LN43 was used as undiluted culture supernatant and will be referred to as the lamin B2 antibody.
- 41CC4 (IgM), recognizing lamins A and C and not reacting with human B-type lamins, will be referred to as lamin A/C antibody [4] and was used as undiluted culture supernatant.
- R27 (IgM), recognizing lamins A and C was used as undiluted culture supernatant in immunoblotting [44].
- 133A2 (IgG3) was a kind gift of Dr. Y. Raymond (Montréal, Canada). It was raised against the carboxy-terminus of 98 amino acids present in lamin A and absent from lamin C. This antibody recognizes lamin A, reacting with the epitope consisting of amino acids 598-611 [19] and will be referred to as the lamin A antibody. Antibody 133A2 (mouse ascitis) was diluted 1:2500 for immunofluorescence and 1:1000 for immunoblotting.

Indirect immunofluorescence technique

The indirect immunofluorescence assay was applied to cell cultures that were grown on coverslips for several days at 30°C. After washing in phosphate buffered saline (PBS, containing 8 g/l NaCl, 0.2 g/l KCl, 1.15 g/l Na₂HPO₄ and 0.2 g/l KH₂PO₄ at pH 7.4) the cells were either immediately fixed, or first detergent extracted (see below) and then fixed in methanol (-20°C for 5 seconds), followed by acetone (room temperature, 3 times five seconds) and air dried. Cells were then incubated with the primary antibody for 30-45 minutes, and after several washes with PBS, incubated with Texas Red-conjugated goat anti-mouse Ig (1:80, Southern Biotechnology

Associates; SBA/ITK; Birmingham, AL/USA) for 30-45 min, or with Texas Red conjugated goat anti-mouse IgM (diluted 1:80, SBA/ITK). Thereafter, cells were washed in PBS, mounted in 90% glycerol containing 0.02 M Tris-HCl pH 8.0, 0.002% NaN_3 , 2% 1,4 diazabicyclo (2,2,2)-octane (DABCO; Merck, Darmstadt, Germany) and 0.5 $\mu\text{g/ml}$ 4',6 diamino-2 phenylindole (DAPI; Merck) for DNA staining. Immunofluorescence micrographs were taken with an automatic camera using Kodak 400 ASA Tri-X pan film.

Preparation of nuclear matrices

Cells grown on coverslips were rinsed three times 5 minutes at 0°C with buffer containing 130 mM NaCl, 5 mM KCl, 7.5 mM MgCl_2 , 0.5 mM phenylmethylsulphonyl chloride (PMSC, Merck) and 5 mM N-ethylmaleimid (NEM, Sigma, St. Louis, MO/USA). Then the cells were submerged in buffer containing 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 1.5 mM MgCl_2 , 0.5 mM PMSC, 5 mM NEM and 300 mM sucrose, and after two minutes of incubation at 0°C a 10% solution of Triton X-100 (BDH, Poole, UK) was added to a final concentration of 0.5%. The slides were incubated with this buffer for 10 minutes at 0°C . The cells were washed twice with buffer containing 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 1.5 mM MgCl_2 , 0.5 mM PMSC and 5 mM NEM (RSB buffer) and subsequently extracted with RSB buffer containing 0.5% sodium deoxycholate (DOC; Merck) and 1% Tween 40 (Sigma). Thereafter the cell remnants were washed twice with RSB buffer and incubated in buffer containing 10 mM Tris-HCl pH 7.4, 110 mM NaCl, 1.5 mM MgCl_2 , 0.5 mM PMSC, 1 mg/ml DNase 1 (Sigma) and 50 $\mu\text{g/ml}$ RNase A (Sigma), and incubated for 20 minutes at RT. Then the cells were washed with RSB buffer and subsequently extracted with 400 mM $(\text{NH}_4)_2\text{SO}_4$, 50 mM Tris-acetate, 2.25 mM MgCl_2 , 0.5 mM PMSC and 5 mM NEM for 10 minutes at 0°C . Finally the obtained nuclear matrices [42] were rinsed with RSB buffer.

Confocal scanning laser microscopy

For confocal immunofluorescence microscopy, the cells were analysed using a MRC600 confocal scanning laser microscope (Bio-Rad, Hemel Hempstead, UK), equipped with an air-cooled Argon-Krypton mixed gas laser and mounted onto an Axiophote microscope (Zeiss). The laser scan microscope was used in the dual parameter set-up, according to the manufacturers specifications, using dual wavelength excitation at 488 nm and 568 nm. Emission spectra were separated by the standard sets of dichroic mirrors and barrier filters. All scans were recorded in the Kalman filtering mode. Stacks of 5-12 confocal images were recorded using a step size of 0.5 - 1 μm . Photographs were taken with an automatic camera using Kodak 400 ASA Tri-X pan film.

