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Identification of Calretinin and the Alternatively Spliced Form Calretinin-22k in Primary Pleural Mesotheliomas and in Their Metastases

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Abstract. *Background: Antibodies against calretinin represent an established, powerful and reliable immunohistochemical marker in the differential diagnosis between mesothelioma and adenocarcinomas. However, in studies published so far, the exact molecular identity of the immunoreactive protein(s) detected in mesothelioma sections has not yet been determined. Materials and Methods: Tissue biopsies from ten mesothelioma samples, primary and metastatic, were analyzed by Western blot analysis and reverse transcriptase-polymerase chain reaction (RT-PCR), in addition to immunohistochemical staining. Results: Besides the selective positive immunoreaction in all 10 samples, calretinin was identified by Western blot analysis and by identification of its mRNA by RT-PCR. In addition, we identified the alternatively spliced form calretinin-22k and the corresponding mRNA, previously detected only in neoplastic cells in colon carcinomas and derived cell lines. Conclusion: Our findings demonstrate that the immunoreactivity observed in the mesotheliomas and their metastases investigated in this report is due to the concomitant presence of calretinin and the alternatively spliced form calretinin-22k.*

Calretinin (CR) and calretinin-22k (CR-22k) are members of the EF-hand family of calcium-binding proteins (1). CR is mainly expressed in specific neurons in the central and peripheral nervous system (2), and in several other organs, *e.g.* testis and ovary. The exact role played by CR in the various cell types remains unknown, but suggested functions include a general role as Ca^{2+}-buffer in non-excitable cells and an involvement in neuroprotection against excitotoxicity (3). In tumor cells, CR has been observed in several colon cancer cell lines (4), in primary colon tumors (5) and antibodies against CR have gained much attention as specific and sensitive markers for mesotheliomas, mainly of the epithelial type (6-8). In combination with other immunohistochemical markers (*e.g.* E-cadherin, cytokeratin 5/6), CR was demonstrated to be useful for the discrimination between mesotheliomas and metastatic adenocarcinomas (9), a distinction which is frequently difficult with conventional histology. CR-22k is an alternative splice product of the CR gene where exons 8 and 9 are deleted. CR-22k is identical to CR up to arginine 178 and due to a frame shift terminates with a C-terminus of 14 amino acids, which share no homology with the full-length protein (10). CR-22k can be identified in immunohistochemical stainings or by Western blot analysis, using a specific antiserum recognizing its 14 C-terminal amino acids (11) that are not present in full-length CR. It has been detected, so far, only in primary colon adenocarcinomas and derived cell lines (11) and in the serum of some cancer patients (12), suggesting a possible role as a marker of malignancy.

The aim of this study was to investigate which forms of CR are present in primary and metastatic mesotheliomas and to observe possible differences in their expression patterns. In previous reports, the antiserum 7696 (13), which recognizes CR as well as CR-22k, was used, not allowing the distinction between the two calretinin isoforms on immunohistochemical stainings. In addition, no biochemical or molecular biological evidence has yet been presented in the previous studies (6-9) that the immunoreactivity observed in the mesotheliomas was actually due to the presence of CR or CR-22k and not to a possible crossreactivity with a closely related protein such as calbindin D-28k (2) or secretagogin (14).

Materials and Methods

Tissue samples. Paraffin-embedded and frozen material from eight tissue samples from primary mesotheliomas and two metastatic mesothelioma samples were retrieved from the Department of

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Key Words: Calretinin, calcium-binding proteins, EF-hand, mesothelioma, tumor marker, differential expression, alternative splicing.

