Contrasting effects of K8 and K18 on stabilizing K19 expression, cell motility and tumorigenicity in the BSp73 adenocarcinoma

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SUMMARY

The co-expression of vimentin and keratin-type intermediate filaments in the same cell was often reported to correlate with increased invasiveness and a more aggressive tumorigenic phenotype. To address the possible physiological relevance of these observations, we transfected simple keratins (K8 and 18) either individually, or in combination, into a tumorigenic but non-metastatic pancreatic adenocarcinoma that expresses vimentin but no keratins. Expression of K8 resulted in the stabilization of endogenous K19 in these cells, and formation of keratin filaments containing K8 and K19. Transfection of K18 yielded unstable K18 protein, but K18 could be stabilized when K8 was co-expressed in the same cells. Clones expressing K18 alone, or together with K8, displayed a reduced ability to grow in soft agar and decreased motility when compared to control, or K8/19 expressing cells. Moreover, K18 expressing cells were dramatically inhibited in their ability to form tumors when injected into syngeneic animals. The extent of suppression in the tumorigenicity of these cells correlated with the level of K18 expressed by these cells. The results show that K18 expression in cells may result in the suppression of the motile and tumorigenic abilities of this adenocarcinoma.

Key words: Simple keratin, Tumorigenesis, Keratin induction

INTRODUCTION

Intermediate filament proteins are expressed in a tissue specific pattern in vivo, and are believed to have a role related to very special aspects of differentiated tissue functions (Fuchs and Weber, 1994). The simple epithelial K8 and K18 keratins are the first intermediate filament proteins to be synthesized during mouse development (Jackson et al., 1980; Oshima et al., 1983), and are present in a variety of adult epithelial cell types including kidney, intestine and the liver (Franke et al., 1981), where they are the only type of intermediate filament protein. Vimentin-containing intermediate filaments appear later, at gastrulation, when primary mesoderm is formed (Franke et al., 1982). Keratins 8 and 18 are co-expressed with vimentin in motile extra embryonic endodermal cells (Lane et al., 1983; Lehtonen et al., 1983), and in the majority of epithelial cells cultured in vitro (Franke et al., 1978; Virtanen et al., 1981; Lane et al., 1982), but this co-expression is very rare in vivo. While the organization (Geiger et al., 1983) and expression in response to environmental conditions that affect cell adhesion differ for the two types of intermediate filament proteins in the same cell (Ben-Ze’ev, 1984, 1985), the physiological role of this co-expression is largely unknown.

Several studies have demonstrated a correlation between the co-expression of vimentin with simple keratins in metastatic cells when compared to their non-metastatic counterparts (Ramaekers et al., 1983; Günther et al., 1984; Ben-Ze’ev et al., 1986; Schaafsma et al., 1993; Oshima et al., 1996). Such correlation was reported for human melanoma (Hendrix et al., 1992; Trejdosievicz et al., 1986; Miettinen and Franssila, 1989) and breast carcinoma cell lines (Thompson et al., 1992), and was suggested to be related to a change in the motile and invasive ness of these cells (Chu et al., 1993, 1996). The mechanism(s) by which such co-expression can affect the motile and tumorigenic properties of tumor cells is not known yet.

To more directly address the possible physiological role of the co-expression of simple keratins with vimentin in cell behavior, we have used a spontaneous pancreatic adenocarcinoma that expresses vimentin, but no keratins (Ben-Ze’ev et al., 1986). A highly metastatic counterpart of this adenocarcinoma was previously shown to express simple epithelial keratins (Ben-Ze’ev et al., 1986). We have studied the consequences of ectopically expressing the simple keratins K8 or K18 alone, or in combination, on the growth, motile and tumorigenic abilities of these cells. We show that transfection with K8 stabilizes the expression of an endogenous K19. K18 when expressed alone, or in combination with K8 affected the motile and anchorage independent growth of these cells, and dramatically suppressed their tumorigenicity in syngeneic animals.