Sample preparation for gel electrophoresis

Cells were rinsed with PBS at RT, scraped from the bottom of the culture flask in PBS and centrifuged at 1200 rpm. The supernatant was discarded and the pellet was stored at -20°C until

usage. Cell pellets were resuspended in buffer containing 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 1.5 mM MgCl₂, 0.5 mM phenylmethylsulphonyl fluoride (PMSF, Merck) and 0.5% Triton X-100 (BDH, Poole, England), and stirred for 10 minutes at 0°C. The cell remnants were collected by centrifugation at 1500 x g at 4°C and the pellet was washed with buffer containing 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 1.5 mM MgCl₂, 0.5 mM PMSF (Merck). Thereafter, the cell remnants were collected by centrifugation at 1500 x g at 4°C, resuspended in buffer containing 10 mM Tris-HCl pH 7.4, 110 mM NaCl, 1.5 mM MgCl₂, 0.5 mM PMSF (Merck), 1 mg/ml DNase 1 (Sigma) and 50 µg/ml RNase A (Sigma), and incubated for 20 minutes at RT. Then the pellet was washed with 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 1.5 mM MgCl₂, 0.5 mM PMSF (Merck). After centrifugation and discarding of the supernatant, the pellet was resuspended in SDS-sample buffer [22] and boiled for 5 minutes.

Gel electrophoresis and immunoblotting

One-dimensional SDS-gel electrophoresis in 10% polyacrylamide (Bio-Rad Laboratories, Hercules, CA/USA) slab gels containing 0.1% SDS (Merck), was performed according to Laemmli [22]. Gels were run on the Mini-Protein II system (Bio-Rad Laboratories) for approximately 45 minutes at 200 V.

Western blotting was performed essentially according to Towbin et al. [41] using the Mini Trans-Blot Cell (Bio-Rad Laboratories) at 100 V for 1 hour. Proteins were blotted onto nitrocellulose (BA85, Schleicher and Schüll, Dassel, Germany), the filters were air-dried and blocked for 1 hour at RT in buffer containing 10 mM Tris-HCl pH 7.6, 0.35 M NaCl and 3% BSA (Sigma). Thereafter the blots were incubated with the primary antibodies in appropriate dilutions for 30-60 minutes at RT in buffer containing 10 mM TRIS-HCl pH 7.6, 0.15 M NaCl, 0.3% BSA, 1% Triton X-100 (BDH), 0.5% DOC (Merck) and 0.1% SDS (Merck). The blots were washed three times with PBS containing 0.5% Triton X-100 (BDH), and incubated for 30-60 minutes at RT with the secondary peroxidase conjugated rabbit anti-mouse Ig antibody (DAKO A/S, Glostrup, Denmark), diluted 1:1000 in PBS containing 0.5% Triton X-100 (BDH) and 0.5% BSA (Sigma). Blots were extensively washed with PBS containing 0.5% Triton X-100 (BDH) and peroxidase activity was detected by chemiluminescence (ECL-kit, Amersham, Buckinghamshire, UK). RX Fuji medical X ray films (Fuji, Tokyo, Japan) were used for the visualisation of the luminescent signals.

Results

Examination of lamin-GFP transfected CHO cells by confocal scanning laser microscopy showed that lamin A-GFP was localized in the nuclear lamina of transfected cells (fig. 1). Apparently, linkage of GFP to the amino-terminus of lamin A does not interfere with incorporation of the fusion protein into the lamina and does not disturb lamina formation in these cells.

Figure 1. Confocal scanning laser microscopy showing optical sections of transfected CHO cells expressing lamin A-GFP (a) and lamin A Δ 10-GFP (b).

To obtain further information on the expression levels of lamin A-GFP over endogenous lamin A, transfected CHO cultures were subcloned. Several clones were selected and used for gel electrophoresis and immunoblotting. The results show that the expression levels of lamin A-GFP in the selected subclones were high as compared to the endogenous levels of lamin A, and that the fusion proteins had the expected molecular weight (fig 2). The results of lamin A Δ 10-GFP transfected CHO cells were similar to those of lamin A-GFP transfected cells. Lamin A Δ 10-GFP was localized in the nuclear lamina of transfected CHO cells (fig. 1) and the expression levels of lamin A Δ 10-GFP in subclones were comparable to those of lamin A-GFP (fig 2).

Figure 2. Immunoblotting results of untransfected CHO cells (lane 1), CHO cells transfected with lamin A-GFP (lane 2), and CHO cells transfected with lamin A Δ 10-GFP (lane 3), incubated with the lamin A antibody 133A2.

