Distribution of adenosine deaminase complexing protein in normal and neoplastic cells

Citation for published version (APA):

Dinjens, W. N. M. (1988). *Distribution of adenosine deaminase complexing protein in normal and neoplastic cells*. [Doctoral Thesis, Maastricht University]. Rijksuniversiteit Limburg.
<https://doi.org/10.26481/dis.19881215wd>

Document status and date:

Published: 01/01/1988

DOI:

[10.26481/dis.19881215wd](https://doi.org/10.26481/dis.19881215wd)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

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Het verschijnen van dit proefschrift werd mede mogelijk gemaakt door financiële steun van de Jan Dekkerstichting & Dr. Ludgardine Bouwmanstichting.

Distribution of adenosine deaminase complexing protein
in normal and neoplastic cells

Distribution of adenosine deaminase complexing protein in normal and neoplastic cells

Proefschrift

ter verkrijging van de graad van doctor
aan de Rijksuniversiteit Limburg te Maastricht,
op gezag van de Rector Magnificus, Prof.dr. F.I.M. Bonke,
volgens het besluit van het College van Dekanen,
in het openbaar te verdedigen
op donderdag, 15 december 1988 om 16.00 uur

door

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geboren te Maastricht in 1956

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The work described in this thesis was performed as a collaborative research project of the Department of Pathology, University of Limburg (Prof.dr. F.T. Bosman) and the Section Enzyme and Somatic Cell Genetics of the Department of Human Genetics, University of Leiden (Prof.dr. P. Meera Khan). This project was financially supported by the Netherlands Cancer Foundation "Het Koningin Wilhelmina Fonds", grant IKW 85-82.

De uitgave van dit proefschrift werd financieel ondersteund door de Stichting Bevordering Klinische Pathologie Limburg.

Zetwerk en druk: Groenevelt bv, Landgraaf
Cover: Juliëtte Dinjens

Ter nagedachtenis aan mijn vader
Aan mijn moeder
Aan Juliëtte en Bas

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List of abbreviations

ADA	Adenosine deaminase
ADA-L	Large molecular weight form of ADA
ADA-S	Small molecular weight form of ADA
ADCP	Adenosine deaminase complexing protein
BSA	Bovine serum albumin
CD	Cluster of differentiation
CEA	Carcinoembryonic antigen
DAB	Diaminobenzidine tetrahydrochloride
DMSO	Dimethyl sulfoxide
ELISA	Enzyme linked immunosorbent assay
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
G/R FITC	Fluorescein isothiocyanate labeled goat anti-rabbit IgG
HAT	Hypoxanthine, aminopterin, thymidine
Ig	Immunoglobulins
i.p.	Intraperitoneal
i.s.	Intrasplenic
i.v.	Intravenous
l.n.	Lymph node
MNC	Peripheral blood mononuclear cells
MoAb	Monoclonal antibody
MW	Molecular weight
NaBT	Butyric acid sodium salt
NRS	Normal rabbit serum
PE	Phycoerythrin
PEG	Polyethylene glycol
PBL-T	Peripheral blood T lymphocytes
PBS	Phosphate buffered saline
PBT	PBS + 0.05% (v/v) Tween 20
PLP	Periodate-L-lysine-paraformaldehyde
PVDF	Polyvinylidene difluoride
RA	Retinoic acid
RIA	Radioimmunoassay
RT	Room temperature
SC	Secretory component
s.c.	Subcutaneous
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SI	Sucrase-isomaltase

Chapter 1

General introduction*

1.1 Introduction

The adenosine deaminase complexing protein (ADCP) (1,2) was first described by Nishihara *et al.* (3) as a conversion factor with the capacity to convert the small molecular form of the enzyme adenosine deaminase (ADA-S) into a large form (ADA-L).

ADCP is a dimeric glycoprotein consisting of two identical subunits of about 110 kD each (4-9). In some studies a subunit of about 70 kD in addition to the 110 kD subunit has been described. This subunit has been regarded as a degradation product (10), as a non-glycosylated form (11) and recently as a substantive mature subunit (12). One molecule of ADCP has the capacity to bind two molecules of ADA-S, both of 43 kD, forming one ADA-L molecule of about 280 kD (Fig. 1)

Fig. 1. Relationship between the ADA isozymes; MW = molecular weight.

* Based on: Adenosine deaminase complexing protein in cancer studies, Ten Kate J, Dinjens WNM, Meera Khan P, Bosman FT. Anticancer Res. 6: 983-988, 1986.

Fig. 2. Schematic representation of ADA isozymes observed in various human tissues seen in starch gel after electrophoresis at pH 6.5. 1, 1' and 1'' are bands of ADA-S, phenotype 1; ADA-L, a, b, c, d indicate different isozymes of ADA-L. ADA-e: an approximately 440,000 D molecular weight form (from ref. 14).

(see ref. 4 for a review). The ADA-L occurs in four different isozymes which are separable by electrophoresis in starch gels. They have been designated as ADA a, b, c, and d, in decreasing order of electrophoretic mobility (11, 13, 14). The isozymes a, b, and c are glycosylation derivatives of d (5, 14). ADA e, another isozyme of about 440 kD occurs exclusively in the intestine (5) and contains ADA-S as well as ADCP antigenic sites (14). In an electric field ADA e migrates cathodal in contrast to ADA d (Fig. 2) (5, 13, 14).

ADCP was initially detected as a soluble protein (1, 3, 15) but subsequently shown to occur in high quantities in plasma membranes (6, 7, 14, 16-18). It occurs in membrane bound and cytoplasmic form in most human tissues. Andy and Kornfeld (19) proposed that soluble ADCP results from proteolysis of the membrane bound form. Highest concentrations of ADCP are found in the human prostate and kidney (14). ADCP expression has been found to be markedly decreased or completely absent in virus transformed skin fibroblasts and in certain human cancer derived cell lines (15, 20-22). Early studies indicated a striking decrease up to total deficiency of ADCP in primary carcinomas of lung, colorectum, liver and kidney (3, 23).

However, our work on colorectal adenocarcinomas failed to confirm a consistent ADCP deficiency in cancer tissues (15, 24, 25). Subsequent studies have demonstrated considerable intercellular heterogeneity in the expression of ADCP (17) and suggested this might be related to direction and level of cellular differentiation. This chapter briefly reviews the current knowledge on certain relevant aspects. The physicochemical characteristics of human ADCP were extensively reviewed elsewhere (4, 14).

1.2 Possible physiological role of ADCP

ADCP has been found in frog (26), chicken (27) and all investigated mammalian species (8, 9, 12, 28-37) except rodents (38, 39). The physiological function of ADCP is still obscure. It has been suggested that its capacity to bind ADA-S might represent its biological function. The enzyme ADA, which converts adenosine and deoxyadenosine into inosine and deoxyinosine respectively, plays an important role in the development of the immune system. Since deficiency of the enzyme is consistently associated with severe combined immunodeficiency disease (1, 14, 40) it has been assumed that ADA activity is necessary for T-lymphocytic differentiation in man. It was suggested that complexed ADA-L is more thermostable than free ADA-S (41). Trotta and Balis (42) proposed that ADA-L could be a storage form of the enzyme and suggested that its presence might reflect a differentiated cell state.

Andy and Kornfeld (16) demonstrated in fibroblasts that membrane bound ADCP can bind ADA-S. More recently, Houghton *et al.* (43) described specific binding of ADA-S to cultured melanocytes, which contain membrane associated ADCP. *In vivo* studies in the rabbit by Schrader *et al.* (44) showed that intravenously injected ADA-S is cleared from the blood by binding to the proximal convoluted tubules in the kidney. These studies provide indirect circumstantial evidence in favor of binding of ADA-S to membrane associated ADCP. It was suggested by some investigators that membrane bound ADA protects cells from the toxic effects of extracellular adenosine and deoxyadenosine by extracellular metabolism (6, 44, 45). Others have proposed that membrane bound ADCP might serve as a receptor for the recovery or removal of free ADA-S (7, 44). The need for removal of ADA-S from the circulation is not self-evident since normal human plasma contains relatively abundant quantities of ADCP and the traces circulating ADA always occur in the complexed form ADA-L (10, 14, 46).

Alternatively, the association between ADCP and ADA-S has been regarded as artificial after it was demonstrated that in plasma and various tissues the quantities of ADA-S and ADCP were not positively correlated (16, 46, 47). Moreover, in neurons in the rabbit brain it was demonstrated by immunohistochemistry that ADA-S and ADCP do not co-localize (48). The occurrence of ADCP in human plasma, saliva, tears, urine (10, 46, 49), semen (own unpublished observation) and in exocrine glands of the rabbit led to the suggestion that ADCP is a secretory protein (18).

1.3 ADCP expression and *in vitro* cell transformation

Inwood *et al.* (20) investigated by starch gel electrophoresis the isozyme patterns of ADA in the soluble fraction of primary human fibroblasts. Cells harvested at confluency were shown to contain the ADA-L isozyme, whereas SV40 transformed human fibroblasts were completely devoid of ADA-L and only contained ADA-S. These results indicate that the expression of ADCP is impaired in virus transformed fibroblasts.

Herbschleb-Voogt *et al.* (15) quantified ADCP by electrophoretic analysis and radioimmunoassay in primary skin fibroblasts, *in vitro* transformed cell lines and tumor derived cell lines. They observed that ADCP is present in large quantities in fibroblasts but absent or nearly absent in transformed fibroblasts or tumor derived cell lines. More recently, ten Kate (14) found decreased membrane bound ADCP in these cells.

The consistent impairment of ADCP expression in all the *in vitro* virus transformed human cell lines led to speculations about the underlying mechanisms. Table I (from ref. 14) lists several possible mechanisms proposed to explain the decrease of ADCP. Cell hybridization experiments (2) suggest that B (Table I) could be the most plausible explanation, but this result was obtained from only one hybrid cell clone. Defective incorporation of ADCP in the plasma membrane (Table I, C.3) can be ruled out, since the expression of soluble as well as membrane bound ADCP was decreased. Alterations in cellular glycoproteins after transformation are extensively discussed by Hynes (50), and may be caused by one or more of the following mechanisms: reduced synthesis, reduced binding and increased rates of degradation. Decreased glycosylation may influence the quantity of a given protein as was demonstrated by Olden *et al.* (51) who reported that glycosylation may protect against proteolytic degradation. Finally, it is conceivable that ADCP is shed into tissue culture medium by cultured cells.

Table I Possible mechanisms for an apparent decrease of ADCP in cellular transformation

-
A.	Somatic mutation in the structural gene of ADCP, leading to
1.	the production of an unstable protein
2.	the formation of a protein which is not recognized by the anti-ADCP antiserum and can not bind ADA-S
B.	Somatic mutation in a hypothetical regulatory gene
C.	Alteration in glycosylation or other mechanisms of epigenetic origin
1.	rendering the protein in A.2
2.	giving rise to proteolytic degradation
3.	affecting the incorporation of ADCP in the plasma membrane
4.	leading to hyper-secretion or a leak of ADCP into the medium
-

1.4 Is there a regulatory mechanism involved in the expression of ADCP ?

The ADCP encoding gene was assigned to human chromosome 2 in rodent-human somatic cell hybrids (2, 52, 53). The claim that chromosome 6 is also involved in the synthesis of ADCP, as reported by Koch and Shows (54) did not get further support (2, 52). Somatic cell hybrids derived from fusions between normal human fibroblasts and rodent cells (E36, a3 or RAG) express human ADCP, only when the human chromosome 2 material carrying the ADCP gene is retained. If the rodent parent happens to be a mouse L-cell derivative A9 or B82 the ADCP is not expressed (52). It is well documented that the human HeLa cell line and its derivatives do not express ADCP (2, 23). One of the hybrids between HeLa and mouse C1DA₁L cells was found to express human ADCP (2). These observations, however scanty, indicate that a reversible regulatory phenomenon might be responsible for the apparent decrease in the level of expression of ADCP. Knowledge on the regulation of ADCP expression might shed some light on the mechanisms behind heterogeneous ADCP expression in cancer tissues reported by ten Kate *et al.* (17).

1.5 ADCP in cancer studies

Nishihara *et al.* (3) found no ADCP in extracts of carcinoma tissue of human lung and liver, organs which normally contain considerable amounts of ADCP. In 1978 Trotta and Balis (23) detected a profoundly decreased ADCP expression in carcinomas of kidney and colon as compared to the expression in normal kidney and colon. These authors suggested that decreased ADCP expression might be associated with cancer. Their findings could not be confirmed by more recent studies (17, 24, 25, 55, 56). Although quantitative differences in ADCP expression were observed between tumor tissues and their respective adjacent normal mucosae a uniform decrease or deficiency of ADCP in colorectal adenocarcinomas was not found. The investigated carcinomas showed decreased, unaltered or increased levels of ADCP. Because the presence of ADCP in the human kidney is restricted to the epithelial cells lining the proximal convoluted tubules, it was suggested that the expression of ADCP in renal tumors might be a parameter for the classification of these neoplasms (57, 58, 59). Recently it was claimed that cell surface expression of ADCP is associated with melanocyte transformation (43). Membrane associated ADCP reactivity was observed in epidermal melanocytes and benign junctional nevi and no reactivity was observed in premalignant dysplastic nevi and primary and metastatic melanoma lesions.

In order to investigate ADCP expression at the cellular level, normal and cancer tissues were analyzed immunohistochemically using a specific anti-human ADCP antiserum. ADCP was localized mainly in the apical regions of the luminal epithelium in the small and large intestine and in the proximal convoluted tubules of the kidney

(17). These findings were in agreement with results of earlier studies, in which ADCP was found to be predominantly associated with apical membranes (6, 7, 16, 55). Striking intercellular heterogeneity in the intracellular distribution of ADCP in colorectal carcinomas was observed (2, 17). In these tumors membrane associated, granular cytoplasmic, diffuse cytoplasmic or negative staining of ADCP was observed. These staining patterns were found to occur within single tumors either alone or in combinations (17). The membrane associated and diffuse cytoplasmic staining patterns showed a high positive correlation with staining for carcinoembryonic antigen (CEA) and secretory component (SC), two tumor associated antigens (60-63). Immunoreactivity for SC was reported to be of prognostic significance (61-63). These findings led us to hypothesize that ADCP might have similar potentials as a tumor associated antigen. Furthermore, since ADCP is an apical membrane associated protein, its expression as a marker of differentiation may be used in tumor classification.

Whether or not ADCP is released from plasma membranes of epithelial cells into body fluids is not known at present. Studies on ADCP levels in sera from patients with cancer originating from tissues with high amounts of ADCP are of particular interest in this respect. High serum ADCP levels are claimed to be found in patients with renal adenocarcinomas containing high ADCP levels (N.H. Bander, personal communication). Recently it was reported that the level of ADCP in urine can be used as a diagnostic marker for renal proximal tubular injury in renal transplant and cancer patients under chemotherapy (49, 64-68).

1.6 Aims of the present study

From the studies discussed in the previous paragraphs it is clear that several characteristics of ADCP suggest that it might be used as a marker in studies on cellular differentiation and tumor classification. Studies to this effect require the availability of specific antibodies, which would allow the localization of ADCP in tissue sections by immunohistochemistry and the quantitative determination of ADCP in tissue homogenates by immunoassay. With this goal the experiments described in chapter 2 were performed. They resulted in a method to generate specific polyclonal and monoclonal antibodies against minute amounts of ADCP, suitable for immunohistochemistry. In chapter 3 immunohistochemical and biochemical experiments on the ADCP expression in murine tissues are described. The suggested absence of ADCP in rodents as concluded from the demonstrated lack of ADA-L in these species is questioned by our results, which again led to speculations on the physiological significance of ADCP. Subsequently we established the distribution of ADCP in human tissues. These studies led to speculations on the physiological function of ADCP, as discussed in chapter 4. Studies concerning the possible physiological significance of the ADA binding capacity of ADCP are described in chapter 5. In these studies the presence of ADA and ADCP on human T-lymphoid cells

was determined. Human T-lymphoid cells were used because it is generally accepted that ADA plays a crucial role in T-lymphoid differentiation since a deficiency of ADA has been causally associated with a severe impairment in T-lymphoid differentiation. As described in chapter 6, ADCP expression is investigated in a number of normal and neoplastic epithelia including gastrointestinal, renal, endometrial, mammary and ovarian neoplasms and metastases. These neoplasms were selected in view of the high levels of ADCP expression in the corresponding normal tissues. Because high concentrations of ADCP were found in the prostate we investigated the ADCP expression patterns in 30 prostatic adenocarcinoma biopsies and compared these patterns with the presence of metastases as outlined in chapter 7. Studies on the expression pattern of ADCP in normal and neoplastic intestinal epithelia led to the suggestion that the presence of ADCP might be a marker for the columnar (absorptive) enterocytic cell lineage and that the pattern of expression of ADCP might be indicative for the degree of differentiation of these cells. In order to further investigate the value of ADCP expression for assessment of the level of differentiation, these patterns were studied in 4 human colorectal carcinoma derived cell lines, as described in chapter 8. The cells were cultured using various differentiation modulating conditions and they were also grown as xenografts in athymic nude rats. The expression patterns of ADCP were compared with those of villin and sucrase-isomaltase, two markers of columnar (absorptive) enterocytic differentiation. Finally, in chapter 9, the results of the preceding chapters are discussed. Especially the physiological function of ADCP and the possible value of ADCP as a parameter for classification and assessment of the biological behavior of neoplasms is reviewed.

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Chapter 2

Solid phase adsorption of antigens for efficient production of antibodies reactive with native and fixed tissue antigens*

2.1 Introduction

The production of antibodies for use in routine immunocytochemistry is often complicated by limited availability of the antigen of interest, poor immunogenicity of the antigen, or lack of reactivity of the antibodies with the target antigens after routine tissue fixation and embedding. The limited availability and poor immunogenicity of the antigen can be alleviated by the use of immunostimulating adjuvants (1, 2), chemical coupling of the antigen with a potent immunogenic carrier (3-8), intrasplenic immunization (6, 8, 9) or adsorption of the antigen to a solid phase (8). Lack of immunoreactivity of an antibody with the antigen in a tissue bound form can be alleviated by immunization with appropriately processed antigen in combination with selection of antibodies for reactivity with the antigen as it is present in processed tissues. An important disadvantage of the usual hybridoma supernatant screening by enzyme linked immunosorbent assay (ELISA) is that antibodies detected with this technique do not necessarily react with the antigen in different physical or chemical conditions (*e.g.* in solution (10) or in fixed and processed tissue (11)). To circumvent this problem hybridoma supernatant screening should be performed with the technique in which the obtained antibody will be eventually used.

We have attempted to generate polyclonal and monoclonal antibodies against adenosine deaminase complexing protein (ADCP), a homodimeric glycoprotein of 220 kD (12), which would allow detection of this protein in routinely processed normal and neoplastic human tissues by immunohistochemistry. In the present paper we describe a procedure for the generation of monoclonal and polyclonal antibodies against nanogram quantities of purified native or denatured ADCP, which detect the antigen in sections of formalin fixed and paraffin embedded human kidney, prostate and small intestine.

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2.2 Materials and Methods

2.2.1 Antigen purification and processing

ADCP was purified from human renal cortex as described previously by ten Kate *et al.* (13). For immunization 20 ng of ADCP dissolved in 20 μ l phosphate buffered saline (PBS) were applied onto nitrocellulose membrane (Schleicher and Schuell, Dassel, FRG). Alternatively, polyvinylidene difluoride (PVDF, Immobilon, Millipore, Bedford MA, USA) membranes were used as solid phase. Prior to application of the antigen, triangular membrane pieces were pretreated in methanol and washed in PBS. ADCP was applied to the wet membrane (2 μ g in 10 μ l PBS). This was subsequently fixed in formalin, embedded in paraffin by routine methods, dewaxed and rehydrated, exposed to methanol with 0.5% (v/v) hydrogen peroxide (as is customary prior to immunostaining of tissue sections) and washed in PBS. After this processing ADCP was still present on the membrane as determined with a protein specific dye (Aurodyne forte, Janssen, Olen, Belgium). The processed antigen bearing membrane fragments (app. 5 mm²) were implanted either subcutaneously (s.c.) or intrasplenically (i.s.) in female Balb/c mice. Intrasplenic implantations were performed as described by Nilsson *et al.* (8). Briefly, the spleen is carefully exposed and with the edge of a needle a small slit is made in the splenic capsule through which the membrane is inserted into the spleen. Hemostasis is accelerated by covering the spleen with a small piece of thrombin impregnated paper (Spongostan, Ferrosan, Denmark).