Table I. *Summary of analyzed cases.*

Case N°	Age	Gender	Site	Asbestos exposure	Western blot	RT-PCR CR / CR-22k
1 A9845	78	M	Pleural	yes	++	degr.
2 2703/00	62	M	Pleural	yes	+	+ / +
3 10700/99	55	M	Pleural	yes	+++	+ / +
4 63/95	64	F	Pleural	no	++	+ / +
5 787/00	35	F	Pleural	no*	++	+ / +
6 1549/94	76	M	Tunica vaginalis	no	++	degr.
7 432/96	68	M	Tunica vaginalis	no	++	degr.
8 398/91	74	F	Peritoneum	no	+++	degr.
9 M1	51	M	Submand. meta.	yes	++	+ / +
10 M2	51	M	Submand. meta.	yes	+	+ / +
controls						
11 1687/99	58	M	Stomach Adca	n. d.	-	degr.
12 3360/99	65	F	Rectum Adca	n. d.	+/-	degr.
13 3388/99	62	M	Colon Adca	n. d.	+/-	degr.
14 1139/99	56	F	Breast IDC	n. d.	+	degr.
15 1397/99	58	M	Renal cell carc.	n. d.	n. d.	- / -

Tissue samples analyzed for the presence of CR or CR-22k. Cases 1 – 8 are from primary mesotheliomas, cases 9 and 10 from two metastatic mesothelioma samples. Cases 11 –15 served as control samples. Abbreviations: Adca: adenocarcinoma; carc: carcinoma; degr.: mRNA degraded; IDC: invasive ductal carcinoma; n. d.: not determined; Submand. meta.: submandibular metastasis. CR expression levels in the mesothelioma samples were considered high (+++), intermediate (++) or low (+). The tumor tissue in samples 11 – 15 was negative, and the weak CR-ir in the colon and rectum carcinomas (+/-) is probably derived from CR-ir nerve endings. The weak positive signal in the Western blot of sample 14 is derived from reactive stroma. * Previous RX for HD

Pathology, San Martino Hospital Belluno, Italy and Cantonal Hospital of Fribourg, Switzerland. In all cases the diagnosis of malignant mesothelioma was based on the characteristic clinical, histopathological and immunohistochemical features (*e.g.* CR +++, CD15 -, Ber Ep4 -, CK5/6 +). The clinical and pathologic data of these cases, as well as of other neoplastic specimens utilized as controls, are summarized in Table I. After surgical removal, the samples were cryopreserved in liquid nitrogen and stored at -80°C. Small pieces of tissues (0.5 - 1 cm in diameter) were cut with a razor blade and used for the different analyses.

Western blot analysis. Soluble proteins were isolated from small tissue samples (0.1 - 0.5 cm^3) as described previously (13, 15). Extraction buffer (10 mM Tris, pH 7.4, 2 mM EDTA, 1 mM β-mercaptoethanol) containing a cocktail of different protease inhibitors (Roche, Mannheim, Germany) was added and soluble proteins extracted by ultrasonication (Kontes, Germany, 3 x 20 s, 4°C) and prior homogenization (Polytron, Kinematica GmbH, Lucerne, Switzerland). The suspension was centrifuged (12,000 x g, 4°C, 20 min) and from the supernatant identical amounts (40 μg) were separated by SDS-PAGE. Proteins were transferred onto nitrocellulose membranes and CR was specifically detected with the CR antibody 7696, diluted 1:1000 in blocking buffer (TBS, pH 7.3 + 10% bovine serum).

In order to enrich CR-22k from the soluble protein fractions from metastases M1 and M2, 3 mg of total protein were applied to a Superdex 75 column (Amersham Biosciences, Switzerland) equilibrated in 10 mM Tris, 120 mM NaCl, 4.8 mM KCl, 1 mM β-mercaptoethanol, pH 7.4. The eluted protein fractions were separated by SDS-PAGE (12.5%) and analyzed as above. The membranes were further processed by the avidin-biotin method (as described under immunocytochemistry), using 4-chloronaphtol/ hydrogen peroxide as a chromogen.