It is likely that the lamin-GFP proteins interact with the endogenous A-type lamins to associate with the nuclear membrane. In order to study the mutual dependence of the individual A-type lamins for this association we performed the same studies with a cell line known to have extremely low levels of endogenous lamin A, i.e. GLC-A1 cells [27]. Lamin A was in general not detected at the nuclear membrane when these cells were examined with the lamin A antibody 133A2, but intranuclear aggregates were stained. We set out to examine the behaviour of transfected A-type lamins in these cells. For this purpose cDNA fusion constructs of lamin A, lamin A Δ 10 and lamin C with GFP were used for transfection of GLC-A1 cells.

ected GLC-A1 cells expressing lamin A-GFP (a,b,g,h), lamin C-GFP (c,d,i,j) stained with lamin A antibody 133A2 (b,d,f) and lamin A/C antibody 41CC4 (g,i,k). In b and f the additional perinuclear aggregates are seen for lamin A-GFP (a,g), lamin C-GFP (c,i) and lamin AΔ10-GFP (e,k), detected with the lamin A antibody (b,d,f). In b and f the additional perinuclear aggregates are seen for lamin A-GFP and lamin AΔ10-GFP. In g and i the additional perinuclear aggregates are seen for the lamin A/C antibody (h,j,l) in transfected as well as untransfected cells.

of transfected GLC-A1 cells, expressing either lamin A-GFP, lamin C-GFP, showed that these fusion proteins localized to the nuclear envelope (Fig 3a,c,e). When transfected cells were stained with the lamin A antibody 133A2, perinuclear aggregates were detected (Fig 3b,d,f), which were not seen for cells transfected with lamin AΔ10-GFP (Fig 3a,c,e). In case of lamin AΔ10-GFP the presence of these aggregates is not due to the relatively high expression of lamin A and lamin C, as suggested previously [29]. The lamin A antibody 133A2 recognizes both endogenous lamin A and does not discriminate between GFP and lamin AΔ10-GFP, therefore the lamina of transfected cells

was stained with the lamin A antibody (Figure 3b and 3f). The presence of intranuclear aggregates in these cells was nevertheless obvious. The lamin A antibody does not react with lamin C-GFP allowing a separate detection of endogenous lamin A and lamin C-GFP. Lamin C-GFP localizes to the nuclear lamina, while endogenous lamin A is detected in the intranuclear aggregates (Figure 3c,d). When GLC-A1 cells were stained with the lamin A/C antibody 41CC4 (Figure 3h,j,l) a perinuclear fluorescence was seen in all cells, due to the endogenous lamin C present in the cells [27, 29]. Intranuclear aggregates of endogenous lamin A are not detected with this antibody because of the relatively low expression levels of lamin A as compared to lamin C in these cells. In transfected cells lamin A-GFP, lamin A Δ 10-GFP and lamin C-GFP were shown to colocalize with the endogenous lamin C in the nuclear lamina (Figure 3g,i,k). As demonstrated by staining of transfected cells with the lamin B2 antibody, a perfect colocalization of the endogenous lamin B2 and transfected lamin A-GFP (Fig. 4a,b), lamin A Δ 10-GFP and lamin C-GFP (Fig. 4c,d) was seen.

Figure 4. Confocal sections of GLC-A1 cells transfected with lamin A-GFP or lamin C-GFP. The distribution of lamin A-GFP (a) and lamin C-GFP (c) colocalizes with lamin B2 immunofluorescence (b,d).

Furthermore, we showed that lamin-GFP proteins are part of the nuclear matrix fraction (Fig. 5), since they were resistant to extractions with detergents, nucleases and high salt. In situ preparations of the nuclear matrix revealed no apparent differences in the extractability of the individual lamin-GFP proteins. This confirmed that in GLC-A1 and CHO cells the GFP-fusion products are not only correctly localized to the nuclear lamina, but presumably also properly incorporated into this structure.

Figure 5. In situ nuclear matrix preparations of GLC-A1 cells transfected with lamin C-GFP (a) and lamin A Δ 10-GFP (c). Lamins A and C were immunocytochemically counterstained with the lamin A/C antibody 41CC4 (b) and the lamin B2 antibody LN43 (d).