2.2.2 Immunization protocols

The various immunization protocols are outlined in Table I. Nine mice were immunized each 5 times at monthly intervals by 20 ng of native ADCP in PBS per immunization. The antigen was administered intravenously (i.v.) (A), intraperitoneally (i.p.) (B) or s.c. (C) without adjuvant (A) or with 50% (v/v) Freund's complete adjuvant (FCA, Difco, Detroit MI, USA) in the first immunization and with 50% (v/v) Freund's incomplete adjuvant (FIA) in the 4 subsequent immunizations (B and C). Seven mice were immunized s.c. 5 times with 20 ng of native ADCP bound to a nitrocellulose membrane (D and E). Six of these mice were boosted 4 days prior to fusion s.c. (2 mice), i.p. (2 mice) or s.c. and i.p. (1 mouse) with 20 ng native ADCP bound to a nitrocellulose membrane or i.v. (1 mouse) with 20 ng ADCP in PBS (D). One mouse was boosted i.p. with a lysate of human kidney cortex (200 μ g protein per boost), 7, 4, 3, 2 and 1 day before fusion (E).

In protocol F 3 mice were immunized with 2 μ g Immobilon adsorbed and processed ADCP, twice s.c. and twice i.s. (2 mice) or twice i.s. (1 mouse). Of these 3, one mouse was boosted i.p. with a lysate of paraffin embedded and subsequently rehydrated human renal cortex (200 μ g protein per boost), 7, 4, 3, 2 and 1 day prior to fusion.

Table 1 Immunization protocols

Protocol	No. of mice ¹	Immunizations				Prefusion boosting ³
		No. ²	Site	Antigen amount	Antigen condition	
A	4	5	i.v.	20 ng	native/liquid	—
B	3	5	i.p.	20 ng	native/liquid 50% FCA/FIA	—
C	2	5	s.c.	20 ng	native/liquid 50% FCA/FIA	—
D	6	5	s.c.	20 ng	native/nitro-cellulose bound	1 × 20 ng; liquid i.v.; nitro-cellulose bound s.c. or i.p.
E	1	5	s.c.	20 ng	native/nitro-cellulose bound	5 × 200 µg native kidney cortex lysate i.p.
F	3	4(2) ⁴	s.c. and/or i.s.	2 µg	processed/Immobilon bound	5 × 200 µg processed kidney cortex lysate i.p.

¹ Female Balb/c mice, 6-8 weeks of age at first immunization

² Intervals monthly

³ Boosting performed one month after the last immunization

⁴ 2 mice were immunized twice s.c. and twice i.s.

1 mouse was immunized twice i.s.

Of all mice serum was collected 7 days after the second and the last immunization and at fusion.

2.2.3 Monoclonal antibody production

Splenic leucocytes were fused with SP 2/0 myeloma cells in a ratio of 2:1 with polyethylene glycol (PEG 1500) and the fusate was seeded in 360 wells of microtiter plates in hypoxanthine, aminopterin, thymidine (HAT) supplemented medium. Balb/c mouse peritoneal macrophages were added as feeder cells.

Prefusion sera and the hybridoma culture supernatants were tested for anti-ADCP antibodies by indirect immunoperoxidase methods on 4 µm cryostat sections, which had been fixed in periodate-lysine-paraformaldehyde (PLP) fixative (14), and on 4 µm sections of formalin or PLP fixed and routine paraffin embedded human kidney

cortex, prostate and small intestine. The sera and supernatants were also applied on sections of processed tissues, which were preincubated with 0.1% (w/v) trypsin (Difco, Detroit MI, USA), 0.1% (w/v) CaCl_2 in water, pH 7.8 for 30 min at RT. An indirect immunoperoxidase technique was used as described by ten Kate *et al.* (15). As conjugate we used rabbit anti-mouse Ig (H + L) immunoglobulins labeled with horse-radish peroxidase (Dakopatts, Glostrup, Denmark). The specificity for ADCP of the immunoreactivity was determined by comparison of the reactivity pattern with that obtained with a well documented specific rabbit anti-human ADCP antiserum (13) applied on sections of PLP fixed and paraffin embedded human kidney cortex, prostate and small intestine. The large scale screening of the hybridoma supernatants was performed by immunohistochemistry on sections of human kidney cortex, prostate and small intestine. The three different tissues were embedded close to each other two times in one paraffin block. Five sections were mounted on one microscope slide in such a way that consecutive sets of tissue were at multichannel micropipet outlet distance. Before applying 10 supernatants, the slide was divided in 10 segments, each containing one set of tissues, by circling with a solid piece of paraffin to prevent admixture of the supernatants.

Antibody specificity was further evaluated by starch gel (16) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) after immunoprecipitation (17). For starch gel evaluation the antibodies were added to purified human ADCP and to a lysate of human kidney cortex. After incubation the antibodies were precipitated with Protein A coated Sepharose CL-4B (Pharmacia, Uppsala, Sweden). Bovine ADA-S (Sigma, St. Louis MO, USA) was added to the supernatants and these mixtures were subjected to starch gel electrophoresis with subsequent *in situ* staining for ADA activity as described previously (18).

For SDS-PAGE evaluation normal human skin fibroblasts in cell culture were metabolically labeled with ^{35}S -methionine (Amersham, UK), after which the cells were lysed. This lysate was used for the immunoprecipitation with Protein A coated Sepharose. The precipitated labeled proteins were separated by SDS-PAGE (19) blotted to a nitrocellulose membrane (20) and visualized by autoradiography.

2.3 Results

The results of the various immunization protocols are summarized in Table II. Immunization procedures A, B and C did not result in specific antibody titers in the serum. In contrast, procedures D and E resulted in specific antibody titers as determined by immunohistochemistry on cryostat and PLP fixed and paraffin embedded human tissue sections. Sufficient titers (1:500) were already obtained after the second immunization which increased only slightly after 3 subsequent immunizations. The 6 mice immunized according to protocol D were used in fusion experiments and although we obtained good fusion efficiencies (hybridomas were growing in more than 80% of the wells) no specific antibody producing clones were found. After im-

Table II Immunization results

Protocol	Anti-ADCP specific antiserum titer			Serum reactivity on processed tissues	No. of MoAb ¹	MoAb reactivity on processed tissues
	Immunization		Prefusion serum			
	Second	Last				
A	0	0	—	no	—	—
B	0	0	—	no	—	—
C	0	0	—	no	—	—
D	1:500	1:1000	1:1000	no	0	—
E	1:500	1:1000	1:3000	no	4	no
F	1:200 (1:500) ²	1:500	1:1500	yes ³	1	yes ³

¹ MoAb = monoclonal antibody² Mouse immunized twice i.s.³ Reactivity after trypsin pretreatment of tissue sections

munization according to protocol E increased prefusion serum antibody titers were found (1:3000) but with considerable non-ADCP related immunoreactivity (Fig. 1A). When immunostaining by rabbit anti-mouse IgM (Fig. 1B) peroxidase and rabbit anti-mouse IgG peroxidase conjugates (Fig. 1C) were compared, ADCP specific staining was clearly detectable by anti-IgG conjugates only. Fusion after protocol E resulted in 4 different ADCP reactive monoclonal antibodies (1071, 1072, 1079 and 1080). Specificity was proven by demonstrating the disappearance of ADCP in a purified ADCP containing solution and in a human kidney lysate after immunoprecipitation. This was performed by starch gel electrophoresis of the supernatants followed by specific *in situ* staining for ADA activity (Fig. 2).

Specificity was also determined by immunoprecipitation in a membrane extract of ³⁵S-methionine labeled human fibroblasts and SDS-PAGE of the precipitated proteins. Representative results are shown in Fig. 3. Two proteins of molecular weight 109 kD and 43 kD are specifically precipitated by the monoclonal antibody. None of the (polyclonal or monoclonal) antibodies generated against native ADCP reacted with this antigen in formalin fixed paraffin embedded tissues, not even after a wide variety of proteolytic, glycolytic or lipolytic treatments. After immunization with ADCP adsorbed to Immobilon membrane and processed according to routine tissue processing protocols, however, immune sera were obtained which reacted with ADCP in routinely formalin fixed and paraffin embedded tissues but only after trypsin treatment. After processing, ADCP no longer reacted with anti-native ADCP

Fig. 1.

Fig. 1. Prefusion reactivity of mouse anti-ADCP antiserum (immunized according to protocol E) on 3 serial sections of a PLP fixed human kidney cortex.

- A. Reactivity detected with rabbit anti-mouse total Ig. Immunoreactivity is observed in the cytoplasm of the proximal and distal tubular epithelial cells. The proximal tubular epithelial cells also show weak apical membrane associated reactivity (immunoperoxidase, 120 $\times$).
- B. Reactivity of the same serum detected with rabbit anti-mouse IgM. Immunoreactivity is observed in the cytoplasm of the distal and proximal tubular epithelial cells. The proximal tubular epithelial cells do not show apical membrane associated reactivity (immunoperoxidase, 120 $\times$).
- C. Reactivity of the same serum detected with rabbit anti-mouse IgG. Strong apical membrane associated immunoreactivity is observed in the proximal tubular epithelial cells. The distal tubules are negative (immunoperoxidase, 120 $\times$).

antibody but reactivity was obtained with antibodies generated against processed ADCP (not shown). Fusion of spleen cells of an immune mouse resulted in a monoclonal anti-ADCP antibody after boosting with a lysate of processed human renal cortex. Using this procedure (Table I, F) one monoclonal antibody was obtained which reacted with paraffin embedded kidney, prostate and small intestine but only after trypsin treatment. However, reactivity appeared to be restricted to those, high ADCP containing, tissues.

2.4 Discussion

The described procedures allow the generation of polyclonal and monoclonal antibodies against ng amounts of purified antigen (ADCP) reactive with native and fixed tissue antigens. Our results demonstrate that administration of ng quantities of ADCP adsorbed to a solid phase is highly effective for the generation of polyclonal antisera, whereas administration of the same amount of ADCP in solution is not sufficient to obtain specific antibodies in the serum. This can be due to an immunostimulating effect of the nitrocellulose membrane. Other possibilities are that immobilized ADCP has undergone conformational changes, increasing its immunogenicity, or that immobilized ADCP is less susceptible to proteolysis leading to a persistent local high concentration of ADCP as was suggested by others (7). Combinations of these possibilities can not be ruled out. This procedure theoretically has the advantage that small quantities of antigens are more likely to induce antibodies with high affinity for the antigen.

Our failure to generate monoclonal antibodies after immunizing mice according to procedure D is in line with the findings of Stähli *et al.* (21). These investigators demonstrated that repeated immunization with μ g quantities of antigen is adequate to obtain high serum antibody titers but is insufficient to generate monoclonal antibodies. They as well as others (6) suggested that this is due to a lack of sufficient numbers of antigen specific blast cells in the spleen. In our experiments this pro-

Fig. 2. Starch gel patterns of ADA isozymes in the supernatant, after immunoprecipitation with monoclonal antibody 1071, of a lysate of human kidney cortex. O = origin.

Lane 1 calf ADA-S.

Lane 2 human kidney lysate + calf ADA-S.

Lane 3 immunoprecipitation with monoclonal antibody (ascites) 1:100. Before electrophoresis of the supernatant calf ADA-S is added.

Lane 4 as 3 but antibody dilution 1:200

Lane 5 as 3 but antibody dilution 1:500

Lane 6 as 3 but antibody dilution 1:1000

Lane 7 as 3 but antibody dilution 1:2000

Note the gradual appearance of ADA-L (ADCP) in the supernatant with increasing antibody dilution.

posed small number of antigen specific blast cells in the spleen can be explained by the subcutaneous administration of solid phase adsorbed antigen because the spleen is known to respond primarily to blood-borne antigens. Successful production of monoclonal antibodies was achieved after repeated boosting of the mice (5 times in 7 days prior to fusion) with high amounts (200-400 μ g) of purified antigen. Our results demonstrate that i.v. boosting prior to fusion can be effectively performed with impure antigen preparations. By boosting with an ADCP containing lysate the specific antibody titer in the serum increases together with generation of a non-ADCP specific antibody titer. With the rabbit anti-mouse IgG and IgM peroxidase conjugates it is possible to discriminate between ADCP specific and non specific reactivity. This result is in agreement with the generally accepted concept that with the applied boosting procedure (procedure E) the presence of ADCP in the lysate

Fig. 3. Autoradiogram of immunoprecipitates after SDS-PAGE. Lysate of metabolically labeled human fibroblasts was immunoprecipitated with monoclonal antibody 1071 (ascites) (lane 1) and control ascites (lane 2). Two specific proteins of 109 and 43 kD respectively are precipitated. O = origin.

generates a secondary antibody response of the IgG type, whereas the other immunogenic components in the lysate are responsible for a primary humoral response of the IgM type. The finding that with procedure E the antibody titer in the serum is increased and the observation that only this procedure results in the generation of specific monoclonal antibodies suggests that by boosting with impure antigen preparations it is possible to increase the proportion of antigen specific blast cells in the spleen to a level at which a successful fusion can be performed.

For the generation of polyclonal and monoclonal antibodies which detect ADCP in sections of routinely processed tissues we also used immobilized antigen. The method is based on the tight binding of the antigen to a solid phase (Immobilon membrane) and the subsequent processing of the coated membrane. The Immobilon membrane is very suited for this procedure because of its high binding capacity for proteins (22, 23) and its resistance against organic solvents. The validity of this procedure is indicated by the finding that the sera of all 3 mice immunized with processed ADCP (procedure F) reacted with sections of processed human kidney cortex, prostate and small intestine, whereas none of the rabbit and mouse polyclonal and monoclonal antibodies against native ADCP reacted with these sections,

even after extensive attempts to restore the immunoreactivity (11, 24-26).

We also found that rabbit antiserum against native human ADCP does not react with immobilized processed ADCP and the polyclonal and monoclonal antibodies against processed ADCP show no reactivity on cryostat sections. Obviously, formalin fixation and paraffin embedding of native ADCP results in the modification of native antigen epitopes. It has been reported that formalin fixation causes irreversible alteration of proteins mainly due to inter- and intra protein methylene bridge formation (27). These formalin induced alterations are thought to be responsible for the inability to detect antigens in processed tissues with antibodies directed against the native antigen. With regard to ADCP, however, the combination of formalin fixation and paraffin embedding appears to affect the immunoreactivity. On cryostat sections post-fixed in formalin or embedded in paraffin ADCP specific immunoreactivity was observed with the rabbit antiserum against native human ADCP. However, on cryostat sections passed through both procedures the immunoreactivity was destroyed. We suggest that the necessity of trypsin digestion for restoration of immunoreactivity is due to masking of the epitopes on the ADCP molecule in the sections of processed tissues. This masking is probably caused by fixation and embedding induced interaction of ADCP with other tissue components because the antibodies did react with immobilized and processed purified ADCP. The reactivity of the monoclonal antibody, generated against processed ADCP, was found to be restricted to kidney, prostate and small intestine. This probably points to a concentration dependent reactivity because these tissues are known to contain high amounts of ADCP.

In summary, solid phase adsorption can be a versatile and efficient approach for immunization with ng quantities of antigen. Processing of the adsorbed antigen according to the routine tissue fixation and embedding protocol can result in antibodies which detect the antigen in sections of routinely processed tissues. The combination of primary immunization with solid phase adsorbed pure antigen and boosting with impure antigen is a useful approach for the generation of monoclonal antibodies against minute amounts of pure antigen.

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Chapter 3

Distribution of adenosine deaminase complexing protein (ADCP) in murine tissues*

3.1 Introduction

Adenosine deaminase (ADA, EC no. 3.5.4.4), the enzyme which catalyses the irreversible hydrolysis of adenosine and deoxyadenosine to inosine and deoxyinosine, occurs in 2 major molecular weight forms in a number of vertebrate species including frog, chicken, cattle, rabbit and man (1-4). These 2 ADA forms are designated as ADA-S and ADA-L. ADA-S is a monomer with a molecular weight of 43 kD. ADA-L, with a molecular weight of about 280 kD, is composed of 2 molecules of ADA-S bound to a homodimeric complexing protein (ADA-complexing protein, ADCP) (5). By complexing with ADCP ADA-S can be converted to ADA-L (5). Initially described as a soluble protein, ADCP was subsequently shown by immunohistochemistry and by quantitative biochemical studies to be localized predominantly in plasma membranes (4, 6-12). The physiological need for ADCP is still obscure. As yet, its capacity to bind ADA-S has been the only recognized property of ADCP. The importance of the ADCP binding, however, is not fully established because ADA-S and ADA-L have identical catalytic and regulatory properties (13), although it was claimed that ADA-L is more thermostable than free ADA-S (14). Trotta and Balis (15) postulated that ADA-L could be a storage form of the enzyme and its presence was thought to be characteristic for a differentiated cell state. In a number of studies evidence has been obtained that membrane bound ADCP can bind ADA-S (10, 16, 17) and it was suggested that membrane bound ADA might protect cells from the toxic effects of adenosine and deoxyadenosine by extracellular metabolism (8, 18). Schrader and Bryer (9) and Schrader *et al.* (19) proposed that membrane associated ADA-free ADCP might serve as a receptor for the recovery or removal of the free ADA-S from the circulation. Other studies have suggested that there are no functional relationships between ADA and ADCP and that their association in tissue homogenates is an artifact because in some tissues the quantities of ADA-S and ADCP did not show any correlation (10, 19, 20). Furthermore, Schrader *et al.* (21) clearly demonstrated immunohistochemically that ADA-S and ADCP are not co-localized in the rabbit brain. In biochemical experiments rat and mouse tissues

* Dinjens WNM, ten Kate J, Wijnen JT, Signet CM, Beek C, van der Linden EPM, Meera Khan P, Bosman FT. Submitted for publication.

did not exhibit any ADA-L, which led to the suggestion that rodents lack ADCP (22, 23). In the present paper we report the presence of ADCP immunoreactivity in the mouse and rat, using an anti-human ADCP antiserum which detects ADCP in man, rabbit and cattle (24). The investigated characteristics, in particular the tissue and subcellular distribution, and the electrophoretic mobility in starch and polyacrylamide gels of the protein in the mouse and rat, correspond with those of ADCP with the exception that the protein does not bind ADA-S. Also this finding might argue against the significance of ADCP for the ADA function.

3.2 Materials and Methods

3.2.1 Immunohistochemistry

Human, mouse (Balb/c) and rat (Wistar) tissues were obtained at autopsy and immediately frozen in isopentane at -80°C or fixed in periodate-lysine-paraformaldehyde (PLP) (25). Subsequently the PLP fixed tissues were routinely paraffin embedded. For the immunohistochemical detection of ADCP we used $4\text{ }\mu\text{m}$ paraffin or cryostat sections. The cryostat sections were fixed in PLP for 10 min at room temperature (RT). After deparaffinization and rehydration of the paraffin sections or fixation of cryostat sections, they were washed in phosphate buffered saline (PBS). Endogenous peroxidase activity was inhibited by treating the sections with a 0.5% (v/v) solution of hydrogen peroxide in methanol (30 min, RT). To prevent non-specific binding the sections were incubated with 20% (v/v) normal goat serum (10 min, RT). Thereafter the anti-ADCP antiserum and goat anti-rabbit Ig peroxidase conjugate (Nordic, Tilburg, The Netherlands) were applied 2 and 1 hour respectively at RT. Between each incubation, sections were rinsed (3x5 min) with PBS. All antiserum dilutions were prepared with PBS containing 1% (w/v) bovine serum albumin (BSA, RIA grade, Sigma, St. Louis MO, USA). Peroxidase activity was visualized with 0.01% (v/v) hydrogen peroxide and 0.05% (w/v) diaminobenzidine tetrahydrochloride (DAB, Serva, Heidelberg, FRG) in Tris-HCl buffer (pH 7.6) and the sections were counterstained with hematoxylin.

3.2.2 Starch gel electrophoresis

Mouse kidney cortex samples were cut into small pieces and washed with cold 0.9% NaCl solution. Homogenization was performed as a 50% suspension in 250 mM sucrose, 20 mM imidazole hydrochloride pH 7.0, with an Ystral dispenser X 10/20 (Salm and Kipp, Breukelen, The Netherlands). The 2,000 g (20 min) supernatant of the homogenate was collected and centrifuged at 4°C for 45 min at 18,000 g. The pellet was treated with a buffer consisting of 5 mM sodium phosphate pH 6.4 containing 1 mM disodium EDTA and 1 mM β -mercaptoethanol with 0.125% deoxycholic acid sodium salt and 0.25% Triton X-100, sonicated (with a Sonocone sonica-

tor with energy level adjusted to 7) on ice for 10 seconds and left for 30 min at 37°C. After centrifugation (45 min, 18,000 g, 4°C) the supernatant was regarded as the membrane fraction.