MATERIALS AND METHODS

Cell culture and transfection

AS and ASML cell lines derived from a spontaneous adenocarcinoma
of the pancreas in the BDX rat (BSp73) (Matzku et al., 1983) were grown in RPMI medium plus 10% bovine calf serum (HyClone, Logan, Utah). HeLa and MDCK cells were grown in Dulbecco’s modified Eagle medium supplemented with 10% bovine calf serum. The cells were maintained at 37°C in a humidified atmosphere of 5% CO2 and 95% air.

AS cells were subcloned from a single colony grown in soft agar, and the tumorigenic properties and metastatic abilities of this clone were similar to those reported for the original clone (Matzku et al., 1983). The human keratin 18 was expressed in AS cells by transfection of the pGC1853 plasmid containing the 10 kb intact gene (Kulesh and Oshima, 1988) into the cloned AS cells. Human keratin 8 cDNA was introduced by transfecting the cells with LK442-K8 (Kulesh et al., 1989) or LK444-K8. The later vector originates from LK444 (Gunning et al., 1987), and contains a second transcriptional unit coding for the phosphotransferase enzyme of the neo+ gene. In some transfections, the hygromycin-resistance (hygro) cDNA, driven by the pGK promoter, was used to provide an additional selectable marker. Transfection was carried out by the calcium phosphate precipitation method (Luthman and Magnuson, 1983), and colonies resistant to 400 μg/ml G418 (Geneticin, Gibco Laboratories, Grand Island, NY), or 100 μg/ml hygromycin (Calbiochem, San Diego, CA) were isolated. Cytokeratin-positive clones were identified by immuno-fluorescence screening using anti-cytokeratin antibodies (see below), and maintained in medium containing either hygromycin, and/or G418. Each clone was subcloned at least twice after which all cells stained positively for cytokeratins.

**Immunofluorescence**

Cells were grown on glass coverslips, fixed with methanol (at -20°C) for 10 minutes, and stained with mAbs against K18 (CY-90), K19 (K4.62), K8 (M20) or vimentin (VIM-13.2), or a pan-cytokeratin antibody (K8.13) that recognizes K1,5,6,7,8,10,11, and 18, all from Sigma Chemical Co. (St Louis, MO). Monoclonal antibody TR OMA 1, directed against K8 (Brulet et al., 1980) was used for screening the transfected AS cells. The secondary antibody was Cy3-labeled goat anti-mouse IgG (Fab′2) fragment (Jackson ImmunoResearch Laboratories, West Grove, PA). Double immunofluorescence staining of cytokeratin and vimentin filaments was performed with polyclonal rabbit anti-vimentin antibody and monoclonal anti-K8 and K18 antibodies, followed by FITC-goat anti-rabbit and Cy3-labeled anti-mouse secondary antibodies. Images were obtained with a Bio-Rad MRC 1024 confocal laser microscope.

**Cell fractionation, PAGE, and immunoblotting**

Triton X-100 and high salt soluble and insoluble fractions were prepared by extracting the PBS-washed cell monolayers, at 4°C, with a buffer containing 0.5% Triton X-100, 10 mM Heps, pH 7.4, 0.6 M KCl, 14 mM β-mercaptoethanol, 2.5 mM EGTA, 5 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride and 2.5 μg/ml leupeptin, followed by centrifugation to remove the Triton/high salt soluble fraction. The soluble fractions were cleared by centrifugation for 10 minutes at 14,000 rpm, and the proteins concentrated by ethanol precipitation. The pellets (soluble fractions) and the Triton X-100 insoluble material (insoluble fractions), were solubilized in equal volumes of sample buffer for SDS-PAGE (Laemmli, 1970). Equal volumes of the two fractions, or whole cell lysates, prepared from similar numbers of cells, were analyzed by PAGE, followed by immunoblotting with different monoclonal antibodies. The antigens were visualized by the enhanced chemiluminescence method (Amersham, Buckinghamshire, UK).

Proteins were metabolically labeled with [35S]methionine, 50 Ci/mmol (1,250 Ci/mmol; Amersham, UK), for 16 hours in methionine-free medium containing 10% dialyzed fetal bovine serum. The [35S]methionine-labeled cells were solubilized in O’Farrell's lysing buffer (O’Farrell, 1975), and analyzed by two-dimensional (2-D) isoelectrofocusing and SDS gel electrophoresis, as described (Ben-Ze’ev, 1990).