Discussion

Several mechanisms have been described for the association of lamins with the nuclear membrane. B-type lamins and lamin A are isoprenylated, which provides them with a hydrophobic anchorage site for the nuclear membrane. Lamin C is not isoprenylated, and the mechanism for incorporation of lamin C into the lamina is not known, but there is evidence that lamin C requires progression through mitosis in order to be incorporated into the lamina [18]. It has also been suggested that for incorporation lamin C needs association with isoprenylated lamin A [24]. With respect to the amino-terminus of lamins, it has been demonstrated that the 28 amino-terminal amino acids of lamins are important for polymerization [15, 32]. Microinjected lamin mutants that lack these 28 amino acids showed impaired incorporation in the lamina. However, expression of amino-terminally deleted lamins in transfected cells resulted in proper lamina incorporation [39, 40]. This was probably the result of association with endogenous lamins [21, 39]. We chose to study lamin incorporation by transfecting GFP-tagged lamin constructs into cells. GFP was fused to the amino-terminal side of lamin A and lamin A Δ 10, since the carboxy-terminus of lamin A, and presumably of lamin A Δ 10 as well, is processed. Fusion of GFP to the carboxy-terminus of the protein might interfere with farnesylation and subsequent cleavages. Besides, when the farnesylation and subsequent cleavages would not be disturbed and processing would take place, GFP would be cleaved off during the processing as a part of the carboxy-terminus [16].

The experiments presented in this paper show that lamin A with a considerable extension at the amino-terminus is properly incorporated into the nuclear lamina of transfected CHO cells. The results suggests that lamin A-GFP is properly processed and associates with the nuclear membrane. Furthermore, immunoblotting showed that proteins of the expected molecular mass were recognized by the lamin A antibody. Since the incorporation of GFP fusion proteins in

CHO cells could be the result of interactions between endogenous lamin A and lamin A-GFP the same experimental set-up was used to study A-type lamin incorporation in GLC-A1 cells, a cell line with extremely low levels of endogenous lamin A. Moreover, lamin A is not localized to the nuclear membrane in these cells [27]. Since the transfected GFP-lamins were properly incorporated into the lamina of these cells, it can be deduced that amino-terminally extended A-type lamins do not require interactions with endogenous lamin A for proper incorporation into the lamina. However, heteropolymerization of the amino-terminally extended A-type lamins with B-type lamins cannot be excluded, since the GLC-A1 cells form a normal lamina with endogenous B-type lamins. Transfection studies of GFP alone showed that this protein does not localize to the nuclear lamina, but is dispersed throughout the cytoplasm.

Expression of high levels of lamin C-GFP did not result in abnormal localization of these lamins, although lamin C is missing the anchorage site for the nuclear membrane [17]. Microinjection studies showed that incorporation of lamin C into the lamina is extremely delayed as compared to incorporation of lamin A [13, 18]. The experiments presented here showed that expression of high levels of lamin C-GFP in transfected GLC-A1 cells did not result in abnormal localization of lamin C. Thus, even in cells with very low levels of endogenous A-type lamins, lamin C can be incorporated into the lamina correctly. Therefore, it can be concluded that a balanced expression of lamin A and Δ 10 compared to lamin C is not obligatory for proper incorporation of lamin C into the lamina. Again, interactions with B-type lamins cannot be excluded.

Previously we have suggested [27, 29] that the alternative splicing product lamin Δ 10 might be responsible for the formation of intranuclear lamin A aggregates in GLC-A1 cells. The data presented in this report show that this is not the case. When lamin Δ 10-GFP was expressed in CHO or GLC-A1 cells, a normal perinuclear localization was seen. Furthermore, the intranuclear aggregates remained present in GLC-A1 cells next to the lamina that was formed with lamin Δ 10-GFP. The nature of the intranuclear A-type lamin aggregates remains largely obscure. Since the staining intensity of the intranuclear aggregates in GLC-A1 with the lamin A antibody is weak and the level of endogenous lamin A is low, as detected in immunofluorescence, immunoblotting and northern blotting studies [27, 29], the formation of aggregates may be due to the inability of the cell to form a lamina at low concentrations of A-type lamins. In several other cases very low levels of A-type lamin expression also leads to intranuclear speckles of A-type lamins. For instance, several small cell lung cancer cell lines display very low levels of A-type lamins localized in intranuclear speckles. Furthermore, this phenomenon was seen in HL60 cells that do not contain A-type lamins, but upon induction of differentiation these cells start to express A-type lamins. During the early phase of expression intranuclear speckles were seen. As lamin A concentration rose the speckles disappeared and a normal lamina was formed (J.L.V. Broers, unpublished results). However, the aggregate like localization in GLC-A1 is unlikely to result from a low concentration of lamin A, since in

transfected GLC-A1 cells with high expression levels of the A-type lamins the aggregate like pattern is still detectable next to the normal lamina.

In conclusion, transfected A-type lamins, amino-terminally extended with a GFP-tag, are properly incorporated into the nuclear lamina. Lamin A Δ 10 does not localize in intranuclear aggregates. Furthermore, relatively high levels of lamin C-GFP are incorporated into the lamina independent of balanced expression of lamin A.

