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Nestin expression in normal adrenal gland and adrenocortical tumors

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Summary. Human adrenocortical cells have been shown to express cytokeratins and vimentin. Nestin is an intermediate filament protein that is mainly expressed in the developing nervous system and that has been recently reported in rat adrenal gland as well. Using immunohistochemical and biochemical approaches, the present study demonstrates that nestin is constantly expressed *in situ* in the cortex of normal human adrenal glands. Nestin expressing cells were prevalently located in the zona reticularis but some positive cells could be spotted in the zona fasciculata as well. Moreover, patches of nestin-positive cells have been constantly detected on sections of cortical adenomas. In contrast, adrenal carcinomas displayed a variable number of nestin-immunoreactive cells that in some cases were virtually absent. Samples of renal clear cell carcinoma metastasis in the adrenals were also examined which did not show nestin-immunoreactivity. We propose that a positive nestin-immunoreaction could be useful in differential diagnosis of clear cell tumors in adrenal glands.

Key words: Nestin, Adrenal gland, Intermediate filaments, Neoplasms, Adrenal cortex

Introduction

Intermediate filament proteins (IFps) represent a large family of more than fifty cytoskeletal proteins. Six classes of IFps have been described so far, based on sequence homology and gene structure. They include class I acidic cytokeratins, class II basic/neutral cytokeratins, class III vimentin-like IFs (vimentin, desmin, glial fibrillary acidic protein and peripherin), class IV neurofilaments and α-internexin, class V nuclear lamins, and class VI which is represented exclusively by nestin (Steinert et al., 1999). A few additional IFps (phakinin, filensin, sinemin, paranemin) cannot be included in these classes according to the above-mentioned criteria (Herrmann and Aebi, 2000). IFps polymerize to form a network of cytoplasmic intermediate filaments (IFs). From a functional viewpoint, IFs are considered important for maintaining cell integrity. This is certain at least for some cytokeratins and desmin as knock-out mice models develop skin diseases and myocardial degeneration due to cell fragility (Ku et al., 1995; Milner et al., 1996; Porter et al., 1996). Further functions have been proposed for other classes of IFps, such as a possible role in gene regulation (Traub and Shoeman, 1994; Klimkowsky, 1995) and hormone secretion (Bertelli et al., 2000; Quintanar, 2000; Regoli et al., 2000). In adrenocortical cells, vimentin may be involved in cholesterol metabolism (Sarria et al., 1992; Hall and Almahbodi, 1997) and in the biosynthesis of glycosphingolipids (Gillard et al., 1994).

IFps display a cell-specific pattern of expression in normal tissues. In pathology, differential expression of IFps has been successfully employed as a tool in tumor diagnosis (Ho and Liem, 1996; Prasad et al., 1999). Nestin is a high molecular weight IFp, which was originally described as a marker of central nervous system stem cells (Lendhal et al., 1990), and has also been detected in skeletal and cardiac myogenic cells (Sejersen and Lendhal, 1993; Kachinsky et al., 1995), in odontoblasts (Terling et al., 1995; About et al., 2000), and is transiently expressed in differentiating rat and mouse testis (Fröjdman et al., 1997). On the other hand, nestin expression in the adult and embryonic pancreas is still a subject under debate (Lardon et al., 2002; Klein et al., 2003; Delacour et al., 2004; Street et al., 2004). Based on the above-mentioned examples, however, it seems that nestin is an exceedingly rare occurrence in adult tissues and, to our knowledge, it is restricted to the interstitial cells of Cajal (Tsujimura et al., 2001), Leydig cells (Lobo et al., 2004), adult angiogenic vasculature (Mokry et al., 2004) and sporadically to mature vascular endothelial cells (Lobo et al., 2004; Mokry et al., 2004). We have also recently succeeded in showing nestin expression in rat adult adrenal glands (Bertelli et al., 2002). In order to explore the possibility of using nestin

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as a marker of adrenal cells in human pathology, we performed a biochemical and immunohistochemical study to ascertain nestin expression in normal human adrenal glands and related tumors.

Materials and methods

Patients and tissues

Cases and controls were taken consecutively and retrospectively from the surgical files of the departments of Human Pathology and Oncology in Siena and Florence. Surgical samples were fixed by immersion in 4% buffered formalin for 24-48 hours, sampled and paraffin embedded.