Isolation of mRNA and reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA from a small tissue sample (0.1 - 0.5 cm^3) of eight primary mesotheliomas, two mesothelioma metastasis (M1 and M2), other tumors (Table I) was isolated by the acid guanidinium thiocyanate-phenol-chloroform method (APGC method (16)). Using the Titan™ reverse-transcriptase kit (Roche, Mannheim, Germany), 1 μg of total RNA was used for the synthesis of the first strand of cDNA (30 min, 37°C), which was immediately followed by PCR using different CR oligodeoxynucleotides (Table II). These primers are similar to the the CR primers as described before (10), but the length is approximately 30 nucleotides. Primer sequences are located on different exons, excluding the possibility of obtaining a PCR fragment of the expected size through contamination by genomic DNA. Primers HCR5' and either HCR3' or CR778 were used for all samples. In addition, primer CR22B or CR22C, which are complementary to the 3'-end of exon 7 and the 5'-end of exon 10 (spanning the deleted exons 8 and 9 of the CR-22k mRNA), were used for the detection of the CR-22k mRNA. The difference between CR22B and CR22C is the number of nucleotides complementary to exon 7, namely 3 and 6 nucleotides, respectively (Table II). The RT-PCR protocol used for amplification was as follows: 37°C for 30 min to reverse-transcribe the mRNA, followed by 40 cycles consisting of 94°C for 20 sec (denaturing), 68°C for 80 sec (annealing and extension). Aliquots of each PCR reaction containing the amplified fragments were separated on a 2% agarose gel stained with ethidium bromide.

Immunocytochemistry. Immunocytochemistry (IHC) of paraffin blocks with the CR antiserum 7696 was performed as described before (6). Because the antiserum specific for the isoform CR-22k (25A) does not efficiently work on routinely prepared histological material, a special fixation procedure was utilized. Cryopreserved

Table IIA. *Primers used for RT-PCR.*

Name	Position	Sequence (5' – 3')	Comments
HCR5'	412*-441	GACAGGAGTGGCTACATCGAAGCCAATGAG	5' primer
HCR5'NEW	311-339	AGCACGTGGGCTCCAGCGCCGAGTTTATG	5' primer
HCR3'	685-714	GGCATCCAGCTCATGCTCGTCAATGTAGCC	3' primer
CR778	748-779	CTGTAGTTGGTGAGCTGTTGAATATTCATTTC	3' primer
CR22B	**579-581**, 676-702	ATGCTCGTCAATGTAGCCGCTTCTATC**CGG**	3' primer for CR-22k
CR22C	**576-581**, 676-698	TCGTCAATGTAGCCGCTTCTATC**CGGGAC**	3' primer for CR-22k

• Positions correspond to the published sequence of human cDNA (A of the ATG start codon is position 1).

Table IIB. *PCR fragment sizes with different primer combinations.*

5' primer	3' primer	Fragment size (bp)	Comments
HCR5'	HCR3'	303	For detection of CR mRNA
HCR5'	CR778	368	For detection of CR mRNA
HCR5'	CR22B	197	For detection of CR-22k mRNA
HCR5'NEW	CR22C	294	For detection of CR-22k mRNA

(-70˚C) samples M1 and M2 were immersed in acetone (-70˚C) for one week, followed by a mixture acetone/formalin (40%)/acetic acid/water (90/5/1/4) as described before (17). After paraffin embedding, 15-µm sections were cut and treated as described before (6). Primary antibodies used were CR7696 (1:3000) and 25A (1:1000). Sections were further processed with the ABC kit (VECTOR Laboratories Burlingame, CA, USA) according to the manufacturer's protocol and binding of the peroxidase was visualized by incubating with the substrate 3,3'-diaminobenzidine (DAB)/0.006% hydrogen peroxide in 0.05 M TBS.