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Chapter 8

Summary and General Discussion

Summary and General Discussion

In the first part of this thesis lamin expression in normal and diseased human tissues has been described. A study in normal tissues, performed with a set of well characterized lamin antibodies, showed the differential expression of A-type lamins. The general hypothesis that A-type lamin expression is dependent on the degree of differentiation of cells was generally confirmed by immunostaining results of stratified squamous epithelia, but was not absolute in all tissues. Several highly differentiated cell types, including cells of white pulp of the spleen and cells of several neuroendocrine glands, show no expression of A-type lamins. This indicates that the degree of differentiation is not the only parameter that dictates A-type lamin expression. Furthermore, the general assumption that B-type lamins are constitutively expressed in all cells was found to be true for lamin B2, but not for lamin B1. Lamin B2 was extensively expressed in all examined tissues and cells except for hepatocytes and centrocytes and centroblasts of the reactive lymph nodes, which were partly negative or very weakly positive. Lamin B1 expression was very heterogenous in all tissues. For instance, lamin B1 was absent from muscle cells, Sertoli and Leydig cells, fibroblasts and endothelial cells, basal cells of several epithelia, and epithelial cells with high levels of differentiation. Lamin B1 expression is correlated with proliferation rather than differentiation, but the correlation was not valid for all examined tissues.

Distinct tumour types were investigated for their lamin expression, in order to get more insight into their development. Specimens of testicular germ cell tumours of adolescents and adults showed that carcinoma *in situ*, either adjacent to seminomas or non-seminomas, is positive for lamin B1 and B2 but negative for lamins A and C. This expression pattern resembled that of normal spermatogonia, which together with their morphological similarities suggests a close relationship between these two cell types. Also seminomas express only lamin B1 and B2, and resemble the lamin expression patterns of spermatogonia, suggesting that this tumour type is differentiated along the spermatogenic pathway. Since in several species germ cells specific lamins have been detected it can be anticipated that also in man such subtype may exist. The presence of such lamin subtype in spermatogonia as well as seminomas would confirm the differentiation pathway of these tumours along the spermatogenic lineage. Furthermore, a *Ras* dependent A-type lamin expression was found in seminomas, in that only tumours with a *Ras* mutation showed expression of A-type lamins next to lamin B1 and B2 expression. Similar *Ras* dependent changes in A-type lamin expression have been described *in vitro* in the small cell lung cancer cell line NCI-H249 (Kaufmann et al. (1991), *Cancer Res.* **51**: 581-586), and could also be induced in the colon carcinoma cell line CaCo 2 by transfection with c-Ha-Ras.

Differentiated non-seminomas, including yolk sac tumours, choriocarcinomas and teratomas, which are differentiated along a somatic pathway, consequently express both A- and B-type lamins. Embryonal carcinomas, which are the least differentiated non-seminomas, were found to

express lamin B1, B2 and C, but not lamin A. This implies that lamin C expression precedes lamin A expression during the process of somatic differentiation. Such an intermediate differentiation stage has not been detected in normal human tissues, but differentiating cells displaying only lamin C expression may have been overlooked in normal tissues, because this transient phase of differentiation lasts only for a short time, resulting in only few cells expressing this lamin pattern.

Another tumour type that was studied for lamin expression was Hodgkin's disease. In lymph nodes characterized with Hodgkin's disease all cells were positive for lamin B1 and B2. This was remarkable since in the reactive lymph nodes a specific population of cells, namely the centrocytes and centroblasts, were negative for lamin B2. Such a population was not detected in Hodgkin's disease. Furthermore, only a small fraction of cells in Hodgkin's disease is positive for A-type lamin expression. Many of these cells are CD30 positive, fairly mature B- and T-lymphocytes, whereas a fraction of these A-type lamin positive cells remained unidentified by the used lymphocyte markers. The CD30 positive Reed-Sternberg and Hodgkin cells were also positive for A-type lamin expression, confirming a relatively mature and differentiated phenotype for these cells. In the small lymphocytes surrounding the Reed-Sternberg and Hodgkin cells A-type lamin expression and Ki-67 staining segregate, indicating that A-type lamin expressing lymphocytes do not proliferate. In the malignant Reed-Sternberg and Hodgkin cells A-type lamin expression and Ki-67 positivity may co-occur in one cell. This proves that in malignant tissues mature A-type lamin expressing cells may retain the ability to proliferate.