In pulse-chase experiments, cells were incubated for 45 minutes in methionine-free medium without serum and 150 μCi/ml [35S]methionine, washed and incubated with excess non-radioactive methionine for different time periods. Total cell lysates were prepared in O’Farrell’s lysis buffer. Equal amounts of TCA-insoluble radioactive material from each lystate were analyzed by 2-D gel electrophoresis.

**Northern blot hybridization**

Total RNA was extracted by the guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). Northern blots containing 20 μg, per lane, of total RNA were hybridized with cDNA probes labeled with [32P]dCTP by the random priming technique as described (Ben-Ze’ev et al., 1990). The cDNA for K19 was obtained by labeling an EcoRI fragment from pMAP11 (Bottger et al., 1995), containing human K19 cDNA. K8 cDNA was recovered as a BamHI fragment from the LK442-K8 plasmid.

**Phagokinetic tracks, growth in agar, zymography, and tumorigenicity assays**

Phagokinetic tracks were visualized on coverslips treated with 1% BSA and coated with colloidal gold (Sigma Immunochemicals, St Louis, MO). 103 cells were seeded on each 22 mm × 22 mm coverslip, and after 24 hours the cells were fixed with 3% paraformaldehyde. Tracks produced by the cells were viewed by phase contrast microscopy with a ×6 objective, transformed into computerized images, and the length of 35-40 tracks, for each cell type measured with the NIH image 1.55 software, as described (Rodríguez Fernández et al., 1992a).

Growth in semisolid medium was determined by seeding 250 and 1,000 cells per 35 mm diameter dish in 0.85 ml medium containing 10% serum and 0.3% bacto-agar (Difco, Detroit, MI), on top of a solid layer of 2.5 ml of 5% bacto-agar in the same medium. Plates were incubated for 2 weeks at 37°C. The number of colonies containing >50 cells was determined microscopically.

Zymography on secreted gelatinases was performed on proteins secreted overnight into 1 ml of serum-free medium, by 106 cells of the different clones grown on plastic, or on top of a hydrated type I collagen gel. The medium was analyzed by PAGE under non-reducing conditions (without β-mercaptoethanol), on gels containing 1 mg/ml gelatin. The gels were washed in 0.05 M Tris-HCl, pH 7.5, 5 mM CaCl2, 0.1% Triton X-100 (to remove SDS), and incubated overnight in the same buffer without Triton X-100, followed by staining with Coomassie blue.

Tumorigenicity of the different clones was determined by injecting the cells into the footpad of syngeneic BDX rats (103 and 5×105 cells per animal), as described (Matzku et al., 1983). When tumors reached 3 cm in diameter, the animals were sacrificed.

**RESULTS**

**Transfection of AS cells with K8 and K18**

To express keratin filaments in AS cells, the cells were co-transfected with a plasmid containing the K8 cDNA and the neo+ gene, and with another construct containing the K18 gene. Clones expressing keratin filaments were identified by immunostaining individual G418-resistant colonies with a monoclonal antibody recognizing K8. Confocal immunofluorescence analysis revealed several clones in which filaments were stained with anti-K8 antibody (Fig. 1A, clone AS 9.5). Such clones, however, were not stained with an antibody against K18 (results not shown), but were positive for vimentin (Fig. 1B). Immunofluorescence of the G418-resistant colonies with anti-K18 identified clones that displayed a diffuse staining with anti-K18 antibody (Fig. 1C, clone AS 9.8), and also some filamentous pattern that partially overlapped with vimentin (Fig. 1C,D). These clones did not stain with anti-K8 antibody (results not shown).
Effects of simple keratin expression in tumor cells