Normal adrenal glands were obtained from kidney surgical resection due to renal cell carcinomas, free of metastatic disease and other pathologies (11 cases, 4 males, age range 42-72 years, median 55 years). Cortical adenomas were from 15 different patients (7 males, age range 37-80 years, median 62); 10 cases also presented a rim of normal adrenal tissue which served as an internal control, and to verify the presence and the localization of nestin in atrophic glands. Cortical carcinomas were from 12 different patients (1 male, range 33-76 years, median 51). Differential diagnosis between cortical adenomas and carcinomas was performed according to the standard criteria described by Weiss (Weiss, 1984; De Lellis, 1999). All carcinoma cases presented vascular and capsular invasion, cellular pleomorphism, atypical mitosis, high mitotic rate and a variable amount of necrosis. Renal clear cell carcinoma metastasic disease in the adrenals was examined in 9 cases (7 males, range 46-72 years, median 61 years). Moreover, five primitive clear cell carcinoma of the kidney were also examined.

Immunocytochemistry

Sections of 3-5 µm were cut from each specimen, mounted on electrostatically charged slides, and dried overnight at 37°C. All sections were then deparaffinized in xylene, rehydrated through a graded alcohol series, and washed in Tris Buffered Saline (TBS). TBS was used for all subsequent washes and for dilution of the antibodies. Tissue sections were heated in a microwave oven twice for 5 minutes at 750 W and were subsequently rinsed in 3% hydrogen peroxide to block endogenous peroxidase (Dakopatts, Glostrup, D). Slides were then incubated for 1 hour at room temperature with the primary antibody (working dilution 1:200). Two different anti-human nestin antibodies were used: a rabbit anti-nestin polyclonal antibody (AB5922, Chemicon, Temecula, CA) and a mouse anti-nestin monoclonal antibody (MAB5326, Chemicon). Slides were incubated with the appropriate secondary antibody (Dakopatts) for 30 minutes at room temperature. New fuchsin or diaminobenzidine were the final chromogen, and hematoxylin was used for nuclear counterstaining. Negative controls for each tissue section were prepared by substituting the primary antibody with the corresponding pre-immune serum. All samples were processed under the same conditions. The results of the immunostainings were independently evaluated by two observers.

SDS/PAGE electrophoresis and Western blotting

In order to confirm our immunohistochemical results, Western blotting analysis with anti-nestin antibody was carried out on proteins of the IF-enriched cytoskeletal fraction of normal adrenal cortex, separated by SDS-PAGE. IF-enriched cytoskeletal fractions were prepared as previously reported (Achstaetter et al., 1986; Carapelli et al., 2004), with minor modifications. Briefly: small portions of adrenal cortex were removed in the course of autoptic examinations, quickly minced and placed in 2 ml of homogenization buffer (96 mM NaCl, 8 mM KH_2PO_4, 5.6 mM $Na_2PO_4.2H_2O$, 1.5 mM KCl, 10 mM EDTA, 0.1 mM dithioerythriol (DTT), 2.5 mg/ml Aprotinin, 100 mM PMSF, pH 6.8). Samples were homogenized with Dounce homogenizers and filtered through four layers of gauze. Six ml of very high salt buffer (2 M KCl, 200 mM NaCl 10 mM Tris-HCl, 0.1 mM DTT, 2.5 mg/ml Aprotinin, 100 mM PMSF, pH 7.4) were added to each homogenate and the suspensions were stirred for 30 minutes on ice. Afterwards, samples were homogenized once more in Dounce homogenizers and centrifuged at 10,000g for 20 minutes at 4°C. After removing the supernatants, pellets were resuspended in 3 ml of high salt buffer (10 mM Tris-HCl, 140 mM NaCl, 1.5 M KCl, 5 mM EDTA, 0.5% w/v Triton X-100, 2.5 mg/ml Aprotinin, 100 mM PMSF, pH 7.6) with Dounce homogenizers. Samples were gently stirred for 30 minutes on ice, centrifuged at 10000g for 20 minutes at 4°C and resuspended in 3 ml of high salt buffer. These steps were repeated once more and the final pellets, instead of being resuspended in high salt buffer, were washed in PBS pH 7.4 with 0.1 mM DTT. Pellets, representing the IF-enriched cytoskeletal fraction, were stored at -20°C until use.