Human ADCP was purified from kidney cortex as described previously (24). Starch gel electrophoresis was performed according to Spencer *et al.* (26) with modifications (27). After electrophoresis the whole gel was sliced horizontally into 2 parts. The upper part was stained for ADA activity. The lower part of the gel was used for electrotransfer of the separated proteins to a nitrocellulose membrane (Schleicher and Schuell, Dassel, FRG). Immunodetection of the blotted proteins was performed as follows. After electrotransfer the membrane was washed (3 times 10 min) in PBS + 0.05% Tween 20 (PBT). Subsequently the membrane was incubated overnight (RT) with anti-ADCP antiserum (1:2000 in PBS + 1% (w/v) BSA), washed in PBT and incubated (1h, RT) with goat anti-rabbit Ig alkaline phosphatase conjugate (Nordic, Tilburg, The Netherlands). After washing in PBT alkaline phosphatase activity was visualized with nitro blue tetrazolium chloride monohydrate (Janssen, Beerse, Belgium) and 5-bromo-4-chloro-3-indolylphosphate (Boehringer, Mannheim, FRG). The reaction was terminated by washing the membrane in H₂O. As a specificity control normal rabbit serum was applied on a parallel blot.

3.2.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Metabolic labeling, preparation of membrane extracts, immunoprecipitation and SDS-PAGE were performed according to Andy and Kornfeld (28). In short: cultures of primary mouse and human skin fibroblasts at confluency were labeled for 18 hours with 100 μ Ci ³⁵S-methionine (1213 Ci/mmol; Amersham, UK). Membrane extracts were prepared and precipitated with Protein A Sepharose CL-4B (1 h; Pharmacia, Uppsala, Sweden) and with normal rabbit serum (overnight) and Protein A Sepharose CL-4B (1 h). The supernatants were incubated overnight with rabbit anti-ADCP or normal rabbit serum, and precipitated for 1 hour with Protein A Sepharose. The immunoprecipitates were solubilized and subjected to SDS-PAGE performed according to Laemmli (29) using 7.5% polyacrylamide slab gels. After electrophoresis the separated proteins were blotted onto a nitrocellulose membrane (30). The lane containing the molecular weight standards was cut from the membrane and the standards were visualized by specific protein staining (Aurodyeforte, Janssen, Olen, Belgium). The other part of the membrane was dried and exposed to X-ray film (Kodak X-OMAT) at - 70°C.

3.3 Results

3.3.1 Immunohistochemistry

In mouse and rat tissues very strong immunoreactivity was found in the brush border of the epithelial cells of the proximal convoluted tubules in the renal cortex. Glomeruli, distal tubules, endothelium and erythrocytes were all negative (Fig. 1). In the prostate immunoreactivity was found in the apical membrane and apical cytoplasm of the glandular epithelial cells. Immunohistochemistry was also applied on sections of mouse and rat small and large intestine and liver (not shown). The immunoreactivity patterns in kidney, prostate, small and large intestine and liver (not shown) exactly resemble those observed in human tissues.

Fig. 1. Immunoreactivity of the anti-ADCP antiserum on a cryostat section of mouse kidney cortex (immunoperoxidase, 250 $\times$). Note the strong apical membrane associated reactivity of the proximal tubular epithelial cells.

3.3.2 Starch gel electrophoresis

In Fig. 2A the result of starch gel electrophoresis and *in situ* staining for ADA activity of lysates of mouse kidney cortex and mouse and human erythrocytes are shown. The human hemolysate did not contain ADA-L. Mouse kidney cortex lysate did not exhibit an ADA-L band. Addition of mouse or human hemolysate (as sources of ADA-S) or bovine ADA-S (not shown) to this lysate, did not result in the appearance of an ADA-L band. Also no ADA-L band could be visualized after addition of mouse hemolysate to purified human ADCP. Immunoblotting after starch gel electrophoresis (Fig. 2B) revealed that the mouse kidney cortex lysate contained an ADCP immunoreactive protein with electrophoretic mobility somewhat lower than human ADCP. This band was not found in the mouse hemolysate. Addition of hemolysate resulted in an additional anodal band on immunoblotting.

Fig. 2. Starch gel electrophoresis of purified human ADCP and of lysates of mouse kidney cortex and mouse and human erythrocytes. One gel is sliced horizontally in two parts. The upper part is stained *in situ* for ADA activity (A). The lower part is used for immunoblotting with anti-ADCP antiserum (B). O = origin.

Lane 1 lysate of human erythrocytes

Lane 2 lysate of mouse erythrocytes

Lane 3 lysate of mouse kidney

Lane 4 mixed lysates of mouse erythrocytes and mouse kidney

Lane 5 mixed lysate of mouse kidney and human erythrocytes

Lane 6 lysate of human erythrocytes and purified human ADCP

Lane 7 lysate of mouse erythrocytes and purified human ADCP

Lane 8 purified human ADCP

Note that the lysate of mouse kidney cortex contains ADCP-reactive protein (B, lane 3, 4 and 5) that does not bind mouse and human ADA-S (A, lane 3, 4 and 5). Mouse ADA-S does also not bind to human ADCP (lane 7).

3.3.3 SDS-PAGE

To determine the molecular weight of the mouse ADCP immunoreactive protein immunoprecipitates of membrane fractions of metabolically labeled confluent mouse skin fibroblasts were analyzed by autoradiography after SDS-PAGE and electroblotting. As shown in Fig. 3, the anti-ADCP antiserum specifically precipitates from mouse fibroblasts 2 proteins of about 109 and 95 kD, comparable to ADCP precipitated from confluent human fibroblasts.

3.4 Discussion

Trotta and Balis (22) and Trotta *et al.* (23) were the first to demonstrate that in mouse and rat tissues ADA-L does not occur. Considering the binding of ADA-S to ADCP as an important functional characteristic, several investigators concluded

Fig. 3. Autoradiogram of immunoprecipitates after SDS-PAGE. Lysates of metabolically labeled human (lane 1 and 2) and mouse fibroblast (lane 3 and 4), were immunoprecipitated with rabbit anti-human ADCP antiserum (lane 1 and 3) and normal rabbit serum (lane 2 and 4). Two specific proteins of 109 and 95 kD respectively are precipitated. O = origin.

that rodents do not have ADCP (21, 22, 31). By starch gel electrophoresis and *in situ* staining for ADA activity we confirmed the results of Trotta and Balis (22, 23). However, we could detect ADCP immunoreactive material in an electroblot after starch gel electrophoresis of a mouse kidney cortex lysate with an electrophoretic mobility close to that of human ADA-L (ADCP). The applied antiserum recognizes human as well as rabbit and bovine ADCP, indicating the presence of species cross-reactive epitopes in the ADCP molecule. Addition of mouse or human hemolysate (as source of ADA-S) or calf ADA-S to the mouse kidney cortex lysate did not result in an ADA-L band after starch gel electrophoresis and *in situ* staining for ADA activity. These results suggest the presence in rodents of an ADCP like molecule which, however, does not bind ADA-S. In order to further characterize this rodent ADCP analog membrane fractions of metabolically (^{35}S -methionine) labeled mouse and human fibroblasts were immunoprecipitated with our anti-ADCP antiserum. The immunoprecipitates were analyzed by autoradiography after SDS-PAGE and electroblotting. The results unequivocally show that in the mouse 2 proteins occur which are precipitated by anti-human ADCP antiserum and have similar electrophoretic mobility (apparent molecular weights of 109 and 95 kD) to the human proteins. The precipitated 95 kD protein has also been reported in the literature (24, 28) and has been regarded as a degradation product of the mature 110 kD protein. These results support the existence of significant homology between human ADCP and the mouse protein that has no ADA-S binding capacity. Further evidence to support the similarity between human ADCP and mouse

ADCP-like protein is derived from immunohistochemical studies in the mouse and the rat using the anti-human ADCP antiserum. These studies show that the tissue localization of the mouse and rat protein closely corresponds with that of the human ADCP.

In summary, our findings show that in rodents an ADCP immunoreactive protein occurs which corresponds with human ADCP in apparent molecular weight, electrophoretic mobility and cell and tissue localization but which lacks the capacity to bind ADA-S. These findings cast serious doubt on the importance of ADCP binding for the function of ADA. The physiological function of ADCP remains to be established.

3.5 References

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Chapter 4

Distribution of adenosine deaminase complexing protein (ADCP) in human tissues*

4.1 Introduction

The adenosine deaminase complexing protein (ADCP) was found to occur mainly in plasma membranes (1-5). In man, ADCP has been demonstrated by immunohistochemistry to be predominantly localized in the brushborders of the epithelial cells of the proximal convoluted tubules in the kidney (1, 4, 6) and of the small and large intestine (4). In addition, Finstad *et al.* (6) reported the immunohistochemical detection of ADCP in epithelial cells of human placenta, prostate, breast and lung without details on subcellular localization. Quantitative biochemical studies in man showed that ADCP occurs predominantly in the membrane fraction of the homogenates of kidney, lung, liver, spleen, thymus, small intestine (1), placenta (2), and skin fibroblasts (3). The occurrence of ADCP in human plasma, saliva, tears, urine (4-9), semen (Dinjens *et al.*, unpublished observations) and in exocrine glands of the rabbit (5) led to the suggestion that ADCP is a secretory protein.

ADCP attracted some attention in cancer research when it was reported that the protein was decreased or completely absent in cancers of human lung, liver, kidney and colorectum (10, 11). However, our group failed to confirm a consistent decrease of ADCP in colorectal adenocarcinomas (4, 12-14). On the other hand, a profound and uniform decrease of ADCP expression was observed in a number of *in vitro* virus transformed human skin fibroblasts as well as in certain cancer derived cell lines (12, 15-17).

The aforementioned findings prompted us to investigate the normal distribution of ADCP in the human body, both quantitatively and immunohistochemically. The obtained information might shed some light on the physiological function of ADCP and provide a baseline for studies regarding ADCP expression in relation to neoplasia.

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4.2 Materials and Methods

4.2.1 Tissue sources and antiserum

Normal human tissues were obtained from surgically removed specimens or from autopsies performed within 5 hours after death. Specific anti-human ADCP antiserum was raised in a Chinchilla rabbit (14). The animal was immunized with ADCP purified from normal human kidney. Specificity of the antiserum was proven by immunoprecipitation. The anti-ADCP antiserum detects free ADCP as well as ADCP complexed with ADA-S, but does not recognize ADA-S (14).

4.2.2 Radioimmunoassay (RIA)

The fresh tissues were cooled on ice and washed with cold 0.9% (w/v) NaCl solution. Each tissue was cut into smaller pieces and homogenized as a 50% suspension in 250 mM sucrose, 20 mM imidazole hydrochloride pH 7.0, with an Ystral Dispenser X 10/20 (Salm and Kipp, Breukelen, The Netherlands). The 20 min 2,000 g supernatant of the homogenate was collected and centrifuged at 4°C for 45 min at 18,000 g. The supernatant was regarded as the soluble fraction and stored at -80°C. The pellet was treated with a buffer consisting of 5 mM sodium phosphate pH 6.4 containing 1 mM disodium EDTA and 1 mM β -mercaptoethanol with 0.125% deoxycholic acid sodium salt and 0.25% Triton X-100, sonicated (with a Sonocone sonicator with energy level adjusted to 7) on ice for 10 seconds and left for 30 min at 37°C. After centrifugation (45 min, 18,000 g, 4°C) the supernatant was regarded as the membrane fraction, and stored at -80°C. The ADCP specific radioimmunoassay was essentially carried out as described by Daddona *et al.* (18) with modifications (14). In short, ^{125}I labeling of purified human ADCP was performed with the lactoperoxidase technique described by Chechick *et al.* (19) resulting in a specific activity of ADCP of about 11 Ci/g. The sample antiserum mixture was incubated for one night at 4°C. To each sample, 100 μl of iodinated ADCP with 10,000 cpm were added, incubated for 3 days at 4°C and immunoprecipitated with a 1% *Staphylococcus aureus* suspension. The standard curve was made with serial dilutions of a human kidney extract. The detection limit was found to be 11 ± 2 (mean $\pm$ SD) ng/ml with a 95% confidence limit. Samples were measured in duplicate, in 3 different dilutions, such that at least 2 dilutions fell within the range of the standard curve. Protein content was estimated by the method of Lowry *et al.* (20).

4.2.3 Immunohistochemistry

Our anti-ADCP antiserum did not detect ADCP in sections from routinely formalin fixed and paraffin embedded tissues. Therefore we used 4 μm sections from periodate-lysine-paraformaldehyde (PLP) (21) fixed and routinely paraffin embedded tissues or 4 μm cryostat sections which were fixed in PLP for 10 min at room

temperature (RT). This PLP fixation of cryostat sections was found to be superior with respect to tissue morphology as compared with acetone fixation. From each tissue type, samples of at least 3 different individuals were examined. The procedure for the indirect immunoperoxidase technique was followed as described by ten Kate *et al.* (4). In short, after deparaffinization and rehydration of paraffin sections or fixation of cryostat sections they were washed in phosphate buffered saline (PBS). Endogenous peroxidase activity was inhibited by treating the sections with a 0.5% (v/v) solution of hydrogen peroxide in methanol (30 min, RT). To prevent non-specific binding the sections were incubated (10 min, RT) with 20% (v/v) normal goat serum. Thereafter the anti-ADCP antiserum and goat anti-rabbit Ig peroxidase conjugate (Nordic, Tilburg, The Netherlands) were applied 2 and 1 hour respectively at RT. Between each incubation, sections were rinsed (3x5 min) with PBS. All antiserum dilutions were prepared with PBS containing 1% (w/v) bovine serum albumin (RIA grade, Sigma, St. Louis MO, USA). Peroxidase activity was visualized with 0.01% (v/v) hydrogen peroxide and 0.05% (w/v) diaminobenzidine tetrahydrochloride (DAB, Serva, Heidelberg, FRG) in Tris-HCl buffer (pH 7.6) and the sections were counterstained with hematoxylin. Specificity of the immunolocalization was assessed by applying preimmune serum on parallel sections as negative control and by using human kidney cortex sections, with a well documented ADCP immunoreactivity pattern, as positive control. In order to enhance the intensity of the DAB stain 0.01 M imidazole was added to the DAB substrate solution (22).

4.3 Results

4.3.1 RIA

The soluble ADCP content was quantified in 18 different tissues derived from up to 9 individuals (Table I). We observed a considerable donor to donor variation in the ADCP content of several tissues. For instance, in the duodenum the values ranged from 42 to 462 and in ileum even from 14 to 1,017 ng/mg protein. The prostate showed the highest specific content of ADCP in its cytosol (4,177 ng/mg protein). The next highest was the renal cortex (1,882 ng/mg protein). Rather high quantities of soluble ADCP were observed also in the renal medulla, the skin and the mucosa of the intestinal tract. Very low amounts, around the detection limit, were seen in breast tissue and erythrocytes.

A comparison of the quantities of ADCP in the soluble and membrane fractions in various tissues is shown in Table II. The ADCP quantity in the membrane fraction was always considerably higher than that observed in the cytoplasm. This ranged from a factor of 2 in the stomach to a factor of about 15 in colon and spleen. Very high quantities of ADCP were found in the membrane fractions of renal cortex and prostate. The amount of ADCP in the membrane fraction of erythrocytes was below

Table I Soluble ADCP in human tissues

Tissue	ADCP ng/mg protein			Range	
	Mean	SD	N		
Skin	128	123	(5)	25-	303
Kidney					
cortex	1,882	327	(7)	1,596-	2,393
medulla	362		(2)	97-	627
Liver	66		(3)	51-	77
Stomach	23	22	(4)	8-	39
Duodenum	180	132	(9)	42-	462
Jejunum	236	201	(8)	78-	680
Ileum	295	294	(8)	14-	1,017
Cecum	237	297	(8)	38-	920
Colon	69	14	(8)	11-	143
Rectum	109	165	(9)	16-	547
Lung	54	60	(5)	0-	156
Pancreas	51	20	(5)	22-	68
Prostate	4,177	5,532	(9)	184-	17,989
Endometrium ¹					
proliferative	59	51	(5)	19-	146
secretory	381	182	(4)	204-	602
Urinary bladder	18		(3)	6-	29
Mammary gland	7		(2)	5-	9
Spleen	34	7	(5)	27-	43
Thymus	47	20	(4)	23-	71
Erythrocytes	nd		(2)		

SD = Standard deviation

N = Number of samples from different individuals

¹ Total (soluble + membrane) ADCP ng/mg protein

nd = Not detectable

the detection limit. The influence of *post mortem* autolysis was investigated by immunohistochemistry applied on human kidney cortex samples. After storage for up to 30 h at RT the ADCP specific immunoreactivity pattern was still observed (not shown).

4.3.2 Immunohistochemistry

Skin

Immunoperoxidase staining revealed ADCP reactivity in the luminal membranes of acinar cells of serous sweat glands. Dermal fibroblasts were also positive. Melanocytes, sebaceous glands and hair follicles were negative.

Kidney

Very strong immunoreactivity was found in the brushborders of the epithelial cells

Table II ADCP quantities in the soluble (S) and membrane (M) fractions of different tissues collected *post mortem* from one autopsy

Tissue	ADCP (ng/mg protein)	
	S	M
Skin	303	3,119
Kidney		
cortex	1,882 $\pm$ 327 (7) ¹	10,498 $\pm$ 5,360 (7) ¹
medulla	97	723
Liver	51	280
Stomach	30	66
Duodenum	158	2,014
Jejunum	321	2,146
Ileum	397	2,802
Cecum	134	787
Colon	143	2,141
Rectum	63	405
Lung	156	323
Pancreas	64	310
Prostate	4,177 $\pm$ 5,532 (9) ¹	14,573 $\pm$ 15,696 (9) ¹
Urinary bladder	20	63
Spleen	37	508
Erythrocytes	nd	nd

¹ Mean $\pm$ SD of (n) samples. All the other tissues were obtained from a single autopsy
nd = Not detectable

of the proximal convoluted tubules in the cortex. Glomeruli, distal tubules, endothelium and erythrocytes were negative (Fig. 1).

Liver

In the hepatocytes the immunoreactivity showed a diffuse cytoplasmic as well as a membrane associated pattern. Bile canaliculi in the central lobular areas were positive. Bile ducts and blood vessels were not stained (Fig. 2).

Small intestine

In duodenum, jejunum and ileum the striated border of columnar cells on the villi stained intensely for ADCP. The staining was apparent from the top to the base of the villi. Immunoreactivity was also observed in large cytoplasmic granules in the mucosal columnar cells. Goblet cells were negative. Immunoreactivity was also observed in mononuclear cells in the lamina propria (Fig. 3).

Fig. 1. Brushborder associated ADCP immunoreactivity in renal proximal tubular epithelium (paraffin section, immunoperoxidase, 180 $\times$).

Fig. 2. Bile canicular ADCP immunoreactivity (arrow) in liver (frozen section, immunoperoxidase, 400 $\times$).

Fig 3. Brushborder associated ADCP immunoreactivity in ileal mucosa (paraffin section, immunoperoxidase, 160 $\times$).

Fig. 4. Columnar cell apical membrane associated ADCP immunoreactivity in colonic mucosa (frozen section, immunoperoxidase, 300 $\times$).

Fig. 5. Luminal membrane associated ADCP immunoreactivity of the serous glands in the trachea (frozen section, immunoperoxidase, 250 $\times$).

Fig. 6. Diffuse cytoplasmic and membrane associated ADCP immunoreactivity in type I (arrow) and type II (arrowhead) pneumocytes in the lung (paraffin section, immunoperoxidase, 550 $\times$).

Fig. 7. Apical membrane, apical cytoplasm and secretory vesicle associated ADGP immunoreactivity in prostatic glandular epithelial cells (paraffin section, immunoperoxidase, 200 $\times$).

Fig. 8. Luminal membrane associated ADGP immunoreactivity in the breast (frozen section, immunoperoxidase, 400 $\times$).

Fig. 9. Luminal membrane associated ADGP immunoreactivity in secretory endometrium (frozen section, immunoperoxidase, 450 $\times$).

Fig. 10. Absence of ADCP immunoreactivity in proliferative endometrium (frozen section, immunoperoxidase, 450 $\times$).