Results

Western blot analysis. Soluble proteins were extracted from all eight mesothelioma samples as well as from the two submandibular metastases M1 and M2 from the same patient. In all samples a clear and strong band at approximately 29 kDa was observed with the antibody CR7696 on the membranes migrating at the same position as purified recombinant human CR or native CR present in extracts from the colon carcinoma cell line WiDr (4) (Figure 1A). The slightly larger size of the recombinant CR band as compared to the ones from the specimen is due to the 6xHis-affinity tag at its amino terminus. The stronger intensity of the band in the metastasis sample M1 compared to M2 (Figure 1B) corresponds well with the percentage of tumour cells *vs.* non-tumour cells, which was larger in M1 than in M2 (data not shown). In the sample 63/95, two faint bands (arrows) at 22 and 44 kDa, indicative of monomers and dimers of CR-22k, respectively, were detected. In order to confirm the presence of CR-22k, a larger amount of cytosolic proteins (3 mg) was isolated from the largest available sample M1. The proteins were separated by gel filtration and eluted fractions were analyzed by Western blot analysis (Figure 1C). In all fractions [1-5] a band corresponding to CR (Mr 29 kDa) was detected, and in fractions 4 and 5, two additional bands corresponding to the monomeric (Mr 22 kDa) and dimeric (Mr 44 kDa) forms of CR-22k can be seen (Figure 1C). Also in samples M2 and A9845, the presence of CR-22k was confirmed (not shown). The additional lower band in sample 398/91 is probably a degradation product of CR as the result of poor preservation of this specimen. The size of the lower protein band does not correspond to one of the two known alternatively spliced forms, CR-22k or CR-20k (10), but it can not be excluded that other, yet unknown, CR splice variants may exist. More probably, the band corresponds to a proteolytic breakdown product, since CR has been previously shown to contain a region around amino acid 60 that is particularly sensitive to proteolytic enzymes such as trypsin (18) and the larger fragment (amino acids 61-271) has a size of approximately 25 kDa, as the size of the band shown in Figure 1A. In support of the degradation hypothesis, in the same sample, the RNA was almost completely degraded (see below).

Additionally, we analyzed cytosolic extracts from selected adenocarcinomas from the stomach, rectum, colon and breast. Immunohistochemistry of these samples did not reveal staining of the tumor tissue with the CR antiserum (not shown). While the stomach adenocarcinoma sample gave no positive signal at the position of CR (a weak crossreactivity with a higher molecular weight is visible in Figure 1A), a faint signal corresponding to CR was observed in the adenocarcinoma samples from the colon, rectum and breast. In these tissues, the immunoreactivity is most probably

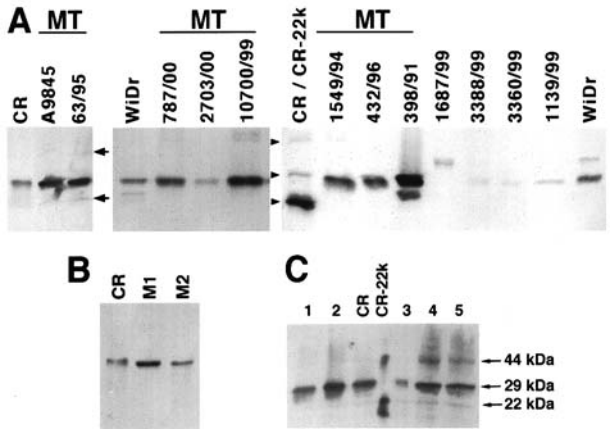

Figure 1. *Western blot from soluble proteins with CR7696. A) Soluble proteins (40 μg) isolated from various samples. MT: Mesothelioma samples; positive controls: WiDr cell extract (WiDr); recombinant CR and CR-22k. The faint bands in sample 95/63 (arrows) indicate the presence of the monomeric (lower band) and dimeric (upper band) form of CR-22k. The slightly larger size of the recombinant proteins CR (M_r 29 kDa; arrowhead, middle) and CR-22k (arrowheads; lower band: 22 kDa, monomeric form; upper band: 44 kDa, dimeric form) is due to the 6xHis affinity tag at their amino termini (11). The other samples are: 1687/99 (adenocarcinoma stomach); 3388/99 (adenocarcinoma rectum); 3360/99 (adenocarcinoma colon); 1139/99 (carcinoma breast).*
B) Proteins (40 μg) isolated from the submandibular metastasis samples M1 and M2.
C) From the sample M1, 3 mg of soluble proteins was separated by gel filtration. Eluted fractions probed with CR7696. Arrows mark the positions of CR (M_r 29 kDa) and CR-22k (M_r 44 kDa, dimeric form; 22 kDa, monomeric form).