Earlier studies on lung cancer showed that the lamin expression patterns are dependent on the degree of differentiation and on the subtype of lung cancer. Furthermore, aberrant localizations of the A-type lamins were seen in adenocarcinomas of the lung. The second part of the thesis focusses therefore on the molecular background of this aberrant lamin expression in lung cancer cells by *in vitro* studies. In the adenocarcinoma cell line of the lung GLC-A1 an abnormal aggregate-like intranuclear staining is seen when stained with an antibody to lamin A. Furthermore, immunoblotting of these cells showed that very little lamin A was detectable in these cells, which was confirmed by Northern blotting showing extremely low levels of lamin A mRNA. For lamin C slightly higher levels of protein and mRNA were detected, but they were still low compared to other cell lines. Lamin C, however seemed to be incorporated into the lamina in a normal way, since no abnormal localization was detected in immunofluorescence when the cells were stained with antibodies recognizing both lamins A and C. GLC-A1 is a cell line that is known to display neuroendocrine characteristics in a subpopulation of the cells and may therefore not be regarded as a fully differentiated adenocarcinoma. This cell line may be another example of cells that are interrupted in their differentiation process and therefore show a transient expression pattern of the A-type lamins, by analogy of the embryonal carcinoma cells described above.

Molecular biological analysis showed that GLC-A1 cells expressed an as yet unknown alternative splicing product of the lamin A/C gene. This alternative splicing product was

identical to lamin A except for exon 10, which was completely missing in the newly found splicing product, and therefore it was designated as lamin A Δ 10. Further analysis learned that this product was a common product in many cell lines and tissues. A major difference between GLC-A1 and many of the other cells that were tested for lamin A Δ 10 expression, was the ratio of lamin A Δ 10 over lamin A. Most cells express high levels of lamin A when compared to the levels of lamin A Δ 10, whereas in GLC-A1 the expression of lamin A Δ 10 is relatively high due to the very low expression of lamin A. In order to study the influence of high levels of lamin A Δ 10 and to investigate the localization of lamin A Δ 10, transfections with cDNA encoding lamin A Δ 10 were performed. Since none of the antibodies to A-type lamins could discriminate between lamin A Δ 10 and lamin A, the transfected lamins were tagged with green fluorescent protein (GFP). In this way transfected lamin A Δ 10 could be recognized by its green fluorescence. An additional advantage was that now endogenous A-type lamins could be distinguished from transfected lamins. The study of lamin A Δ 10-GFP showed that this fusion protein localized to the nuclear membrane, and is likely to incorporate properly into the lamina, since it was shown to be part of the nuclear matrix.

Fusion of GFP to the aminotermminus of lamin A, lamin C or lamin A Δ 10 did not prevent or disturb the incorporation into the nuclear lamina. Also high expression levels of lamin-GFP fusion proteins in GLC-A1 cells did not result in abnormal localization or disturbed incorporation. This was especially remarkable for lamin C, since lamin C has no isoprenylation site at the carboxyterminus, and was expected to be unable to incorporate into the nuclear lamina without interaction with lamin A. In GLC-A1 cells incorporation of lamin C-GFP cannot be aided by interactions with lamin A, since lamin A is almost absent from these cells. Therefore we conclude that lamin C is capable of incorporating into the lamina without help of lamin A. This was also confirmed by the normal laminar localization of lamin C in embryonal carcinomas, which also lack lamin A and in which no abnormal localization of lamin C was seen. Furthermore, varying ratios of lamin A over lamin C do not influence the incorporation of lamin C into the lamina. However, it has to be noted that the cells that form a lamina with lamin C in absence of lamin A contain lamin B1 and B2. Since it has been shown that heterodimer formation of lamins can occur, it is possible that lamin C is incorporated into the lamina via interaction with the B-type lamins.

Samenvatting

weinig lamine A aanwezig is in deze cellen. Dit werd bevestigd in Northern blotting studies die aangaven dat er extreem lage niveaus van lamine A mRNA aanwezig waren. Lamine C werd door deze cellijn iets meer tot expressie gebracht, maar vergeleken met andere cellijnen was dit niveau ook nog erg laag. Lamine C werd echter wel ingebouwd in de kernlamina, want als de GLC-A1 cellen werden gekleurd met antilichamen die lamine A en C herkennen, dan werd een zwakke, maar normale lamina-kleuring gezien. Omdat GLC-A1 een adenocarcinoom-celijn is die weinig klierbuis differentiatie vertoont en waarvan bekend is dat een subpopulatie van de cellen ook neuro-endocriene eigenschappen bezit, kan deze cellijn worden betiteld als een slecht gedifferentieerde adenocarcinoom cellijn. Deze cellijn kan een voorbeeld zijn van cellen die niet volledig zijn gedifferentieerd of cellen die hun differentiatie-route niet hebben voltooid en mogelijk in een overgangsfase van ongedifferentieerd naar gedifferentieerd verkeren. Daarom is het mogelijk dat deze cellen ook een overgangsvorm van lamine-expressie laten zien, geen c.q. weinig lamine A maar wel lamine C, zoals bij de hierboven beschreven embryonaalcel carcinomen.