Two-dimensional gel electrophoresis of [35S]methionine-labeled cells separated by PAGE (Fig. 3A-C), revealed that clone AS 9.5 (Fig. 1A), and another clone, AS 9.20, that presented filamentous staining with anti-K8, but no staining with anti-K18, expressed K19 (Fig. 2C and 3C), and no K18 (Figs 2C and 3B). Clone AS 9.8 displaying mainly a diffuse staining for K18 (Fig. 1C), expressed K18, but no K8 or K19 (Figs 2B and 3A-C). These results imply that the filaments stained with anti-K8 in clone AS 9.5 (Fig. 1A) contained K8 and K19, and have no K18. This was further confirmed by the staining of filaments in AS 9.5 cells with anti-K19 antibody (Fig. 1E). Western blot analysis of lysates from these clones with a pan cytokeratin antibody (K8.13), did not detect additional keratins to those shown in Fig. 3A-C (data not shown).

**Stabilization of K19 expression in K8 transfected AS cells**

Since the parental AS cells expressed no detectable K19 (Fig. 3C), we have examined the possibility that they express K19 RNA, and that the type I K19 protein is stabilized against degradation when the type II K8 was introduced into these cells. Northern blot hybridization showed that AS cells, and all the different transfected clones derived from it, express K19 RNA (Fig. 3D), while only clone AS 9.5 cells also express K8 RNA (Fig. 3E). Hybridization with K18 cDNA revealed that AS 9.8 and 9.1 cells express this RNA (results not shown), while the other clones do not, in agreement with the expression of K18 protein in these clones (Fig. 3B).

Based on previous studies (Kulesh and Oshima, 1988; Kulesh et al., 1989) expression of K18 in the absence of a type II keratin partner was expected to result in an unstable K18 protein. Pulse chase experiments have indeed demonstrated that the K18 expressed in clone AS 9.8 has a short half life, and was rapidly degraded to undetectable levels by 2 hours after the initiation of the chase (Fig. 3G, compare to F), while K18 was stable when co-expressed with K8 in control, MDCK cells (Fig. 3H, compare to I).

**Transfection of K8 into AS cells expressing K18**

To obtain AS cells that express keratin filaments composed of K8 and K18, the AS 9.8 clone expressing K18 was co-trans-
Fig. 2. Two-dimensional gel analysis of keratins synthesized in AS clones transfected with K8 and K18. Cells were labeled for 16 hours with [35S]methionine, and equal amounts of radioactive-proteins were analyzed. (A) untransfected AS cells; (B) clone AS 9.8 expressing K18; (C) clone AS 9.5 synthesizing K8 and K19; (D) control MDCK cells. V, vimentin; a, actin. The unlabeled arrowhead points to a reference protein migrating close to K18. The position of the various keratins on the gel were confirmed by immunoblot analysis with the relevant antibodies (not shown).

Fig. 3. Immunoblot, northern blot hybridization and degradation rates of keratins expressed by various AS clones transfected with K8 and K18. G-418 resistant AS clones co-transfected with K8 and K18 were analyzed for keratin expression by reacting western blots of gels loaded with equal amounts of protein with antibodies against: (A) K8; (B) K18; and (C) K19. RNA extracted from control AS cells, neo' AS cells (AS neo), and different AS clones transfected with K8 and K18 was separated on agarose gel and hybridized with 32P-labeled cDNA to: (D) K19; (E) K8. HaCaT, is a human keratinocyte cell line. AS 9.8 (F,G) and MDCK cells (H,I) were labeled with [35S]methionine for 45 minutes (F,H), followed by washing and incubation in medium containing excess unlabeled methionine for 2 hours (G) or 8 hours (I). Equal amounts of radioactive protein were analyzed by 2-D gel electrophoresis. The arrow marks the position of a protein migrating close to K18; a, actin.
Effects of simple keratin expression in tumor cells

9.8 and 9.1) contained significant levels of K18 and all the cellular vimentin, it is possible that K18 formed mixed filaments with vimentin in such cells (see Fig. 1C,D).