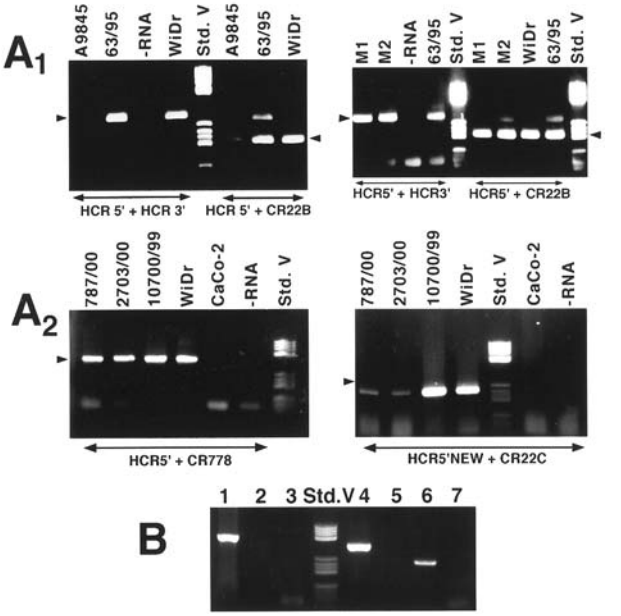

Figure 2. *RT-PCR with CR and CR-22k-specific primers. A) Total RNA (1 μg) from mesotheliomas (95/63, A9845; A_1, 787/00, 2703/00 and 10700/99; A_2) and from submandibular metastases (M1 and M2; A_1) were used. The presence of CR mRNA results in a fragment of 303 bp or 368 bp (upper arrowheads). The CR-22k mRNA-specific primer combination HCR5' and CR22B or HCR5'NEW and CR22C gives rise to fragments of 197 bp and 294 bp, respectively (lower arrowheads). Positive control RNA from WiDr cells; negative controls: CaCo-2 RNA or omission of any input RNA (but including all other solutions).*
B) RT-PCR from the CR-negative renal cell carcinoma 1397/99. Conditions and primers as in A). Primers and conditions for human GAPDH RT-PCR, which served as a positive control for the RT reaction have been described before (27). Lanes 1 – 3: RT-PCR from sample 1397/99 with primers for GAPDH (1), CR (2) and CR-22k (3). Lanes 4 –7 are positive and negative controls for CR (4, 5) and CR-22k (6, 7), respectively. For the positive controls CR and CR-22k cDNA plasmids (10) was used as template, while mouse liver total RNA was used for the negative controls.

derived from nerve endings of CR-positive myenteric neurons in the human colon (19) and reactive stromal cells in the breast sample contained in the isolated tissue that were observed in the corresponding immunohistochemical assay.

RT-PCR from total RNA of primary or secondary mesotheliomas. From all ten samples, total RNA was isolated and used to detect the mRNAs for CR and CR-22k by RT-PCR. The PCR product amplified with primers HCR5' and HCR3' (fragment size: 303 bp) or HCR5'NEW and CR 778 (fragment size: 368 bp) indicative of the full-length mRNA was detected in the samples 63/95, M1 and M2 or 787/00, 2703/00 and 10700/99, respectively (Figure 2A). No signal was seen with A9845, 1549/94 and 398/91, but analysis of the RNA from these samples (tissues taken at autopsy) showed severe RNA degradation evidenced on a formaldehyde/agarose gel (not shown), which explains the negative result in the PCR reaction. Although RNA from sample 432/96 was mostly degraded, a very faint band at the position of the CR mRNA was observed (not shown). Total RNA from WiDr cells (4) was used as a positive control and RNA isolated from the CR-negative colon carcinoma cell line CaCo-2 (4) or a PCR reaction mix without RNA served as negative controls. As further negative controls, total RNA isolated from a CR-negative tumor sample (1397/99; renal cell carcinoma) was used. No specific signals for CR were detected, while the RT-PCR reaction for the control marker GAPDH resulted in a positive signal in this sample (Figure 2B). In addition, the primer combinations HCR5' and CR22B (fragment size: 197 bp) or HCR5'NEW and CR22C (fragment size: 294 bp) were used to specifically detect the alternatively spliced mRNA coding for CR-22k (Figure 2 A1 and A2). A fragment of the correct size was detected in all samples, in which the integrity of the RNA was maintained. With the primer combination HCR5' and CR22B we had observed a faint band of larger size (*e.g.* visible in samples 63/95 and M2 (Figure 2A1), which is the result of the annealing of primer CR22B also to the full-length CR mRNA as described before (10). This leads to a PCR amplicon, which is 94 nucleotides