Large intestine

In the mucosa of different parts of the large intestine (cecum, colon, rectum) ADCP was detected mainly in the brushborder region of the luminal columnar epithelial cells. In the crypt epithelium and in goblet cells no immunoreactivity was found. Again, mononuclear cells in the lamina propria were positive (Fig. 4).

Trachea

ADCP immunoreactivity was found in the serous tracheal glands confined to the luminal plasma membranes of the epithelial cells. The epithelium of mucinous glands and ciliated epithelium were negative (Fig. 5).

Lung

In the lung type I pneumocytes showed diffuse cytoplasmic staining in combination with membrane associated immunoreactivity, whereas the surfactant producing pneumocytes type II, identified by the periodic acid Schiff (PAS) reaction, showed strong cytoplasmic and weak membrane associated immunoreactivity (Fig. 6).

Pancreas

ADCP immunoreactivity in the pancreas was confined to the luminal membranes of the epithelial cells of the intra- and interlobular ducts. Islets of Langerhans and pancreatic acini were negative.

Prostate gland

Very strong ADCP immunoreactivity was found in the apical membrane and apical cytoplasm of the glandular epithelial cells and in the secretory vesicles and secretory fluid. Flattened glandular epithelial cells, obviously inactive, showed no or only weak ADCP immunoreactivity. The fibromuscular stroma was negative (Fig. 7).

Salivary gland

In the salivary gland the luminal membranes of the serous glands showed ADCP immunoreactivity whereas the mucinous glands were negative.

Gall bladder

Staining in the wall of the gall bladder was confined to the luminal plasma membrane of the diverticular epithelial cells. No immunoreactive material was found in the fibromuscular layer.

Mammary gland

ADCP immunoreactivity was found in the luminal membranes of the epithelial cells of the lobular acini and ducts (Fig. 8).

Uterus

In secretory endometrium ADCP immunoreactivity was found in most endometrial glands. The staining was confined to the luminal membranes of secretory epithelial cells and to the secretory fluid (Fig. 9). No staining was observed in proliferative endometrium (Fig. 10). The endometrial stroma and the myometrium were negative. In the cervix the endocervical glandular epithelial cells showed strong immunoreactivity at the luminal membranes.

Placenta

Weak apical membrane associated immunoreactivity was observed in the syncytial trophoblasts.

Other tissues

No ADCP specific immunoreactive staining was found in stomach, testis, seminal vesicles, thyroid gland, urinary bladder or ovary.

4.4 Discussion

The physiological function of ADCP is still obscure. It has been suggested that its capacity to bind ADA-S may be its major role (23). We (4, 24) and others (1-3, 5, 23) have demonstrated that membrane associated ADCP is not completely saturated with ADA-S (1, 25, 26). It has been shown that this membrane bound ADCP still has the capacity to bind ADA-S (3, 24, 25). Membrane bound ADCP therefore, by binding ADA to the plasma membrane, may function in the metabolism of adenosine and deoxyadenosine (1-3). Membrane ADCP might also serve as a receptor for the recovery or removal of free ADA-S released into the circulation by events such as cell turnover (1, 7, 25). It has been shown that in various tissues (1, 25, 26), including human T-lymphoid cells (24), a correlation between the quantities of ADA-S and ADCP does not exist. By immunohistochemistry ADA-S and

ADCP were not co-localized in rabbit brain neurons (27).

Against this background we decided to analyze quantitatively as well as qualitatively the presence of ADCP in various human tissues. In some tissues considerable interindividual variation in the soluble ADCP content was demonstrated as determined by RIA. This may be an artefact, due to differences in autolysis between various tissue samples, as is exemplified by the wide range of values found in the different parts of the small intestine, an organ with very rapid autolysis. Alternatively, it is conceivable that between different individuals differences in ADCP levels exist. Finally, the detected variation might be a reflection of tissue heterogeneity. Our histochemical results did show tissue heterogeneity *e.g.* in the mammary gland and in the pattern of bile canalicular staining in the liver. On this basis different samples of the same tissue may show different amounts of ADCP.

Table II shows that ADCP is mainly associated with membranes. The amounts observed in the membrane fractions invariably appeared to be much higher than in the soluble fractions. The immunohistochemical observations supported the predominant membrane associated localization of the protein. These results are in good agreement with other immunohistochemical and biochemical findings in human kidney (1, 4, 6), lung (1, 6), liver (1), spleen (1), thymus (1), prostate (6), small and large intestine (4, 6), breast (6), placenta (2, 6) and skin fibroblasts (3). However, in contrast to the literature, ADCP was not detected in skin melanocytes (28). This can be due to the presence of a different molecular form of ADCP in these cells which is not recognized by our antiserum.

Our immunohistochemical and biochemical data demonstrate that ADCP is predominantly present in absorptive epithelia and in exocrine glands, especially at the absorptive or secretory apex of the cell. The fact that ADCP has been found in human secretions such as saliva, tears, urine, plasma (7-9) and semen (Dinjens *et al.*, unpublished results); our immunohistochemical and biochemical findings that in the endometrial glands ADCP is present in secretory epithelium exclusively during the secretory phase; and our immunohistochemical demonstration of extensive ADCP expression in the prostatic secretory vesicles and secretory fluid, supports the suggestion that ADCP is a secretory protein (5, 7). The expression of ADCP in the cytoplasm was not only demonstrated biochemically (Table I and II) but also immunohistochemically *e.g.* in lung, liver and prostate. These results contest the suggestion by Andy and Kornfeld (17) that soluble ADCP results from proteolysis of the membrane form, during preparation of the tissue extracts.

These findings are largely in agreement with ours in mouse and rat (29) and with those of Schrader and West (15), in the rabbit. Some differences were apparent, which point to species differences in ADCP expression. In favor of this suggestion evidence was presented by Schrader and Bryer (1) and Schrader *et al.* (23). They demonstrated that using the same goat anti-rabbit ADCP antiserum the human glomeruli did not show immunoreactivity whereas the rabbit glomeruli were positive. An alternative possibility might be the existence of molecular differences between human and rabbit ADCP. This explanation is supported by our inability to

get completely identical immunoreactivity patterns with our anti-human ADCP antiserum on rabbit tissues to those described by Schrader and West (5) with their anti-rabbit ADCP antiserum (results not shown).

In summary, we found membranous localization of ADCP in high quantities in the human kidney, prostate and intestinal tract by biochemical as well as by immunohistochemical methods. The immunoreactivity patterns largely correspond with those in the rabbit, mouse and rat. Our findings indicate that the presence of ADCP might be related to the secretory and/or absorptive process. Its physiological function, however, remains obscure.

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Chapter 5

Immunocytochemical demonstration of adenosine deaminase (ADA) and adenosine deaminase complexing protein (ADCP) on human T-lymphoid cells*

5.1 Introduction

Adenosine deaminase (ADA, EC no. 3.5.4.4), the enzyme which catalyses the irreversible conversion of adenosine and deoxyadenosine to inosine and deoxyinosine respectively, plays an important role in the development of the immune system. The enzyme ADA is probably involved in both B and T-lymphocyte differentiation in man because an inherited ADA deficiency is causally associated with severe combined immunodeficiency disease (1). Increased amounts of the substrates of ADA are generally thought to play a role in the etiology of this disease (2).

Two electrophoretically distinguishable molecular weight forms of ADA are known to occur in man and they are designated as ADA-S and ADA-L. The small isozymic form (ADA-S) is a monomer with a molecular weight of about 43 kD. The large form (ADA-L) with a molecular weight of about 280 kD is composed of one homodimeric molecule of ADA complexing protein (ADCP) and two molecules of ADA-S. ADA-S can be converted to ADA-L by complexing with ADCP (3). ADCP was initially described as a soluble glycoprotein (4-6) and subsequently found to be predominant in plasma membranes (7-11).

In a number of studies ADA was reported to be partly associated with the plasma membrane (8, 11-16) and evidence was presented that membrane ADA displays its enzymatic activity extracellularly (17, 18). The main function assigned to ADA in lymphoid cells is the protection of these cells from the toxic effects of elevated levels of adenosine and deoxyadenosine (19). In the present study we investigated the occurrence of ADA and ADCP on the outer surface of human T-lymphoid cells by immunocytochemistry.

* Based on: Dinjens WNM, van der Boon J, ten Kate J, Zeijlemaker WP, De Bruijn CHMM, Bosman FT, Meera Khan P (1986). Cell surface adenosine deaminase (ADA) and its complexing protein (ADCP) in human T-lymphoid cells. *Adv. Exp. Med. Biol.* 195B: 407-414.

5.2 Materials and Methods

5.2.1 Preparation of cell suspensions

Human peripheral blood mononuclear cells (MNC) were isolated from the blood of normal healthy donors by centrifugation over Percoll ($d = 1.076 \text{ g/cm}^3$; Pharmacia, Uppsala, Sweden). Contaminating platelets were removed by two low-speed washing steps and erythrocytes were lysed with ice-cold ammoniumchloride solution. Human peripheral blood T lymphocytes (PBL-T) were obtained by nylon wool filtration of the MNC (20). The final cell suspension contained more than 90% E-rosette-forming cells.

Human thymocytes were prepared as described by Astaldi *et al.* (21). Thymus tissue was obtained from children undergoing cardiac surgery. After removal of fat, connective tissue and blood vessels, the fragments were cut into small pieces and gently pressed through a metal sieve. The thymocytes were washed in phosphate buffered saline (PBS).

5.2.2 Antisera and monoclonal antibodies

Specific anti-human ADA-S and anti-human ADCP antisera were raised in rabbits (22, 23). The anti-ADA-S antiserum detects free ADA-S as well as ADA-S bound to ADCP (ADA-L). The anti-ADCP antiserum detects free ADCP as well as ADCP complexed with ADA-S, but does not recognize ADA-S (23). Fluorescein isothiocyanate labeled goat anti-rabbit IgG immunoglobulins (G/R FITC) were obtained from Nordic Immunological Laboratories, Tilburg, The Netherlands. Phycoerythrin (PE) conjugated monoclonal antibodies anti-Leu-1 (anti-CD5), anti-Leu-2a (anti-CD8) and anti-Leu-3a (anti-CD4), reactive with all T-cells, T-cytotoxic/suppressor cells and T-helper/inducer cells respectively, were purchased from Becton and Dickinson, Sunnyvale CA, USA.

5.2.3 Flowcytometry

About 10^6 cells were incubated with normal rabbit serum (NRS), anti-ADA or anti-ADCP antiserum for 1 h at 4°C . The cells were washed once and incubated (1 h, 4°C) with a combination of G/R-FITC and PE conjugated anti-CD5, anti-CD8 or anti-CD4 after which the cells were washed twice. Optimal dilutions of the antisera, G/R-FITC and monoclonal antibodies were prepared in PBS containing 0.5% (w/v) bovine serum albumin (RIA grade; Sigma, St. Louis MO, USA) and 0.01% (w/v) sodium azide. The cells also were washed in this solution.

To investigate the binding of ADA-S to lymphoid cells we incubated these cells with a lysate of human erythrocytes before incubation with antiserum. We used the erythrocyte lysate because human erythrocytes contain only ADA-S and are completely devoid of ADCP (and ADA-L) (24).

Fluorescence intensity of viable lymphocytes was measured with a fluorescence activated cell sorter (FACS IV, Becton and Dickinson). Contaminating non-viable lymphocytes, monocytes and cell debris were gated out electronically. The percentage of fluorescence positive cells was calculated from the fluorescence histogram as follows. For the histograms obtained from incubation with anti-CD5-PE, anti-CD8-PE or anti-CD4-PE a clear distinction between positive and negative cells was possible (Figs. 1-3) and the threshold was set at the minimum between the two populations. The cells with fluorescence intensity exceeding the threshold were regarded as positive. For the calculation of the percentage anti-ADA and anti-ADCP positive cells the histogram obtained with NRS was used to determine the threshold. Double fluorescence (FITC and PE) was measured by gating out the PE negative cells and determining the FITC fluorescence intensity of the PE positive cells.

5.2.4 Statistical evaluation

The data were statistically analyzed with Student's t-test.

Fig. 1. Fluorescence histograms
A. MNC incubated with anti-CD5-PE; B. CD5 positive population; C. NRS control of B;
D. Anti-ADA of B; E. Anti-ADCP of B.

Fig. 2. Fluorescence histograms

A. MNC incubated with anti-CD8-PE; B. CD8 positive population; C. NRS control of B; D. Anti-ADA of B; E. Anti-ADCP of B.

Fig. 3. Fluorescence histograms

A. MNC incubated with anti-CD4-PE; B. CD4 positive population; C. NRS control of B; D. Anti-ADA of B; E. Anti-ADCP of B.

5.3 Results

The results of a representative experiment are shown in Figs. 1-3. In Fig. 1A the fluorescence histogram of MNC incubated with anti-CD5 is shown. In this experiment 75% of the cells were CD5 positive. Of the CD5 positive population (Fig. 1B) the histograms obtained after incubation with NRS, anti-ADA or anti-ADCP antiserum are shown in Figures 1C, 1D and 1E respectively. Of the CD5 positive cells 15% was anti-ADA and 48% was anti-ADCP positive. The MNC population contained 28% CD8 positive cells (Fig. 2A) and of this population (Fig. 2B) 12% of the cells were anti-ADA (Fig. 2D) and 29% anti-ADCP positive (Fig. 2E) in comparison with the NRS control (Fig. 2C). Of the MNC, 48% was CD4 positive (Fig. 3A and 3B). The CD4 positive population contained 14% anti-ADA (Fig. 3D) and 54% anti-ADCP positive cells (Fig. 3E). In Fig. 4 the results are shown of 8 experiments with human MNC. The MNC populations contained an average percentage of 71 ± 8 PBL-T (CD5 positive); 47 ± 4 being of T-helper/ inducer type (CD4 positive) and 27 ± 5 of T-cytotoxic/suppressor type (CD8 positive). The PBL-T populations contained a percentage of 21 ± 6 membrane ADA positive cells and 47 ± 10 membrane ADCP positive cells.

Fig. 4. Percentage anti-ADA (dotted bar) and anti-ADCP (cross-hatched bar) positive PBL - T (n = 8), T helper/inducer (n = 8), T cytotoxic/suppressor (n = 8) and thymocytes (n = 4)

☆ significantly different p < 0.001
 ★ significantly different p < 0.001

A highly significant ($p < 0.001$) difference was observed between the percentage membrane ADCP positive T-helper/inducer cells (55 ± 7) and T-cytotoxic/suppressor cells (28 ± 5), whereas in both populations a percentage of 17 ± 5 of the cells displayed membrane ADA expression (Fig. 4). In 4 experiments with human thymocytes a percentage of 20 ± 7 of the CD5 positive cells was membrane ADA positive. The percentage thymocytes expressing membrane ADCP (14 ± 8) was significantly different ($p < 0.001$) from the percentage membrane ADCP positive PBL-T (47 ± 10) (Fig. 4). In 4 experiments in which isolated PBL-T were incubated with a lysate of human erythrocytes the percentage of anti-ADA positive viable cells increased from 19 ± 7 to 51 ± 11 whereas no difference in ADCP expression was found. The results of a representative experiment are shown in Fig. 5.

Fig. 5. Fluorescence histograms

- A. PBL-T preincubated with PBS and incubated with NRS
- B. PBL-T preincubated with a lysate of human erythrocytes and incubated with NRS
- C. PBL-T preincubated with PBS and incubated with anti-ADA antiserum
- D. PBL-T preincubated with a lysate of human erythrocytes and incubated with anti-ADA antiserum

5.4 Discussion

The enzyme ADA is known to play a role in T-lymphocyte differentiation and ADA concentrations are reported to be subject to developmental control in cells of the T-lymphocyte lineage (25-27) and in epithelium of the gastrointestinal tract (27, 28). Protection of lymphoid cells from the toxic effects of elevated levels of adenosine and deoxyadenosine has been proposed as the main function of ADA in these cells (19). Although ADA occurs as a soluble protein, it has also been suggested to appear in a plasma membrane associated form in different mammalian cell types, displaying its enzymatic activity extracellularly (17, 18). It was postulated that membrane bound ADA protects the cells from toxic effects of adenosine and deoxyadenosine by extracellular metabolization (11, 29). As known from inherited ADA deficiency, human T-lymphoid cells are extremely sensitive to adenosine and deoxyadenosine. Therefore, we investigated the capacity of these cells to degrade these substrates by extracellular metabolization. To this end we determined the occurrence of ADA on the outer surface of different subsets of human T-lymphoid cells. In man, ADA occurs in two major molecular forms: single or associated with the high molecular weight glycoprotein ADCP (3). In some human tissues ADCP was found predominantly associated with the plasma membrane (7-11), and could still bind ADA-S (7). Therefore, we also investigated the presence of ADCP in the membranes of human T-lymphoid cells and the ADA-S binding capacity of these cells. Cells with membrane bound ADA and cells with membrane bound ADCP were found in all investigated T-lymphoid cell populations but in varying numbers. At the population level the quantities of plasma membrane associated ADA and ADCP were not correlated. Whether or not these proteins occur on the same cells or on different cell populations remains to be determined by double labeling studies. However, the finding that the percentage of membrane ADA positive cells never exceeds the percentage of membrane bound ADCP positive cells together with the finding that after incubation with a lysate of human erythrocytes the percentage of membrane ADA positive T lymphocytes increases to that of the membrane ADCP positive cells suggests that membrane bound ADA is associated with membrane bound ADCP.

In human and rabbit tissues no unambiguous correlation between membrane bound ADCP and membrane bound ADA has been reported either (8, 16, 30, 31). These results and the finding that in erythrocytes ADA is partly attached to the plasma membrane (18), indicate that ADA in the plasma membrane is partly ADCP-free (ADA-S). Andy and Kornfeld (7) demonstrated that ADCP present in isolated membranes of fibroblasts has the capacity to bind ADA-S. It was suggested that membrane ADCP serves as a receptor for the recovery or removal of free ADA-S (8, 30). Recently Schrader *et al.* (30) suggested that in the rabbit intravenously infused ADA-S is cleared from the blood by binding to brushborder associated ADCP in the epithelial cells of the proximal convoluted tubules in the kidney. We found that intact PBL-T also have the capacity to bind ADA-S. As neither anti-ADA nor

anti-ADCP antiserum inhibits the binding of ADA-S to ADCP we were not able to investigate whether ADA-S is anchored in the membranes by association to ADCP. T-helper/inducer cells significantly more often contained membrane associated ADCP than T-cytotoxic/suppressor cells. The membrane ADCP content therefore appears to be a new quantitative difference between these cell populations.

It has been suggested that membrane bound ADCP aids in the extracellular detoxification of adenosine and deoxyadenosine by positioning ADA on the plasma membrane. In this respect it would be interesting to study the sensitivity for adenosine and deoxyadenosine of the ADCP positive and negative subpopulations. However, our observation that very few human thymocytes which are extremely vulnerable to adenosine and deoxyadenosine toxicity, and have high ADA activity, show membrane ADCP expression does not support the role of ADCP in the extracellular detoxification of adenine nucleosides.

In summary, we found membrane bound ADCP more often in T-helper/inducer than in T-cytotoxic/suppressor cells, without a clear quantitative relationship with membrane bound ADA. The role of ADCP in the physiological function of ADA therefore remains unclear.

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Chapter 6

Adenosine deaminase complexing protein (ADCP) immunoreactivity in adenocarcinomas*

6.1 Introduction

The adenosine deaminase complexing protein (ADCP) is present in all human cells with the exception of erythrocytes. By quantitative biochemical and qualitative immunohistochemical studies we demonstrated high concentrations of this protein in the intestinal tract, kidney, prostate, secretory endometrium and mammary gland (1). ADCP has been regarded as a potentially interesting tumor associated antigen because in colorectal adenocarcinomas the quantity and localization were altered (2-7). We demonstrated that the expression of ADCP in colorectal adenocarcinomas correlates with that of secretory component (7), of which the expression correlates with tumor stage and prognosis (8-10). Furthermore, in prostatic adenocarcinomas the pattern of ADCP immunoreactivity is associated with the presence of metastases (11). In colorectal and prostatic adenocarcinomas the cellular localization of ADCP might be used as a parameter for the assessment of the degree of differentiation of the tumor (7, 11).

These results prompted us to study ADCP expression by immunohistochemistry in gastrointestinal, renal, endometrial, mammary and ovarian neoplasms and metastases. Ovarian neoplasms were included because the tumor cells of mucinous cystic neoplasms of the ovary can show intestinal characteristics (12).