Growth and motile properties of keratin-transfected AS cells

The growth of the different AS clones was examined on plastic and in soft agar, in the presence and absence of serum. On plastic, the various AS clones showed very similar growth kinetics, growing slower in the absence of serum, but did not differ significantly from each other (results not shown). The plating efficiency of these clones on plastic was also very similar (Fig. 7, open bars), but when cultured in soft agar, cells expressing K18 either alone (clones AS 9.1 and 9.8), or in combination with K8 (clone AS C6), showed a 2- to 4-fold decrease in cloning efficiency. In contrast, clone AS 9.5, expressing K8/19, displayed a similar cloning ability in soft agar to that of control AS cells (Fig. 7).

This difference between AS clones expressing K18 and those that do not was also observed when the length of phagokinetic tracks, on colloidal gold-coated coverslips, was examined (Fig. 8). The control AS neo and the AS 9.5 clone expressing K8/19 were significantly more motile (between 2.5- and 4-fold) when compared to AS 9.1, 9.8 and C6, that either express K18 alone, or in combination with K8 (Fig. 8).

The ability to secrete proteases by these clones, when cultured on plastic or hydrated collagen, showed that while on collagen all clones secreted increased levels of gelatinases, there were no significant differences among the different clones in this property (results not shown).

The tumorigenic ability of AS clones transfected with different keratins

Previous studies have indicated that co-expression of vimentin and keratin-type intermediate filaments, in certain cell types, correlated with increased tumorigenicity and invasiveness of the cells (Hendrix et al., 1992). We have therefore examined the tumorigenic ability of the different keratin-transfected AS clones in syngeneic rats. The results summarized in Table 1 show that while control AS and AS neo, as well as AS 9.5 cells that express K8/19, develop tumors that reached 3 cm in diameter after 35-40 days, all clones expressing keratin 18, either alone (AS 9.1 and 9.8), or in combination with K8 (clone AS C6), displayed a dramatic delay in the development of tumors. Clone AS C6 that expressed the highest level of K18 (Fig. 6B), was unable to form tumors. In some of the animals injected with an AS clone expressing K18 (AS 9.1) small, regressing tumors developed that disappeared at later times (Table 1).

DISCUSSION

This study has demonstrated that the expression of K8 can stabilize the expression of K19 by enabling the formation of intermediate filaments containing K8 and K19. Keratin filaments are always composed of type I and type II pairs (Eichner et al., 1984). Although such filaments can be formed in vitro by copolymerization of almost any type I with type II keratin (Hatzifeld and Franke, 1985), this study is the first to demonstrate the formation of keratin filaments in cells expressing K8/19, in

Fig. 4. Transfection of K8 into AS 9.8 cells containing K18. AS 9.8 cells were co-transfected with K8 and the hygro r cDNAs, and expression of K8 was determined using anti-K8 antibody on western blots from individual hygro r clones (lanes 2-7). (A) Immunoblot with anti-K8; (B) the same blot reprobed with anti-K18.

The solubility of the transfected keratins in Triton X-100 and high salt was compared in the different clones to that of vimentin, co-expressed in the same cells (Fig. 6). Vimentin displayed a complete resistance to solubilization by detergent and high salt, in all the cell lines tested (Fig. 6A). In contrast, variable amounts of the transfected keratins were found to be soluble under such conditions (Fig. 6B-D), in both transfected AS cells, as well as in various epithelial cell lines including HeLa, MDCK and ASML (a highly metastatic variant of the AS clone; Matzku et al., 1983). K8 was mostly insoluble when co-expressed with either K19 in clone AS 9.5, or with K18 in clone AS C6 (Fig. 6C). In contrast, K18 was mainly in the Triton/high salt soluble fraction when expressed alone in clones AS 9.8 and AS 9.1, but the majority of K18 was shifted to the Triton-high salt insoluble fraction in cells co-expressing K8 and 18 (clone, AS C6; Fig. 6B). It is noteworthy that in AS C6 cells that formed well developed keratin filaments (Fig. 5B,D), a relatively high level of soluble K18 was present (Fig. 6C). The majority of K19 was insoluble in AS 9.5 and control MDCK cells, but not in ASML cells (Fig. 6D). Since the Triton/high salt insoluble fraction of K18 transfected cells (AS

Nonionic detergent and high salt solubility of K8, 18 and 19

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the absence of K18. In addition, we showed that a type II keratin (K8) can stabilize the expression of a type I keratin (K19). The occasional expression of an unpaired keratin (Kulesh et al., 1989) is likely responsible for the apparent induction of a complementary keratin by forced expression of a type II keratin (Giudice and Fuchs, 1987). In our study, the expression of K8 in AS cells stabilized K19 expression since these cells expressed endogenous K19 RNA. This, in turn, enabled the formation of keratin filaments that contained K8 and K19 which most probably stabilized K19 against degradation.