Moleculair biologische analyse bracht aan het licht dat GLC-A1 cellen een tot nu toe onbekend alternatief splicingsproduct van het lamine A/C gen tot expressie brengen. Dit alternatief splicingsproduct is identiek aan lamine A, met uitzondering van exon 10, dat afwezig is. Daarom werd dit nieuwe product lamine AΔ10 genoemd. Analyse van andere cellijnen en weefsels leerde dat lamine AΔ10 een splicingsproduct is dat in alle geteste cellen en weefsels voorkomt. Het grote verschil tussen GLC-A1 en de andere cellijnen was de verhouding tussen de expressie niveaus van lamine A en lamine AΔ10. In de meeste andere celtypen was de lamine A expressie hoog in vergelijking tot het expressie niveau van lamine AΔ10. In GLC-A1 is juist het expressie niveau van lamine AΔ10 relatief hoog in vergelijking tot het expressie-niveau van lamine A. Om de invloed van hoge lamine AΔ10 niveaus te bestuderen en om de lokalisatie van lamine AΔ10 te onderzoeken werden transfectie studies uitgevoerd met cDNA constructen coderend voor lamine AΔ10. Omdat geen van de voorhanden zijnde antilichamen kon discrimineren tussen lamine A en lamine AΔ10, werden de getransfecteerde lamines gelabeld met 'green fluorescent protein' (GFP). Op deze manier konden de getransfecteerde lamine A of lamine AΔ10 herkend worden aan hun groene fluorescentie. Tevens konden nu getransfecteerde lamines onderscheiden worden van endogene lamines. Uit het transfectie onderzoek met lamine AΔ10-GFP bleek dat het fusieproduct van lamine AΔ10 en GFP normaal in de lamina gelocaliseerd was. Verder kon worden aangetoond dat het lamine AΔ10-GFP fusieproduct goed ingebouwd was in de lamina, omdat het niet te extraheren was met detergentia of hoog zout. Aminoterminal koppeling van GFP aan lamine A, lamine C of lamine AΔ10 heeft kennelijk geen invloed op de lokalisatie en incorporatie van deze lamines in de kernlamina. Dit was opmerkelijk voor lamine C, omdat deze lamine in tegenstelling tot lamine A niet geïso-prenyleerd wordt en verwacht werd dat deze lamine niet goed naar de kernmembraan gebracht en ingebouwd wordt zonder de hulp van lamine A. Omdat in GLC-A1 cellen bijna geen lamine A aanwezig is kan de inbouw van lamine C in de lamina dus niet lamine A

gemedieerd zijn in deze cellen. Daaruit kon worden geconcludeerd dat inbouw van lamine C onafhankelijk is van aanwezigheid van lamine A. Dit was eigenlijk ook al duidelijk uit de normale localisatie van lamine C in embryonaalcel carcinoemen, die eveneens geen lamine A tot expressie brengen. Blijkbaar hebben de verhoudingen tussen lamine A en lamine C geen effect op de incorporatie van lamine C in de lamina. Men dient echter wel in gedachten te houden dat alle cellen die een lamina vormen met lamine C in afwezigheid van lamine A, endogeen lamine B1 en B2 tot expressie brengen. Omdat vorming van heterodimeren tussen A-type en B-type lamines mogelijk is, kan niet worden uitgesloten dat inbouw van lamine C in afwezigheid van lamine A gebeurt via interactie met één of beide B-type lamines.

Curriculum Vitae

Barbie Machiels werd geboren op 2 december 1965 te Maastricht. In 1985 behaalde zij haar atheneum B diploma aan het Henric van Veldeke College te Maastricht. In datzelfde jaar begon ze met haar studie Moleculaire Wetenschappen aan de Landbouw Universiteit te Wageningen. Tijdens haar studie liep ze drie maanden stage bij het Department of Chemical Engineering, Division Bioseparations van de University of Washington te Seattle, USA, onder leiding van Prof. Dr. R.R. Fisher. Daarna deed ze in 1989 een afstudeeronderzoek van zes maanden bij de vakgroep Moleculaire Biologie van de Landbouw Universiteit Wageningen, onder leiding van R. Verkerk en Dr. P. Zabel. Een tweede afstudeeronderzoek van zes maanden heeft ze uitgevoerd binnen de sectie CP-Intermediates van DSM Research B.V., onder leiding van Dr. R.J. Vijn (DSM Research) en Prof. Dr.Æ. de Groot (Organische Chemie, Landbouw Universiteit Wageningen). In juni 1990 behaalde ze het doctoraal diploma Moleculaire Wetenschappen binnen de biologisch-chemische oriëntatie. Vanaf november 1990 heeft ze een half jaar als vrijwilliger gewerkt bij de vakgroep Biochemie van de Universiteit Maastricht. In juni 1991 is ze als OIO gestart bij de vakgroep Moleculaire Celbiologie van de Universiteit Maastricht binnen het door STW gefinancierd 'proteasomen-project'. Dit project, onder leiding van Prof. Dr. F.C.S. Ramaekers, heeft geresulteerd in enkele wetenschappelijke publicaties. Sinds september 1993 is ze ook bij het lamine-onderzoek van de vakgroep Moleculaire Celbiologie betrokken, onder leiding van Dr. J.L.V. Broers en Prof. Dr. F.C.S. Ramaekers, wat geleid heeft tot het proefschrift: Differential expression of nuclear lamins in normal and diseased human cells.