6.2 Materials and Methods

6.2.1 Antibodies

Two ADCP reactive monoclonal antibodies (clones 1071 and 1072) were obtained by immunizing mice subcutaneously with purified native human ADCP adsorbed to a nitrocellulose membrane (Schleicher and Schuell, Dassel, FRG). During 7 days prior to fusion the mice were boosted 5 times intravenously with a lysate of human kidney cortex (13).

* Dinjens WNM, van der Linden EPM, Lenders MHJH, ten Kate J, Meera Khan P, Bosman FT. Submitted for publication.

Specificity of the 2 monoclonal antibodies was demonstrated by immunoprecipitation followed by SDS-PAGE or starch gel electrophoresis as described previously (11). The well documented polyclonal anti-human ADCP antiserum was raised in a Chinchilla rabbit. The rabbit was immunized with 40 μ g of purified native human ADCP emulsified in Freund's complete adjuvant. Serum was collected after 3 booster injections with 40 μ g of ADCP emulsified in Freund's incomplete adjuvant. The specificity of the antiserum was tested by immunoprecipitation and SDS-PAGE (5).

6.2.2 Tissue panel

Reactivity of the monoclonal antibodies with a panel of normal and neoplastic human tissues was investigated. Because the monoclonal antibodies did not show reactivity on sections of formalin fixed and paraffin embedded human tissues we used cryostat sections. We examined tissue specimens of at least 3 different individuals including normal kidney, prostate, mammary gland, endometrium, stomach and small and large intestine. The investigated neoplasms were 24 renal cell carcinomas, 5 primary breast adenocarcinomas and 2 lymph node metastases of breast adenocarcinomas, 23 endometrial adenocarcinomas, 15 gastric adenocarcinomas, 1 adenocarcinoma of the small intestine and its lymph node metastasis, 13 ovarian carcinomas and 7 ovarian adenomas, 1 omentum metastasis from an ovarium carcinoma, 16 colorectal adenocarcinomas, 2 liver, 2 lymph node and 1 skin metastases of colorectal adenocarcinomas and 4 colorectal adenomas. Staging of the renal cell carcinomas was performed according to Robson (14).

6.2.3 Immunohistochemistry

Cryostat sections (4 μ m) were air dried and fixed in periodate-lysine-paraformaldehyde (15) for 10 min at room temperature (RT). After rinsing in phosphate buffered saline (PBS) endogenous peroxidase activity was inhibited by treating the sections with a 0.5% (v/v) solution of hydrogen peroxide in PBS for 30 min at RT. The procedure for the indirect immunoperoxidase technique was followed as described elsewhere (3). Prior to application of polyclonal antibodies the sections were preincubated with 20% (v/v) normal goat serum to reduce non-specific binding. Ultimately peroxidase activity was visualized by using diaminobenzidine tetrahydrochloride (DAB, Serva, Heidelberg, FRG) as substrate. The sections were counterstained with hematoxylin.

6.3 Results

6.3.1 Reactivity in normal human tissues

Table I summarizes the reactivity of the ADCP reactive antibodies on normal human tissues. In the kidney cortex immunoreactivity was confined to the brushborder region of the epithelial cells of the proximal convoluted tubules. In the prostate strong immunoreactivity was found in the apical membrane and apical cytoplasm of the glandular epithelial cells and in the secretory vesicles and fluid in the glandular lumen. In the mammary gland reactivity was found along the luminal pole of the epithelial cells of lobuli and ducts. Proliferative endometrium did not show reactivity with the investigated antibodies. However, in secretory endometrium immunoreactivity was found confined to the apical membranes of the secretory epithelial cells of most endometrial glands. In the stomach reactivity with monoclonal antibodies 1071 and 1072 was found associated with the apical membranes of the columnar cells lining the foveolae and gastric glands. The foveolar epithelial cells also showed diffuse cytoplasmic immunoreactivity (Fig. 1). In the stomach no immunoreactivity with the polyclonal antiserum was found. Strong immunoreactivity with all antibodies was observed in the small and large intestine corresponding with the brushborder region of the epithelial cells. The polyclonal antiserum did not react with goblet cells (Fig. 2) whereas both monoclonal antibodies showed diffuse cytoplasmic reactivity with goblet cells (Fig. 3) but with striking heterogeneity, ranging from negative to intense staining.

Table I Reactivity of ADCP reactive antibodies with normal human tissues

Tissue	Polyclonal antiserum	1071	1072
Kidney	+	+	+
Prostate	+	+	+
Breast	+	+	+
Endometrium			
proliferative	—	—	—
secretory	+	+	+
Stomach	—	+	+
Small intestine	+	+	+
Large intestine	+	+	+

6.3.2 Reactivity in neoplasms

The reactivity of ADCP specific polyclonal and monoclonal antibodies with human neoplasms is shown in Table II.

Fig. 1. Reactivity of MoAb 1071 in gastric mucosa showing diffuse cytoplasmic and apical membrane associated staining of the columnar cells lining the foveolae (frozen section, immunoperoxidase, 200 $\times$).

Fig. 2. Reactivity of polyclonal anti-ADCP antiserum in jejunal mucosa showing brushborder associated staining (frozen section, immunoperoxidase, 180 $\times$).

Fig. 3. Reactivity of MoAb 1071 in jejunal mucosa showing reactivity with mucin vacuoles in addition to brushborder associated reactivity (frozen section, immunoperoxidase, 300 $\times$).

Table II Reactivity of ADCP reactive antibodies with human neoplasms¹

Neoplasm	N	Polyclonal antiserum	1071	1072
Mammary adenocarcinomas	5	2	2	2
lymph node metastases	2	0	0	0
Endometrial adenocarcinomas	23	13	13	13
Ovary:				
clear cell carcinoma	1	0	0	0
granulosa cell tumor	1	0	0	0
serous cystadenocarcinomas	9	1	1	1
serous cystadenoma	1	1	1	1
omentum metastasis	1	0	0	0
mucinous cystadenocarcinomas	2	2	2	2
mucinous cystadenomas	6	5	5	5
Gastric adenocarcinomas	15	2	9	7
Small-intestinal adenocarcinoma	1	1	1	1
lymph node metastasis	1	1	1	1
Colorectal adenocarcinomas	16	10	11	12
lymph node metastases	2	1	1	1
liver metastases	2	1	1	1
skin metastasis	1	0	1	1
Colorectal adenomas	4	4	4	4

¹ Results are expressed as the number of positive specimens

N = Number of specimens

Breast adenocarcinomas

Two out of 5 investigated infiltrating ductal carcinomas focally showed weak diffuse cytoplasmic and apical reactivity with all tested antibodies, the other 3 were negative.

Endometrial adenocarcinomas

The polyclonal antibodies reacted with 13 of 23 investigated endometrial adenocarcinomas. With both monoclonal antibodies 13 tumors showed reactivity. Intercellular heterogeneity was observed in all neoplasms. Solid tumor masses were usually negative, glandular structures were often locally positive.

Ovarian neoplasms

All antibodies reacted weakly with only 1 of 9 serous cystadenocarcinomas in an apical pattern. A metastasis in the omentum of a negative tumor was also negative. The same reactivity pattern, but stronger, was observed in the single investigated serous

Fig. 4. Mucinous cystadenocarcinoma of the ovary stained with MoAb 1071 showing apical membrane associated and mucin vacuole reactivity (frozen section, immunoperoxidase, 200 $\times$).

cystadenoma. The two mucinous cystadenocarcinomas both were positive with all antibodies. All antibodies reacted with 5 out of 6 mucinous cystadenomas. In the mucinous cystic neoplasms the reactivity was always focal and apical membrane associated with or without diffuse cytoplasmic reactivity. In addition, both monoclonal antibodies reacted with mucin vacuoles (Fig. 4). In two other ovarian neoplasms, a clear cell carcinoma and a granulosa cell tumor, no immunoreactivity was found.

Gastric adenocarcinomas

The polyclonal antiserum reacted with 2 of 15 gastric adenocarcinomas. Monoclonal antibody 1071 reacted with 9 tumors and 1072 with 7. The common patterns of reactivity, apical or diffuse cytoplasmic staining, local and single or in combinations were encountered. Five gastric tumors were classified as diffusely infiltrative adenocarcinomas. They all showed diffuse cytoplasmic reactivity with 1071 and 1072.

Adenocarcinoma of the small intestine

One adenocarcinoma of the small intestine and its lymph node metastasis both showed apical staining in glandular structures with polyclonal antibodies and 1071 and 1072. Solid tumor fields were negative.

Colorectal neoplasms

In 10 out of 16 colorectal adenocarcinomas reactivity of the polyclonal antibodies was observed, in 11 reactivity with monoclonal antibody 1071 and in 12 with 1072. All 4 colorectal adenomas reacted with all applied antibodies. The reactivity was invariably local and in an apical, diffuse cytoplasmic, or granular cytoplasmic pattern. These patterns were also found in combinations. The polyclonal antibodies did

not react with goblet cells whereas 1071 and 1072 showed diffuse cytoplasmic and mucin associated reactivity in this cell type. The 4 colorectal adenomas showed apical staining with the polyclonal antibodies. Staining with 1071 and 1072 furthermore showed diffuse cytoplasmic (2 cases) or granular cytoplasmic (2 cases) reactivity. One lymph node metastasis of an ADCP negative primary colorectal adenocarcinoma was negative for all the antibodies whereas another lymph node metastasis showed apical reactivity with all the antibodies. One liver metastasis of a colorectal adenocarcinoma reacted with an apical pattern. A skin metastasis of a colorectal adenocarcinoma showed focal apical staining with 1071 and 1072.

Renal cell carcinomas

Table III summarizes the reactivity of the ADCP specific polyclonal antibodies on renal cell carcinomas. The tumors were divided in non-metastatic stage I-III, stage I-III tumors that developed a metastasis and stage IV tumors. Four non-metastatic stage I-III tumors exclusively showed membrane associated reactivity (Fig. 5) whereas 6 stage I-III tumors showed in addition focally diffuse cytoplasmic staining (Fig. 6). All 4 of the stage I-III tumors that developed a metastasis focally showed diffuse cytoplasmic and membrane associated reactivity. From 10 stage IV tumors 5 were completely negative, 1 exclusively showed diffuse cytoplasmic staining and 4 reacted with the antiserum in a focal pattern, diffuse cytoplasmic as well as membrane associated.

Table III   Reactivity of ADCP specific polyclonal antibodies with renal cell carcinoma¹

Pattern	Stage		
	I-III	I-III ²	IV
Exclusively membranous	4	0	0
Focal membranous and cytoplasmic	6	4	4
Exclusively cytoplasmic	0	0	1
Negative	0	0	5

¹ Results are expressed as the number of positive specimens

² Stage I-III tumors that developed a metastasis

6.4 Discussion

The reactivity patterns of the 2 monoclonal antibodies on normal human tissues largely matched the pattern observed with the polyclonal antiserum. Several differences, however, were observed. Monoclonal antibodies 1071 and 1072 did react with

Fig. 5. Renal cell carcinoma stained with polyclonal anti-ADCP antiserum showing membrane associated reactivity (frozen section, immunoperoxidase, 200 $\times$).

Fig. 6. Renal cell carcinoma stained with polyclonal anti-ADCP antiserum showing membrane associated and cytoplasmic reactivity (frozen section, immunoperoxidase, 400 $\times$).

the epithelial cells of the gastric foveolae and glands, which did not stain with polyclonal antiserum. Both monoclonal antibodies stained goblet cells and mucin vacuoles in the intestinal tract, which did not stain with the polyclonal antiserum. The additional reactivity of the monoclonal antibodies was retained when the antibodies were diluted in 2 M NaCl, 2% (v/v) Tween 20 or both and therefore is probably not a result of non-specific interactions. These findings question the specificity of the monoclonal antibodies or the sensitivity of the polyclonal antiserum. We demonstrated previously that both monoclonal antibodies react with purified ADA-

free ADCP from human kidney cortex and with ADCP in lysates of human kidney cortex and cultured human skin fibroblasts (13). These results suggest that different molecular forms of ADCP exist, all present in the kidney cortex but not all recognized by the polyclonal antiserum. However, the polyclonal antiserum as well as the monoclonal antibodies were generated against ADCP purified from kidney cortex. None of the described ADA isozymic forms shows a tissue distribution comparable to the reactivity of the monoclonal antibodies (16, 17). The observed differences in reactivity may be related to the species in which the antibodies were generated (rabbit versus mouse) or may be a result of the different preparations chosen for ADCP immunization, soluble for the generation of polyclonal antiserum versus solid phase adsorbed for the generation of monoclonal antibodies. Another possibility is that the monoclonal antibodies recognize epitopes on the ADCP molecule which are also present on other molecules (*e.g.* mucins) but which are not recognized by the polyclonal antiserum. More extensive tests for reactivity of the monoclonal antibodies are required to resolve this issue. As yet we regard these monoclonal antibodies as ADCP reactive and they can not be considered as ADCP specific.

In all the investigated normal human tissues the immunoreactivity patterns demonstrated epithelial cell polarity. ADCP reactivity localized predominantly in exocrine glands and absorptive epithelia especially at the secretory or absorptive apex of the epithelial cells (1, 18). This finding led to the suggestion that ADCP is a secretion and/or absorption associated protein. ADCP expression in endometrial glands exclusively during the secretory phase and confined to the secretory edge of the epithelial cells points to a relationship between the presence of ADCP and the secretory process. However, the occurrence of ADCP in intestinal and proximal tubular epithelial cells and cultured fibroblasts illustrates that ADCP is not an exclusively secretory protein.

In neoplasms immunoreactivity with the ADCP reactive antibodies could roughly be discriminated into apical membrane associated, diffuse cytoplasmic, granular cytoplasmic and negative. These patterns often appeared focally in the tumor and are comparable to the reactivity patterns described for the polyclonal antiserum in colorectal and prostatic adenocarcinomas (3, 7, 11). The presence or absence of ADCP in tumors might be associated with type and degree of differentiation. The differences in ADCP expression patterns observed in tumors, compared to their normal tissue counterparts, can be due to differences between normal and neoplastic cells in posttranslational mechanisms in which the ADCP molecule is involved. These mechanisms include posttranslational modification of the protein as described for *e.g.* spectrin (19), villin (20), sucrase-isomaltase (21) and mucins (22, 23) or modified catabolism as described *e.g.* for spectrin (20) and sucrase-isomaltase (21). The possibility that ADCP expression might be correlated with tumor behavior was studied in 24 well staged renal cell carcinomas. Staging is a reflection of the tumor spread, including degree of invasion and presence or absence of metastases and as such is an indication of the biological behavior of the tumor. All 4 tumors with membrane ADCP expression, as in the normal kidney, were non-metastatic

stage I-III tumors and all 5 ADCP negative tumors were stage IV tumors. These findings resemble the observations in prostatic adenocarcinomas where strong membrane associated staining occurs in non-metastatic tumors. These results justify further exploration of ADCP expression as an indication for tumor progression in selected tumor types.

The use of ADCP expression in a neoplasm for histogenetic tumor classification or for determination of the primary in metastases of unknown origin is limited because ADCP occurs in endodermal (intestinal tract), ectodermal (endometrium, breast) and mesodermal (kidney) tissues. However, within one organ, ADCP expression may be used as a parameter to determine the cell of origin or the degree of differentiation. This is illustrated in the kidney where the expression of ADCP in renal cell carcinomas supports the view that these tumors originate from proximal tubular epithelial cells. Along the same line, ADCP expression in mucinous cystadenocarcinomas and gastric adenocarcinomas in a pattern comparable to that in intestinal epithelium fits the hypothesis that in these neoplasms the epithelium shows intestinal differentiation (12, 24).

In a number of endometrial and 1 adenocarcinoma of the small intestine reactivity patterns were found in histological well differentiated areas, comparable to the patterns found in the normal tissue counterparts. Also in colorectal adenomas and mucinous cystadenomas of the ovary normal patterns of reactivity were observed. These results favor the suggestion that a reactivity pattern in a tumor, resembling the pattern in normal tissue, is indicative for the presence of highly differentiated cells in the neoplasm. Comparable to what was reported in colorectal adenocarcinomas (7) we found that the reactivity of the polyclonal antiserum correlated with the presence of secretory component in endometrial, intestinal, mammary and ovarian neoplasms (data not shown). In colorectal tumors expression of secretory component appeared to be of prognostic significance (8-10) but at present there is no evidence that the pattern of ADCP expression correlates with prognosis. The occurrence of metastases in both ADCP positive and negative primary tumors questions the value of ADCP as a prognostic indicator. Studies concerning the relationship between ADCP expression and biological behavior of neoplasms are severely hampered by the inability of our antibodies to react with routinely processed tissue specimens.

In summary, the reactivity patterns of the ADCP reactive monoclonal antibodies on normal and neoplastic human tissues largely matches the pattern observed with the polyclonal antiserum. In normal tissues ADCP expression demonstrates epithelial cell polarity. The deviation of this expression pattern in neoplasms can be an indication for loss of polarity and as such for absence of or incomplete differentiation. Our results suggest that the expression of ADCP might be a useful parameter in the assessment of the histogenesis and type and degree of differentiation of tumors. Future studies should focus on the relationship between the regulation of the ADCP expression and biological behavior of the tumor.

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Chapter 7

ADCP expression and metastatic potential in prostatic adenocarcinomas*

7.1 Introduction

In the human body the highest quantities of the adenosine deaminase complexing protein (ADCP) are present in the prostate, as determined by radioimmunoassay of tissue extracts (1). ADCP has been claimed to be a transformation sensitive protein in view of the finding that ADCP expression is markedly decreased or completely absent in the cytosol of some *in vitro* transformed human skin fibroblasts and human cancer derived cell lines (2-5). An immunohistochemical survey of 91 human primary colorectal adenocarcinomas revealed markedly heterogeneous ADCP expression. Diffuse and granular cytoplasmic as well as membrane bound immunoreactivity occurred (6). Membrane associated ADCP immunoreactivity was found more frequently in well differentiated colorectal adenocarcinomas compared to undifferentiated tumors. In these adenocarcinomas the expression of ADCP appeared to be correlated with that of the secretory component, which has been proven to be of prognostic significance. These findings suggested that the expression of ADCP in colorectal adenocarcinomas might also have prognostic significance. Against this background we investigated ADCP expression in 30 prostatic adenocarcinomas by immunohistochemistry. The observed ADCP expression patterns were correlated with the grade of the tumors. The ADCP expression patterns were also investigated in 7 human prostatic tumor cell lines grown as xenografts in athymic nude mice.

7.2 Materials and Methods

Raising, testing and specificity of the applied rabbit anti-human ADCP antiserum have been described previously (7). By radioimmunoprecipitation testing the antiserum appeared to detect two monomeric ADCP units of 94 and 109 kD. Our anti-ADCP antiserum did not detect ADCP in sections of routinely formalin fixed and paraffin embedded tissues. Therefore all prostatic adenocarcinoma specimens, obtained at biopsies or from transurethral resections, were fixed in periodate-lysine-

* Dinjens WNM, ten Kate J, Kirch JAJM, van der Linden EPM, Tanke H, Jonas U, van den Ingh HFGM, Meera Khan P, Bosman FT. Submitted for publication.

paraformaldehyde (PLP) (8) and embedded in paraffin following routine procedures. On 4 μ m sections the procedure for the indirect immunoperoxidase technique was followed as described by ten Kate *et al.* (6). In short, after deparaffinization and rehydration of paraffin sections they were washed in phosphate buffered saline (PBS). Endogenous peroxidase activity was inhibited by treating the sections with a 0.5% (v/v) solution of hydrogen peroxide in methanol for 30 min at room temperature (RT). To prevent nonspecific binding the sections were incubated (10 min, RT) with 20% (v/v) normal goat serum. Thereafter the anti-ADCP antiserum and goat anti-rabbit Ig peroxidase conjugate (Nordic, Tilburg, The Netherlands) were applied 2 and 1 h respectively at RT. Between each incubation sections were rinsed (3x5 min) in PBS. All antiserum dilutions were prepared with PBS containing 1% (w/v) bovine serum albumin (Sigma, St. Louis MO, USA). Peroxidase activity was visualized with 0.05% (w/v) diaminobenzidine tetrahydrochloride (DAB, Serva, Heidelberg, FRG) and 0.01% (v/v) hydrogen peroxide in Tris-HCl buffer (pH 7.6) and the sections were counterstained with hematoxylin. Specificity of the immunolocalization was assessed by applying normal rabbit serum on parallel sections as negative control, and by using sections of human renal cortex, with a well documented ADCP immunoreactivity pattern, as positive control. On sections stained with hematoxylin and eosin the histological grade of the tumors was assessed independently by 2 pathologists, based on architectural and cyto-nuclear characteristics. The tumors were classified as well, moderately to well, moderately to poorly and poorly differentiated. The presence of metastases was determined by bone scan. Suspicious "hot spots" were re-investigated by directed X-ray. From patients with "hot spots" serum alkaline phosphatase, prostatic acid phosphatase and total acid phosphatase activities were determined. Balb/c nude mouse xenografts of 7 different human prostatic tumors were a generous gift of Dr. G.J. van Steenbrugge, Department of Urology, Erasmus University, Rotterdam, The Netherlands. Characteristics of these cell lines are listed in Table II (compilation from ref. 9, with permission). ADCP specific immunohistochemistry of these xenografts was applied on cryostat sections, post-fixed in PLP (10 min, RT), following the procedure described for paraffin sections.