IF-enriched cytoskeletal fractions were separated by electrophoresis through a 6% polyacrylamide gel as described by Laemmli (1970), and transferred to nitrocellulose in a Bio-Rad Transblot apparatus (BioRad, Segrate, Italy). A 5% (w/v) solution of skimmed milk in TBS was used to quench non-specific protein binding of the antibodies. Membranes were incubated overnight at room temperature with 1:2000 anti-nestin antibody in 5% (w/v) solution of skimmed milk in TBS. Specific bands were detected using an electrochemiluminescense kit (Roche Diagnostics, Milano, Italy)

Results

SDS/PAGE electrophoresis and Western blotting

In order to confirm that the immunocytochemical reaction was specific for nestin, we carried out western

blotting analysis with anti-nestin antibodies on samples of proteins of the IF-enriched cytokeletal fraction of 6 normal adrenal cortex sampled in the course of autoptic examinations. Western blotting experiments (Fig. 1) revealed a major band migrating at the expected molecular weight (~280 kDa) according to Sultana et al., (1998). A weaker band, migrating at ~260 kDa, was also detectable, consistent with the previously reported existence of nestin doublet protein bands (Messam et al., 2000). Both bands have been detected with variable intensity in all samples tested with the exception of one case where post-mortem degradation was in a too advanced stage.

Nestin expression in normal human adrenal glands

Positive nestin-immunoreaction was observed in all normal adrenal glands examined, mainly restricted to the reticular layer (Fig. 2a). Peripheral cortical areas, i.e. the glomerular cell layer, were negative in all samples examined, while fascicular layers presented focal positive cellular areas (Fig. 2a). The positive reaction was present in the cytoplasms most frequently in a diffuse form, yet in some cases dots of stains were evident (Fig. 2b). Such aspects were present either in completely normal adrenal glands or in hypotrophic normal glands close to cortical adenomas. All adrenal medulla cells were always completely negative (Fig. 2a). The two different antinestin antibodies used gave the same results.

Nestin expression in adrenocortical tumors

All examined cortical adenomas presented different amounts of nestin-positive neoplastic cells. Positivity was either diffuse or dot-like in the cytoplasm, yet the stained cells were only a fraction of the neoplastic ones (Fig. 2c, d). In fact, in the majority of the tumors examined, focal areas of positivity were mixed with negative neoplastic cells. Such positive foci could be found in all areas of the tumor, i.e. in both the central part and the boundary regions.

Cortical carcinomas did not have a constant appearance. Seven different cases presented an immunoreaction comparable to cortical adenomas (Fig. 2e), in that the positivity was restricted to focal areas, while five cases were completely negative (Fig. 2f).

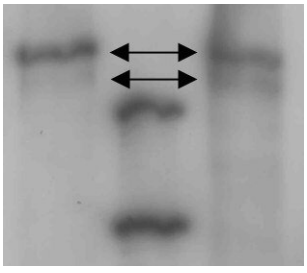

Fig. 1. Western blotting with anti-nestin antibody of the IF-enriched cytoskeletal fraction of adrenal proteins separated by 6% SDS-PAGE. Lanes 1, 3: samples from two different adrenals. Lane 2: molecular weight standards (250 kDa, 150 kDa). Two bands (double arrows), migrated approximately at 280 and 260 kDa, are unveiled by the electrochemiluminescense reaction.

All the examined clear cell carcinomas of the kidney, either samples from the primitive tumor in the kidneys or secondary metastatic disease in the adrenals, were completely unstained (Fig. 2g,h). Both antibodies employed in this study gave similar results.

Discussion

In the eighties and nineties, we witnessed a profusion of studies aimed at describing IFps expression patterns in virtually all tissues and organs. The immunohistochemical investigations were mainly prompted by the possibility of employing this family of proteins as tissue and cell markers in tumour diagnosis. Cytokeratins and vimentin are the only IFps detected so far in the human adrenal cortex and related tumors. Vimentin, which is detectable only in a few scattered cells of normal adrenal glands and heterogeneously expressed in cortical adenomas (Cote et al., 1990; Haak and Fleuren, 1995), has consistently been found to be highly expressed in adrenocortical carcinoma (Cote et al., 1990; Miettinen, 1992). Cytokeratins, which occur in normal adrenal glands, have been irregularly detected in cortical adenomas (Cote et al., 1990; Haak and Fleuren, 1995) whereas a tendency towards the loss of cytokeratin reactivity has been observed in adrenocortical carcinoma (Cote et al., 1990; Miettinen, 1992). However, some carcinomas and adenomas still maintain high levels of cytokeratin expression (Miettinen, 1992; Haak and Fleuren, 1995).