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Chapter 2, figure 4. Immunoperoxidase staining of liver (A), pancreas (B), parathyroid (C) and vagina epithelium (D-F), using the lamin B2 antibody LN43 (A, D), the lamin B1 antibody (B,E) the lamin A antibody (C) and the lamin A/C antibody 41CC4 (F). Note the very weak staining of hepatocytes as compared to bile duct cells using the lamin B2 antibody (A). In pancreas lamin B1 staining shows large variation in intensities between different acinar cells (B). Parathyroid cells are only very weakly stained with the lamin A antibody, while surrounding stromal and vascular cells are strongly positive (C). In vagina (D-F), all epithelial and stromal nuclei are stained with the lamin B2 antibody (D). In contrast, the lamin B1 antibody stains few basal cells and all parabasal cells. Staining intensity is clearly decreased in the upper intermediate layer and superficial layer of epithelium (E). Lamins A/C are (very) weakly expressed in basal cells and staining intensity increases with level of differentiation (F). Bar represents 50 µm.

Chapter 3, figure 2. Representative illustrations of immunostainings of parenchyma adjacent to testicular germ cell tumours (a-c) and testicular germ cell tumours (d-h). (a) Testicular parenchyma adjacent to testicular germ cell tumours containing carcinoma in situ, stained with lamin B1 antibody 119D5; Sertoli (arrowhead) and Leydig (arrow) cells are negative for lamin B1. (b) Testicular parenchyma adjacent to embryonal carcinoma containing carcinoma in situ, stained with lamin B2 antibody X223; Sertoli cells (arrows) are positive for lamin B2. (c) Testicular parenchyma adjacent to a testicular germ cell tumour containing carcinoma in situ, stained with lamin A/C antibody R27; spermatogonia (arrowhead) and carcinoma in situ (arrows) are negative for A-type lamins. (d) Ras- seminoma stained with lamin B2 antibody X223; both normal (lymphocytes, arrow) and tumour cells are positive for lamin B2. (e) Ras- seminoma stained with lamin A/C antibody R27; tumour cells are negative for lamin A and C, while focal spots of normal tissue (lymphocytes, arrow) are positive for lamin A and C. (f) Embryonal carcinoma stained with lamin A antibody 133A2; tumour cells are negative for lamin A. (g) Embryonal carcinoma stained with lamin A/C antibody R27; both tumour cells and normal tissues are positive. (h) Teratoma stained with lamin A/C antibody R27; the inset shows a higher magnification of the teratoma illustrating the lamina staining.

Chapter 4, figure 1. Reactive lymph node stained with antibody R27, showing that A-type lamins are present in a few cells of the follicle centre and a population of cells in the paracortex.

Chapter 4, figure 2. Staining of the reactive lymph node with antibody 119D5 showing that cells of the follicle centre and the surrounding areas exhibit lamin B1.

Chapter 4, figure 3. The reactive lymph node after antibody LN43 staining, showing that lamin B2 is not or very weakly expressed in the cells of the follicle centre.

Chapter 4, figure 4. Double-label fluorescence microscopy of a reactive lymph node showing that centroblasts of the follicle centre express the proliferation marker Ki-67 (in green) but not lamin B2 (in red), while the majority of the surrounding cells in the mantle zone, paracortex and medulla show the reverse staining pattern. Centrocytes were negative for both Ki-67 and lamin B2. cc: centrocyte region; cb: centroblast region.

Chapter 4, figure 5. Reed-Sternberg/Hodgkin cells do express lamin B2, as shown after staining with LN43. Note that surrounding cells stain more strongly.

Chapter 4, figure 6. Hodgkin lymphoma stained with antibody R27 shows that Reed-Sternberg and Hodgkin cells express A-type lamins.

Chapter 4, figure 7. Double-label fluorescence microscopy of Hodgkin lymphoma with the proliferation marker Ki-67 (in green) and antibody R27 for A-type lamins (in red), showing that in general Ki-67 and A-type lamin staining are mutually exclusive. DNA was counterstained with DAPI (blue).

Chapter 4, figure 8. Triple-label fluorescence microscopy of a CD30-positive Reed-Sternberg cell (in blue) expressing A-type lamins as detected with antibody R27 (in red) and the proliferation marker Ki-67 (in green).

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