7.3 Results

In Fig. 1 the pattern of immunoreactivity in the normal human prostate is shown. The staining appeared to be confined to the apical membrane and apical cytoplasm of the secretory glandular cells. Also secretory vesicles and the contents of tubular lumina showed strong ADCP immunoreactivity. Flattened glandular epithelial cells, presumably inactive, showed no or only weak ADCP immunoreactivity. Myoepithelium also was negative.

In 30 prostatic adenocarcinomas we observed four distinct ADCP expression patterns: diffuse cytoplasmic (Fig. 2), strong membranous (Fig. 3), mixed cytoplasmic

A

B

Fig. 1A and B. ADGP immunoreactivity in PLP fixed normal prostatic tissue. Note the staining of the apical membrane and apical cytoplasm of the glandular epithelial cells and of secretory vesicles (immunoperoxidase, A: 200 $\times$, B: 550 $\times$).

Fig. 2. ADGP immunoreactivity in PLP fixed prostatic adenocarcinoma tissue. Note diffuse cytoplasmic staining (immunoperoxidase, 400 $\times$).

Fig. 3. ADCP immunoreactivity in PLP fixed prostatic adenocarcinoma tissue. Note strong apical membrane associated staining (immunoperoxidase, 400 $\times$).

Fig. 4. ADCP immunoreactivity in PLP fixed prostatic adenocarcinoma tissue. Note the mixed cytoplasmic and membranous staining (immunoperoxidase, 400 $\times$).

Fig. 5. ADCP immunoreactivity in PC-EW cells, xenografted in a nude mouse. Note the membrane associated expression of ADCP (frozen section, immunoperoxidase, 200 $\times$).

Table I ADCP immunoreactivity patterns in 30 prostatic adenocarcinomas

Patient	Grade of differentiation ¹	ADCP immunoreactivity		Metastases
		Membranous	Cytoplasmic	
1	W	++	—	a
2	M	++	—	a
3	M	++	—	a
4	M	++	—	a
5	M/W	++	—	a
6	W	++	—	a
7	M/W	++	—	a
8	W	++	—	a
9	W	++	—	a
10	W	++	—	a
11	M	+	—	a
12	M	+	++	a
13	M	+	++	p ^o
14	M	—	++	p*
15	M	—	++	p*
16	M	—	++	p*
17	M	—	++	p*
18	M	—	++	p*
19	P	—	++	p*
20	P	—	++	p ^o
21	M	—	++	a
22	M	—	++	a
23	M	—	++	a
24	M	—	++	a
25	P/M	—	++	a
26	P/M	—	—	px
27	M	—	—	p*
28	M	—	—	p ^o
29	M	—	—	p*
30	P/M	—	—	a

¹W = Well differentiated; M = Moderately differentiated; P = Poorly differentiated; — = no; + = weak; ++ = strong immunoreactivity; a = absent; p = present; * Bone metastasis; ^o Lymph node metastasis; x Lymph node, bone and lung metastases.

and membranous (Fig. 4) and no ADCP expression. Only very limited intercellular heterogeneity was observed but this might be due to the fact that most investigated specimens were small needle biopsies. As shown in Table I the 7 well or moderately to well differentiated tumors all showed exclusively membrane associated ADCP expression. In none of these patients metastases were observed. In contrast, in 5 tumors graded as poorly or poorly to moderately differentiated no membrane ADCP reactivity was found and 3 showed diffuse cytoplasmic ADCP expression. Of these 5 patients 3 appeared to have developed metastases. Of the 18 moderately

differentiated tumors 3 were found to express exclusively membrane associated ADCP, 2 showed weak membrane associated and strong cytoplasmic staining, 9 showed exclusively diffuse cytoplasmic staining and in 3 specimens no ADCP staining could be observed. In none of the primary tumors of the 12 patients with metastases strong membrane associated ADCP expression was observed.

The results of ADCP immunohistochemistry on prostatic adenocarcinoma cell line xenografts is shown in Table II. The 2 moderately differentiated xenografts showed apical membrane associated ADCP expression (Fig. 5), whereas the 5 poorly differentiated xenografts were all negative for ADCP.

Table II ADCP immunoreactivity in xenografts of human prostatic carcinomas

Tumor	References	Origin ¹	Xenograft grade of differentiation	ADCP expression pattern
PC-82	(10)	primary tumor	moderately	apical
PC-EW	(11)	metastasis (l.n.)	moderately	membranous
PC-3	(12)	metastasis (bone)	poorly	apical
PC-93	(13)	primary tumor	poorly	membranous
PC-133	(9)	metastasis (bone)	poorly	—
PC-135	(9)	primary tumor	poorly	—
LNCaP	(14)	metastasis (l.n.)	poorly	—

¹l.n. = lymph node; — = no expression.

7.4 Discussion

In a study of a rather limited number of cases Trotta and Balis (15) claimed that in cancers of kidney and colon decreased ADCP expression occurred. In a large series of colorectal adenocarcinomas, however, we failed to find a uniform decrease of ADCP expression. Investigation of ADCP expression at the cellular level by radioimmunoassay (7) and immunohistochemistry (6, 16) revealed striking intercellular heterogeneity in colorectal carcinomas. In view of the finding that the expression of ADCP in colorectal adenocarcinomas was correlated with that of secretory component we suggested that the ADCP expression might be of prognostic value in these tumors (6). In prostatic cancer patients the extent of tumor spread as reflected in the occurrence of metastases is an important prognostic variable. It would therefore be of great importance to find markers that might predict the biological behavior (e.g. formation of metastases) of the primary prostatic carcinoma. With

this aim we investigated the expression pattern of ADCP in prostatic adenocarcinomas and compared this with the occurrence of metastases.

In our series of 30 prostatic adenocarcinomas we observed four distinct patterns of expression of ADCP. In 12 carcinomas we observed mainly cytoplasmic ADCP expression, in 11 mainly membranous expression was seen, in 2 we found cytoplasmic and membranous expression whereas in 5 no immunoreactivity was observed. Correlation of these expression patterns with the presence of metastases revealed that strong membranous ADCP expression occurred only in primary tumors without metastases, whereas in metastatic tumors no or mainly cytoplasmic ADCP immunoreactivity was found. These results indicate that the expression pattern of ADCP in human prostatic adenocarcinomas might predict whether or not metastases are likely to occur.

It is generally assumed that undifferentiated tumors behave more aggressively and show a higher tendency for metastatic spread than well differentiated tumors. The polar membranous pattern of ADCP expression in prostatic adenocarcinomas, which corresponds to the pattern of expression in the normal prostate, might indicate that these carcinomas are more highly differentiated. The finding that all the non-metastatic well or well to moderately differentiated tumors showed strong membranous ADCP expression is in agreement with this assumption. In contrast, poorly differentiated carcinoma cells, which have a higher metastatic tendency, lose their polar characteristics and show diffuse cytoplasmic or no ADCP expression. In line with this view is the observation that in the prostatic carcinoma cell line xenografts apical membrane associated ADCP was found in the 2 moderately differentiated xenografts whereas 5 poorly differentiated xenografts were negative. However, one of these ADCP expressing moderately differentiated xenografts was established from a lymph node metastasis of a primary prostatic tumor. We could not study ADCP expression in the metastasis but the histological grade of the metastasis appeared to be comparable to the grade of the xenografts as was also the case for PC-82 (9). Most likely these results indicate that also tumors with membrane associated ADCP expression can metastasize. However, the possibility that in the metastasis of an undifferentiated tumor re-differentiation occurs can not be ruled out.

Comparison of the ADCP expression patterns with ultrastructural characteristics of cellular differentiation and determination of the ADCP expression patterns in metastases of prostatic adenocarcinomas can validate the suggestion that the expression pattern of ADCP is a parameter of differentiation and as such a predictor of the biological behavior of prostatic adenocarcinomas.

In summary, we demonstrated that in human prostatic adenocarcinomas membranous ADCP staining occurs preferably in tumors without metastases. Conversely, metastases seem to occur preferably in cases with no or only cytoplasmic ADCP staining. These observations suggest that immunohistochemical detection of ADCP might predict the biological behavior of prostatic cancer. This possibility should be confirmed in larger series of patients with sufficiently long follow-up.

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Chapter 8

Expression of adenosine deaminase complexing protein (ADCP) in human colorectal carcinoma cell lines*

8.1 Introduction

Adenosine deaminase complexing protein (ADCP) is a ubiquitous glycoprotein predominantly present in plasma membranes with high concentrations in the prostate and in brushborders of epithelial cells of the proximal convoluted tubules in the kidney and of the intestinal tract (1-4). In the intestinal tract ADCP immunoreactivity was observed to be confined to the apical plasma membrane of the columnar epithelial cells. Goblet cells were negative (2, 4). In colorectal carcinomas a correlation was found between expression of ADCP and secretory component which led to the hypothesis that ADCP might be a marker for intestinal columnar cell differentiation (5). Furthermore, evidence was obtained that the ADCP expression pattern might be a reflection of the degree of differentiation of the epithelial cells (2, 5-7).

In the present study we investigated the relationship between ADCP expression and intestinal columnar epithelial cell differentiation in 4 human colorectal adenocarcinoma derived cell lines. We used the cell lines HT-29 and Caco-2 because they can express enterocytic differentiation features (enterocyte = brushborder bearing resorptive columnar cell in the small intestine) (8). We also used the recently described cell lines 5583-E and 5583-S which were established from a single human colonic adenocarcinoma (9). The cell lines were cultured under several potentially differentiation modulating conditions (8, 10). These include addition of glucose, sodium butyrate (NaBT), dimethyl sulfoxide (DMSO) or retinoic acid (RA), omission of serum or replacement of glucose by inosine. The cell lines were also grown as xenograft in nude rats. As parameter for columnar (absorptive) enterocytic differentiation the expression of two microvillus associated proteins, villin and sucrase-isomaltase, was used (11-14). By immunocytochemistry we compared the expression of ADCP with that of villin and sucrase-isomaltase.

*Dinjens WNM, Lenders MHJH, van der Linden EPM, Meera Khan P, ten Kate J, Bosman FT. Submitted for publication.

8.2 Materials and Methods

8.2.1 Chemicals and antibodies

α -D-(+)-glucose, butyric acid sodium salt (NaBT), RA and inosine were purchased from Sigma (St. Louis MO, USA). DMSO was obtained from Merck (Darmstadt, FRG). Diaminobenzidine tetrahydrochloride (DAB) was from Serva (Heidelberg, FRG). Anti-human sucrase-isomaltase monoclonal antibody (HBB2/614/88) was a generous gift of Dr. H.P. Hauri, Biozentrum der Universität Basel, Switzerland (14, 15). Anti-human villin monoclonal antibody was from Serotec (Blackthorn, UK) (16). Furthermore a rabbit anti-human ADCP antiserum was used (17).

8.2.2 Cell lines

The cultured cells were established lines derived from human colorectal adenocarcinoma. The cell lines HT-29 and Caco-2 were obtained from Dr. J. Fogh (Memorial Sloan Kettering Cancer Center, Rye, NY). Cell lines 5583-E and 5583-S were established at our institute and described elsewhere (9). In short 5583-E and 5583-S were established from a single primary colonic adenocarcinoma. 5583-E cells grow in epithelial layers, are clonogenic in soft agar and form poorly differentiated tumors in athymic mice. 5583-S cells grow in suspension in clusters, are not clonogenic in soft agar and form moderately to well differentiated tumors in athymic mice.

8.2.3 Culture conditions

The cells were cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM, Flow Laboratories, Herts, UK) supplemented with 10% inactivated (56°C, 30 min) dialyzed fetal bovine serum (Hyclone, Logan UT, USA). The cells were seeded in plastic flasks (650 cm³ Falcon, Oxnard CA, USA) and cultured at 37°C in an atmosphere of 92.5% air – 7.5% CO₂. The treatment of cells with glucose, NaBT, DMSO or RA was started in the exponential growth phase by supplementing the medium with glucose (20 mM; final concentration 25 mM), NaBT (2 mM), DMSO (2% v/v), or RA (35 μ M). Cells were also cultured in serum free medium and in glucose free medium supplemented with 2.5 mM inosine. The culture media were changed daily. Three different effects of the culture medium on cell growth were observed:

1. growth of the cells was not or only slightly affected
2. growth of the cells was strongly inhibited but the cells remained viable (assessed by microscopic examination and trypan blue dye exclusion) and
3. cells did not remain vital.

When cell growth was not affected the cells were harvested after 10 passages, in exponential and in confluent growth conditions. When cell growth was strongly inhibited but the cells remained viable, cells were harvested after 20-30 days. The cells

were passaged by standard trypsinization. After trypsinization of cells grown in serum free medium, the cells did not adhere to the culture flask when re-seeded in serum free medium. Addition of serum restored the adhering capacity of the cells. Therefore cells were cultured in serum free medium only for 20-30 days and harvested in the exponential growth phase.

8.2.4 Tumors in nude rats

Harlem Sprague Dawley athymic nu/nu male rats of 8-10 weeks old were inoculated subcutaneously with 2×10^6 tumor cells in PBS. Tumors were obtained from all 4 cell lines. Tumors were excised depending upon the size (max 1 cm³) 20-50 days after inoculation and snap frozen in isopentane, quenched in dry-ice (-80°C), for immunohistochemistry.

8.2.5 Immunocytochemistry

The cultured cells were harvested by gentle scraping with a rubber policeman. Attention was paid to preserve the intercellular adhesions as much as possible. The cells were fixed in 70% ethanol in PBS for 20 min at 4°C. After washing the pellet was resuspended in 5% (v/v) agar in PBS (at 56°C) and immediately cooled to 4°C. The agar block was subsequently embedded in paraffin according to routine procedures. Paraffin sections (4 µm) were rehydrated prior to immunocytochemistry. Cryostat sections (4 µm) of the xenografts were dried and fixed in periodate-lysine-paraformaldehyde (18) for 10 min at RT. After washing in PBS endogenous peroxidase activity was blocked by incubating the sections for 30 min at RT in PBS containing 0.5% (v/v) hydrogen peroxide. The standard procedure for the indirect immunoperoxidase technique was followed. Prior to application of the polyclonal anti-ADCP antiserum the preparations were preincubated with 20% (v/v) normal goat serum to reduce non-specific binding. Peroxidase activity was visualized using diaminobenzidine hydrochloride (DAB) as the substrate and the preparations were slightly counterstained with hematoxylin.

8.2.6 Electronmicroscopy

After harvesting, the cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphatebuffer pH 7.2, postfixed 1 h in 1% osmium tetroxide, dehydrated in a graded series of alcohols and embedded in Epon. Ultrathin sections were stained with uranylacetate and leadcitrate.

8.3 Results

In Table I the effects of the culture conditions on growth and viability of the cells

Table I Effect of culture conditions¹ on growth and viability of the cells²

Cell line	- glucose + inosine		NaBT		DMSO		Serum deprived	
	G	V	G	V	G	V	G	V
HT-29	+	+	+	+	-	+	+	+
Caco-2	+	+	-	+	-	+	+	+
5583-E	-	+	-	+	-	+	+	+
5583-S	-	+	+	+	-	+	-	-

¹ For details see Materials and Methods

² Growth (G) and viability (V) are indicated as + or -

are listed. Cell growth and viability were not affected by addition of glucose or RA. Substitution of glucose by inosine reduced growth but not the viability of 5583-E and 5583-S cells. Similarly, addition of NaBT in Caco-2 and 5583-E and of DMSO in all cell lines reduced growth but not viability. Growth and viability of 5583-S cells were reduced by serum deprivation.

The results of the immunocytochemical studies on cell lines grown *in vitro* are listed in Table II. Villin and sucrase-isomaltase reactivity was observed exclusively along the apical plasma membrane of the cells. Strong cytoplasmic ADCP expression was always accompanied by membrane associated expression. In multilayered cell groups only the superficial cell layer expressed the membrane associated antigens. In confluent HT-29 cells cultured in the presence of inosine formation of intracellular lumina was observed. These lumina showed marginal plasma membrane staining for villin, sucrase-isomaltase and ADCP (Fig. 1). Ultrastructurally, these cells showed intracytoplasmic lumina with a well developed brush border-like margin

Fig. 1. HT-29 cells at confluency, grown in the presence of inosine, showing a small intracytoplasmic lumen with marginal reactivity for ADCP (arrow). Also weak apical membrane associated ADCP immunoreactivity is observed (arrowhead) (immunoperoxidase, 750 $\times$).

Table II Villin, sucrose-isomaltase (SI) and ADCP immunoreactivity^{1, 2} in 4 human colorectal adenocarcinoma derived cell lines

culture conditions	HT-29						Caco-2						5583-S					
	Exponentially growing			Confluent			Exponentially growing			Confluent			Exponentially growing			Confluent		
	Villin SI		ADCP	Villin SI		ADCP	Villin		ADCP	Villin SI		ADCP	ADCP		Villin	SI		ADCP
	2	2	1 2	2	2	1 2	2	2	1 2	2	2	1 2	1	2	2	2	1 2	1 2
standard	-	-	+	-	-	+	+	-	-	++	++	++	-	-	-	-	+	+
+ glucose	-	-	+	-	-	+	+	-	-	+	+	++	*	-	-	-	+	+
- glucose																		
+ inosine	+	+	+	++	+	++	++	+	-	++	++	++	-	*	-	-	-	+
+ NaBT	+	-	++	++	+	++	++	*	*	++	++	++	*	*	-	-	-	+
+ DMSO	*	*	*	+	-	-	*	*	*	++	++	+	*	*	+	+	+	+
+ RA	-	-	+	+	-	+	+	+	++	++	+	++	-	-	-	-	-	++
- serum	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	x x

¹ Diffuse cytoplasmic
² Apical membrane associated
³ 5583-S cells grow in suspension
 - = no; + = weak; ++ = strong immunoreactivity
 * Under growth inhibiting culture condition the cultures are regarded as confluent
 x = Growth and viability are reduced

Fig. 2. HT-29 cells at confluency, grown in the presence of inosine, showing an intracytoplasmic lumen with a well developed brush border-like margin (8000 $\times$).

Fig. 3. HT-29 cells at confluency, grown in the presence of inosine, showing surface microvilli with characteristic axonemata (arrow) (26400 $\times$).

(Fig. 2) and a surface structure characterized by microvilli with characteristic axonemata (Fig. 3). In HT-29 cells cultured in the presence of glucose surface microvilli were rare (not shown). Except for HT-29 cells cultured in the presence of DMSO, villin reactivity was always accompanied by ADCP expression. Reactivity of sucrase-isomaltase was always found in combination with villin and ADCP expression. However, ADCP reactivity was not always accompanied by villin or sucrase-isomaltase reactivity and similarly villin was observed without sucrase-isomaltase expression. Under standard culture conditions and at confluency Caco-2 showed

expression of ADCP (Fig. 4), villin (Fig. 5) and sucrase-isomaltase, HT-29 did not express villin and sucrase-isomaltase and 5583-E and 5583-S showed only weak ADCP expression. Compared to the level of expression in the exponential growth phase, confluent Caco-2 cells showed an increased expression of ADCP, villin and sucrase-isomaltase whereas in 5583-E cells only ADCP expression increased. Antigen expression in cells cultured under growth inhibitory conditions was compared with the expression in cells grown under standard conditions and harvested at confluency. Replacement of glucose by inosine or addition of NaBT resulted in an increase in villin, sucrase-isomaltase and ADCP expression in HT-29 cells compared to standard culture conditions, whereas in 5583-S cells only the cytoplasmic expression of ADCP increased. Under these conditions only marginal differences were observed in Caco-2 cells, and in 5583-E cells the expression of ADCP decreased. Addition of DMSO induced in 5583-E cells a weak expression of membrane associated villin (Fig. 6) and sucrase-isomaltase. DMSO reduced the sucrase-isomaltase and ADCP reactivity in Caco-2 cells whereas ADCP expression in HT-29 cells was completely abolished. Addition of glucose reduced or did not alter the expression of villin, sucrase-isomaltase and ADCP in Caco-2, HT-29 (Fig. 7) and 5583-E cells. However, in 5583-S cells glucose enhanced the cytoplasmic ADCP expression. RA had an inhibiting effect on expression of sucrase-isomaltase and villin in Caco-2 cells and on ADCP reactivity in 5583-E cells. On the contrary RA strongly enhanced the expression of ADCP in Caco-2 and 5583-S cells. Serum deprivation reduced villin expression in Caco-2 cells and ADCP expression in HT-29 and 5583-E cells. However, after 22 days of serum deprivation HT-29 cells demonstrated production of mucins (Fig. 8). Immunohistochemistry applied on nude rat xenografts of the cell lines revealed that in all xenografts apical membrane associated reactivity with anti-ADCP (Fig. 9) and anti-villin occurred, whereas in the 5583-E xenograft no reactivity with anti-sucrase-isomaltase was observed and in 5583-S xenografts only weak membrane associated reactivity (Table III).