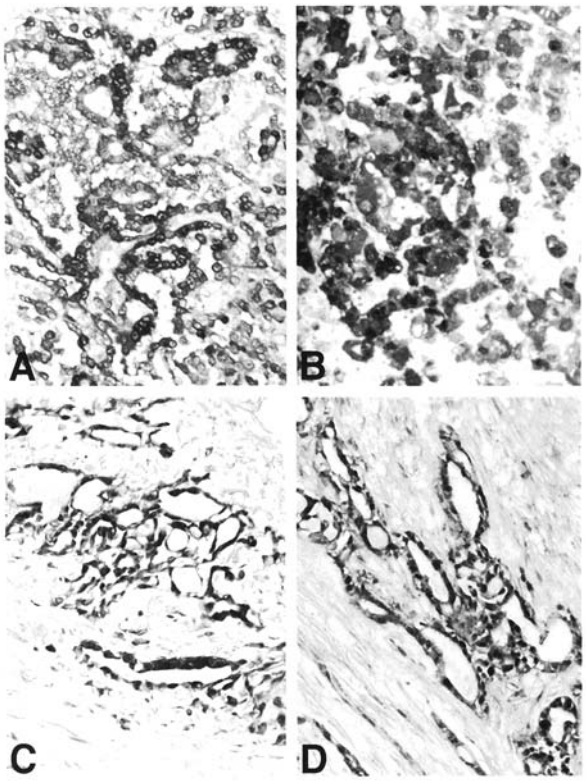

Figure 3. *Immunohistochemistry on tissue sections from two primary mesotheliomas (95 63/1 and A9845) and two metastases (M1 and M2) from a patient with a mesothelioma with CR-antiserum 7696. In the selected regions of tissue 63/95 (A) and A9845 (B) the majority of tumor cells are strongly stained with the antiserum CR7696. The immunoreactivity is present in the cytoplasm and in the nucleus. In the sections from the mesothelioma metastases, CR-immunoreactivity is abundant in the tubular parts of samples M1 (C) or M2 (D). Sections A and B are faintly hematoxylin-stained.*