Apparently, no type II keratin was expressed in AS cells, since expression of the transfected K18 in AS cells did not result in the formation of keratin filaments (a pan cytokeratin antibody did not detect additional keratins on western blots)

Fig. 5. Organization of keratins and vimentin-type intermediate filaments in transfected AS C6 cells. Double immunofluorescence confocal images of AS C6 cells stained with polyclonal anti-vimentin (A,C), and monoclonal anti-K18 (B) and anti-K8 (D) antibodies. (E), Composite image showing partial overlap of the two intermediate filament systems. Bar, 10 μm.

Fig. 6. Nonionic detergent and high salt solubility of K8 and K18 in transfected AS cells. Different AS clones, and HeLa, MDCK and the highly metastatic ASML cell line, were extracted with Triton X-100 and high salt. Equal volumes of the Triton/high salt soluble (S) and insoluble (I) fractions were separated by PAGE and western blots incubated with antibodies to: (A) vimentin; (B) K18; (C) K8; and (D) K19. The MDCK sample in D was also incubated with anti-K18, after K19 detection.
From such cells. The singly expressed K18 had a short half life, in agreement with previous studies (Kulesh and Oshima, 1988; Kulesh et al., 1989; Lu and Lane, 1990), and did not induce K19 expression, as these type I keratins cannot form polymers with each other. While K8 and K19-containing filaments could be formed in stably transfected cells (Fig. 1A and E), their organization was less well developed when compared to AS cells expressing K8 and K18 (Fig. 5B and D), which are the specific keratin pairs co-expressed in cell lines and tissues. Furthermore, in K8 and K18 expressing cells, K19 was not expressed at detectable levels, supporting the view that the more efficient co-polymerization of K18 with K8, depleted all the available K8 molecules from polymerizing with K19. This result could explain previous observations on K19 expression that is quite variable in cell lines (Darmon, 1985; Nagle et al., 1987) and in vivo (Bader et al., 1988; Nagle et al., 1991), where mosaic expression is common, even in cells containing K8 and K18. Perhaps, the instability of the K8/K19 pair may account for the K19 mosaicism.

Stabilization of K18 against degradation by the expression of K8 was also demonstrated. When individual K18 expressing clones were analyzed for levels of a transfected K8, the highest level of K18 in such clones correlated with the highest expression of K8. These results are in agreement with another study showing that in fibroblasts expressing an unstable K8, transfection of K18 causes stabilization of the K8 protein (Lu et al., 1993). Complementary stabilization may thus constitute

**Table 1. Tumorigenicity of AS cells transfected with keratins**

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*The size of tumors is expressed as the higher outer diameter. Five rats from each AS clone were injected with 10⁵ cells per animal. When the tumors reached 3 cm in diameter, the animals were sacrificed.*

**Fig. 7. Growth properties of K8 and K18 transfected AS cells in soft agar.** Different AS clones were either seeded on plastic dishes at 200 cells/10 cm plate, to determine plating efficiency on plastic (PE), or 250 and 1,000 cells on 35 mm diameter dishes in agar, to determine colony formation in soft agar. Colonies containing >50 cells were counted, in duplicates, after 2 weeks.
a general mechanism for regulating the assembly of keratin filaments in a variety of epithelial cells.