8.4 Discussion

The human colon carcinoma cell lines HT-29 and Caco-2 have the potential to express characteristics of enterocytic differentiation *in vitro* and *in vivo* (14, 19-23). These characteristics include the presence of junctional complexes and the development of cell polarity, more specifically the occurrence of a brushborder and the expression of structural (villin) or functional (sucrase-isomaltase, aminopeptidase N, dipeptidylpeptidase IV, lactase, alkaline phosphatase) brushborder associated proteins (16, 21-23). Brushborders and their associated proteins normally occur in small intestinal columnar cells, which, together with the emergence of small intestinal types of mucin (sialomucin) in colon cancer (24), underlines the aberrant nature of epithelial cell differentiation in colon carcinoma cells. Nevertheless, HT-29 and Caco-2 cells are useful models for the study of small intestinal epithelial differentiation (19, 25, 26).

Fig. 4. Caco-2 cells at confluency stained for ADCP showing strong apical membrane associated and weak cytoplasmic reactivity (immunoperoxidase, 1200 $\times$).

Fig. 5. Caco-2 cells at confluency stained for villin showing strong apical membrane associated reactivity (immunoperoxidase, 750 $\times$).

Fig. 6. 5583-E cells cultured in the presence of DMSO showing weak apical membrane associated villin expression (arrow) (immunoperoxidase, 400 $\times$).

Fig. 7. HT-29 cells at confluency, grown in the presence of 25 mM glucose. Note that growth occurs in multicell layers. Weak cytoplasmic ADCP reactivity is observed (immunoperoxidase, 500 $\times$).

Fig. 8. Diamine / Alcian Blue staining of HT-29 cells grown without serum. Mucin vacuoles indicating the emergence of goblet-like cells can be noted (arrow) (750 $\times$).

Fig. 9. Rat xenograft of HT-29 stained with anti-ADCP antiserum showing strong luminal membrane associated and weak cytoplasmic reactivity for ADCP (immunoperoxidase, 250 $\times$).

Table III Villin, sucrase-isomaltase (SI) and ADCP immunoreactivity^{1,2} in rat xenografts

Cell line	Villin		SI		ADCP	
	1	2	1	2	1	2
HT-29	+	++	+	++	+	++
Caco-2	+	++	+	++	+	++
5583-E	+	++	-	-	+	++
5583-S	+	++	-	+	+	++

¹ Diffuse cytoplasmic² Apical membrane associated

- = no; + = weak; ++ = strong reactivity

Caco-2 cells show enterocytic differentiation at late confluency under standard culturing conditions whereas HT-29 cells sustain this type of differentiation in medium without hexoses (8, 20, 21, 23, 27). After serum deprivation a subpopulation of HT-29 cells differentiates into goblet-like cells (28) and also into Paneth-like cells, as evidenced by the production of lysozyme (29, 30). Other agents, such as NaBT, DMSO and RA induce HT-29 cells to display one or more of these characteristics of enterocytic differentiation (10, 31-33). In contrast, culturing of HT-29 cells in medium containing 25 mM glucose has been shown to induce an undifferentiated phenotype (23).

Our data with regard to villin and sucrase-isomaltase expression in HT-29 and Caco-2 cells under various conditions, are in agreement with the literature (14, 19-23). We did, however, not observe cytoplasmic villin reactivity in undifferentiated HT-29 cells as reported by others (34). The reduced expression of sucrase-isomaltase and villin by Caco-2 cells as induced by glucose, DMSO and RA and the enhanced expression of villin in NaBT treated HT-29 cells are new findings. It is clear that growth inhibition in Caco-2 cells (either in confluency or after addition of NaBT or DMSO) is not invariably followed by differentiation. When grown under standard conditions 5583-E cells are undifferentiated whereas 5583-S cells express a more differentiated goblet cell like phenotype although columnar cells with well developed microvilli occur also (9). Evidently, the induction of differentiation requires from cell line to cell line different specific signals.

In 5583-E cells DMSO induced enterocytic differentiation with expression of villin and sucrase-isomaltase. In 5583-S cells immunocytochemistry failed to reveal enterocytic differentiation, despite the fact that some of these cells showed microvillous surface differentiation by electron microscopy (9). Conceivably, in these cells the level of expression of villin is so low that it can not be detected by immunocytochemistry. It is also possible that an altered protein is produced, which is not recognized by the anti-villin monoclonal antibody. Le Bivic *et al.* (35) also reported the presence of microvilli on undifferentiated HT-29 cells without concomitant villin immunoreactivity.

The mechanisms by which the various culture conditions modulate cellular differentiation are not well understood, however, a number of effects have been described. High glucose concentrations in the medium are reported to result in high intracellular concentration of UDP-N-acetylhexosamines (22). An increase of these compounds has been shown in undifferentiated cells to reflect an impairment of protein glycosylation (8). Also a close relationship between energy metabolism and intracellular protein breakdown has been demonstrated. Glucose deprivation prevented lysosomal protein breakdown (8). NaBT is known to induce hyperacetylation of histones through inhibition of histone deacetylase and it has been suggested that this process is involved in the regulation of gene expression (36). DMSO alters membrane permeability and microviscosity and causes single-strand breaks in the DNA (37). Retinoids are thought to act like steroid hormones by forming a complex with a binding protein which is translocated into the nucleus causing alterations in gene expression (38).

Both 5583-E and 5583-S cells, grown as xenografts in nude rats, expressed villin whereas only 5583-S showed membranous sucrase-isomaltase reactivity. This suggests that 5583-S cells have reached a higher level of differentiation, assuming that along the process of differentiation sucrase-isomaltase expression is acquired later than microvillus formation. This corresponds with the earlier reported morphological characteristics (9).

The expression of villin and sucrase-isomaltase in neoplastic colorectal epithelia are well accepted characteristics of enterocytic differentiation (14, 16). ADCP immunoreactivity has been previously described in columnar cells in the large and small intestine (4) and its apical membrane associated reactivity was suggested to be indicative for polar, microvilli bearing cells (2, 4, 5). To elucidate the usefulness of ADCP as a marker of enterocytic differentiation we correlated its expression with that of villin and sucrase-isomaltase on the 4 cell lines grown *in vitro* and *in vivo* in nude rats. The finding that membrane associated sucrase-isomaltase reactivity is always accompanied by membranous villin expression was expected, because membranous localization of sucrase-isomaltase has been described exclusively in cells containing well developed microvilli (11-14). Except for HT-29 cells cultured with DMSO, villin expression was always found in combination with ADCP expression but ADCP reactivity also occurred without villin expression. The strongest membrane associated ADCP reactivity always occurred in well differentiated cells but usually in combination with cytoplasmic reactivity. In normal intestinal cells ADCP reactivity is not found in the cytoplasm whereas in colorectal carcinoma cytoplasmic ADCP expression is frequently observed (2, 4-6). Cytoplasmic ADCP expression may indicate loss of cell polarity. Cell polarity is generally regarded as a characteristic of the differentiated state. In reviewing these data it is tempting to speculate that the expression of villin, sucrase-isomaltase and ADCP might represent different levels of enterocytic differentiation.

The xenograft experiments demonstrate that all 4 cell lines when exposed to an appropriate environment have the potential to differentiate as enterocytes. Recently

it has been shown that xenografts of Caco-2 contain columnar, goblet and neuroendocrine cells. HT-29 xenografts only showed columnar and neuroendocrine cells (39). *In vitro*, HT-29 cells can also differentiate as goblet cells (28) and Paneth cells (29, 30). Cell lines 5583-E and 5583-S grown as xenografts showed goblet and columnar cell differentiation, neuroendocrine and Paneth cells were not observed (39). These results indicate 1. that under *in vitro* conditions these cell lines show a more limited repertoire of differentional characteristics than under *in vivo* conditions and 2. all 4 cell lines harbor relatively undifferentiated cells which have retained the propensity to differentiate into different directions. These cells might be multipotent "tumor stem cells" at various levels of maturation. Recently Kirkland (40) demonstrated that a single human rectal adenocarcinoma cell can differentiate into columnar, goblet and endocrine cells. Her results support the unitarian stem cell hypothesis for cell renewal in colorectal epithelium. The exploration of refined markers for stages of differentiation of different types of intestinal cells will increase our understanding of normal and neoplastic intestinal epithelial cell differentiation. This knowledge might lead to biologically relevant as well as clinically useful new classifications of colorectal carcinoma. In the search for such differentiation markers cell lines with different differentiating capabilities might prove helpful.

In summary, these studies show that :

1. 5583-E and 5583-S cells show a more limited propensity towards enterocytic differentiation than Caco-2 and HT-29 cells;
2. *In vivo* these cell lines show a more extensive repertoire of differentional capabilities than *in vitro*;
3. ADCP is predominantly expressed in cells with columnar differentiation and is probably an earlier appearing marker in this cell lineage than the endstage markers villin and sucrase-isomaltase.
4. Growth and differentiation are separately regulated cellular processes in colorectal carcinoma cell lines.

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Chapter 9

General discussion

9.1 Introduction

In this final chapter the results of the investigations described in the previous chapters are discussed, particularly in relation to the possible physiological function of ADCP and its significance as a marker in neoplastic transformation.

9.2 Physiological function of ADCP

ADCP was discovered about 15 years ago by biochemical investigation devoted to the enzyme adenosine deaminase (ADA) (1). The observed binding property of ADA to ADCP (2) has led to the speculation that the physiological function of ADCP is related to its binding to ADA (3-8). However, as yet the various theories concerning an ADA related function of ADCP have not been proven. None of these theories consider the possibility that ADCP might have an enzymatic activity related to adenosine deamination. Several multienzyme complexes have been described and they all facilitate the passage of reaction intermediates directly from one enzyme to another (9). For ADA a purine 5'nucleotidase complex would be a candidate. This dimeric enzyme converts adenosine monophosphate into adenosine and inorganic phosphate and it has a predominant plasma membrane associated localization. However, its apparent subunit molecular weight is below the ADCP subunit molecular weight (10, 11). All the theories of an ADA related function of ADCP are based on the tight binding capacity of ADCP for ADA. This tight binding of ADCP to ADA, so far only proven in cell lysates, need not necessarily point to a physiological role for this binding. Tight and specific interactions have been described between physiologically independent proteins (12).

Evidence is being accumulated at present, that the function of ADCP is not related to ADA. This suggestion is derived from the observed lack of co-localization of ADCP and ADA as determined by immunohistochemical investigations (13-16). The evidence with regard to the occurrence of ADCP in murine tissue extracts that do not show ADA binding capacity, as described in chapter 3, also argues against an ADA related function for ADCP. The question remains: What is the function of ADCP? Though its capacity to bind ADA has not been proven to be of physiological significance, this possibility can not be completely excluded. The fact that the protein is glycosylated suggests that its function is plasma membrane associated

and/or associated with the cellular process of secretion. Its predominant association with plasma membranes and its presence in secretory fluids (6, 8, 14, 15-18) is in accordance with this suggestion. The expression of ADCP in endometrial glands exclusively during the secretory phase, and the expression in epithelial cells at the secretory pole of the cells (*e.g.* prostate, salivary and sweat glands) strongly suggests a secretion related function of ADCP (18). On the other hand, abundant ADCP expression also occurs in cells without a secretory function (*e.g.* epithelial cells of the proximal convoluted tubules of the kidney and fibroblasts) whereas some secretory cell types (*e.g.* mucus secreting cells in the gastrointestinal tract) do not express ADCP. Absence of ADCP was also reported for some normal cell types (such as erythrocytes) and for tumor derived and virus transformed cell lines (19-22). Therefore, ADCP is neither an obligatory membrane protein nor an obligatory secretory protein. It is not unlikely, however, that when ADCP is present in secretory cells its function might be related to the secretory process. Because of abundant ADCP expression in absorptive epithelial cells at the absorptive pole of the cells (intestine, renal proximal convoluted tubules) (18) it is tempting to speculate that in these cells the function of ADCP is related to the absorptive process. In non-secretory and non-absorptive cells its function might be related to the maintenance of structural or functional integrity of the membrane. These assumptions would imply a multifunctional physiological role for ADCP. Multifunctional proteins are well known and include hormones, enzymes and growth factors. It has to be stressed here that these reflections on the function of ADCP are mainly based upon immunohistochemical identification of this protein. With this technique low levels of ADCP might escape detection, which limits the validity of speculations solely based upon immunohistochemical observations.

9.3 ADCP and neoplastic transformation

During the last decades many efforts have been made to find tumor associated antigens with diagnostic, prognostic or therapeutic usefulness in the management of cancer patients. The establishment of the hybridoma technique for the production of monoclonal antibodies (23) created expectations that new tumor antigens would be discovered with high tumor specificity. The assumption behind these thoughts was based on the theory of Ehrlich, proposed at the beginning of this century, that cells in the process of neoplastic transformation acquire tumor specific antigens (24). However, despite immense effort, no truly tumor specific antigens have been found as yet. Although tumor specific antigens are probably present in some tumors (*e.g.* proteins encoded by viral genes in tumors induced by virus infection or neo-antigens encoded by hybrid genes generated by cancer specific intergenic recombinations can be regarded as tumor specific (25-29)), malignant transformation is more and more regarded as a process of disturbed normal regulation rather than the acquisition and expression of new genetic information. According to this theory

malignant transformation would not be accompanied by the expression of neo-antigens but rather by quantitative and/or distributional changes in normal (including fetal) antigens. These changes might be the result of alterations on the transcriptional, translational or posttranslational level of protein synthesis.

Because most if not all (plasma cells, T lymphocytes) of the somatic cells of an individual are genetically equal malignant transformation of a cell can result in expression of genes far deviated from what is observed in normal cells. This is demonstrated by the extensively studied expression of the intermediate filament protein family. The expression of different intermediate filament proteins has been demonstrated to parallel established principles of embryonic development and classical histology (30, 31). These findings led to the suggestion that intermediate filament protein expression can provide information useful in tumor diagnosis and determination of tumor histogenesis (30). Indeed intermediate filament proteins appeared to be quite valuable in this respect, but this value is somewhat limited by the occurrence of tumor cells which do not express or conversely co-express 2 or 3 different intermediate filament proteins, which in the corresponding normal cells are cell type specific (32). From these observations it can be anticipated that not the unique expression of a neo-antigen but rather aberrant levels, or patterns of expression or combinations, of normal cellular constituents might approximate the described goal of tumor specificity. Exploration of this possibility and its potential value would require elaborate studies on the expression of well defined antigens on large series of tumors with sufficiently long patient follow-up.

What could be the value of the determination of ADCP in tumors in this perspective? Neither the absence of ADCP — contrary to what has been postulated by others (33) — nor the presence of ADCP in neoplastic cells is in any way tumor specific (17, 33-39). Expression of ADCP is not tissue nor cell type specific (18) and therefore its presence in metastatic lesions does not provide clues with regard to the origin of the primary tumor. In a neoplasm in a particular organ or cell system the level of expression or its subcellular distribution might be an indicator of the differentiation processes in the neoplastic cells and as such might somehow predict the biological behavior of the neoplasm. In this respect biochemical analysis of tumor extracts is of limited value because this does not provide information on the frequently observed intercellular heterogeneity within a single neoplasm (36, 38, 39). Immunohistochemical analysis yields essential information because it provides a possibility to study antigen expression at cellular and even subcellular level.

ADCP is secreted into body fluids (5, 40-41), therefore quantitative ADCP determination in these fluids in patients with an ADCP producing cancer may have diagnostic value or may provide information useful for patient follow-up after treatment. Such applications can be envisioned in prostatic or renal adenocarcinomas. Because the function of ADCP is unknown, the predictive value of its cellular expression *per se*, and its subcellular localization for the biological behavior of the tumor is speculative. The described apical membrane associated expression of ADCP in a number of normal epithelia (18) reflects normal cell polarity and epithelial cell

polarity can be regarded as a sign of appropriate differentiation. Consequently, deviation of the normal ADCP expression pattern in tumor cells can be regarded as an indicator of a less differentiated phenotype. This was substantiated in prostatic and renal adenocarcinomas where ADCP expression patterns correlated with metastatic propensity which is generally considered to be higher in poorly differentiated cells (39, 42). It would be a major breakthrough if the actively proliferating and metastasizing cells in a tumor could be identified on the basis of specific patterns of antigen expression. For such purposes ADCP can hardly be considered a suitable candidate because its expression has been found to reflect a relatively high level of differentiation (38, 39) and absence of a marker should be regarded as insufficient for positive identification of a cell.

9.4 Future perspectives

As discussed in the previous paragraph in general, determination of ADCP expression in tumors and secreted body fluids will be of limited value because at best ADCP is a marker for relatively well differentiated cell populations, the biologically least harmful tumor cells. However, the observed relationship between ADCP expression and tumor grade in renal adenocarcinomas (39) and ADCP expression and metastatic propensity in prostatic adenocarcinomas (43) is potentially important. The validity of these findings should be confirmed in larger series of patients with sufficiently long follow-up. Further exploration of the role of ADCP in neoplasia requires elucidation of its function. This would involve determination of the amino acid sequence of ADCP, studies on physicochemical properties of the molecule and identification of the ADCP gene and artificial (de-) regulation of its expression under a wide range of physiological conditions in different cell types. After identification of the ADCP gene homologies to other genes with known functions can be determined. Such studies were clearly beyond the scope of this thesis. Studies on the expression of ADCP in neoplasia are not likely to provide essential new information unless the physiological function of ADCP is more clearly resolved and the regulation of its expression understood.

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Summary

This thesis focusses on the distribution of the adenosine deaminase complexing protein (ADCP) in normal and neoplastic cells. In CHAPTER 1 the properties of the protein and its possible behavior during malignant transformation are discussed. Some of the reviewed data indicate that the expression of ADCP might be specifically altered during malignant transformation. It has been suggested that ADCP can be used as a marker in studies on cellular differentiation and tumor classification. Studies to this end require the availability of specific antibodies which would allow the qualitative determination of ADCP in tissue sections by immunohistochemistry and the quantitative determination of ADCP in tissue homogenates by radioimmunoassay (RIA). These methods require the availability of highly specific anti-ADCP antibodies.

In CHAPTER 2 a method is described for the generation of antibodies against minute amounts of ADCP. The method is based on the adsorption of ADCP to a solid phase (nitrocellulose membrane) and the subsequent implantation of this protein-coated membrane in a mouse. This procedure resulted in high serum antibody titers against native ADCP. In some experiments we used a polyvinylidene difluoride (PVDF) membrane. In order to generate antibodies which would allow the detection of ADCP in routinely fixed and processed tissues, PVDF membranes carrying ADCP were fixed and processed according to routine tissue processing protocols before implantation. This procedure resulted in the generation of antisera which allowed the detection of ADCP in routinely processed tissues. For the generation of monoclonal antibodies the immunized mice received booster injections with higher quantities of impure ADCP preparations, unfixed or after fixation and processing. With this procedure a panel of ADCP reactive monoclonal antibodies was obtained.

It has been suggested that mouse and rat lack ADCP because in these species exclusively the small molecular weight form of adenosine deaminase (ADA-S) is found. This suggestion is based on the assumption that the ADA binding capacity is an inherent functional characteristic of ADCP. CHAPTER 3 reports on the presence of ADCP immunoreactivity in mouse and rat determined with a species cross-reactive polyclonal anti-ADCP serum. In the mouse the tissue and subcellular distribution and the electrophoretic mobility in starch and polyacrylamide gels of the protein correspond with those of ADCP but it does not bind ADA-S. It is suggested that the function of ADCP is not ADA-related.