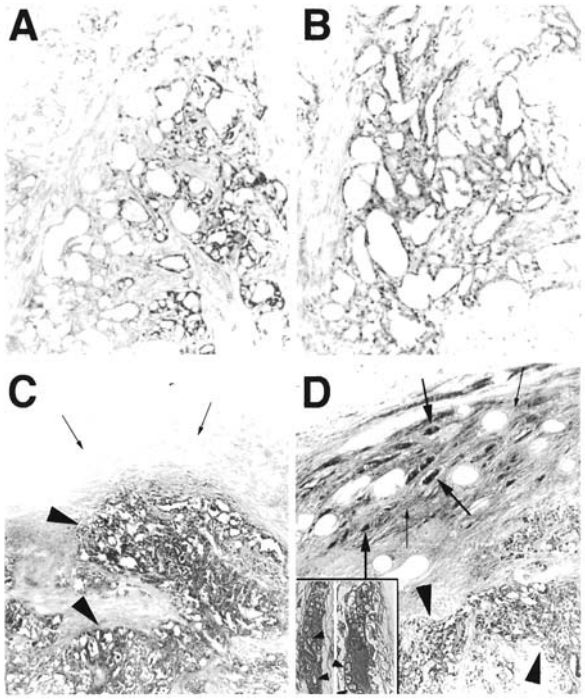

Figure 4. *Comparison of the distribution of CR and CR-22k with CR7696 and the CR-22k-specific antiserum 25A in tissue biopsies from metastases (M1 and M2). Two sections from the same specimen M1 were stained with either CR7696 (A) recognizing preferentially CR or with the antiserum 25A (B) recognizing the 14 C-terminal unique amino acids of CR-22k. Staining of sample M1 with the antiserum CR7696 (A) is similar in most regions when compared to the staining with 25A (B). In the sections derived from the sample M2 (C, D), the staining of the compact mass of tumor cells (arrowheads) is equivalent with the antisera CR7696 (C) and 25A (D). In addition, staining is seen with 25A in connective tissue areas (thin arrows) and in multinucleated, giant cells (D, thick arrows). Two of these cells are shown in the inset at higher magnification. In each cell, multiple unstained nuclei (small arrowheads) are visible.*

larger than the CR-22k-specific PCR fragment. Synthesis and testing of several novel primer pairs led to the optimized combination of HCR5'NEW and CR22C, with which the upper band was not produced (Figure 2A2) and is thus the preferred primer combination. As for CR, the CR-22k RT-PCR reaction was negative for the renal cell carcinoma used as negative control (Figure 2B). These results indicate that the mRNAs for both CR and CR-22k can be specifically detected in samples from mesotheliomas or metastases derived from these tumors.

Immunohistochemistry. In all mesothelioma samples analyzed by immunohistochemistry (A9845, 63/95, M1 and M2), the antiserum CR7696 against CR strongly labelled epithelial cells decorating the alveolar-like structures of the tumor (Figures 3 and 4). The shape of the immunolabelled cells varied from flat to cuboidal, in different parts of the same tumor or of the metastases. The CR-22k-specific antiserum 25A stained exactly the same structures (Figure 4) but, in addition, a positive reaction was found in some regions of the interstitium and particularly in isolated cells (Figure 4D). In all cases the immunoreactivity was cytoplasmic and nuclear and homogeneously distributed. Control sections of tissues incubated without the primary antibodies (7696 and 25A) did not exhibit immunostaining.

Discussion

The distinction between mesotheliomas and metastatic adenocarcinomas involving the pleura or the peritoneum is frequently difficult, from a purely histological point of view. Until recently, the discrimination between these two types of tumors was based on a panel of antibodies (*e.g.* B72.3, CEA, Ber-Ep4, MOC-31 or CD15, (9)) which stain positive only for adenocarcinomas and yield a negative result for mesotheliomas. For several years, a positive marker for the identification of mesotheliomas was lacking. In several reports

by different groups, an antibody against the Ca^{2+}-binding protein calretinin (antiserum CR7696 (13)) was shown to specifically label reactive mesothelial cells and mesotheliomas, especially of the epitheloid type (6-8), while in almost all cases adenocarcinomas were negative. Anti-CR antibodies have also been described to be useful for the differential diagnosis of reactive and neoplastic mesothelium *versus* metastatic tumors in effusion cytology (20, 21).