We have recently been able to detect nestin in rat adrenal glands (Bertelli et al., 2002). This finding prompted us to investigate its expression in human adrenal glands as well as in related tumours. The present investigation reports for the first time that nestin is expressed in a subpopulation of human adrenocortical cells. The expression of nestin is confirmed in immunocytochemical experiments by the use of two different antibodies which gave similar results. Moreover, western blotting analysis of adrenal homogenates confirms the specificity of the reactions. In normal adrenal glands nestin is detectable in the cortex, mostly in the *zona reticularis*, whereas in cortical adenomas nestin immunoreactivity appears confined to patches of tissue and in adrenocortical carcinomas it persists only in few scattered cells. The patterns of staining in normal and pathological adrenal glands prompt us to consider the functional role played by IF in adrenals, which probably goes beyond the mere structural reinforcement of cells. In particular, the functions of nestin and vimentin are strictly related, as nestin seems to be obliged to co-assemble with vimentin (Eliasson et al., 1999; Steinert et al., 1999). In adrenocortical cells, vimentin IFs have been shown to be associated with lipid droplets and are thought to keep lipid droplets apart from mitochondria, thus modulating the first step of steroidogenesis (Hall and Almahbodi, 1997). According to this hypothesis, a partial de-polymerisation of IFs, leading to the approach of lipid