In CHAPTER 4 studies concerning the distribution of ADCP in the human body are described. This was investigated quantitatively by ADCP specific RIA of soluble and plasma membrane fractions and qualitatively by immunohistochemistry. In these studies a specific rabbit anti-human ADCP antiserum was used. In 18 investigated tissues ADCP was found in the soluble as well as in the membrane fractions. In all tissues the membrane fractions contained more ADCP than the soluble frac-

tions. ADCP immunoreactivity was found predominantly in exocrine glands and absorptive epithelia especially at the secretory or absorptive apex of the cell. This led to the suggestion that the function of ADCP is related to the secretory and absorptive processes.

Because of the important role of ADA in human T-lymphoid differentiation the presence of ADA and ADCP on human T-lymphoid cells was studied, as described in CHAPTER 5. Of all investigated T-lymphoid cells about 18% expressed membrane ADA. Membrane ADCP expression was demonstrated on 47% and 14% of the peripheral blood T-lymphocytes (PBL-T) and thymocytes (anti-CD5 positive) respectively. The T-helper/inducer (anti-CD4 positive) and T-cytotoxic/suppressor (anti-CD8 positive) subpopulations contained 55% and 28% membrane ADCP positive cells respectively. When the PBL-T were incubated with a lysate of human erythrocytes (as source of ADA-S) about 50% of the cells was found to display ADA at the membrane. It is likely, that ADA-S binds to membrane associated ADCP.

In CHAPTER 6 the ADCP expression in various normal and neoplastic human tissues is described. In these studies a polyclonal anti-ADCP antiserum and 2 ADCP reactive monoclonal antibodies (1071 and 1072) were used. The reactivity patterns of the 2 monoclonal antibodies did not completely match the patterns observed with the polyclonal antiserum. This finding might imply more molecular heterogeneity of ADCP than hitherto known. The patterns of expression of ADCP in renal cell carcinomas were found to correlate with the stage of the tumor. The patterns of staining in various other carcinomas suggested that ADCP might be an additional parameter in the assessment of the histogenesis and degree of differentiation of neoplastic cells but with limited significance.

A more detailed study of ADCP expression in human prostatic adenocarcinomas is described in CHAPTER 7. The anti-ADCP antiserum showed 4 distinct staining patterns: diffuse cytoplasmic, membranous, both cytoplasmic and membranous and no ADCP expression. When a membranous staining pattern was observed no metastases were found, whereas metastases occurred only in cases with no or only cytoplasmic ADCP staining. This suggests that immunohistochemical detection of ADCP might predict the biological behavior of prostatic cancer. However, the occurrence of membrane ADCP staining in the xenograft of a cell line derived from a prostatic carcinoma metastasis indicates that this is not an absolute fact.

The results of studies concerning the relationship between ADCP expression and intestinal epithelial cell differentiation are reported in CHAPTER 8. Because in the digestive tract ADCP expression occurs only in absorptive enterocytes its pattern of expression was compared with those of villin and sucrase-isomaltase, two highly specific markers for enterocytic differentiation. As model system 4 human colorectal carcinoma derived cell lines were used, grown *in vivo* and *in vitro* under differentiation modulating conditions. Villin expression was almost always found in combination with membranous ADCP expression whereas ADCP expression also occurred without villin expression. Sucrase-isomaltase expression was also found in

combination with villin. The strongest membrane ADCP reactivity was observed in well differentiated cells, but usually in combination with cytoplasmic reactivity. Because cytoplasmic ADCP reactivity was not observed in normal enterocytes this cytoplasmic ADCP reactivity is probably a hallmark of incomplete enterocytic differentiation. It is suggested that expression patterns of villin, sucrase-isomaltase and ADCP might represent different levels of enterocytic differentiation. Finally, in CHAPTER 9 the possible physiological function of ADCP and its role in understanding the process of neoplastic transformation are discussed. Considerations on future perspectives for ADCP research conclude this chapter.

Samenvatting

In dit proefschrift zijn de resultaten van onderzoek naar het vóórkomen van het adenosine deaminase complexerend proteïne (ADCP) in normale en neoplastische cellen beschreven.

In HOOFDSTUK 1 wordt een overzicht gegeven van de relevante literatuur betreffende ADCP. Uit dit overzicht blijkt dat het expressie patroon van ADCP in cellen mogelijk verandert tijdens maligne ontaarding. In de literatuur wordt tevens gesuggereerd dat de expressie van ADCP gebruikt kan worden als een merker voor cellulaire differentiatie en tumor classificatie. Dit zou onderzocht kunnen worden met behulp van antilichamen die ADCP specifiek kunnen aantonen. Met deze antilichamen kan ADCP zichtbaar worden gemaakt in weefselcoupes met behulp van immunohistochemische technieken. Tevens kan ADCP kwantitatief worden bepaald in weefsel homogenaten door middel van radioimmunoassay (RIA).

In HOOFDSTUK 2 wordt een procedure beschreven waarmee polyclonale en monoclonale antilichamen kunnen worden verkregen wanneer slechts zeer kleine (nanogram) hoeveelheden ADCP beschikbaar zijn. Deze methode is gebaseerd op de binding van ADCP aan een vaste drager (nitrocellulose membraan) waarna deze drager wordt geïmplantéerd in een muis. Na immunisatie van muizen met nanogram hoeveelheden (natief) ADCP gebonden aan een nitrocellulose membraan werden antiseren verkregen die reageren met natief ADCP. In een aantal experimenten werd als vaste drager een polyvinylideen difluoride (PVDF) membraan gebruikt. Na binding van ADCP en vóór implantatie werd het PVDF membraan behandeld overeenkomstig de procedure die weefsel routinematig ondergaat vóór toepassing van de immunohistochemische techniek (dat wil zeggen fixatie met formaline, insluiten in paraffine, ontparaffineren en remmen van de endogene peroxidase activiteit). Na toepassing van deze procedure werden antiseren verkregen waarmee ADCP aangetoond kan worden in routinematig behandelde weefselstukjes afkomstig van nier, prostaat en dunne darm. Ter verkrijging van monoclonale antilichamen kregen de geïmmuniseerde muizen extra injecties met homogenaten van weefsel dat ADCP bevat. Deze procedure leverde een aantal monoclonale antilichamen op die met ADCP reageren.

In de literatuur is gesuggereerd dat muizen en ratten geen ADCP bezitten omdat uit biochemische experimenten bleek, dat in deze diersoorten geen vorm met een hoog molecuulgewicht van het enzym adenosine deaminase (ADA-L) aanwezig is. Deze suggestie is gebaseerd op de veronderstelling dat binding van de vorm met een laag molecuulgewicht van adenosine deaminase (ADA-S) door ADCP een functionele en inherente eigenschap van ADCP is. In HOOFDSTUK 3 worden experimentele resultaten beschreven die aantonen dat in muizen en ratten een ADCP immunoreactief eiwit vóórkomt. Zowel de weefsel- en subcellulaire distributie als de elektroforetische mobiliteit in zetmeel en polyacrylamide gels van dit eiwit komen nauw overeen met die van ADCP. Echter, dit ADCP immunoreactief eiwit vertoont geen affiniteit

voor ADA. Geponereerd wordt dat deze bevinding een aanwijzing is voor een fysiologische functie van ADCP die niet gerelateerd is aan ADA.

De resultaten van experimenten naar de expressie van ADCP in normale humane weefsels zijn beschreven in HOOFDSTUK 4. Met behulp van een specifiek konijne anti-humaan ADCP antiserum is ADCP kwantitatief aangetoond met een RIA en kwalitatief met de immunoperoxidase techniek. Met de RIA werd ADCP gevonden zowel in de cytosol- als de membraanfracties van de onderzochte weefsels. In elk weefsel werd meer ADCP (per milligram eiwit) gevonden in de membraanfractie dan in de corresponderende cytosolfraction. Dat ADCP voornamelijk membraan geassocieerd voorkomt werd bevestigd door de resultaten van de immunohistochemische studies. De bevinding dat ADCP met name werd gevonden in het secretair of resorptief gedeelte van de cellen van exocriene klieren en resorptieve epithelen leidde tot de suggestie dat ADCP een secretie of resorptie geassocieerd eiwit is.

Het enzym ADA speelt een belangrijke rol bij de differentiatie van T-lymfocyten. Daarom werd het voorkomen van ADA en ADCP op intacte humane T-lymfoïde cellen bestudeerd. De resultaten van deze flowcytometrische studie zijn beschreven in HOOFDSTUK 5. Uit perifere bloed werden de totale T-lymfocyten populatie (anti-CD5 positief) en de T-helper/inducer (anti-CD4 positief) en T-cytotoxische/suppressor (anti-CD8 positief) subpopulaties onderzocht. Tevens werden thymocyten onderzocht. In elke onderzochte subpopulatie bleek ongeveer 18% van de cellen membraan geassocieerd ADA te bezitten. Membraan geassocieerd ADCP werd gevonden in 55% van de T-helper/inducer cellen, in 28% van de T-cytotoxische/suppressor cellen en in 14% van de thymocyten (anti-CD5 positief). In de totale T-lymfocyten populatie van het perifere bloed bleek 47% van de cellen membraan geassocieerd ADCP te bezitten. Na incubatie van deze populatie met een lysaat van humane erythrocyten (als bron van humaan ADA-S) werd op ongeveer 50% van de cellen ADA aangetoond. Deze bevinding leidde tot de veronderstelling dat dit ADA-S gebonden is aan membraan geassocieerd ADCP.

In HOOFDSTUK 6 worden de resultaten beschreven van immunohistochemische ADCP bepalingen in een aantal normale en neoplastische humane weefsels. In deze studies werd gebruik gemaakt van een konijne polyclonaal anti-ADCP antiserum en twee met ADCP reagerende muise monoclonale antilichamen. De reactiviteitspatronen van de twee monoclonale antilichamen komen niet volledig overeen met het reactiviteitspatroon van het polyclonale antiserum. Deze bevinding duidt wellicht op, tot nu toe onbekende, moleculaire heterogeniteit van ADCP. In een kleine serie niertumoren werd gevonden dat de ADCP expressiepatronen correleren met de staging van de tumoren. Gesuggereerd wordt dat dit mogelijk wijst op een relatie tussen de ADCP expressiepatronen en het biologisch gedrag van de niertumoren. Bepaling van de ADCP expressiepatronen in andere tumoren leidde tot de veronderstelling dat deze patronen parameters kunnen zijn voor bepaling van de histogenese en mate van differentiatie van epitheliale tumoren.

In HOOFDSTUK 7 wordt beschreven dat in prostaattumoren een relatie werd gevonden tussen de ADCP expressiepatronen en de aanwezigheid van metastasen.

Geen metastasen werden gevonden wanneer de primaire tumor, zoals normaal prostaatweefsel, membraan geassocieerde ADCP immunoreactiviteit vertoonde. Metastasen werden uitsluitend gevonden in patiënten waarbij in de primaire prostaattumor ADCP diffuus in het cytoplasma of niet werd aangetoond. Uit deze resultaten werd geconcludeerd dat de immunohistochemische ADCP bepaling in prostaattumoren van belang kan zijn voor de voorspelling van het biologisch gedrag van de tumor.

HOOFDSTUK 8 behandelt een onderzoek naar de relatie tussen ADCP expressie en de differentiatie van enterocyten. De expressiepatronen van ADCP werden vergeleken met de expressiepatronen van de eiwitten villine en sucrase-isomaltase, twee markers voor enterocytair differentiatie. Omdat een aantal colorectale tumorcellijnen geïnduceerd kan worden tot enterocytair differentiatie zijn vier humane colorectale tumorcellijnen (HT-29, Caco-2, 5583-E, 5583-S) als model gebruikt. Deze werden gekweekt *in vivo* als xenotransplantaat en *in vitro* onder een aantal differentiatie modulerende omstandigheden. Op één uitzondering na werd villine steeds in combinatie met membraan gebonden ADCP expressie gevonden, doch ADCP kon tevens worden aangetoond in afwezigheid van villine expressie. Sucrase-isomaltase werd uitsluitend aangetoond in combinatie met villine. De sterkste membraangebonden ADCP immunoreactiviteit werd gevonden in goed gedifferentieerde cellen, echter meestal in combinatie met cytoplasmatische reactiviteit. In normale darmepitheelcellen werd nooit ADCP in het cytoplasma aangetoond. Wellicht wijst deze cytoplasmatische ADCP expressie op niet volledig voltooide enterocytair differentiatie. Gesuggereerd wordt dat expressie patronen van villine, sucrase-isomaltase en ADCP verschillende differentiatie niveau's in de enterocytair differentiatie vertegenwoordigen.

In HOOFDSTUK 9 wordt gespeculeerd over de fysiologische functie van ADCP en over de waarde van de bepaling van ADCP en andere antigenen in het algemeen voor het vergroten van het inzicht in het proces van neoplastische transformatie. Het proefschrift eindigt met een aantal overwegingen voor verder onderzoek aan het eiwit ADCP.

Dankwoord

Bij de totstandkoming van dit proefschrift ben ik dank verschuldigd aan velen. In de eerste plaats dank ik mijn ouders, dat ze mij de gelegenheid hebben geboden om te studeren. Zij en mijn oma hebben mij gestimuleerd en mijn studie met belangstelling gevolgd. Ik betreur het zeer dat mijn vader en mijn oma het verschijnen van dit proefschrift niet meer meemaken.

De bijdragen van Prof.dr. F.T. Bosman aan dit proefschrift zijn onontbeerlijk geweest. Beste Fré, jouw kennis van zaken, enthousiasme en optimisme waren voor mij uitermate inspirerend. De wijze waarop en de snelheid waarmee jij de manuscripten corrigeerde hebben mij gestimuleerd. Jouw adagium "Het komt wel goed" heeft zelfs op een Maastrichtenaar indruk gemaakt.

Dr. J. ten Kate, beste Joop. Jij hebt niet alleen de basis gelegd voor het onderzoek, beschreven in dit proefschrift, maar je bent ook nauw bij de uitvoering betrokken geweest. Jouw ideeën, adviezen en suggesties met betrekking tot zowel het experimentele werk als de manuscripten hebben een essentiële bijdrage geleverd.

Het onderzoek, beschreven in dit proefschrift, is uitgevoerd in samenwerking met Prof.dr. P. Meera Khan, afdeling Anthropogenetica, Rijksuniversiteit Leiden. Beste Meera, jou dank ik voor de vrijheid die ik kreeg bij de uitvoering van het onderzoek en voor de stimulerende gesprekken die wij voerden.

Dr. Wim Zeijlemaker (afdeling Klinische Immunobiologie) en Dr. Dirk Roos (afdeling Bloedcelchemie), Centraal Laboratorium van de Bloedtransfusiedienst (CLB) van het Nederlandse Rode Kruis te Amsterdam, en Prof. dr. Chris De Bruijn, Technische Universiteit Eindhoven, dank ik voor de begeleiding van mijn voormalig onderzoek op het CLB, waar de basis is gelegd voor het in hoofdstuk 5 beschreven onderzoek.

Een groot deel van de immunohistochemische experimenten is uitgevoerd door Edith van der Linden. Beste Edith, jouw inzet en nauwgezette manier van werken hebben mij herhaaldelijk uit de onderzoeksdalen gehaald waarin ik was terecht gekomen.

De inzet van Marie-Hélène Lenders is met name in de eindfase van het onderzoek van cruciale betekenis geweest.

Aan Juul Wijnen, afdeling Anthropogenetica, Rijksuniversiteit Leiden, ben ik dank verschuldigd voor het zuiveren van ADCP en het uitvoeren van de RIA's en de elektroforetische eiwitscheidingen in zetmeel gels.

Claire Bollen en Marianne Rikers dank ik voor het snel invoeren van de manuscripten in de computer. Beste Claire en Marianne, jullie bleven onder hectische omstandigheden toch steeds perfect werk afleveren.

Caspar Signet is betrokken geweest bij de productie van de monoclonale antilichamen. Hij en Cor Beek hebben een aantal elektroforetische eiwitscheidingen nauwkeurig uitgevoerd.

Bij de fotografie en elektronenmicroscopie heb ik veel hulp gehad van Paul Bomans en Marc Stuart.

Op Birgit Engelen en Margriet Pijls heb ik nooit tevergeefs een beroep gedaan voor het snijden van weefselcoupes.

Dank ben ik verschuldigd aan de velen die weefsel voor mij hebben verzameld en ter beschikking gesteld: Sjaak Joosten, Piet op de Beek en Jacques Baltis; Drs. Jan Kirch, afdeling Urologie en Prof.dr. G.J. Fleuren, afdeling Pathologie, Rijksuniversiteit Leiden; Dr. J. Koudstaal, afdeling Pathologie en Drs. Marc Snijders, afdeling Gynaecologie, De Wever ziekenhuis, Heerlen; Dr. Harry van den Ingh, afdeling Pathologie, St. Clara ziekenhuis, Rotterdam en Dr. Gert Jan van Steenbrugge, afdeling Urologie, Erasmus Universiteit Rotterdam en Drs. Willem Rinsema, afdeling Algemene Heelkunde, Academisch Ziekenhuis, Maastricht.

Dr. Hans Tanke en Drs. Paul Oljans, afdeling Histochemie en Cytochemie, Rijksuniversiteit Leiden, zijn behulpzaam geweest bij de uitvoering van de flow-cytometrische bepalingen beschreven in hoofdstuk 5.

Dr. H.P. Hauri, Biozentrum der Universität Basel, danke ich herzlich für das zur Verfügungstellen von anti-Sucrase-Isomaltase monoklonalen Antikörpern.

Van Peter Dormans, vakgroep Medische Microbiologie, kreeg ik waardevolle adviezen voor de uitvoering van de immunoprecipitatie experimenten.

Drs. Adriaan de Bruïne dank ik voor het graderen van de prostaattumoren.

De medewerkers van de dienst Centrale Proefdier Voorziening, met name Frans Weekers, Eep van Dam en Peter Kelderman dank ik voor de assistentie bij het werk met proefdieren.

Prof.dr. G.H. Blijham, Prof.dr. J. Drukker, Prof.dr. F. ten Hoor, Prof.dr. C.H.M.M. De Bruijn (Technische Universiteit Eindhoven) en Prof.dr. D.J. Ruiter (Katholieke Universiteit Nijmegen) ben ik erkentelijk voor de beoordeling van dit proefschrift.

Lucia Geurts en Dr. Ton de Goeij dank ik voor de correctie van de Nederlandse teksten.

Alle medewerk(st)ers van de vakgroep Pathologie en de in het Biomedisch Centrum werkzaam zijnde leden van de vakgroep Interne Geneeskunde dank ik voor de fijne en collegiale sfeer waarin mijn onderzoek heeft plaatsgevonden.

Lieve Juliëtte, jouw steun en geduld waren onontbeerlijk.

Curriculum vitae

Winand Dinjens werd op 13 januari 1956 geboren te Maastricht. Vanaf 1968 tot 1975 bezocht hij het Henric van Veldeke-College in Maastricht. Hier behaalde hij in 1973 het diploma HAVO en in 1975 het diploma Atheneum B. In 1975 begon hij met de studie Biologie aan de Katholieke Universiteit Nijmegen en behaalde in december 1978 het kandidaatsexamen B1g. In april 1982 legde hij het doctoraalexamen af met als hoofdvak Zoölogie II (Dierfysiologie, onder leiding van Prof.dr. S.E. Wendelaar Bonga) en als bijvakken Anthropogenetica (onder leiding van Prof.dr. C.H.M.M. De Bruijn) en Zoölogie I (Ontwikkelingsbiologie der dieren, onder leiding van Prof.dr. J.M. Denucé en Prof. dr. C.H.M.M. De Bruijn). Tevens behaalde hij onderwijsbevoegdheid in Biologie. Vanaf mei 1982 tot juni 1985 was hij werkzaam binnen een FUNGO project dat werd uitgevoerd op de afdeling Klinische Immunobiologie van het Centraal Laboratorium van de Bloedtransfusiedienst van het Nederlandse Rode Kruis te Amsterdam (onder leiding van Dr. W.P. Zeijlemaker) en op het Anthropogenetisch Instituut van de Katholieke Universiteit Nijmegen (onder leiding van Prof.dr. C.H.M.M. De Bruijn).

Sinds augustus 1985 verricht hij onderzoek in het kader van een KWF project bij de afdeling Pathologie van de Rijksuniversiteit Limburg te Maastricht (onder leiding van Prof.dr. F.T. Bosman).