An important point to consider in all immunohistochemical methods used as a diagnostic tool is the specificity of the antibodies. It has been demonstrated that the antiserum CR7696 recognizes CR from many different species and does not crossreact with the closely related protein calbindin-D28k (13) under most conditions. But the Ca^{2+}-bound form of calbindin-D28k, if present at high concentration, was shown to crossreact with the CR antiserum (22, 23). To date, although calretinin has been widely accepted as one of the most reliable positive mesothelioma markers, a definitive characterization of the molecular target of the CR antiserum in mesothelioma specimens is still lacking. In this study we identify and characterize the immunoreactive protein in mesotheliomas by analyzing a series of primary and metastatic mesotheliomas. In all cases, an immunoreactive band of 29 kDa was identified co-migrating with human recombinant CR on Western blots. In addition, the mRNA coding for CR was identified by RT-PCR, supporting the results from the Western blot analysis experiments. Immunohistochemistry on the tissue sections with the antiserum CR7696 revealed that the intracellular localization of CR was cytosolic and nuclear, as was described in the initial reports on CR expression in mesotheliomas (6, 7). In recent reports some authors emphasized nuclear CR staining as specific for mesotheliomas (24, 25). However, nuclear CR-ir has been described also in colon tumor cell lines *in vitro*, and in some colon cancer *in vivo*. Furthermore, a change from cytosolic to nuclear CR staining can be induced by treatment of colon cancer cell lines with sodium butyrate (26), suggesting that the nuclear localization of CR cannot be considered a mesothelioma-specific effect. Analysis of the CR primary amino acid sequence has not yet revealed any "classical" nuclear localization signal (NLS), thus it is currently unknown by which mechanism CR is transported and maintained in the nucleus.

In previous studies on colon carcinoma cell lines expressing CR, we identified a CR isoform with a molecular mass of 22 kDa, CR-22k (11), which is the result of alternative splicing of the CR mRNA (10). Because CR-22k was present in neoplastic but not in normal intestinal epithelia, we suggested that this isoform is linked to malignancy and thus decided to look for CR-22k expression in mesothelioma samples. Since CR-22k levels are generally much lower than CR levels, we made use of two of the samples (one mesothelioma, one metastasis), where the amount of tissue was sufficiently large. The presence of CR-22k in these samples was confirmed by Western blot analysis. Immunohistochemistry on the metastasis samples with a CR-22k-specific antiserum revealed that the pattern of immunoreactivity was similar to that obtained with CR7696, suggesting that both proteins, CR and CR-22k are mainly co-expressed. However the slightly different localization in some areas (especially in the connective tissue and in isolated cells) is an indication that the levels of these two proteins are probably differently regulated in various cells, suggesting distinct functions for the two isoforms. The finding that the currently available CR-22k antisera are not well suited for routine formalin-fixed paraffin sections restricted our investigation to the metastasis samples M1 and M2, for which a special fixation protocol was performed. Either new epitope retrieval methods or novel CR-22k antibodies will need to be developed in order to analyze this isoform in standard pathological samples. In addition to identifying CR-22k at the protein level, RT-PCR of selected cases of mesotheliomas revealed the CR-22k mRNA to be present in these samples. The unambiguous identification of the CR-22k mRNA was further facilitated by the use of a novel primer set (HCR5'NEW and CR22C), which was less prone to erroneously amplify also the full-length CR mRNA.

The ratio between CR and CR-22k varies significantly (*e.g.* in the colon adenocarcinoma cell lines WiDr or COLO205 (11)) and the addition of sodium butyrate, an inducer of differentiation in colon carcinoma cell lines, leads to a transient accumulation of CR-22k in the nucleus of WiDr cells (26). Furthermore, CR-22k has been detected in the serum of several patients with colon tumors or necrotising inflammation of the gut (12), while full-length CR was not detected in these cases. Taken together, these findings support the hypothesis of distinct roles for CR and CR-22k, but it remains to be demonstrated if the presence of one or both of these proteins is directly linked to the transformed phenotype of the tumor cells expressing them. Evidence in support of this hypothesis was presented by Marilley *et al.* (27), who demonstrated that CaCo-2 cells transfected with either CR or CR-22k plasmids were more resistant towards sodium butyrate-induced differentiation than the untransfected or mock-transfected Caco-2 cells.

It is interesting to note that, in all samples analyzed in this study, not only CR, but also CR-22k was present, a finding which is in agreement with those shown in colon carcinoma cell lines, where CR and CR-22k were always concomitantly expressed (11). The presence of both proteins is not confined to primary mesotheliomas, but was also detected in metastases from mesotheliomas. In conclusion, this study identifies, for the first time, full-length CR and CR-22k at the protein and the RNA level in mesothelioma and further validates CR as a reliable marker for the positive identification of mesotheliomas and their metastases.

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