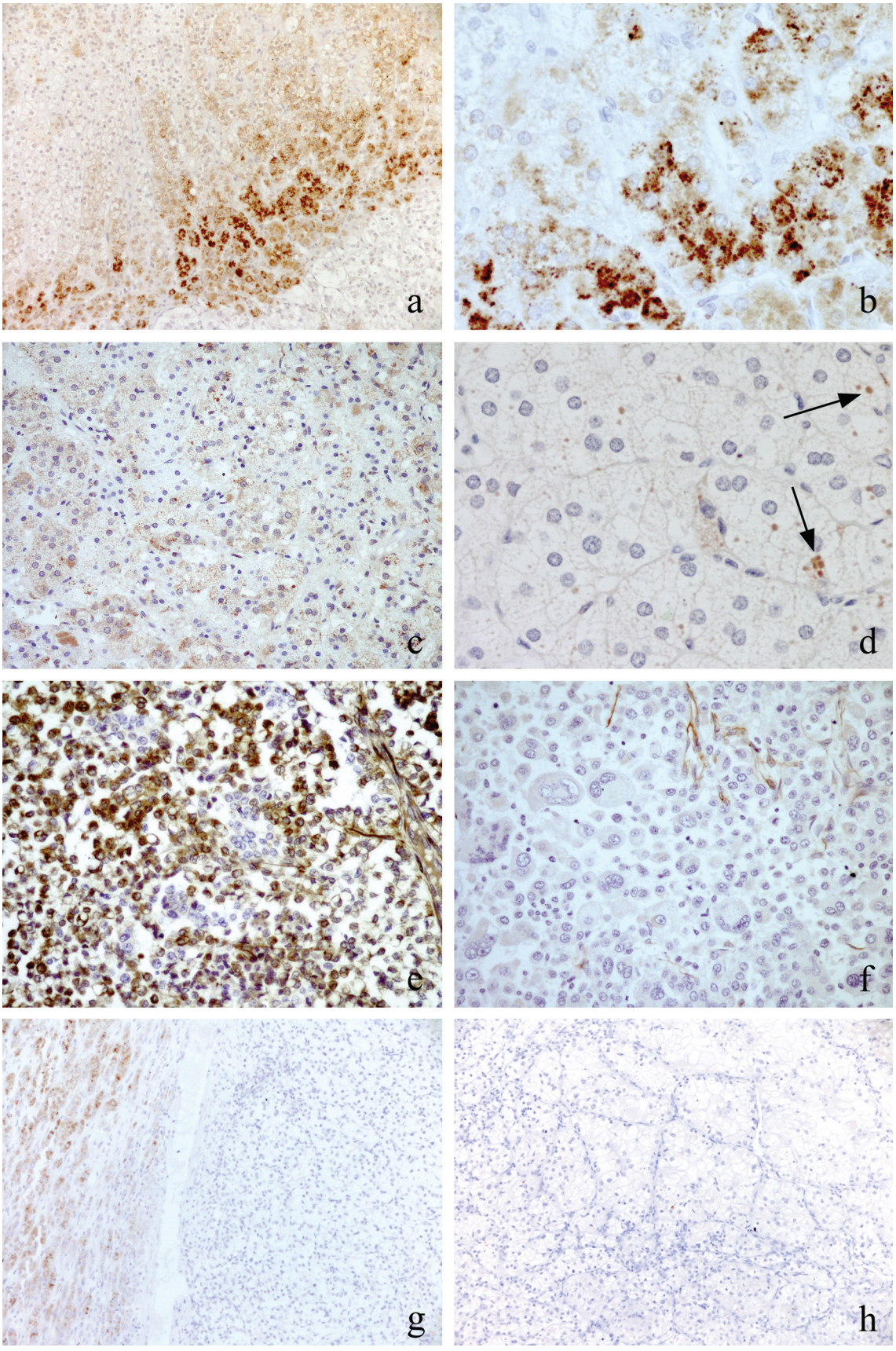

Fig. 2. Immunohistochemical reactions with anti-nestin antibodies on sections from normal and tumoral adrenal glands. Normal adrenal gland **(a, b)**. **a.** The cytoplasm of the cortical cells in the reticular layer is strongly immunostained. The adrenal medulla (M) is completely unstained, wheras in the upper left corner scanty cells of the fascicular layer are stained. **b.** Higher magnification shows diffuse and dotted immunoreaction in the cytoplasm of reticular and fascicular layer cells. Cortical adenoma **(c, d)**. **c.** Neoplastic cells show intense focal reaction, easily visible at low magnification. **d.** Small dots of stain may be present in isolated cells even in areas featuring negativity at low magnification. Cortical carcinoma **(e-h)**. **e.** A tumor showing nestin-positivity. **f.** A tumor from a different patient is completely negative. Adrenal metastasis of clear cell carcinoma of the kidney **(g)** and primitive clear cell carcinoma of the kidney **(h)** are completely negative. On the left side of the adrenal metastasis of clear cell carcinoma **(g)** residual cortical cells show nestin immunoreaction (internal positive control). a, e, f-h, x100; b,d, x 200; c, x 60

droplets to mitochondria, would represent the first step in the adrenal steroidogenesis. The limited nestin immunoreaction in normal adrenal glands (restricted to the *zona reticularis*) and in related tumors may be due to masking effects that vary according to adrenal zone and/or to different functional involvement of the cells. Down-regulation of nestin expression could possibly be the reason for the loss of immunoreactivity in adrenal tumors, but an up-regulation of vimentin (Miettinen, 1992; Haak and Fleuren, 1995) could also bring about the same result, changing the ratio between the two IFps and masking all nestin antigenic sites. Additional studies will be needed to address this issue.

So far, nestin immunoreactivity has been reported in a limited number of tumors, most of them deriving from neuroectodermal cells. Gastrointestinal stromal tumors (Miettinen and Lasota, 2001; Tsujimura et al., 2001), schwannomas (Sarlomo-Rikala et al., 2002), astrocytomas (Ehrmann et al., 2005), malignant melanomas (Ehrmann et al., 2005), neuroblastomas (Thomas et al., 2004) and meningiomas (Tohyama et al., 1992) have almost constantly been reported to express nestin. Additional nestin-positive tumors are capillary haemangiomas (Ehrmann et al., 2005), childhood rhabdomyosarcomas (Kobayashi et al., 1998), ependymomas and glioblastoma multiforme (Almqvist et al., 2002). Finally, weak and inconstant nestin immunoreactivity has been detected in phaeochromocytomas, neurinomas and carcinoids (Ehrmann et al., 2005). Interestingly, in many cases, nestin expression seems to correlate with malignancy. As a matter of fact, high grade astrocytomas display a higher level of nestin expression than low grade astrocytomas do, and nestin seems to be heavily involved in cell proliferation and motility of neuroblastoma cells as well (Thomas et al., 2004). This is apparently in contrast with our findings that point toward a progressive loss of adrenocortical nestin immunoreactivity in parallel with the increase of cell malignancy. However, as above-mentioned, a decrease of nestin antigenic sites available for antibody binding could explain this paradox.

Positive nestin immunoreactivity may be of some use in differential diagnosis of clear cell tumors. In the present study, clear cell carcinomas of the kidney are consistently negative for nestin, while cortical adenocarcinomas did not present a constant feature. Positive immunoreactivity for nestin may therefore help to diagnose cortical adenocarcinoma along with other markers, such as melan-A103, inhibin A and synaptophysin (Pelkey et al., 1998; Renshaw and Granter, 1998). Unfortunately, negativity is not able to give relevant information. At any rate, to obtain a definitive conclusion, the present study needs to be confirmed in a more broad selection of cases.

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