A significant amount of the transfected simple keratins were soluble in nonionic detergent and high salt, in contrast to vimentin that was totally insoluble under these conditions. This soluble fraction was observed when the keratins were expressed in pairs (i.e. K8 with K18, K8 with K19) in a variety of cell types, in addition to AS cells (Fig. 6). Other cell lines (Franke et al., 1987; Chou et al., 1993), and Xenopus oocytes and eggs (Gall and Karsenti, 1987) were also shown to contain a soluble fraction of simple keratins. The role of this soluble pool of simple keratins is yet to be determined. When K18 was expressed alone, the majority of the protein was soluble under these conditions. However, the K18 found in the Triton/high-insoluble fraction of such cells could possibly interact with vimentin to form some mixed filaments, as indicated by their co-localization using immunostaining (Fig. 1C,D).

We have examined several biological properties of the keratin-transfected AS cells. The expression of the different keratins had no apparent effect on the kinetics of AS cell growth on plastic in the presence or absence of serum. In contrast, the growth of clones expressing K18 alone, or in combination with K8, was significantly decreased in soft agar, suggesting that K18 expression can partially inhibit the anchorage independence of these tumor cells. Interestingly, K8 expression did not elicit a similar result. It is likely that those clones which expressed K18 in the absence of a complementary type II keratin, express quite high levels of K18 mRNA to permit the detection of undegraded and soluble (unpolymerized) protein. When mouse K8 is removed from normal cells by gene targeting, the endogenous levels of mouse K18 is not sufficient to overcome the degradation rate (Baribault et al., 1993).

The motile properties of the K18 expressing AS clones was also dramatically reduced when compared to control, or K8 expressing clones. Finally, the tumorigenic ability of K18 transfected cells was suppressed, and in a clone expressing the highest level of K18 (AS C6), this suppression was the most dramatic, and no tumors appeared even 5 months after the animals were transplanted.
injected with these cells. As K18 in such cells was mainly present in the Triton/high salt insoluble fraction, and in the other K18 positive clones Triton-insoluble K18 was present (Fig. 6B), the formation of some mixed filaments between K18 and vimentin (Fig. 1C,D) could play a role in this tumor suppressive activity.

These results were rather unexpected, based on previous observations showing an increase in the motile and the tumorigenic ability of cell lines co-expressing vimentin with simple keratins, when compared to cells expressing only one type of intermediate filament system (Chu et al., 1993; Thompson et al., 1992; Hendrix et al., 1992; Ben-Ze’ev et al., 1986). Since pancreatic adenocarcinomas normally express K8 and K18 (Schussler et al., 1992), including the metastatic counterpart of AS cells (Ben-Ze’ev et al., 1986), restoration of their expression in AS cells may suppress a previous abnormality. Expression of simple keratins may thus contribute to malignancy only in those cell types in which they are not normally expressed, such as the melanomas described above.

In addition, when comparing high- and low-metastatic counterparts, at least in the pancreatic BSp73 adenocarcinoma used in this study, additional differences to those in intermediate filament proteins were documented: for example, the highly metastatic ASML counterpart of AS cells, while expressing simple keratins, lacked vinculin (Raz et al., 1986), and restoration of vinculin expression suppressed their tumorigenic and metastatic ability (Rodríguez Fernández et al., 1992b). Furthermore, a different splice variant of CD44 was detected in the highly metastatic ASML cells that, when introduced into the non-metastatic AS cells, conferred the malignant metastatic ability (Günther et al., 1991). Therefore, the differences that are often observed in the molecules expressed by pairs of high- and low-metastatic variants should be interpreted with caution, before conclusions can be reached on their direct role in the invasive and metastatic ability of these cells.

While simple keratins were not yet directly linked to a human disease, the elimination of simple keratin expression by inactivation of K8 in mice, by genetic knock out, results in embryonic lethality (Baribault et al., 1993), and in a different genetic background, cause female sterility and colorectal adenomatous hyperplasia (Baribault et al., 1994), or an earlier onset of mammary gland tumors in MMTV-polyoma middle T transgenic mice (Baribault et al., unpublished). In addition, keratins were identified as candidate tumor suppressor genes by subtractive hybridization (Lee et al., 1991), and were shown to confer multidrug resistance when transfected into fibroblasts (Bauman et al., 1994). These results are consistent with the present study implying a suppressive role for simple keratins in the progression towards tumorigenicity, but the mechanism(s) by which simple keratins can fulfill such functions is yet to be determined.

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